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Molecular Epidemiology of Microorganisms

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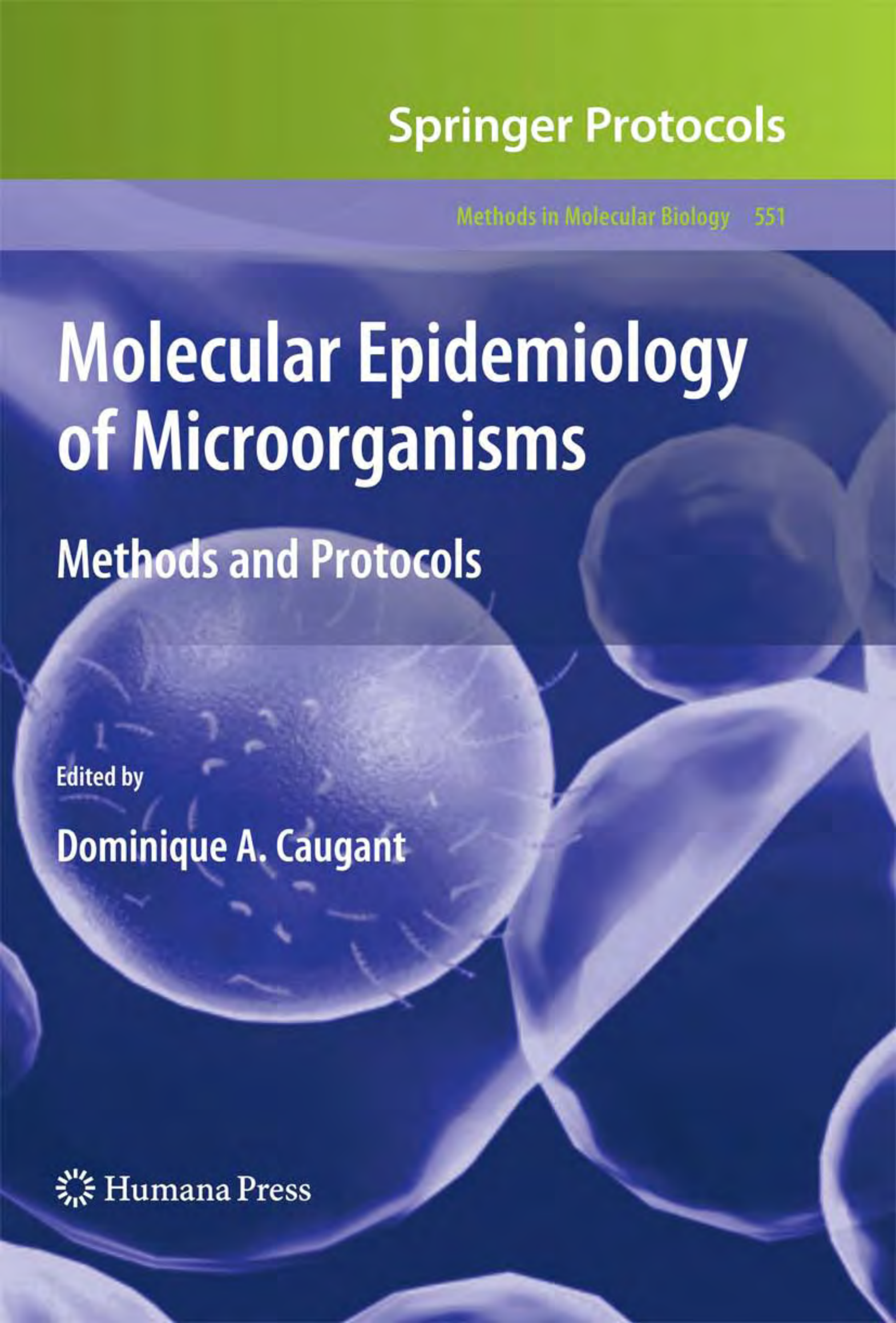
Edited by

Dominique A. Caugant



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Series Editor
John M. Walker
School of Life Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

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Dominique A. Caugant

Norwegian Institute of Public Health and University of Oslo, Oslo, Norway

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Editor
Dominique A. Caugant
Norwegian Institute of Public Health and University of Oslo
Oslo, Norway
Dominique.Caugant@fhi.no

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Preface

The development and rapid implementation of molecular genotyping methods have revolutionized the possibility for differentiation and classification of microorganisms at the subspecies level. Investigation of the species diversity is required to determine molecular relatedness of isolates for epidemiological studies. Methods for molecular epidemiology of microorganisms must be highly reproducible and provide effective discrimination of epidemiologically unrelated strains.

A wide range of techniques has been applied to the investigation of outbreaks of transmissible disease, and these have been critical in unraveling the route of spread of pathogens for humans, animals, and plants. The choice of a molecular method will depend on the type of questions to be addressed, on the degree of genetic diversity of the species to be analyzed, and on the mechanisms responsible for generation of the diversity. The applications of molecular methods, singly or in combination, have greatly contributed in the past two decades to basic microbial science and public health control strategies.

Molecular Epidemiology of Microorganisms: Methods and Protocols brings together a series of methods-based chapters with examples of application to some of the most important microbes. Both traditional and novel techniques are described, and the type of information that can be expected to be obtained by their application is indicated.

I am indebted to all internationally respected colleagues who have provided state-of-the-art chapters for inclusion in this book. I am very grateful for their outstanding contributions, enthusiasm for the project, and friendship. I would like to thank John Walker at Humana Press for the invitation to put this book together and his continuous encouragement.

Dominique A. Caugant

Contents

Contributors

David Michael Aanensen • *Department of Infectious Disease Epidemiology, Imperial College London, London, UK*
Pablo Bifani • *Pasteur Institute, Scientific Institute of Public Health, Belgium*
Lawrence B. Blyn • *Ibis Biosciences, Carlsbad, CA, USA*
Jon S. Brazier • *Anaerobe Reference Laboratory, NPHS Microbiology Cardiff, University Hospital of Wales, Heath Park, Cardiff, UK*
Eleanor M. Cottam • *Institute for Animal Health, Pirbright, Surrey, UK*
Jeffrey R. Driscoll • *Division of Tuberculosis Elimination, Centers for Disease Control and Prevention, Atlanta, GA, USA*
David J. Ecker • *Ibis Biosciences, Carlsbad, CA, USA*
Mark W. Eshoo • *Ibis Biosciences, Carlsbad, CA, USA*
Tamara Feldblyum • *Food and Drug Administration, Center for Devices and Radiological Health, Office of In Vitro Diagnostic Devices Evaluation and Safety, Rockville, MD, USA*
Alexander W. Friedrich • *Institute for Hygiene, University of Münster, Münster, Germany*
Norman K. Fry • *Respiratory and Systemic Infection Laboratory, Health Protection Agency, Centre for Infections, London, UK*
Ibtissem Grissa • *Department of Genetics and Microbiology, University of Paris XI, Orsay, France*
Thomas A. Hall • *Ibis Biosciences, Carlsbad, CA, USA*
Marie Hallin • *Laboratoire de Référence MRSA-Staphylocoques, Department of Microbiology, Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium*
William Paul Hanage • *Department of Infectious Disease Epidemiology, Imperial College London, London, UK*
James C. Hannis • *Ibis Biosciences, Carlsbad, CA, USA*
John P. Hays • *Erasmus MC, University Hospital Rotterdam, Department of Medical Microbiology and Infectious Diseases, Unit Research and Development, Rotterdam, The Netherlands*
Kelli L. Hiett • *Poultry Microbiological Safety Research Unit, Russell Research Center, Agricultural Research Service, U.S. Department of Agriculture, Athens, GA, USA*
Steven A. Hofstadler • *Ibis Biosciences, Carlsbad, CA, USA*
Carol Holm-Hansen • *Division of Infectious Disease Control, Norwegian Institute of Public Health, Oslo, Norway*
Stephan Huehn • *Federal Institute for Risk Assessment, National Salmonella Reference Laboratory, Berlin, Germany*
Ana Belén Ibarz Pavón • *Department of Zoology and Peter Medawar Building for Pathogen Research, University of Oxford, Oxford, UK*

Timothy J. Johnson • *Department of Veterinary and Biomedical Sciences, University of Minnesota, St. Paul, MN, USA*
Keith A. Jolley • *Department of Zoology and Peter Medawar Building for Pathogen Research, University of Oxford, Oxford, UK*
Donald P. King • *Institute for Animal Health, Pirbright, Surrey, UK*
Nick J. Knowles • *Institute for Animal Health, Pirbright, Surrey, UK*
Barry Kreiswirth • *Tuberculosis Centre, Public Health Research Institute, Newark, NJ, USA*
Ed J. Kuijper • *Reference Laboratory for Clostridium difficile, Medical Microbiology Department, LUMC, Leiden, and The National Institute for Public Health and Environment, Bilthoven, The Netherlands*
Natalia Kurepina • *Tuberculosis Centre, Public Health Research Institute, Newark, NJ, USA*
Martin C. J. Maiden • *Department of Zoology and Peter Medawar Building for Pathogen Research, University of Oxford, Oxford, UK*
Burkhard Malorny • *Federal Institute for Risk Assessment, National Salmonella Reference Laboratory, Berlin, Germany*
Christian Massire • *Ibis Biosciences, Carlsbad, CA, USA*
Barun Mathema • *Tuberculosis Centre, Public Health Research Institute, Newark, NJ, USA*
Lisa K. Nolan • *Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA, USA*
Tansy M. Peters • *Health Protection Agency, Centre for Infections, London, UK*
Christine Pourcel • *Department of Genetics and Microbiology, University of Paris XI, Orsay, France*
Rangarajan Sampath • *Ibis Biosciences, Carlsbad, CA, USA*
Paul H. M. Savelkoul • *Department of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam, The Netherlands*
Bruce S. Seal • *Poultry Microbiological Safety Research Unit, Russell Research Center, Agricultural Research Service, U.S. Department of Agriculture, Athens, GA, USA*
Marc J. Struelens • *Laboratoire de Référence MRSA-Staphylocoques, Department of Microbiology, Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium*
Hervé Tettelin • *Institute for Genome Sciences, Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD, USA*
Michel Tibayrenc • *IRD Representative Office, French Embassy, Bangkok, Thailand*
Kirsti Vainio • *Division of Infectious Disease Control, Norwegian Institute of Public Health, Oslo, Norway*
Alex van Belkum • *Erasmus MC, University Hospital Rotterdam, Department of Medical Microbiology and Infectious Diseases, Unit Research and Development, Rotterdam, The Netherlands*
Renate J. van den Berg • *Anaerobe Reference Laboratory, NPHS Microbiology Cardiff, University Hospital of Wales, Heath Park, Cardiff, UK*
Elisabeth van Pelt-Verkuil • *Hogeschool Leiden, Department of Post Graduate Courses, Leiden, The Netherlands*

GILLES VERGNAUD • *DGA/D4S–Mission pour la Recherche et l'Innovation Scientifique (MRIS), Armées, and Department of Genetics and Microbiology, University of Paris XI, Orsay, France*
PAOLO VISCA • *Department of Biology, University Roma Tre, and Molecular Microbiology Unit, National Institute for Infectious Diseases "Lazzaro Spallanzani," Rome, Italy*
JEMMA WADSWORTH • *Institute for Animal Health, Pirbright, Surrey, UK*
XIAO-MING WANG • *Pasteur Institute, Scientific Institute of Public Health, Belgium*

Chapter 1

Microbial Molecular Epidemiology: An Overview

Michel Tibayrenc

Abstract

In this introductory chapter, I stress one more time the urgency to better connect molecular epidemiology and evolutionary biology. I show how much population genetics and phylogenetic analyses can confer a considerable added value to all attempts to characterize strains and species of pathogens. The problems dealing with the mere definition of basic concepts, such as species, subspecies, or strains, are briefly summarized. Last, I show the important contribution of molecular epidemiology to our knowledge of the basic biology of pathogens and insist on the necessity not to separate the studies dealing with pathogens from those that concern the hosts and the vectors, in the case of vector-borne diseases.

Key words: Cladistics; molecular marker; phylogenetic analysis; population genetics; species; strain typing.

1. Introduction

This introductory chapter is definitely not a comprehensive survey of what molecular epidemiology is today. It instead aims at putting the field into perspective, with its promises fulfilled or let down, its practical implications in terms of public health, its unsolved challenges, and its future potential with the burgeoning of advanced technologies. For more complete overviews of the field, refer to recent reviews *(1*, *2*). The present text is something of a political claim. Other authors of this book may not share the same views.

The field covered by this book is undeniably a topical one: A Medline search with the key words "molecular epidemiology" produces more than 5,000 references. For the sole year 2007,

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the number approaches 1,000. Of these references, roughly 10% cover a different field, which should instead be called genetic susceptibility to diseases. The rest are related to the very theme of this book, which I try to define below. This definition reflects my own views and again may be not shared by the other authors of the book.

1.1. An Attempt to Define Molecular Epidemiology

The Centers for Disease Control and Prevention in Atlanta, Georgia, or more exactly its branch that specializes in transmissible diseases, the National Center for Infectious Diseases, can be considered the mecca of molecular epidemiology. In 1994, this institute issued the following definition of microbial molecular epidemiology: "the various biochemical and molecular techniques used to type and subtype pathogens" *(3)*. This definition is strictly a technology-based one. As developed in this chapter, I feel it is indispensable to broaden and enrich this definition. First, technology is not enough to characterize pathogens, and its exclusive use could prove to be grossly misleading. The use of evolutionary concepts makes molecular epidemiology considerably more efficient and makes it possible to gather precious knowledge of the basic biology of the organisms under study. Second, identifying pathogens is too narrow a goal for molecular epidemiology. The so-called downstream studies *(4)* aim at evaluating the impact of the genetic diversity of pathogens on their relevant medical properties (pathogenicity, antigenic diversity, and drug and antibiotic resistance). These reflections have led me to propose a broad definition of molecular epidemiology *(1)*: (1) the definition, identification, and tracking of relevant pathogen species, subspecies, strains, clones, and genes by means of molecular technology and evolutionary biology; and (2) the evaluation of the impact of a pathogen's genetic diversity on its relevant medical properties.

1.2. Increasing Importance of the Field and Advanced Technologies

The field of molecular epidemiology has experienced a rapid growth year after year, from fewer than ten references in Medline before 1981 to close to 1,000 for the sole year 2007. It is striking, when doing a retrospective search, to see techniques, such as multilocus enzyme electrophoresis (MLEE), that have been earlier considered gold standards vanish in favor of the new stars: microarrays, real-time polymerase chain reaction (PCR), and especially multilocus sequence typing (MLST). There is undoubtedly something of a fad here. The treasured old techniques did not prove to be unworthy, and they still deserve recognition for certain of their uses (*see* my chapter on MLEE in this book). Moreover, the new stars, although they are very powerful, are by no means panaceas.

It is not my purpose here to denigrate the new technologies. They have undoubtedly contributed considerably to the progress

made in the field. For example, MLST is incomparable in finely dissecting the impact of recombination in microbes (*see* **Subheading 2.5**). I can only repeat here what I have said many times: There are no good and bad techniques; there are only techniques that are better designed to answer given questions. Still the fact remains that, all things being equal, a paper that relies on the hottest technique in fashion will be more easily published than another one based on MLEE or restriction fragment length polymorphism (RFLP).

1.3. What Is Molecular Epidemiology Good for?

In the heroic times of molecular epidemiology (late 1970s), hopes were high that it would become a routine diagnostic tool like enzyme-linked immunosorbent assay and indirect immunofluorescence. This happened only partially. The practical contribution to daily patient care remains limited and mainly consists of species identification using PCR techniques, which is still limited to specialized laboratories. Where strain typing (i.e., characterization at the subspecific level) is concerned, it is not used as a routine analysis.

It can be said that in the present state of the art, molecular epidemiology is more a research tool than a significant contribution to routine clinical medicine *(5)*. In this perspective, many papers apply the current state of knowledge to epidemiological surveys. Many tools are quite standardized and can be successfully applied to various situations. Spoligotyping for the identification of *Mycobacterium tuberculosis* strains is a typical example. Since it was designed more than 10 yr ago *(6)*, hundreds of papers using this technique have been published. Each of them now has a limited added value, restricted to the analysis of specific, local situations. At the other end of this scale, articles developing the most advanced research are paving the way for the molecular epidemiology of tomorrow (*see* **refs**. *7–11*, among others).

1.4. The Distressing and Persistent Gap Between Molecular Epidemiologists and Evolutionists/Population Biologists

I can say that my entire career has been devoted to spreading propaganda in favor of uncompartmentalizing molecular epidemiology and population genetics/evolution. To a large extent, this has proved to be a failure. A recent article again focusing on this need *(1)* amounted to preaching in the desert and is among my least-cited articles. Many evolutionists are attracted to the fascinating models offered by transmissible diseases and coevolution between hosts, pathogens, and vectors. This is the case for the authors of the cited masterpiece papers. However, as a rule, they adhere to a vision of evolutionists, could have very speculative approaches (which is welcome in basic research), and sometimes do not heed the potential applicability of their research in terms of public health. This makes most of these papers simply unreadable for clinicians, public health managers, and even scientists involved in applied research. Notable exceptions can be found in the recent literature. Some evolutionists and phylogeneticians do their best to make themselves accessible to nonspecialists *(9,12)*.

On the other hand, many contributions related to molecular epidemiology and strain typing do not say a word about the possible contribution of evolutionary biology to this discipline. This is true even for very recent papers, some of them published in high-impact journals, supposedly the state-of-the-art in the field *(13–15)*. These articles, although they contain extremely valuable information and may propose innovative concepts, entirely miss an evolutionary interpretation of the data. Bacterial populations are simply considered a set of eternal clones with no recombination among them, which is a glaring mistake for many bacterial species, if not all. Hybrid papers that underline the contribution of evolutionary studies to molecular epidemiology, and remain accessible to nonevolutionists, are the exception rather than the rule *(16)*.

2. The Targets of Molecular Epidemiology: Relevant Species, Subspecies, Strains, Clones, and Genes

The first, basic goal of molecular epidemiology is to identity, characterize, and follow those entities (units of analysis) that are relevant to the clinician and the epidemiologist. This again emphasizes the crucial role of evolutionary biology since these entities are extremely difficult or impossible to characterize and even to define without the help of the concepts from this discipline.

2.1. Species

The concept of species is a typical example of how difficult it is to define and delimit the units of analysis for molecular epidemiology. This has been discussed at length in another article *(17)*, and I only review the many challenges raised by the problem.

Intuitively, pathogen species look like solid entities that should be easy to characterize and follow. However, an entity that is not clearly defined is like a vanishing mirage. A personal anecdote illustrates how misleading it can be to adhere to the unfounded belief that species made official with a Latin name are engraved in stone. Years ago my laboratory was asked to determine the species of a *Leishmania* strain from Latin America (*Leishmania* are the kinetoplastid parasitic protozoa responsible for leishmanioses). Using MLEE and comparison with a set of reference strains, we identified the strain as *Leishmania panamensis*. The colleague who sent the strain responded that the identification was glaringly wrong. He had a counteranalysis done by another laboratory, which identified the strain as another species, *Leishmania guyanensis*. Puzzled by these contradictory results, we performed a broad survey of many strains of both species. The conclusion was crystal clear: If a blind approach was used, by MLEE analysis *L. panamensis* and *L. guyanensis* strains showed

no differences. In other words, these two supposedly separate species had been described first on geographical (phenotypical) grounds, but from a phylogenetic point of view, they could not be distinguished from each other.

For so-called higher organisms, the species concept is already a headache, although species of mammals, birds, and insects do exist and are confirmed by recurrent observations. If pathogen species are concerned, the definition of *species* is a "mission impossible," as confirmed by the abundant literature devoted to it. This led some evolutionists to consider that a definition of the species was hopeless and useless, except in birds ***(18)***. However, scientists working in applied research, clinicians, health professionals, and decision makers cannot accept such an extreme and puristic view: It is an obligation to define the targets of medicine and control measures. Malaria is *not* caused by *Escherichia coli*, and *Leishmania* parasites are *not* transmitted by tsetse flies. Thousands of species are described and used in the world of pathogens. When designing molecular epidemiology tools to try to characterize them, it is crucial to know which upstream concept has been used to define these species. Many microbial species have been defined on epidemiological or medical bases; this is a special case of the phenotypic species concept, according to which species are defined on phenotypic characteristics. For example, *Leishmania infantum* is the causative agent of infant leishmaniosis in the Mediterranean basin, *M. tuberculosis* is the agent of tuberculosis, and so on. When targeting such species with molecular epidemiology, it is necessary to verify that they correspond, at least to some extent, to discrete collections of genetically related genotypes. If this is not obtained, as in *L. panamensis* and *L. guyanensis*, for example, it will be impossible to characterize such species as a whole and to distinguish them from other species. An extreme and classical example is *Shigella* bacteria, which have been assigned the rank of a specific genus by clinicians due to their striking pathogenic properties (they cause severe dysentery). Yet a phylogenetic analysis reveals that *Shigella* are merely a bunch of *E. coli* clones, which are not even monophyletic (they do not constitute a specific, unique evolutionary line). Characterizing all *Shigella* as a discrete genetic entity is therefore hopeless. The only means to specifically track *Shigella* strains is to characterize those pathogenicity genes that make them so virulent.

To handle the species concept for pathogens, the operational view I have defended ***(17)*** states that (i) the world of pathogens in a genetic view is not level and undifferentiated. It has clear discontinuities, even if their borders are not always sharply defined. It is therefore desirable to use the phylogenetic species concept to describe pathogen species ***(19)***, but using a very flexible approach, since the genetic discontinuities that exist in the pathogen world many times do not correspond to sharply defined phylums

(see the discussion of the concept of discrete typing unit). ***(ii)*** One should by all means try *not* to describe new species. In the case of pathogens, it is clear that the definition of a species is really a matter of convenience. One describes species when it is relevant for applied research, clinical practice, and health policy, not when it gives the opportunity to publish a new paper. Let us stop the species inflation.

2.2. Subspecies

Subspecies are subdivisions of a given species that are given a triname. For example, the zebu, long considered a species that was different from the European ox (*Bos indicus* vs. *Bos taurus*), has been made a simple subspecies of the latter (*Bos taurus indicus*), which is logical since absolutely no mating barriers exist between the two formerly described species. In so-called higher organisms, subspecies are defined as geographic morphological variants of a given species. They do exist, as shown by recurrent observations ***(2)***. If pathogens are concerned, nothing clear emerges. One can say that scientists describe subspecies on the same grounds as species (phenotypic or phylogenetic criteria or both) when they dare not describe a species. A pathogen subspecies is something like a timid species—not a very operational concept. It would be wise to drop this practice with pathogens. Either the entity deserves to be defined and is given the rank of species or it does not.

2.3. Strains

The term *strain* is one of the most widely used and the most confusing in the literature dealing with pathogens. In laboratory jargon, a strain is no more than the collection of parasites you handle in Petri dishes or culture flasks. The right term here should be *stock*. Specialists (myself included) often speak about a reference strain, which is a cell line isolated from a given host at a given time in a given place. The correct name should be *isolate*. If molecular epidemiology is concerned, people seek to characterize strains with molecular tools. In this case, one refers to multilocus genotypes, which immediately opens two closely related Pandora's boxes: how to delimitate multilocus genotypes and the problems about defining the notion of clonality.

2.4. Clones, Clonal, Clonality

There is great confusion in the use of the terms *clones*, *clonal*, and *clonality*. When speaking about a clonal species, many authors actually refer to a species whose genetic diversity is either weak or null ***(14)***. Many sexual species have very low genetic variability, while some species with no genetic recombination are genetically extremely diversified. This has nothing to do with the mode of reproduction. Rather, a species whose genetic diversity is very low is simply assumed to have a recent common ancestor, whatever its mating system. Other authors limit the term *clonal* to only mitotic propagation. My articles dealing with clonality have been

frequently misunderstood because of this confusion. A clonal species should instead refer to a species in which descendants are genetically identical to the parent. This gives a genetic definition to the clone. Many cases of uniparental reproduction produce genetic clones, not only mitotic propagation, but also apomictic parthenogenesis, gynogenesis in some fish species, and self-fertilization in haploid organisms. Extreme cases of homogamy will also lead to the production of genetic clones since only those cells that are genetically identical or extremely similar will mate together *(4)*.

Even if clonality is properly defined in this way, in population genetic terms, it does not mean that our trouble is over. First comes the problem of properly characterizing clones. Let us imagine a species that is perfectly clonal, that is, in which gene exchange is totally absent. As we discussed in this **Section 2.4**, this probably does not exist in the world of pathogens. But even if it did, let us characterize clones of this purely clonal species with one of the stars of the fashionable techniques available today, MLST *(11)*. The strains that share identical MLST alleles are referred to as *sequence types*. Can they be considered clones? In other words, are they really genetically homogeneous? The answer is no. The promoters of the MLST approach themselves soon discovered (M. Achtman, personal communication) that RFLP based on a few antigen genes added to MLST considerably improved its resolution power. In other words, the clones identified with MLST are genetically heterogeneous. This is true for any technique. The concept of *clonet* was forged to overcome this problem (*see* **Subheading 3**.).

2.5. Not-So-Clones and Not-So-Clades

The first expression, not-so-clones, is a joke by my witty friend B. Levin; the second, not-so-clades, is a plagiarism of my own. After the successful clone concept was born *(20)*, it was soon evidenced that many bacterial species did not amount to a mere collection of eternal clones *(21)*. Actually, most pathogen species are capable of both clonal propagation and genetic exchange *(7)*. The contribution of each varies between species and, more surprisingly, may vary within the same species between different populations, transmission cycles, and ecosystems *(22)*. These considerations are not relevant only to evolutionists. They have considerable implications for molecular epidemiology/strain typing. Although genetic exchange in pathogens may have various faces, such as conjugation; transformation; transfection in bacteria; meiotic recombination in *Trypanosoma brucei*, the agent of sleeping sickness *(23)*; and nonmeiotic hybridization in *Trypanosoma cruzi*, the agent of Chagas' disease *(10)*, its consequences on population structure are similar. When genetic exchange is frequent, multilocus genotypes are ephemeral, and clades no longer deserve the name since different genetic lineages are only imperfectly separated from each other.

3. Concepts that Proved to be Only Partially Successful: Discrete Typing Units, Tags, and Clonets

Let us summarize the headache: Clades in pathogen species mate with each other from time to time. Even worse, some clades have two ancestors instead of one, as is the case for the remarkable hybrid genotypes recorded in *T. cruzi* ***(10)***. Wisely, Hall and Barlow ***(12)*** called for great caution when performing phylogenetic analysis in those species for which genetic exchange is frequent. Indeed, careless use of such analyses could prove to be grossly misleading. Clades are evolutionary lineages that have one ancestor and are genetically isolated from each other ***(24)***. With this clean definition, for example, even in the case of a species such as *T. cruzi*, in which clonal evolution is preponderant, the genetic subdivisions do not deserve the term of clade ***(22)***. Still in many pathogen species, it is clear that genetic variation is not evenly distributed, and that unambiguous, stable subdivisions are apparent. Such units may be characterizable for epidemiological tracking and may have different relevant properties in terms of ecological distribution, pathogenicity, and so on. Should one renounce the attempt to describe these subdivisions only because the concept of clade is ineffective? Other terms have been proposed but are not satisfactory: "Cluster" is only a visual description of subdivisions within a dendrogram. It is as informative as saying that a cake is divided into slices. "Line" and "lineage" are utterly vague. Mammals are a lineage. The Bourbon kings of France are a lineage as well.

I have proposed the term *discrete typing unit* (DTU) to refer to these stable genetic subdivisions within pathogen species: collections of genotypes that are genetically more similar to each other than to any other collections of genotypes, that appear to persist in space and time, and that can be characterized in common by specific markers, or tags ***(25)***. Although the proposal was well accepted in several congresses, it has proved to be particularly successful only among scientists working on *T. cruzi*. The rest of the literature struggles with a tangle of vague concepts: not-so-clades, lineages, and clusters. This is distressing since DTUs constitute a highly reliable target for molecular epidemiology, and tags are identified and designed with the very goal of characterizing the DTUs specifically.

The clonet ***(26)*** is another partial success story. A *clonet* is a set of genotypes that appear to be identical with a given set of genetic markers in a clonal species. As explained, even in those species that are highly clonal, a clone characterized by a given set of genetic markers may not be a real clone but more probably a family of genetically related clones. The nuance is considerable. Clones characterized with a given technique, for example, a few

primers for randomly amplified polymorphic DNA analysis, might be perfect optical illusions. Depending on the resolution power of the technique used, the common ancestor of the clone might be either a few weeks or months old, which is quite relevant to epidemiological follow-up, or hundreds of years old, which is relevant to the evolutionist but not to the epidemiologist. Before aiming at characterizing clones, it is therefore indispensable ***(i)*** to ascertain that the species and the population under study are truly clonal, which can be done by reliable population genetics analysis only; and ***(ii)*** to scale the resolution power of the markers to be used to the goal of the study.

4. Targeting Relevant Genes Rather than Whole Organisms

The object of molecular epidemiology is chiefly to survey what is medically relevant. What matters for health professionals is pathogenicity and resistance to treatments. Designing sophisticated phylogenies (even so-called not-so-phylogenies), characterizing acutely multilocus genotypes is highly relevant to the evolutionist. It may be less so to the health professional if the genes that drive medically relevant properties evolve largely independently from phylogenies and multilocus genotypes. In the case of bacteria, many important genes are harbored by plasmids, which are able to jump from a bacterial cell to another. Even nuclear genes could jump frequently from one genome to another (horizontal gene transfer), especially if they undergo great selective pressure. In medical research, it is therefore crucial to identify the genes to be followed and to design specific markers for them. Needless to say, however, ***(i)*** this is also very important to the evolutionist, and ***(ii)*** elaborating a sophisticated population genetics framework for the entire species remains quite informative for the follow-up of these culprit genes, precisely to see how independent they are from the general evolution and demography of the host species.

5. The Great Contribution of Evolutionary Biology to Our Knowledge of the Basic Biology of Pathogens

Evolutionary studies still have much to tell us about the world of pathogens. However, a consensus picture has emerged on the reproductive strategy of microbes: Many species play on a double keyboard and are capable of both sexual recombination and clonal propagation. This is wise from an evolutionary point

of view: Sexual recombination serves to quickly generate new genetic combinations able to respond to new selective challenges, and in turn, clonal propagation makes it possible to stabilize in long-term favorable genetic combinations.

The vast majority of pathogens have recombination as a side mechanism, but it is not at all mandatory for their reproduction. It is only a useful last resort on an evolutionary scale to allow successful genotypes to make an appearance. *Trypanosoma cruzi* is an illustrative example. Two of these genotypes that appear to be hybrids behave as conquistadores and, once generated by recombination of two ancestors, now propagate themselves clonally over vast geographical ranges, mainly in human transmission cycles. In spite of these occasional bouts of recombination, the species as a whole is profoundly structured into six persistent DTUs ***(27,28)***, found over the entire geographical reach of the species.

To some extent, this is also true in the case of *E. coli*. Although the subdivisions visible within this species might be less sharply defined than *T. cruzi* DTUs are, it is remarkable that those uncovered by the pioneer isoenzyme work by Ochman and Selander ***(29)*** (A, B1, B2, and D) have been recognized by recent studies relying on totally different molecular tools ***(30,31)***, making these subdivisions perfectly honest DTUs. The population genetics framework thus elaborated for *E. coli* provides a remarkable evolutionary tool for the study of medically relevant features [*Shigella* strains, pathogenicity islands, mutator genes ***(32)***, and antibiotic resistance genes].

Plasmodium falciparum, the agent of the most malignant form of malaria, is another example of the relevance of population biology studies to biomedical research. It has been long considered ***(33)*** the paradigm of a panmictic organism (a species is panmictic when genetic exchange occurs at random with no other obstacles than geographical distance or isolation by time). The cautious proposal that some populations of *P. falciparum* might undergo some kind of uniparental propagation ***(34)*** has received a flurry of blows. However, many, if not most, populations of this parasite show a strong linkage disequilibrium (nonrandom association of genotypes occurring at different loci), which indicates a severe inhibition to recombination in these populations ***(35)***. Whatever the final explanation, the rough data show that many *P. falciparum* populations are by no means panmictic.

It is not an exaggeration to say that evolutionary studies have revolutionized our views on pathogen population biology and dynamics, with considerable payoffs in terms of medical research. It is all the more distressing that evolution science is still not considered a built-in component of molecular epidemiology, as it should be.

6. The Future

It will always be useful to trace pathogen genes and genotypes responsible for epidemics, especially those genotypes designated as "superspreaders," causing the majority of infections in a given species. However, this classical and restricted conception of molecular epidemiology is bound to be outshined. Technological progress makes it possible to envisage a thorough characterization of pathogens, integrating genomic, proteomic, metabolomic, clinical, and epidemiological data. This is rendered accessible by automatic sequencing, microarrays, and geographical information systems. It is the concept of pathogen profiling (***15***). Integrating complex sets of data will be made possible by the emerging Web portals and portals of portals (MLSTNet for MLST data, PulseNet for pulsed-field gel electrophoresis data, among others). It would be a pity not to interpret these abundant and complementary data in terms of evolutionary biology, which could lead to the population genomics and population proteomics needed.

Finally, as already advocated many times (***25***,***36***), research investigating the pathogen, its host, and in the case of vector-borne diseases, its vector, should not be artificially compartmentalized when obviously these organisms do not evolve separately and on the contrary follow a pattern of coevolution. The host is a characteristic of the pathogen, and vice versa, and the same is true for pathogens and vectors. Pathogen profiling could not be complete without parallel evolutionary studies on the host and the vector. The MEEGID (Molecular Epidemiology and Evolutionary Genetics) congresses and the journal *Infection, Genetics and Evolution* are the privileged tribunes for this integrated approach.

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Chapter 2

Multilocus Enzyme Electrophoresis for Parasites and Other Pathogens

Michel Tibayrenc

Abstract

In this chapter, I expose the main properties and theoretical background of a somewhat out-of-fashion technique, multilocus enzyme electrophoresis (MLEE). I show that the remarkable properties of this marker—clear Mendelian inheritance, codominance, strong phylogenetic signal—are still valid, although of course more modern markers now are able to yield far more refined results. MLEE can still be used in many circumstances when a cheap and reliable marker is required. I summarize what have been the main contributions of MLEE to the study of parasites and other pathogens.

Key words: Co-dominance, electrophoresis, Mendelian inheritance, molecular epidemiology, phylogenetic analysis, population genetics, strain typing.

1. Introduction

Writing a chapter on isoenzymes in 2008 is an interesting challenge. Is this technique not relegated to the status of an ancient prototype? In the age of real-time polymerase chain reaction (PCR), automatic sequencing, and microarrays, it may seem strange to continue to use this peculiar cuisine with its many toxic coloring agents and questionable recipes. Still, the wealth garnered from the isoenzyme era is indeed remarkable, if only for its historical significance, and it deserves to be reviewed. Moreover, the advantages offered by isoenzymes when the technique was born remain valid. This inexpensive and hardy technology can therefore be a salvation for laboratories with few resources, making it possible to conduct very reliable research at low cost. Another reason for not condemning isoenzymes to the dungeon of science

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is the utility of their dual vision and data congruency. When genetic characterization of pathogens is involved (the topic of this book), it is reassuring that different types of markers provide convergent results: This is the *congruence principle (1)*. Using two different types of markers will therefore augment the reliability of the results. Moreover, all things being equal, experience shows that the resolution power of the analysis is greater when doing this: Five enzyme-coding loci and five restriction fragment length polymorphism (RFLP) loci give better results than ten RFLP loci alone.

Since the nature of this technology, and even sometimes the mere existence of it, is virtually unknown to many young scientists, I feel it will be useful to start at the very beginning and to expound the main features that make the technique relevant. This will have the advantage of stamping out many preconceived ideas. It is presented as answers to frequently asked questions, drawing on only the main points. For a more comprehensive review, see *(2)*.

2. The Nature of Isoenzymes and Their Main Properties

2.1. What Is an Isoenzyme?

The term *isoenzyme (3)* actually has a purely technical definition. Isoenzymes or isozymes are different molecular forms of the same enzyme that have different migration speeds in electrophoresis. It should be emphasized that there is nothing genetic behind this definition. **Figure 1** shows different isoenzyme forms of the same enzyme, glucose phosphate isomerase (GPI). It can be seen that the differences in migration are considerable (+ is at the top of the gel, following the tradition of isoenzyme studies), including in the same species. An isoenzyme is therefore an enzyme and hence a protein.

2.2. Why Do Isoenzymes Migrate Differentially in Electrophoresis?

In the electrophoresis chamber, the experimenter installs an electrical field from the cathode to the anode. Biological molecules disposed on conductive materials migrate according to their electrophoretic charge. Other parameters may interfere, for instance, the shape of the molecule, the molecular weight, and the filter effect of certain media, such as starch or polyacrylamide. On other media, such as cellulose acetate, the electric charge is virtually the only factor that acts on the migration. Isoenzymes have different migration speeds because they have different overall electric charges.

2.3. What Is the Origin of a Protein's Electric Charge?

A protein's electric charge is a result of the individual electric charge of each amino acid comprising the protein. Some amino acids are positive, others are negative, and many are neutral. The overall

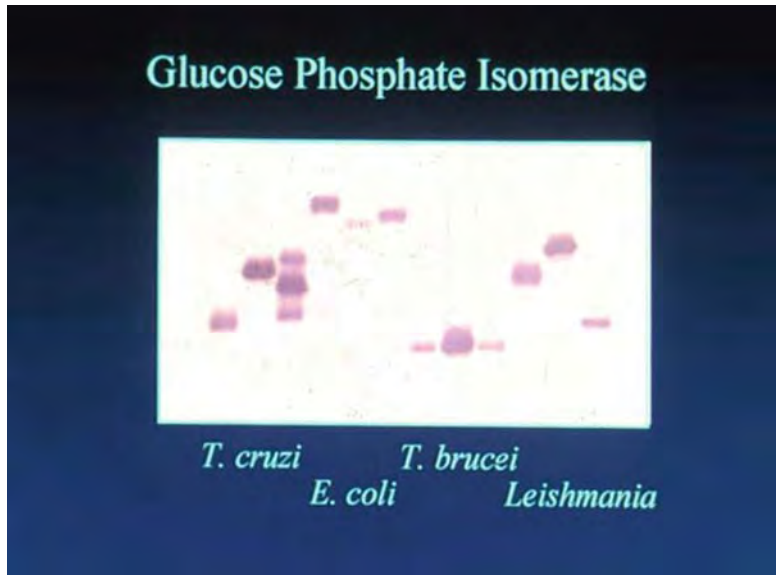

Fig. 1. An isoenzyme survey with the enzyme system glucose phosphate isomerase of various pathogen species. From left to right: Three stocks of *Trypanosoma cruzi*, the parasitic protozan responsible for Chagas' disease; three stocks of the bacterium *Escherichia coli*; two stocks of the African trypanosome *Trypanosoma brucei*; three stocks of *Leishmania* spp., the parasites responsible for leishmanioses. This experiment demonstrates the polyvalence of the isoenzyme tool.

electric charge of a protein therefore directly reflects the primary sequence of amino acids.

2.4. How Are Isoenzymes Revealed and Stained?

On an electrophoretic gel, thousands of proteins will migrate together. If one uses a nonspecific marker (e.g., Coomassie blue), hundreds of protein bands will be stained, and the gel will not be readable. The promoters of the isoenzyme technique in the 1960s came up with the idea of associating a staining reaction with the specific substrate of the enzyme. Enzymes are catalyzers that lower the energy required for a given metabolic chain. Each enzyme is associated with a specific substrate. For example, lactate dehydrogenase specifically metabolizes lactic acid. With a specific staining reaction associated with lactic acid, only lactate dehydrogenase molecules will be revealed on the gel. This is a true biochemical probe.

Hundreds of staining procedures have been designed, and many different enzyme systems can be used. This chapter recounts the general principles of the method but does not provide a catalogue of these protocols. Detailed recipes are provided in *(2)* and *(4)*. Each of these recipes makes it possible to reveal the activity of only one enzyme, as a rule one genetic locus.

2.5. How Many Enzyme Systems Should Be Used?

To have a representative sample of the genome of the organism under study, I would say 15–25 enzyme systems should be used. This is the number used in our laboratory for characterizing parasite strains. Experience shows that the results obtained with this number (corresponding to roughly the same number of genetic loci) are highly reliable and have been fully confirmed by the use of more sophisticated molecular techniques (*see* **Chapter 11**). When one enzyme system has a strong phylogenetic signal, used alone, it can constitute what we call a tag, a specific marker for a given unit of analysis (*see* **Chapter 1**).

2.6. What Are the Principal Techniques Used?

For the most part, three media are used for isoenzyme characterization: polyacrylamide, starch, and cellulose acetate. As in all techniques, each has advantages and drawbacks. Cellulose acetate consists in small gels that are sold ready to use (Helena® Laboratories, Beaumont, TX). One has only to soak the gel in the appropriate buffer just before running the electrophoresis. The experiment is very short (20–30 min), and once dried, the gel is as easy to keep and store as a playing card and can be sent by mail. I have some gels dating from my early career 30 years ago that are as readable as the first day. Polyacrylamide and starch gels are more time consuming, but their resolution is generally better. Polyacrylamide would be the best from this point of view. Thick starch gels can be sliced to reveal three or four different enzyme systems with the same run, which saves a great deal of time.

2.7. How Are the Samples Prepared and Preserved?

Preparation and preservation of samples are the main drawbacks of the technique. Enzymes are hydrosoluble proteins that are extremely thermosensitive. A convenient cold chain is therefore imperative. Samples (e.g., pellets of parasite cells) must be preserved in a deep freezer at –70°C. A cell pellet should not be used directly; cells should be disrupted by a mechanical means and centrifuged so that only the liquid supernatant is used for electrophoresis. Once defrosted, the sample should be kept on an ice bed until use. By comparison, DNA techniques are a dream since DNA is a very resistant molecule, and dried samples kept at room temperature still have a great deal of DNA material that is usable for analysis.

2.8. Are Isoenzymes Mere Phenotypes?

Isoenzymes are definitely not mere phenotypes if it is understood by this that isoenzymes are something like artifacts that have little or nothing to do with genetic variation. Strictly speaking, isoenzymes are phenotypes. However, they have a very clear genetic background, accounting for their great importance in population genetics. As mentioned, the differential electrophoretic migration of isoenzymes is an effect of their differential electric charges, resulting from the individual charge of their amino acids. Different migration speeds are directly related to the primary

sequence of isoenzymes and therefore to the sequence of the genes that encode them. Post-translational modifications may interfere, but it is considered that 90% of the isoenzyme diversity directly reflects a genetic variability.

2.9. Are All Types of Genetic Variation Reflected by Isoenzyme Variability?

Obviously, all types of genetic variation are not reflected by isoenzyme variability. Isoenzyme variability concerns genes coding for hydrosoluble proteins only.

2.10. Are All Mutations Able to Generate Isoenzyme Diversity?

Only mutations that change the primary structure of the protein will have an impact on isoenzyme variability. Silent mutations will not.

2.11. Do Isoenzymes Indicate All Proteic Variation?

Isoenzymes do not indicate all proteic variation. Approximately 70% of amino acid changes do not modify the electric charge of a protein *(5)*. Two proteins with the same electrophoretic mobility may also have different primary sequences. However, if the technique has sufficient discriminatory power, two enzymes that have the same migration have a high probability of having either an identical or a similar amino acid sequence.

2.12. What Organisms Can Be Analyzed Using Isoenzymes?

All organisms that have enzyme systems can be analyzed by isoenzymes. Empirically, certain categories of organisms are particularly well suited for this technique. Insects that are easy to crush, such as mosquitoes and sandflies, are examples. Of course, microbes have to be bulk cultured for isoenzyme analysis. This presents the risk of culture bias *(6)*. Many hosts are infected by several different genotypes of a given pathogen. When sampling an isolate, it is very frequent that this isolate is itself composed of several genotypes. Some of them might be selected by the culturing process, to the detriment of others, so that the genotype composition of the culture changes over time.

Apart from this drawback, isoenzymes are a fine generalist marker *(6)*, usable for virtually all organisms, so that different species can be compared. For example, when similar isoenzyme techniques are used, it is possible to reliably compare the variability or population structure of African and American trypanosomes.

2.13. Do Isoenzymes Have a Clear Mendelian Inheritance?

Isoenzymes have a clear Mendelian inheritance, and this is the method's most relevant point for population genetics. In the late 1960s and 1970s, an incredible number of isoenzyme studies were published. Virtually the entire living reign was surveyed *(1)*. The Mendelian inheritance of isoenzymes is therefore extremely well known, even more so since many mating experiments were conducted on laboratory animals (e.g., fruit flies, mice); genealogies were abundantly tested as well.

Many isoenzyme variants can be equated to alleles or at least families of alleles. The term *allozyme* or *alloenzyme* has been coined for these variants *(7)*. Isoenzyme variability is codominant, meaning that as a rule, heterozygous patterns differ from homozygous patterns (see **Figs. 2** and **3**). Heterozygous genotypes often show very typical patterns; their genetic background is explained in **Figs. 2** and **3** for monomeric and dimeric enzymes. A typical heterozygous pattern for a diploid organism, in the case of a dimeric enzyme, is three banded, with the central band more intensely stained than the two other bands (**Fig. 3**). The third sample from the left in **Fig. 1** illustrates this pattern.

As a rule, one enzyme's biochemical system reveals the activity of one genetic locus. When referring to the enzyme system from a biochemical point of view, the enzyme is abbreviated as

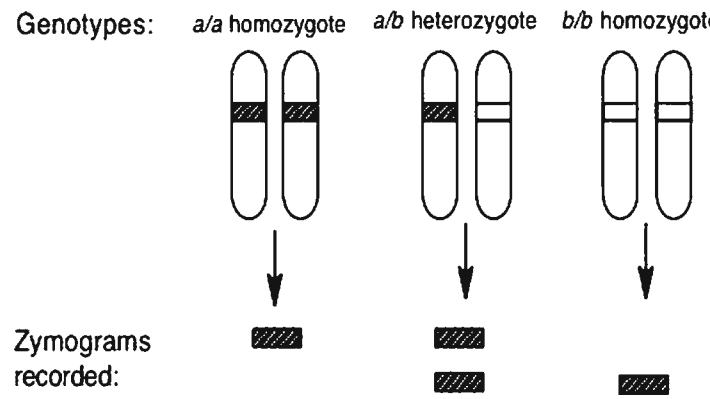

Fig. 2. Genetic background of zymograms attributable to monomeric enzymes.

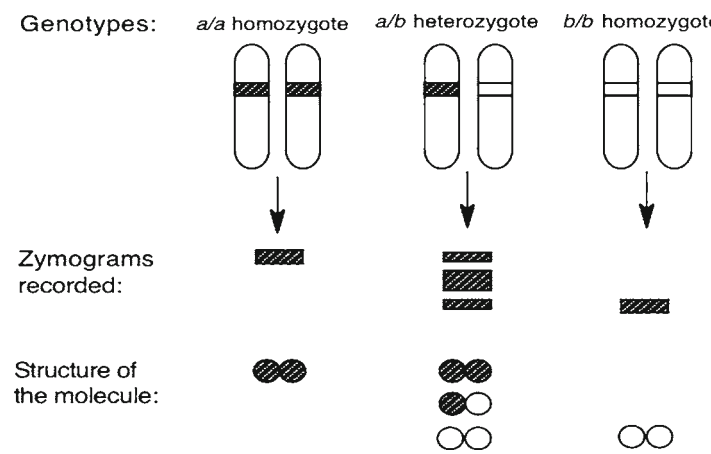

Fig. 3. Genetic background of zymograms attributable to dimeric enzymes.

GPI for glucose phosphate isomerase. When referring to the genetic locus, it becomes *gpi*. Different allozymes (alleles) of a given locus are termed *gpi* 1, *gpi* 2, *gpi* 3, and so on from the electrophoretically fastest to the slowest, respectively.

Some enzyme systems reveal the activity of two genetic loci or more.

2.14. Do Isoenzymes Undergo Natural Selection?

Despite thousands of studies in the 1970s and 1980s *(1)*, there is still no final answer to the long-debated question of whether isoenzymes undergo natural selection. The current view is that isoenzymes undergo little or no natural selection, but there are exceptions. At the very least, the selective pressure they receive is far weaker than antigenic variation or antibiotic resistance genes in pathogens.

2.15. Do Isoenzymes Show Much Homoplasy?

Whether isoenzymes show much homoplasy is an eternal criticism of the technique. Logically speaking, it is warranted; practically speaking, it is not really. *Homoplasy* refers to when the possession of an identical character does not stem from common ancestry. Reversion, convergence, and parallelism are sources of homoplasy. In the case of isoenzymes, on a given gel, there cannot be hundreds of different bands. The space is limited, and as stated, sometimes two enzymes with the same migration pattern may have different primary sequences. The risk is higher when the experiment surveys organisms that are phylogenetically quite distant. The molecular clock of the marker is not adapted to that application. In a broad sense, *molecular clock* refers to how fast the genomic part that codes for the variability of a given marker evolves. The molecular clock of the genes that code for isoenzyme variation is acceptable when comparing different genotypes of *Trypanosoma cruzi* (already its upper limit) or different species of New World *Leishmania*. It is totally inadequate when comparing African and American trypanosomes, or even worse, trypanosomes and *Plasmodium*. When surveying organisms corresponding to a suitable evolutionary scale, the impact of homoplasy is considerably lowered by ***(i)*** using isoenzyme techniques that have a fine resolution power and ***(ii)*** relying on a sufficient number of loci. With two or three loci, the impact of homoplasy is bound to be extensive. When more than 20 loci are used, the homoplasy component decreases due to the congruence principle *(1)*. Phylogenies designed from isoenzyme studies are perfectly robust and fully confirmed by newer molecular techniques. When one aims at finely dissecting specific traits, such as patterns of recombination in a given species (not only the rough rate of recombination), it is advisable to rely on more discriminatory approaches, such as multilocus sequence typing *(8)*.

3. What Good Have Isoenzyme Studies Done for Parasitological Research?

Now that the main questions on isoenzymes have been answered, let us consider the contribution of the technique to our field of parasitological research.

3.1. Strictly Speaking Parasites

The contribution of isoenzymes to parasitology has been considerable. Many pioneering studies have been conducted since the late 1970s. It is not the purpose of this chapter to provide a comprehensive review of everything accomplished in the field. Instead, I expand on a few remarkable examples.

Early on, isoenzymes were used—like many markers have been and still are used—as a mere means of identification, for typing purposes. Pioneers in the field noted that some sets of stocks of parasites shared exactly the same isoenzyme profile. These categories were called *zymodemes* ***(9–11)***. No genetic interpretation was used in this approach. However, implicitly or intuitively, the authors inferred that stocks ranked in the same zymodeme shared some common ancestry, were a convenient target for epidemiological tracing, and even could have in common some relevant medical properties, such as pathogenicity ***(12)***. Therefore, behind this empirical interpretation was the perception that zymodemes were the result of a common clonal descent. This intuitive perception of strains was very strong and is still present in recent articles with no population genetics background ***(13)***. In the late 1970s and 1980s, hundreds of papers were published on isoenzyme typing of parasites, including African trypanosomes ***(14,15)***, cattle species ***(16)***, American trypanosomes ***(17–19)***, *Leishmania* ***(20–22)***, *Toxoplasma gondii* ***(23)***, *Entamœba histolytica* ***(24)***, *Giardia intestinalis* ***(25)***, *Naegleria* ***(26)***, and *Trichomonas* ***(27)***.

This abundance of studies greatly clarified the subspecific variability of the species surveyed. As an example, the existence of three main subdivisions within *T. cruzi* was soon recognized ***(18)***. Similarly, the existence of a specific group of *Trypanosoma* brucei linked to human infection in West Africa (*T. brucei gambiense* group I) was inferred from isoenzyme characterization ***(28)***. *Leishmania* species that had first been described by epidemiological and ecological criteria were corroborated by isoenzyme analysis ***(29)***. Last, the three groups of *Toxoplasma gondii* that had been recovered by many studies were first seen with isoenzymes ***(23)***. Many other examples of the contribution of isoenzymes to parasite-subspecific and -specific taxonomy could be cited.

However, it was distressing that the descriptive and empirical interpretation used in all these studies missed the major advantage of the isoenzyme technology: its very clear genetic background. Since isoenzymes have been used to analyze virtually all living organisms, their Mendelian inheritance was perfectly

known. This made it possible to develop comparative genetics that could be used for organisms such as parasites whose formal genetics was poorly known.

A very clean phylogenetic and cladistic approach of *Leishmania* isoenzyme taxonomy by Rioux and colleagues made it possible to considerably improve our knowledge on the subspecific and specific classification of the agents of leishmanioses *(30–32)*. The existence of genetic exchange and hybridization in these parasites was first suspected from isoenzyme studies *(33)*.

Genetic interpretation of zymograms made it possible to propose the iconoclastic hypothesis that African trypanosomes have sex *(34)*. The existence of this unexpected biological trait was fully confirmed by later experiments *(35)*. However, occasional genetic exchange does not render populations panmictic (*panmixia* is the occurs when genetic exchange is random). Quantitative population genetics analyses relying on the frequency of multilocus genotypes and the estimation of linkage disequilibrium (the nonrandom association of genotypes occurring at different loci) are indispensable for forming a clear idea of the frequency of recombination since they are specifically designed for that. Isoenzyme data suit perfectly this kind of analysis since genetic variability at many different loci can be analyzed individually. This specific quantitative population genetics approach has been championed by our group. Based on the analysis of our own isoenzyme data and that of many other teams, it has been possible to show that many parasitic protozoa have a clonal population structure, with occasional bouts of genetic recombination *(36)*. The term *clonal* is used here with its population genetics meaning and refers to when multilocus genotypes propagate themselves unchanged over space and time. This can be seen not only in the case of mitotic propagation, but also in several cases of parthenogenesis, of self-fertilization, and of extreme homogamy *(37)*. Most microparasites therefore play on a double keyboard and combine both genetic recombination (not much in most cases) and clonal propagation.

It is not the place here to rekindle the clonality/sexuality debate or to write a comprehensive review of parasite population genetics. My goal is only to show, through a few examples, that the contribution of the isoenzyme technology to our knowledge on the subspecific variability, basic biology, population genetics, and phylogeny of microparasites has been considerable.

Where phylogenetic analysis is concerned, it can be seen that isozenzymes may provide very strong phylogenetic signals. **Figure 4** hows the very typical isoenzyme patterns that signal the main genetic subdivisions that have resulted from many recurrent observations in *T. cruzi (38)*. Interestingly, three of these subdivisions correspond to the three principal zymodemes identified nearly 30 years ago by the pioneering studies of Ready

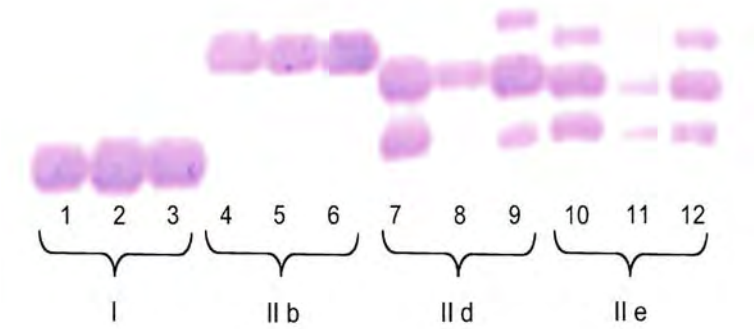

Fig. 4. Specific isoenzyme patterns of the main genetic subdivisions within the species *Trypanosoma cruzi* illustrating the strength of the phylogenetic signal of isoenzymes.

and Miles *(18)*. This is a clear demonstration of the permanency of these genotypes in space and time.

3.2. Fungi and Bacteria

While the current usage restricts the term *parasites* to pathogenic worms and protozoa, from an evolutionary and medical point of view, pathogenic fungi and bacteria are also parasites. A word should be said about isoenzyme studies dealing with these pathogens. Viruses could be considered parasites as well but are inappropriate for isoenzyme studies.

Our group has always defended the vision of a comparative population genetics approach between different kinds of pathogenic microorganisms. This has been severely attacked based on the reasoning that protozoa, fungi, and bacteria are totally different organisms. However, they all have isoenzymes, and health professionals and biochemical researchers working on them all need to type their strains. Comparisons are therefore not only legitimate but also extremely informative *(37)*.

To some extent, isoenzyme research on pathogenic fungi and its results show strong similarities with those seen in pathogenic protozoa. Natural populations of these pathogens show a wide array of population structures with different relative impacts of recombination and uniparental propagation. Many of these results have been attained by isoenzyme analysis (for a comprehensive review, *see* **ref. 39**).

Where bacteria are concerned, a wealth of isoenzyme studies have been generated since the pioneering studies of Milkman *(40)* and Selander and Levin *(41)*, and many papers have been published on almost all pathogenic bacterial species. Isoenzyme analyses provided for the first time a very clear idea of the population structure of bacteria, and more sophisticated approaches

were able to fully confirm the main results. The clonal paradigm *(42)*, which seemed to be confirmed by many isoenzyme studies *(41,43)*, has been challenged for some species by studies relying on the same technique *(44–46)*.

Population genetic studies on pathogens are not only a matter of basic research. Although they provide precious insights into the basic biology of microbes, they also have strong implications for applied research. Molecular epidemiology, the very topic of this book, mainly relies on the characterization of multilocus genotypes. If frequent genetic recombination renders these multilocus genotypes unstable, strain characterization is impossible (*see* **Chapter 1**). Within this perspective, pathogenic protozoa, fungi, and bacteria raise exactly the same type of problem, and as we have seen, they show striking similarities in their patterns of population structure. Much information has been gathered through isoenzyme analyses, although more recent techniques (microsatellites, multilocus sequence typing) now make it possible to explore these problems in greater depth.

4. Present and Future of Isoenzyme Analysis in Parasitology and Medical Microbiology

There is no doubt that the glorious time of isoenzymes is behind us. I would not give a student a research program based exclusively or mainly on this technique. Molecular DNA approaches, especially those that rely on PCR amplification, provide outstanding advantages. The future of molecular epidemiology rests more on population genomics and pathogen profiling (*see* **Chapter 1**) than on isoenzyme analysis. However, the advantages of the method that made it the gold standard of population genetics for two decades have not vanished with the advent of more recent techniques; it is still a marker with perfectly known Mendelian inheritance, which permits multilocus analysis and is applicable to most living organisms. In addition, it is relatively inexpensive.

For population genetics and phylogenetic studies, using two very different kinds of genetic markers is invaluable since they will explore different parts of the genome, having different evolutionary patterns. According to the congruence principle *(1)*, as was emphasized at the start of this chapter, a line of results is strongly supported when it is corroborated by several different approaches. Specific population genetic tests have been developed that rely on this dual vision. It is therefore perfectly convenient to corroborate a study based on, say, microsatellites, by good old isoenzyme analysis.

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Chapter 3

Plasmid Replicon Typing

Timothy J. Johnson and Lisa K. Nolan

Abstract

To facilitate the study of plasmids and their roles in human and animal health, environmental processes, and microbial adaptation and evolution, plasmid classification has been an important focus of plasmid biologists over the years. Initial schemes were based on the ability of a plasmid to inhibit F fertility, but due to certain limitations, these methods were superseded by incompatibility or Inc typing. Inc typing classifies plasmids by their ability to stably coexist with other plasmids in the same bacterial strain, a trait that is dependent on their replication machinery. Coresident plasmids are incompatible when they share the same replication mechanisms. Since plasmid replicon type determines Inc group, the terms *Inc* and *Rep* type to describe plasmid types are used interchangeably. Initially, Inc typing relied on introduction of a plasmid into a strain carrying another plasmid and determining whether both plasmids were stably maintained in the progeny. However, physical Inc typing is time consuming and not easily used in large-scale applications. Some of these shortcomings were addressed through development of a classification scheme based on identification of basic replicons using DNA hybridization and of a polymerase chain reaction (PCR)-based method of replicon typing enabling plasmid typing on a large scale. Here, we elaborate on a recently described PCR-based typing method that streamlines the typing of plasmids occurring among the Enterobacteriaceae; we believe the method will prove applicable to the study of plasmids on a large scale.

Key words: Inc typing, plasmid typing, plasmids, replicon typing.

1. Introduction

Plasmids are self-replicating, extrachromosomal units of DNA that encode nonessential but often valuable traits for their host bacterium *(1)*. Plasmids are a type of mobile genetic element (MGE), and as such, they are important agents of horizontal gene transfer (HGT). HGT of plasmids and other MGEs comprises an important source of genetic information for their bacterial

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hosts. In an avian pathogenic *Escherichia coli* (APEC) strain from which the genome was recently sequenced, it was shown that about 10% of the total genetic information was contained within four plasmids *(2)*. The traits encoded by these four plasmids included virulence factors, fitness factors, and genes encoding resistance toward antibiotics, disinfectants, and heavy metals *(3,4)*. In addition, these plasmids contained genes encoding for their dissemination and stability *(3,4)*. In general, such a pool of mobile genes (the mobilome) likely plays a crucial role in microbial evolution, providing bacteria a means to compensate for their lack of sexual reproduction, the major mechanism of genetic innovation in higher organisms *(5)*. Acquisition of such "ready-made" genes on plasmids and other MGEs enables the host bacterium to respond quickly to environmental changes, such as introduction of disinfectants and antibiotics. This would not be the case if bacterial fitness were solely reliant on *de novo* evolution *(5)*.

Plasmids contain genes necessary for initiation and control of replication and include accessory genes that encode a wide variety of phenotypes that help their bacterial hosts exploit and adapt to their environments *(6,7)*. These traits are considered accessory functions and include antibiotic and heavy metal resistance, metabolic properties, and pathogenicity. Such phenotypes have important implications for human and animal health, environmental processes, and microbial adaptation and evolution. In recognition of the importance of plasmids in these processes and to facilitate their study, plasmid classification has been an important focus of plasmid biologists over the years. Initial schemes were based on the ability of a plasmid to inhibit F fertility *(8)*, but due to certain limitations, these methods were superseded by incompatibility or Inc typing in the 1970s *(8)*. *Incompatibility* typing classifies plasmids by their ability to stably coexist with other plasmids in the same bacterial strain. Incompatibility is defined as the inability of two plasmids to be stably inherited in the absence of external selection *(1)*. Plasmids that are incompatible with one another are assigned to the same incompatibility or Inc group, while those that can exist together generally belong to different incompatibility groups. Coresident plasmids are defined as incompatible when they share the same replication mechanisms. Since plasmid replicon type determines Inc group, the terms *Inc* and *Rep* type to describe plasmid types are used interchangeably *(1)*.

Initially, Inc typing relied on introduction of a plasmid into a strain carrying another plasmid and determining whether both plasmids were stably maintained in the progeny *(1)*. Unfortunately, physical Inc typing proved a time-consuming task that was fraught with shortcomings. Couturier *et al.* addressed some of these shortcomings through development of a classification scheme based on identification of basic replicons using DNA:DNA hybridization *(9)*. Sobecky *et al.* showed that this method could

be applied to identify plasmid replicon types among isolates from complex marine microbial communities *(10)*. Similarly, Mainil *et al.* demonstrated that this technique could be used to identify replicon types among the virulence plasmids of enterotoxigenic *E. coli (11)*. However, the use of this technique was still extremely laborious, making typing of plasmids in large bacterial populations problematic. The advent of the polymerase chain reaction (PCR) made new time-saving methods of plasmid typing possible. Carattoli *et al.*'s PCR-based method of replicon typing enabled plasmid typing on a large scale *(12)*. This method employs five multiplex and three simplex PCRs to recognize the major plasmid Inc groups occurring among the Enterobacteriaceae. The utility of this method for epidemiological studies was demonstrated with study of the plasmids encoding resistance to the newer β-lactam antibiotics among *Salmonella* and *E. coli (13,14)*. Recently, we have simplified this replicon typing scheme to efficiently detect the presence of 18 replicon types occurring among the Enterobacteriaceae *(15)*.

The original procedure used by Carattoli *et al.* involved five different multiplex panels each recognizing three replicon types and three simplex PCR reactions for the F, K, and B/O replicon types *(14)*. We have made modifications to this procedure to make it more cost-effective and faster for screening large bacterial populations. First, instead of using a genomic purification kit to prepare template DNA, we used boiled lysates as a source of template DNA, as described by Johnson and Stell *(16)*. This technique is a fast, inexpensive method for producing total DNA template suitable for PCR. The second modification was to reduce the total number of PCR panels used in the assay. **Table 1** lists the primers used within each panel, the target of each primer pair, and the expected amplicon sizes. This protocol screens for 17 gene products.

2. Materials

2.1. Cell Culture and Control Strains

1. MacConkey's agar plates (BD Diagnostic Systems).
2. Luria broth (LB) (BD Diagnostic Systems).
3. Control strains: The original control strains used for this procedure are available on request from Alessandra Carattoli, Istituto Superiore di Sanità, Rome, Italy *(14)*. These controls were created by cloning the PCR product of each replicon type amplified into the pULB plasmid vector. The targets for each plasmid replicon include replication genes, *ori* sites, iteron sequences, and plasmid-partitioning genes specific for

Table 1
Primers and Controls Used in This Procedure

Panel	Inc type	Target	Amplicon size		Primer sequence
1	B/O	RNAI	159	F R	GCGGTCCGGAAAGCCAGAAAAC TCTGCGTTCCGCCAAGTTCGA
1	FIC	*repA2*	262	F R	GTGAACTGGCAGATGAGGAAGG TTCTCCTCGTCGCCAAACTAGAT
1	A/C	*repA*	465	F R	GAGAACCAAAGACAAAGACCTGGA ACGACAAACCTGAATTGCCTCCTT
1	P-1 alpha	Iterons	534	F R	CTATGGCCCTGCAAACGCGCCAGAAA TCACGCGCCAGGGCGCAGCC
1	T	*repA*	750	F R	TTGGCCTGTTTGTGCCTAAACCAT CGTTGATTACACTTAGCTTTGGAC
2	K/B	RNAI	160	F R	GCGGTCCGGAAAGCCAGAAAAC TCTTTCACGAGCCCGCCAAA
2	W	*repA*	242	F R	CCTAAGAACAACAAAGCCCCCG GGTGCGCGGCATAGAACCGT
2	FIIA	*repA*	270	F R	CTGTCGTAAGCTGATGGC CTCTGCCACAAACTTCAGC
2	FIA	Iterons	462	F R	CCATGCTGGTTCTAGAGAAGGTG GTATATCCTTACTGGCTTCCGCAG
2	FIB	*repA*	702	F R	GGAGTTCTGACACACGATTTTCTG CTCCCGTCGCTTCAGGGCATT
2	Y	*repA*	765	F R	AATTCAAACAACACTGTGCAGCCTG GCGAGAATGGACGATTACAAAACTTT
3	I1	RNAI	139	F R	CGAAAGCCGGACGGCAGAA TCGTCGTTCCGCCAAGTTCGT
3	F	RNAI/*repA*	270	F R	TGATCGTTTAAGGAATTTTG GAAGATCAGTCACACCATCC
3	X	*ori*	376	F R	AACCTTAGAGGCTATTTAAGTTGCTGAT TGAGAGTCAATTTTTATCTCATGTTTTAGC
3	HI1	*parA-parB*	471	F R	GGAGCGATGGATTACTTCAGTAC TGCCGTTTCACCTCGTGAGTA
3	N	*repA*	559	F R	GTCTAACGAGCTTACCGAAG GTTTCAACTCTGCCAAGTTC
3	HI2	Iterons	644	F R	TTTCTCCTGAGTCACCTGTTAACAC GGCTCACTACCGTTGTCATCCT
3	L/M	*repABC*	785	F R	GGATGAAAACTATCAGCATCTGAAG CTGCAGGGGCGATTCTTTAGG

See ding IncP primers.

that particular replicon (**Table 1**). In our protocol, we also use a set of *E. coli* control strains that harbors wild-type plasmids in their natural state. Because most of these plasmids occur in low copy, we have found that stronger products are often observed for the cloned pULB controls as compared to the wild-type controls. Therefore, we include wild-type controls to ensure that our sensitivity is high enough to detect naturally occurring low-copy plasmids. We make these wild-type controls available on request.

4. 1.5-mL centrifuge tubes (Fisher Scientific).

2.2. Polymerase Chain Reaction

1. Amplitaq Gold Taq polymerase (Applied Biosystems).
2. dNTP (deoxynucleotide 5′-triphosphate) mix (USB Corp.). The dNTP mix, purchased at a concentration of 10 m*M*, is diluted 1:4 in nanopure water to give a working concentration of 2.5 m*M* and is aliquoted into 1.5-mL microcentrifuge tubes in volumes of no more than 400 μL to avoid repetitive freeze-thaws.
3. Primers (IDT Technologies, Coralville, IA): Oligonucleotides are purchased at the 25 nmol amount with standard desalt. When received, the primers are resuspended to a concentration of 0.1 m*M*. For each PCR panel (*see* **Table 1**), equal amounts of each primer are combined into a pooled primer tube. For example, we typically combine 50 μL of each resuspended primer in the panel.
4. PCR buffer (Applied Biosystems).
5. $MgCl_2$ (Fisher Scientific).
6. PCR tubes (Fisher Scientific).
   Amplitaq Gold Taq, dNTP mix, IDT Technologies primers, PCR buffer are listed because we wish to provide exact details regarding the optimization of this protocol. This is not an endorsement for these products. Other products could be substituted in their place but may require further optimization of the procedure.

2.3. Agarose Gel Electrophoresis of PCR Products

1. SeaKem Agarose (Lonza Bioscience).
2. Tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer (Fisher Scientific).
3. Loading buffer (40% glycerol, 0.4% bromophenol blue in distilled water).
4. Ethidium bromide (Sigma Aldrich Corp.).
5. DNA molecular weight marker (Minnesota Molecular Inc.).

3. Methods

3.1. Day 1

Streak out the pULB control strains, the wild-type strains, and any experimental strains to be tested on MacConkey's agar. Incubate overnight at 37°C.

3.2. Day 2

1. Prepare 1.5-mL centrifuge tubes for each strain containing 1 mL of LB broth.
2. Autoclave the tubes and allow them to cool to room temperature.
3. Inoculate a single colony of each streaked strain into each of the tubes.
4. Incubate overnight with moderate shaking at 37 °C.

3.3. Day 3

Prepare template DNA from the LB cultures using the boiled lysate method *(16)*.

3.3.1. Preparation of Template DNA

1. Preheat a dry heat block to 100 °C.
2. Centrifuge the LB cultures at 12,000*g* for 1 min.
3. Pour off the supernatant and blot the tubes on a dry paper towel.
4. Resuspend the bacterial pellets in 200 µL of nanopure water.
5. Incubate the tubes at 100°C for 10 min.
6. Centrifuge the tubes at 12,000*g* for 1 min.
7. Withdraw the supernatant from each tube and place in a clean 1.5-mL microcentrifuge tube. This is your template DNA to be used in the PCR reaction.

3.3.2. Polymerase Chain Reaction

1. Thaw boiled lysate template, 10X PCR reaction buffer, 2.5 m*M* dNTP mix, primer pools, and nanopure water on ice.
2. Add 2 µL of each boiled lysate template to appropriate 0.2-mL PCR tube.
3. Assemble master mix as follows (volumes are given per reaction):
   2.5 µL 10X PCR buffer
   2.0 µL dNTP mix
   4.0 µL $MgCl_2$
   1.0 µL primer pool
   0.25 µL Taq polymerase
   13.25 µL nanopure water
4. Mix well and add 23 µL of master mix to each reaction tube (hot start not necessary; *see* **Note 1**).
5. Subject tubes to the following cycling conditions in a thermal cycler:
   Step 1. 94°C for 5 min

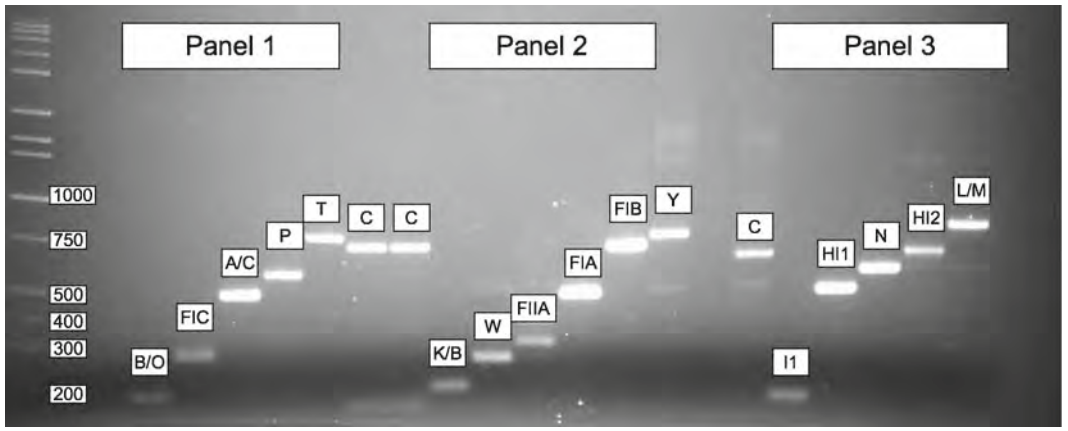

Fig. 1. Agarose gel electrophoresis of control strains for plasmid replicon typing procedure. *C* wild-type control strains. First C depicts *E. coli* strain APEC O1, harboring the FIB replicon in panel 2. Second C depicts *E. coli* strain APEC O2, also harboring the FIB replicon. Third C again depicts APEC O1, harboring the HI2 replicon in panel 3. Note that the F and X amplicons are not included in this image. The use of these primers is optional. The X replicon is extremely rare among Enterobacteriaceae, and the F replicon is an additional option for further confirmation of the presence of an F-type plasmid.

Step 2. 94°C for 30 s

Step 3. 60°C for 30 s

Step 4. 72°C for 90 s

Step 5. Steps 2–4 are repeated 29 times

Step 6. 72 °C for 5 min

3.3.3. Electrophoresis

1. Run 12 μL of each reaction on a 1.5% (w/v) agarose gel to visualize products.
2. Interpret the results by comparing the known controls (**Fig. 1**) to the experimental strains (*see* **Notes 2** and **3** regarding assay specificity and multiple products). It is good to run a linear DNA marker that has bands ranging from at least 50 to 1,000 bp. There should be at least one band per 100 bp on the low end of the marker.

4. Notes

1. In our experience, a hot start PCR is not necessary for this procedure. With our reagents on ice, we assembled the PCR reaction at room temperature without any adverse effects. This procedure was optimized using AmpliTaq Gold DNA polymerase. While other Taq polymerases could be used, their use may require some optimization.

2. This protocol was designed to err on the side of caution *(14,15)*. That is, the primers used here are more likely to miss a product than they are to detect a false-positive reaction. Because plasmids have a high degree of plasticity, this assay is not perfect. Compounding the complexities of plasmid plasticity is the general shortage of whole-plasmid sequences available for analysis. Very recently, several plasmid genome-sequencing projects have increased the number of replicon sequences available for comparative analyses (http://ecoli.cvm.iastate.edu and http://www.sanger.ac.uk). A comparison of the primer sequences in this protocol with those newly available plasmid sequences revealed that the protocol accurately detects most of the sequenced plasmids from each known incompatibility group. However, it is also evident from these analyses that some plasmids may not be detected. Future efforts will need to focus on the further development of this technique to all plasmid variants. At present, though, this protocol is a fast, inexpensive method for determination of most of the plasmid replicon types occurring among large populations of members of Enterobacteriaceae.
3. In our experience, double bands or bands of unexpected size can sometimes occur with the FIIA primers. Also, faint high molecular weight bands are sometimes observed inexplicably. However, these bands do not appear to have an effect on the assay as they have been observed in our sequenced wild-type controls and have not affected the expected products. As mentioned, some replicon types are highly variable. As a result, an occasional false negative could occur due to this variability. The replicons that have a high degree of variability include FIIA, L/M, FIA, and FIC.
4. IncP plasmid replicon: It is important to note that the primers in this procedure only detect the IncP-1 α plasmid replicon type. While the multiplex panels do not detect the IncP-1 β replicon type, other PCR-based protocols exist for its detection *(17)*.

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Chapter 4

The Application of Randomly Amplified DNA Analysis in the Molecular Epidemiology of Microorganisms

Alex van Belkum, Elisabeth van Pelt-Verkuil, and John P. Hays

Abstract

The polymerase chain reaction (PCR) has essentially been designed to amplify specific regions within DNA molecules. This requires knowledge of the local nucleic acid sequence to design primer oligonucleotides. However, to generate DNA fingerprints, the PCR can be modified in a way that facilitates the random amplification of elements for which the precise nucleotide sequence is not known. When DNA is subjected to PCR at relatively low annealing temperatures while using relative short DNA primers of non-specific sequence, amplification is often targeted towards a larger number of domains within the template. Post-PCR analysis of these fragments, usually using electrophoretic technologies, results in strain-specific fingerprints due to small differences in primer annealing sites or the selective presence or absence of certain DNA domains among strains. These procedures are collectively called random amplification of polymorphic DNA (RAPD) analyses and have been very useful in high-speed, high-throughput screening for DNA variation among strains of a wide variety of microbial species and isolates within these species. This chapter describes the basic features of this technology, including an experimental protocol that can essentially be applied to DNA from all species of microorganisms.

Key words: Bacterial typing, DNA fingerprints, electrophoresis, PCR, RAPD.

1. Introduction

The polymerase chain reaction (PCR) allows the specific and exponential synthesis of a predetermined DNA region via the use of two short synthetic oligonucleotides. These primers form the termini of the nucleic acid molecule to be amplified. PCR amplifications are highly specific, specificity being determined by the correct hybridisation of primers to complementary sequences present on the target DNA molecule to be amplified. Since primers used for diagnostic purposes need to be precisely complementary to their target sequences, a certain amount of sequence

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data is required for adequate primer design. Once hybridised to the target DNA, the primers provide the double stranded 3′-hydroxyl terminus required by thermostable DNA-dependent DNA polymerases to initiate the synthesis of a new DNA strand. Moreover, because PCR uses two primers, repeated cycles of primer hybridisation (annealing) and disassociation allows DNA amplification in the 5′-to-3′ direction on both strands to occur, with the primers effectively acting as Okazaki fragments. PCR amplification is a cyclic process, and the DNA is initially denatured by heating in an aqueous environment. Hybridisation of the specific oligonucleotide primers is then achieved by lowering the temperature to the annealing temperature (T_m). After the primer hybridisation step, the temperature is raised to an optimal temperature for thermostable DNA polymerase-mediated DNA strand replication. This series of events is repeated a number of times. PCR principles have been detailed in a large body of international scientific literature ***(1,2)***.

Inter-repeat PCR (also called Rep-PCR) is based on the fact that repetitive DNA sequences may occur as randomly scattered motifs within individual genomes, and that the intervening distances between these sequences in different isolates or individuals may also vary. In this case, PCR amplification from one particular repeat region to another repeat region gives rise to variable fragment lengths and therefore variable genetic fingerprints within different isolates or individuals due to inter-repeat region length variation ***(3)***. It should be noted that the presence and position of the repeat region does not have to be known prior to PCR. To be successful, however, the repeated motifs do need to be present in opposing orientations (i.e. facing in towards each other) so that a single PCR primer can hybridize to both individual repeat sequences and generate a PCR amplimer. In addition, the distance between annealing sites (i.e. repeat units) should be sufficiently short for the DNA polymerase to be able to span the distance. This process is generally referred to as random amplification of polymorphic DNA (RAPD) or arbitrary primed (AP)-PCR. Such RAPD and AP-PCR protocols are usually performed with the help of relatively short primers (approximately ten nucleotides in length), which increases the chance of the primer "finding" and hybridizing to an opposing repeat sequence at a reasonable distance between the annealing sites. To further increase the probability of primer hybridisation, the annealing temperature used in these protocols is kept low (between 25°C and 42°C) so that stretches of DNA that are not 100% identical may also act as sites for primer hybridisation.

RAPD, AP-PCR and indeed inter-repeat PCR protocols generate complex mixtures of amplimers (and hence DNA banding patterns or fingerprints after gel electrophoresis), which need to be adequately separated for successful comparative analysis. In most cases, straightforward agarose gel electrophoresis is

sufficient to generate band pattern profiles (often referred to as *DNA fingerprints*). Densitometric scanning of the fingerprints may facilitate the automated interpretation of the sometimes-dense arrays of DNA fragments. One particular problem encountered using RAPD and AP-PCR protocols is that the reproducibility of the PCR using a particular primer sequence and a specific DNA extract may be variable, and that selection of a suitable primer may be a complicated process involving multiple rounds of trial and error. However, these PCR protocols are convenient, rapid and flexible in the production of DNA fingerprints for genetic identification ***(4)***.

PCR-mediated DNA fingerprinting protocols have been mainly used in epidemiological studies, to determine the evolutionary relatedness of different species (in both the eukaryotic and prokaryotic kingdoms), and to determine genetic polymorphisms between different individuals (paternity testing, forensic examinations, etc.) and may be readily adapted and applied to high-throughput screening strategies. RAPD analysis was extremely popular in the 1990s. However, due to its relatively low degree of interinstitutional reproducibility the popularity of the method waned. To date, RAPD is primarily used for "quick-and-dirty" assessment of bacterial interisolate relatedness, frequently within the framework of nosocomial infection control. This chapter describes the technological intricacies of RAPD and provides some troubleshooting guidelines.

2. Materials

2.1. Bacterial Cell Culture

1. Columbia blood agar plates (bioMérieux, Boxtel, The Netherlands).
2. Alternately, brain heart infusion (BHI) broth: 7.7 g calf brain, 9.8 g beef heart, 10.0 g proteose peptone, 2.0 g dextrose, 5.0 g NaCl and 2.5 g disodium hydrogen phosphate per litre. The pH of the broth should be set at 7.4, and all ingredients can be purchased at Difco Laboratories (Detroit, MI). The broth should be autoclaved at 121°C for 20 min and can subsequently be stored at 4°C. Broth is usually aliquoted into culture tubes or bottles and can be stored for up to 4 wk without an apparent loss in quality.

2.2. Bacterial Lysis and DNA Isolation

1. 10 m*M* Tris-HCl buffer at pH 8.0 containing 10 μg/mL of lysozyme (Sigma, Amsterdam, The Netherlands) for gram-negative bacteria.
2. 10 m*M* Tris-HCl buffer at pH 8.0 containing 10 μg/mL of lysostaphin (Sigma) for gram-positive bacteria.

3. Lysis buffer L6: Dissolve 120 g of guanidine thiocyanate (GuSCN) (Fluka Chemie, Buchs, Switzerland) in 100 mL of 0.1*M* Tris-HCl, pH 6.4. Subsequently, add 22 mL of 0.2*M* ethylenediaminetetraacetic acid (EDTA), pH 8.0 and 2.6 g of Triton X-100 (Packard-Bell, Downers Grove, CA). Homogenise the solution by extensive vortexing and store in the dark at room temperature.
4. Washing buffer L2: Dissolve 120 g of GuSCN (Fluka Chemie) in 100 mL of 0.1*M* Tris-HCl, pH 6.4. Homogenise the solution by extensive vortexing and store in the dark at room temperature. Both buffer L6 and L2 can be stored for at least 3 mo without any drop in quality.
5. 70% ethanol can be made by diluting pure ethanol with double-distilled water in a ratio of 7:3.
6. Elution buffer: 10 m*M* Tris-HCl, pH 8.0. This buffer can be autoclaved for 20 min at 121°C.
7. Diatom suspension for DNA affinity capture: Add 10 g of high-purity analytical-grade Celite (Janssen Chimica, Beerse, Belgium) to 50 mL water to which 500 µL of 32% concentrated HCl is added.

2.3. Polymerase Chain Reaction

1. DNA template: Every individual reaction mixture should contain between 10 and 50 ng of DNA (*see* **Subheading 3.2**.). DNA samples should be thawed shortly prior to amplification, and DNA is usually added in the final step of the PCR sample preparation.
2. PCR buffer: 10 m*M* Tris-HCl, pH 9.0, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.01% (w/v) gelatin, 0.1% (w/v) Triton X-100. This is added to a sample master mix as a 10X concentrated solution. Some of the reactions may require additional or less $MgCl_2$; working concentrations range between 1.5 and 4.0 m*M*.
3. Nucleotide triphosphates (Fermentas, Amsterdam, The Netherlands). These are added at a final concentration of 0.2 m*M* by using fixed amounts of a 2 m*M* stock solution.
4. A single species of primer is added at a final concentration of 0.5–1.0 pmol/µL. Primers are stored at –20°C at a concentration of 50 pmol/µL. Several primer species have been used successfully in the past. These include ERIC1 (5′-ATG TAA GCT CCT GGG GAT TCA-3′), ERIC2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′), AP1 (5′-GGT TGG GTG AGA ATT GCA CG-3′), AP7 (5′-GTG GAT GCG-3′), RAPD10730 (5′-GGC CAT AGA GTG TTG CAG ACA AAC TGC-3′), RAPD1247 (5′-AAG AGC CCG T-3′), RAPD1254 (5′-CCG CAG CCA A-3′), RAPD1281 (5′-AAC GCG CAA C-3′) and RAPD1283 (5′-GCG ATC CCC A-3′). Primers can be synthesised at Eurogentec (Seraing, Belgium), although many other specialised companies can be addressed.

5. Thermostable Tth DNA polymerase (SuperTaq, HT Biotechnology, Cambridge, UK); usually 0.2 units per PCR suffices. The final volume of the sample to be amplified usually is 50 μL.

2.4. Agarose and Polyacrylamide Gel Electrophoresis

1. Layer mix: 2.5 mg/mL bromophenol blue (Merck, Utrecht, The Netherlands), 50% (w/v) glycerol. This is added to the amplified samples.
2. Agarose slab gels: 0.8–3% (w/v) agarose, containing 1 μg/mL ethidium bromide.
3. Agarose electrophoresis buffer system: 40 m*M* Tris–HCl, 20 m*M* sodium acetate, 2 m*M* sodium EDTA. The pH is set at 7.7 using acetic acid. Alternatively, gels may also be run in Tris-borate-EDTA, pH 7.4, buffer systems.
4. Sodium dodecyl sulphate (SDS) polyacrylamide gel buffers. The separation buffer contains 0.3*M* Tris-HCl, 0.4% (w/v) SDS, pH 8.5. The stacking buffer consists of 0.1*M* Tris-HCl, 0.4% (w/v) SDS, pH 6.8. Both buffers can be stored at room temperature.
5. Polyacrylamide gels: 30% acrylamide/bisacrylamide solutions (37.5:1), *N,N,N,N*′-tetramethyl-ethylenediamine (TEMED, Bio-Rad, Veenendaal, The Netherlands) and 10% ammonium persulphate (APS) in water are required for gel preparation. TEMED is best stored in the dark. The APS needs to be aliquoted and stored at –20°C.
6. SDS polyacrylamide gel running buffer: 25 m*M* Tris–HCl, 180 m*M* glycine, 0.5% (w/v) SDS. This buffer can be stored at room temperature.
7. Ethidium bromide: 10 mg/mL stock solution. This is used for gel staining at a working concentration of 1 μg/mL. Ethidium bromide is a potent mutagen and should be handled with appropriate care.

3. Methods

3.1. Bacterial Cell Culture

1. Plate bacteria, either from purified primary cultures or from archival, frozen stocks, onto Columbia blood agar plates. Single colonies need to be available after growth, so cell suspensions need to be either diluted or streaked to single-colony quadrants.
2. Using a 10-μL loop, collect single colonies and inoculate in 1–5 mL BHI broth.
3. After overnight incubation at 37°C, harvest the cells and subject to DNA isolation protocols.

3.2. DNA Isolation Procedures

1. Gram-positive bacteria need to be pretreated with enzymes that digest the bacterial peptidoglycan within the cell wall. The most frequently used enzyme is lysostaphin. When applied for 60 min at 37°C in simple Tris-EDTA buffer, a concentration of 100 ng/mL suffices for complete spheroplast conversion of the numbers of cells mentioned. Spheroplasts are the gram-positive starting cells for DNA isolation process. The reaction can be performed in a volume of 200 µL in Eppendorf tubes. Gram negatives are pretreated for 30 min at 37°C in lysozyme-containing buffer and can be introduced in the protocol described below immediately afterwards.
2. Cells are physically disintegrated by the addition of 400 µL of lysis buffer L6 to the enzyme pre-treated cells. This process is essentially instantaneous, but an incubation of 10 min at room temperature with regular mixing certainly does the job.
3. DNA is subsequently immobilised on silica particles (10 µL of the Celite suspension to be added to the 600 µL lysis mix). Wash using centrifugation and perform the following resuspension steps: twice in 400 µL lysis buffer L6, twice in 400 µL lysis buffer L2, once in 1 mL 70% ethanol, and finally in 1 mL pure acetone. After remnants of acetone have evaporated (10 min at room temperature), the DNA can be liberated from the solid Celite matrix by incubation for 15 min in 100 µL of TE buffer at 56°C. Care needs to be taken that no Celite is included in the final supernatant that contains the DNA. This might lead to recapturing of the DNA to the solid matrix. The supernatant containing the DNA is separated from the Celite by centrifugation (*see* **Note 1**).
4. The DNA concentration is determined by spectrophotometry at 260 nm. After adjusting the DNA concentration to 5 ng/µL, the DNA is stored at –20°C. The stored solution is ready for use in RAPD tests.

3.3. The PCR Thermocycling Regime

1. Preparation of master mix. This mix contains all of the ingredients required for amplification except for the DNA. If the number of DNA samples is n, then the mix contains the following amounts of reagent: $(n + 1) \times 5$ µL 10X concentrated PCR buffer, $(n + 1) \times 5$ µL primer stock, $(n + 1) \times 5$ µL 2 mM dNTPs and $(n + 1) \times 25$ µL water. To this mix $(n + 1) \times 0.15$ units Tth polymerase is added. The volume of the amount of enzyme can be ignored in the overall volume calculation.
2. Template addition: Add 5 µL of each DNA sample to 45 µL of the master mix (*see* **Note 2**).
3. The PCR mixtures are overlaid with 50 µL of mineral oil, and cycling is performed in BioMed PCR machines (BioMed Model 60, Theres, Germany) (*see* **Note 3**).

4. Cycling conditions consist of the following steps: Predenaturation of the DNA for 5 min at 95°C; denaturation of the DNA at 95°C for 1 min; primer annealing at temperatures between 25 and 35°C for periods between 1 and 3 min; extension at 72°C for 1 min. The last three steps are usually repeated for 35–45 times. This is followed by a final extension step for 5 min at 72°C to synthesise all amplified fragments to complete double-stranded hybrids.
5. Immediate post-PCR storage of the amplified material is preferable at 4°C.

3.4. Agarose Gel Electrophoresis

1. Prepare an agarose suspension in water and heat it up to at least 65°C (usually the suspension is heated to boiling point using microwave irradiation) until the agarose is dissolved. Allow the agarose solution to cool 40°C. Then, pour the solution into the casting frame with preset sample slot formers, where it will solidify to an opalescent solid mass. The average agarose gel used for molecular biology purposes contains between 0.8 and 4% agarose (1% = 10 g/L). A 1–2% matrix allows the efficient length-based separation of PCR amplimers of sizes between 100 bp and 10 kb in length (*see* **Note 4**).
2. Agarose gels may be cast in various formats, although major differences in handling are required for horizontal versus vertical gel systems. Gels that are approximately 5 mm thick need to be run at a constant current of 100 mA for at least 2 h. The nature of agarose matrices does not allow the separation of molecules less than approximately 15 nucleotides in length. The type of electrophoresis buffer used may also affect the resolution of DNA separation (*see* **Note 5**).
3. To assist in amplimer size determination, molecular weight markers are usually added to an empty well in the gel. A wide range of these molecular weight markers is available for purchase commercially, and some gels may be purchased with the molecular weight markers already included.
4. Examine the banding patterns on UV transillumination of the ethidium bromide-stained gel. A UV transmission device (Vilber Loumat, Paris, France) coupled to a CCD (charge-coupled device) video camera with online video screen (Fotodyne, Rotterdam, The Netherlands) is sufficient equipment. Data can be stored electronically and on paper using a video thermoprinter (Mitsubishi, Amsterdam, The Netherlands).

3.5. Polyacrylamide Gel Electrophoresis

1. Polymerisation is initiated by the addition of TEMED in combination with the radical-supplying APS to the appropriate amounts of the monomeric acrylamide solutions (depending on gel dimensions and the percentage of acrylamide required). The density of the network is determined by the amount of

acrylamide/bisacrylamide added, with the gel taking approximately 60 min to achieve complete polymerisation. Polyacrylamide gels with a very dense cross-linked structure (achieved using elevated acrylamide concentrations), may be required to separate short DNA fragments that differ in size by only a single nucleotide. Appropriate safety measures should be taken when making polyacrylamide gels and handling acrylamide in particular as acrylamide is a potent neurotoxin. Laboratory coats, safety glasses, gloves and mouth protection should be worn during all manipulations with this chemical.

2. On complete assembly of the gel electrophoresis unit, rinse the slots suited for sample application with electrophoresis buffer and load the samples. The gel can be run overnight (usually at low voltages of 5 V/cm) or during working hours at higher voltage. Dye fronts serve as markers for electrophoresis duration. The optimal time span or migration distance depends on the complexity of the samples and should be determined empirically.
3. Stain the gels as described for the agarose gels (*see* **Note 6**).

3.6. Data Collection and Interpretation

1. DNA bands can be captured using CCD cameras. Banding patterns can be collated in computer programs suited for fingerprint comparison and management.
2. BioNumerics (Applied Maths, St. Martens Latem, Belgium) is a popular tool for fingerprint comparison and quantifying fingerprints and hence analysing strain differences. Bands are selected manually, and for subsequent calculations band position tolerance is usually set at 1–4% (*see* **Note 7**).

4. Notes

1. Many different nucleic acid isolation procedures, both manual and automated, have been described in the past. Choice of a procedure is often dependent on local availability of expertise, automated DNA extraction machines and, last but not least, funding. One of the most important protocols is the one published by Boom and coworkers *(5,6)*. The method described in this protocol is very widely employed (the initial publication has already been cited nearly 2,000 times since its public appearance), and many of the automated DNA isolation robots (e.g., MagnaPure by Roche and EasyMag by bioMérieux) use the same chemistry. The method uses chaotropic salts to disintegrate cells and affinity capture of the DNA on a solid silica matrix. GuSCN is a core component in the protocol.

However, on contact with acids, GuSCN reacts into HCN, which is a highly toxic gas. The GuSCN containing buffers need to be purified prior to use. The materials used to prepare these buffers may be contaminated with DNA, which should be removed by preincubation of the buffers with adequate amounts of Celite. Dry columns and glassware should be autoclaved or heat treated at higher temperatures to physically disintegrate contaminating DNA. However, since for RAPD approaches relatively large amounts of template DNA are included in the PCR, DNA contamination from materials and reagents is usually not a major problem. Washing of the silica particles during the DNA isolation protocol is very important since remnants of GuSCN will completely inhibit PCR amplification.

2. The composition of the PCR mix may vary significantly depending on the nature (and quality) of the heat-resistant thermostable DNA-dependent DNA polymerase used. When SuperTaq is used, the amplification conditions describe guaranteed optimal performance of this specific enzyme. The use of other enzymes will require modification of the experimental PCR protocol, which may lead to different RAPD fingerprints. Low concentrations of detergents, such as Triton X-100, Tween-20, betain or dimethyl sulphoxide (DMSO) may be included in the master mix to help increase the specificity of primer binding. The same compounds may assist in overcoming problems caused by secondary structure or possibly even inhibitory compounds. The composition of the PCR master mix should ideally be optimized for every new PCR protocol developed as any change in the PCR methodology (including changes in primer design, deoxyribonucleotide composition, template nucleic acid and type of thermostable DNA polymerase used) may influence the specificity of amplification. All PCR reaction ingredients should be stored in a freezer in a dedicated "clean" room where strict guidelines are enforced to help prevent possible contamination of reaction mixes and ingredients.
3. Many different brands of PCR machines are currently available, using a range of different principles for temperature control, such as metal Peltier elements (heating or cooling being achieved by passing an electric current through two conductors, the most popular current method), as well as hot water and hot air. In the vast majority of these machines, sample heat exchange occurs via tight contact between the reaction tube and a metal (aluminium or even gold-plated) heating block or via direct contact with heated fluids or air. Peltier element thermocyclers may be purchased in many shapes and sizes, ranging from those containing a single heating block to those

containing two or three individually controlled heating blocks per machine *(7)*. Moreover, these heating blocks are often interchangeable, allowing the same machine to be used for a range of PCR reaction tube sizes, as well as PCR applications (e.g. *in situ* PCR). Peltier-based machines can be obtained from a wide variety of companies (Applied Biosystems, Bio-Rad, Brinkman/Eppendorf, CLP, MatriCal, MJ Research, MWG Biotech, Stratagene, Techne, Thermo Electron and Whatman Biometra). Machines based on principles such as circulating air (Roche Applied Sciences, St. John Associates, Brooks Automation, Corbett Research, Idaho Technology); heating blocks (Stratagene); water baths (Abgene); electrically conducted polymers (Biogene); microfluidics (BioTrove) and ovens (Cepheid) are available as well, and more detailed information can be retrieved from company Web sites.

4. The polysaccharide agarose (poly D-galactose 3,6-anhydro-L galactose) is constructed from multiple disaccharide building blocks and may be purified from marine algae. It is a chemically stable solid compound at room temperature and is commercially available in powder or granular form and in a range of specifications (e.g., for the resolution of low molecular weight amplimers). PCR-amplified DNA may be isolated after gel electrophoresis using commercially available kits (or enzymes such as β-agarase). If the electrophoresis power packs and buffer tanks are not regularly inspected or technical maintenance not performed at regular intervals, then variations in the electrical field strength at different points within the gel may occur. This could possibly result in PCR products migrating in a non-uniform manner through the gel, leading to misinterpretation of amplimer size (PCR specificity) as well as a reduction in gel resolving capacity.
5. Tris-acetate-EDTA buffer provides a better resolution of fragments larger than 4 kb and Tris-borate-EDTA buffer provides a better resolution of 0.1- to 3-kb fragments. Tris-borate-EDTA buffers also tend to have a greater buffering capacity and may be reused a few times before being replaced.
6. Several complete polyacrylamide gel electrophoresis systems are commercially available. For example, the Pharmacia PhastSystem provides a complete package for polyacrylamide gel electrophoresis, allowing various DNA fragments to be analysed in detail and visualised by the silver-staining assay that is included. The gels in this system (either 12.5 or 20% polyacrylamide) are sold in a ready-to-use format, and electrophoresis is completely standardised by using dedicated equipment. All physical parameters, including temperature, power, current, and so on, are automatically controlled. Other commercially available systems perform high-speed analyses

using polyacrylamide, polyethylene or hydroxycellulose carriers within glass capillaries. This "capillary" electrophoresis methodology facilitates the analysis of samples containing low concentrations (in the nanomolar range) of DNA at high electric field strengths of 1,000 V/cm *(8)*.

7. The optimal analyses of the data are essentially defined by the end user. In most cases when RAPD analyses are performed, visual inspection of the gel pictures or straightforward comparison of the fingerprints using commercially available software (e.g., Gelcompar by Applied Maths) suffice to reach reliable conclusions. When nosocomial outbreaks of infection are to be discarded or confirmed simple assessment of fingerprint identity suffices. When fingerprints of epidemiologically related strains are dissimilar, cross infection can essentially be excluded. Note that this type of local usage of RAPD testing is its mainstay. Intercenter data exchange is essentially impossible and should not be pursued *(9)*. When the RAPD technology is to be used for more complex bacterial population analyses, which is beyond the scope of the current chapter, detailed information on computerized processing of the data can be retrieved from the literature.

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Chapter 5

Use of Repetitive Element Palindromic PCR (rep-PCR) for the Epidemiologic Discrimination of Foodborne Pathogens

Kelli L. Hiett and Bruce S. Seal

Abstract

The use of defined primers for polymerase chain reaction (PCR) amplification of interspersed repetitive DNA elements present at distinct locations in prokaryotic genomes is referred to as repetitive element sequence based-polymerase chain reaction (rep-PCR). The initial discovery of repetitive extragenic palindromic (REP) elements occurred in the genomes of *Escherichia coli* and *Salmonella*. The family of REP elements is generally between 33 and 40 bp in length, has 500 to 1,000 copies per genome, and comprises about 1% of the bacterial genomes of *E. coli* or *Salmonella*. The amplified DNA fragments, when separated by electrophoresis, constitute a genomic fingerprint that can be employed for subspecies discrimination and strain delineation of bacteria and fungi. The application of rep-PCR to microbes has proven a discriminatory and reproducible tool for microbial subtype analyses and for microbial ecology investigations.

Key words: Bacteria, BOX, ERIC, REP, Rep-PCR, repetitive elements.

1. Introduction

Repetitive element sequence-based polymerase chain reaction (rep-PCR) is a PCR-based method that targets known, conserved, repetitive DNA sequences that are usually present in multiple copies within bacterial genomes ***(1–5)***. The initial discovery of repetitive extragenic palindromic (REP) elements occurred in the genomes of *Escherichia coli* and *Salmonella*. The family of REP elements is generally between 33 and 40 bp in length, has 500 to 1,000 copies per genome, and comprises about 1% of the bacterial genomes of *E. coli* or *Salmonella* ***(6)***. Another family of interspersed

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repetitive elements common to *E. coli* and *Salmonella* is the enterobacterial repetitive intergenic consensus (ERIC) elements. ERIC elements range from 124 to 127 bp in size and have 30 to 150 copies per genome ***(7)***. ERIC primer sets result in more complex profiles relative to REP primer sets. However, ERIC primer sets appear more sensitive to possible contaminants ***(8)***. The first repetitive intergenic sequence found in a gram-positive species (*Streptococcus pneumoniae*) was the BOX element, approximately 154 bp in length ***(9)***. Use of the BOX primer generates highly complex profiles and is often sufficient for characterization and differentiation of bacterial isolates.

Rep-PCR has proven a discriminatory and reproducible tool for microbial subtype analyses and for microbial ecology investigations. The method uses primers that target noncoding repetitive sequences interspersed throughout the bacterial and fungal genome (1,6). The amplified DNA fragments, when separated by electrophoresis, constitute a genomic fingerprint that can be employed for subspecies discrimination and strain delineation of bacteria and fungi ***(10,11)***.

Initially, DNA is isolated from the organism of interest. Single or multiple defined primers are then used for PCR under high-stringency conditions. The targeted sequences are generally spaced 20–400 bp apart throughout the genome and are rarely located within open reading frames. In addition, the repetitive elements may be present in both orientations. The resulting amplicons are resolved using a gel matrix. Electrophoretic resolution is often performed using larger agarose gels (25 cm in length) with extended run times, up to 18–19 h, to achieve the best resolution. Resolution of amplicons can also be achieved by the initial use of fluorescently labeled primers in a PCR, followed by electrophoresis through a polyacrylamide gel on an ABI sequencer. A third separation technique currently being implemented is the microfluidic Lab-on-a-Chip technology (Agilent Technology, Foster City, CA). Briefly, this technology employs pressure or electrokinetic forces to move small volumes of fluid through a network of channels and wells that are etched onto glass or polymer chips.

The development of a commercially available, semiautomated rep-PCR assay system, the DiversiLab™ System, offers advances in standardization and reproducibility over manual, gel-based rep-PCR ***(12, 13)***. The system allows for archiving of fingerprint patterns using Web-based software, and databases created with characterized strains can be used as reference libraries against which unknown samples can be queried. Regardless of the separation technique employed, analyses of the resulting banding patterns are best achieved using computer-assisted pattern analysis software.

2. Materials

2.1. DNA Extraction

1. UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories Inc.).
2. MO BIO Vortex Adapter (MO BIO Laboratories Inc.).

2.2. Rep-PCR

2.2.1. Rep-PCR of Campylobacter spp

1. AmpliTaq® DNA polymerase with GeneAmp® 10X PCR buffer II and 15 m*M* $MgCl_2$ solution (Perkin-Elmer Applied Biosystems).
2. GeneAmp dNTP (deoxynucleotide 5′-triphosphate) blend, 10 m*M* (Perkin-Elmer Applied Biosystems).
3. UPrime Dt Primer Set (Integrated DNA Technologies).
4. Dimethyl sulfoxide (DMSO).
5. Bovine serum albumine (BSA).
6. Thermal cycler (MJ Research).

2.2.2. Rep-PCR of Clostridium perfringens

1. DiversiLab *Clostridium* DNA Fingerprinting Kit (bioMérieux).
2. AmpliTaq DNA polymerase with GeneAmp 10X PCR buffer II and 15 m*M* $MgCl_2$ solution (Perkin-Elmer Applied Biosystems).
3. Thermal cycler (MJ Research).

2.2.3. Rep-PCR of Salmonella enterica Serotypes

1. DiversiLab *Salmonella* DNA Fingerprinting Kit (bioMérieux).
2. AmpliTaq DNA polymerase with GeneAmp 10X PCR buffer II and 15 m*M* $MgCl_2$ solution (Perkin-Elmer Applied Biosystems).
3. Thermal cycler (MJ Research).

2.3. Resolution of Amplified Products

2.3.1. Resolution of Amplified Products for Campylobacter spp

1. Seakem LE agarose: 1.5% (BioWhittaker Molecular Applications).
2. 1X TAE buffer (Sigma): 0.04*M* Tris-acetate, 0.001*M* ethylenediaminetetraacetic acid (EDTA).
3. Ethidium bromide solution (Sigma).
4. DNA molecular weight marker and loading buffer: Ready-Load™ 1 kb Plus DNA Ladder (Invitrogen).
5. DNA loading dye: 0.25% bromophenol blue, 40% (w/v) sucrose in water.
6. Horizontal electrophoresis chamber: 23 cm wide by 25 cm long (Millipede A6, Owl Separation Systems), including a gel comb with 1.0-mm teeth thickness (MTC, Owl Separation Systems).

7. Computerized video image system (EpiChem³ Darkroom Gel Documentation System, Ultra-Violet Products).
8. Bionumerics software (Applied Maths).

2.3.2. Resolution of Amplified Products for Clostridium perfringens

1. DiversiLab LabChip Kit (bioMérieux).
   a. LabChip microfluidic chips.
   b. Electrode cleaning chips.
   c. Syringes.
   d. DNA ladder.
   e. DNA marker.
   f. DNA dye concentrate.
   g. DNA gel matrix.
   h. Spin filter.
2. Agilient 2100 bioanalyzer.
3. Chip-priming station.
4. Vortex mixer (MS 3 basic) with chip adaptor (IKA®).

2.3.3. Resolution of Amplified Products for Salmonella enterica *Serotypes*

1. DiversiLab LabChip Kit (bioMérieux).
   a. LabChip microfluidic chips.
   b. Electrode cleaning chips.
   c. Syringes.
   d. DNA ladder.
   e. DNA marker.
   f. DNA dye concentrate.
   g. DNA gel matrix.
   h. Spin filter.
2. Agilient 2100 bioanalyzer.
3. Chip-priming station.
4. Vortex mixer (MS 3 basic) with chip adaptor (IKA).

3. Methods

3.1. DNA Extraction

1. Add 1.8 mL of an overnight culture of bacterial culture to a 2-mL microcentrifuge tube and centrifuge at 10,000*g* for 30 s at room temperature. Decant the supernatant and remove any additional media using a pipet tip.
2. Resuspend the cell pellet in 300 μL of microbead solution (UltraClean Microbial DNA Isolation Kit). Transfer the resuspended cells to a microbead tube.

3. Add 50 μL of MD1 solution to microbead tube.
4. Secure the microbead tubes horizontally in the MO BIO vortex adapter or secure tubes horizontally on a flat vortex pad with tape. Vortex at maximum speed for 10 min.
5. Centrifuge the microbead tubes at 10,000*g* for 30 s at room temperature.
6. Transfer the supernatant to a clean 2-mL collection tube.
7. Add 100 μL MD2 solution to the supernatant. Vortex for 5 s and set at 4°C for 5 min.
8. Centrifuge the tubes at 10,000*g* for 1 min at room temperature.
9. Transfer the entire volume of the supernatant to a clean 2-mL collection tube; avoid disturbing the pellet.
10. Add 900 μL of MD3 solution to the supernatant and vortex 5 s.
11. Load approximately 700 μL onto the spin filter and centrifuge at 10,000*g* for 30 s at room temperature. Discard the flow-through, add the remaining supernatant to the Spin Filter, and repeat the centrifugation step.
12. Add 300 μL of MD4 solution and centrifuge at 10,000 *g* for 30 s at room temperature. Discard the flowthrough.
13. Centrifuge at 10,000*g* for 1 min at room temperature to ensure removal of all excess liquid.
14. Being careful not to splash liquid on the spin filter, place the spin filter into a new 2-mL collection tube.
15. Add 50 μL of MD5 solution to the center of the white filter membrane, being careful not to touch the membrane.
16. Centrifuge at 10,000*g* for 30 s at room temperature.
17. Discard the spin filter. The DNA recovered in the tube is now ready for subsequent use.

3.2. Rep-PCR

3.2.1. Rep-PCR of Campylobacter *spp*

1. Use 100 ng of isolated genomic DNA as a template for the rep-PCR (*see* **Note 1**). The total volume of the rep-PCR is 25 μL; thus, the DNA cannot exceed a volume of 12.77 μL.
2. Place 1 μL of genomic DNA (100 ng) into a sterile 0.2-mL microcentrifuge tube.
3. Prepare a master mix by using the volumes of reagents indicated in **Table 1** to a 1.5-mL microcentrifuge tube.
4. Aliquot 24.0 μL of the master mix into each microcentrifuge tube containing 100 ng of genomic DNA.
5. Perform amplification as follows: An initial denaturation is carried out at 96°C for 2 min, followed by 31 cycles of denaturation at 94°C for 3 s, then 92°C for 30 s, annealing at

Table 1
Master Mix for Rep-PCR of *Campylobacter* spp.

Reagent	Volume (μL)	Number of reactions[a]
10X buffer with $MgCl_2$	2.5	$(2n + c^+ + c^- + 1)$
BSA	0.2	$(2n + c^+ + c^- + 1)$
DMSO	2.5	$(2n + c^+ + c^- + 1)$
dNTPs	3.125	$(2n + c^+ + c^- + 1)$
Primers (Uprime-Dt)	1.0	$(2n + c^+ + c^- + 1)$
Water	14.175	$(2n + c^+ + c^- + 1)$
Taq polymerase	0.5	$(2n + c^+ + c^- + 1)$
Total volume	24.0	$(2n + c^+ + c^- + 1)$

[a]*n*, number of samples for analysis; c^+, positive control; c^-, negative control; 1, one extra reaction to adjust for pipeting errors.

40°C for 1 min, and extension at 65°C for 8 min. A final extension step at 65°C for 8 min follows.

6. Store the microcentrifuge tubes at 4°C.

3.2.2. Rep-PCR of Clostridium perfringens

1. Use 1 μL (approximately 100 ng) of isolated genomic DNA as a template for the rep-PCR.
2. Place 1 μL of genomic DNA (100 ng) into a sterile 0.2-mL microcentrifuge tube.
3. Prepare a master mix by adding the volumes of reagents indicated in **Table 2** to a microcentrifuge tube.
4. Aliquot 24.0 μL of the master mix into each microcentrifuge tube containing 100 ng of genomic DNA.
5. Perform amplification as follows: An initial denaturation is carried out at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 70°C for 90 s. A final extension step at 70°C for 3 min follows.
6. Store microcentrifuge tubes at 4°C.

3.2.3. Rep-PCR of Salmonella enterica *Serovars*

1. Use 1 μL (approximately 100 ng) of isolated genomic DNA as a template for the rep-PCR.
2. Place 1 μL of genomic DNA (100 ng) into a sterile 0.2-mL microcentrifuge tube.
3. Prepare a master mix by adding the volumes of reagents indicated in **Table 3** to a microcentrifuge tube.

Table 2
Master Mix for Rep-PCR of *Clostridium difficile*

Reagent	Volume (μL)	Number of reactions[a]
10X buffer with $MgCl_2$	2.5	$(2n + c^+ + c^- + 1)$
Primer mix H	2.0	$(2n + c^+ + c^- + 1)$
Rep-PCR master mix 1	14.175	$(2n + c^+ + c^- + 1)$
Taq polymerase	0.5	$(2n + c^+ + c^- + 1)$
Total volume	24.0	$(2n + c^+ + c^- + 1)$

[a]*n*, number of samples for analysis; c^+, positive control; c^-, negative control; 1, one extra reaction to adjust for pipeting errors.

Table 3
Master mix for Rep-PCR of *Salmonella enterica*

Reagent	Volume (μL)	Number of reactions[a]
10X buffer with $MgCl_2$	2.5	$(2n + c^+ + c^- + 1)$
Primer mix P	2.0	$(2n + c^+ + c^- + 1)$
Rep-PCR master mix 1	14.175	$(2n + c^+ + c^- + 1)$
Taq polymerase	0.5	$(2n + c^+ + c^- + 1)$
Total volume	24.0	$(2n + c^+ + c^- + 1)$

[a]*n*, number of samples for analysis; c^+, positive control; c^-, negative control; 1, one extra reaction to adjust for pipeting errors.

4. Aliquot 24.0 μL of the master mix into each microcentrifuge tube containing 100 ng of genomic DNA.
5. Perform amplification as follows: An initial denaturation is carried out at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 70°C for 90 s. A final extension step at 70°C for 3 min follows.
6. Store the microcentrifuge tubes at 4°C.

3.3. Resolution of Amplification Products

3.3.1. Resolution of Campylobacter *spp. Amplification Products*

1. Prepare a 1.5% (w/v) Seakem LE agarose gel in 1X TAE buffer mixed with 3.0 μg/mL of ethidium bromide (*see* **Note 2**).
2. Allow the gel to solidify for at least 1 h.
3. Following solidification, gently remove the comb and submerge the gel in 1X TAE buffer containing 1.5 μg/mL of ethidium bromide.

4. Add 2 μL of DNA loading dye to 10 μL of each amplified sample.
5. Load three lanes (evenly distributed across the gel) with 1 μL each of Ready-Load 1 kb Plus DNA ladder.
6. Load the amplified samples in the remaining wells.
7. Run the gel at 4°C until the dye front is approximately 20 cm from the wells. This is generally achieved using 120 V for 8 h.
8. Expose the gel to ultraviolet light. Capture the image as a tagged image file format (TIFF) file for import into the Bionumerics software for further analyses.

3.3.2. Resolution of Clostridium perfringens *and* Salmonella enterica *Serovar Amplification Products*

1. Remove the DiversiLab DNA chip supplies and reagents from 4°C and allow contents to warm to room temperature for at least 30 min prior to use (*see* **Note 3**).
2. Turn on the Agilient Bioanalyzer 2100 and allow a warm-up period of at least 30 min prior to use and start the Bioanalyzer software.
3. Prepare the gel-dye mix: Vortex the tubes containing the DNA gel matrix and the DNA dye concentrate and briefly centrifuge. Mix 200 μL of the DNA gel matrix solution and 10 μL of the DNA dye concentrate into a 1.5-mL microcentrifuge tube. Vortex until the solution is homogeneous.
4. Transfer the homogenized solution to a provided spin filter (*see* **Note 4**).
5. Centrifuge at 1,500*g* for 10 min at room temperature. Discard the filter and store solution at 4°C protected from light.
6. Briefly vortex and centrifuge the DNA marker and DNA ladder.
7. Unwrap a new Agilent DNA microfluidic chip and inspect both back and front for defects or fingerprints.
8. Place the Agilent DNA microfluidic chip in the chip-priming station (Agilent) with the base plate in position C. Tightly attach the syringe and the syringe clip in the topmost position. Adjust the syringe plunger to 1.0 mL.
9. Pipet 9 μL of gel-dye mix, using reverse pipeting technique, into the chip-priming well (G) (*see* **Note 5**).
10. Lower the lid of the chip-priming station and lock it in place by pressing down on the silver tab. Depress the syringe plunger and allow the chip to pressurize for exactly 30 s (*see* **Note 6**).
11. After exactly 30 s, release the syringe clip. Allow the plunger to rise by its own pressure until any movement ceases. Gently unlock the lid by lifting the silver tab and remove the DNA chip.

12. Inspect the back of the DNA chip for air bubbles in the channels; bubbles appear as lines. Note any channels that have bubbles as a total chip failure may occur if a bubble is present.
13. Pipet 9 μL of gel-dye mix, using reverse pipeting technique, into the two waste wells (G).
14. Pipet 5 μL of DNA marker, using reverse pipeting technique, into the ladder well and each of the 12 sample wells.
15. Pipet 1 μL of DNA ladder, not using reverse pipeting technique, into the ladder wells.
16. Pipette 1 μL of PCR product or control, not using reverse pipeting technique, into each of the sample wells. Be sure to avoid "blowout" during pipeting.
17. Ensure that vortex is level using a leveling bubble. Place the chip securely into the vortex adapter and vortex for exactly 1 min at a speed of 2,400*g*.
18. Place the DNA chip in the Agilent Bioanalyzer within 5 min of sample loading.
19. Select DiversiLab System V1.4 from the Assay menu of the software.
20. Click Start. Resolution of bands requires approximately 1 h.

4. Notes

1. Using the UltraClean Microbial DNA Isolation Kit, 1 μL of recovered genomic DNA is approximately 100 ng.
2. The thinner the gel is made, the sharper the bands are.
3. Keep the contents of the kit covered so the reagents are not exposed to light.
4. Be careful not to touch the spin filter with your pipet.
5. Be certain to remove any air bubbles with a clean sterile pipet chip.
6. Make sure to allow the chip to pressurize for exactly 30s.

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Chapter 6

Pulsed-Field Gel Electrophoresis for Molecular Epidemiology of Food Pathogens

Tansy M. Peters

Abstract

Foodborne diseases due to well-recognized pathogens have emerged as an important and growing public health problem with a significant impact on health. Molecular methods for subtyping these microorganisms have become a valid adjunct to the traditional techniques employed in most laboratories. One such molecular technique for the detection and identification of food pathogens is pulsed-field gel electrophoresis (PFGE). This method separates large DNA molecules by the use of an alternating electrical field, such that greater size resolution can be obtained when compared to normal agarose gel electrophoresis. PFGE is often employed to track pathogens, such as *Salmonella*, *Shigella*, *Escherichia coli* (including O157), *Campylobacter*, and *Listeria* species through the food chain. The contour-clamped homogeneous electric field (CHEF) PFGE system is considered to be the gold standard for use in epidemiological studies of these organisms.

Key words: Alternating electrical field, CHEF, foodborne pathogen, molecular epidemiology, PFGE.

1. Introduction

The epidemiology of foodborne diseases is constantly changing as bacterial pathogens emerge and increase in prevalence or become associated with new food vehicles. Employing molecular tools in epidemiological investigations is often useful for identifying the routes by which these organisms are transmitted *(1)*. Of the many techniques available, separating mixtures of DNA into different size fragments by electrophoresis is fundamental to the field.

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Conventional agarose gel electrophoresis is unable to resolve fragments exceeding approximately 20,000 bp. Pulsed-field gel electrophoresis (PFGE) was developed for the separation of larger DNA fragments, such as whole chromosomes or large chromosomal fragments *(2)*. In PFGE, the orientation of the electric field relative to the gel is altered, such that the DNA molecules relax as the current is temporarily switched off and elongate when the field is reapplied. By continually changing the field orientation, DNA has to change its conformation to reorient, and with each re-orientation of the field, smaller size fragments will move in the new direction more quickly than the larger fragments. As the larger DNA lags behind, the smaller DNA fragments travel further along the course of migration and ultimately produce the required separation *(3)*.

PFGE has traditionally been used for gene mapping and is commonly considered the gold standard in epidemiological studies of pathogenic organisms *(4–6)*. The following method is used for PFGE of *Salmonella* spp. and *Escherichia* spp. and can be readily modified for other foodborne pathogens.

2. Materials

2.1. Cell Culture and Lysis of Cells in PFGE Plugs

1. Trypticase soy agar (TSA) plates, nutrient agar plates or comparable medium.
2. Cell suspension buffer (CSB): 100 m*M* Tris-HCl, 100 m*M* ethylenediaminetetraacetic acid (EDTA), pH 8.0 (s*ee* **Note 1**).
3. 2% (w/v) agarose in TE (*see* **item 4**) buffer (Megabase from Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK, or SeaKem Gold (SKG), Lonza Wokingham Ltd., Berkshire, UK). These grades of agarose are required for PFGE plugs as they give added strength, thus minimizing plug breakage at later stages of the process.
4. TE buffer: 10 m*M* Tris-HCl, 1.0 m*M* EDTA, pH 8.0.
5. 1% (w/v) sodium dodecyl sulfate (SDS).
6. Proteinase K: Either 20 mg/mL or 50 mg/mL stock solution. Prepare and store as frozen aliquots at –20°C. Only thaw the required amount for your number of samples. Once thawed, any unused solution should be discarded.
7. Lysis buffer: 50 m*M* Tris-HCl, 50 m*M* EDTA, 1% (w/v) sarkosyl, 0.1 mg/mL proteinase K, pH 8.0. Proteinase K is not added until just prior to use.
8. Plug molds: Available commercially (e.g., Bio-Rad).

9. Heating block: For example, Hybaid Dri-Block DB2 (Hybaid Ltd., Ashford, UK).
10. Turbidity meter (Gene-Trak® Systems GT 9316, Diffchamb, Notts, UK), or spectrophotometer to measure OD 1.35 at 650 nm, or McFarland Standard no. 5.

2.2. Washing of PFGE Plugs

1. Sterile ultrapure (reagent grade type 1) water at 55°C.
2. TE buffer: 10 m*M* Tris-HCl, 1.0 m*M* EDTA, pH 8.0. Store at room temperature and equilibrate to 55°C prior to use.
3. Shaking water bath.

2.3. Restriction Digestion of DNA

1. *Xba*I restriction enzyme or other suitable restriction enzymes (Gibco®, Invitrogen Ltd., Paisley, UK, or Roche Diagnostics Ltd., West Sussex, UK). Store at –20°C.
2. Sterile ultrapure, nuclease-free water (Sigma, Poole, UK).
3. Scalpel or similar blade.

2.4. Pulsed-Field Gel Electrophoresis

1. Tris-borate EDTA buffer (TBE): 50 m*M* Tris-HCl, 50 m*M* boric acid, 0.5 m*M* EDTA. Store at room temperature.
2. Agarose (Bio-Rad, pulsed-field certified or SKG).
3. Molecular reference marker strain (lambda ladder pulsed-field gel marker, New England Biolabs, Hitchin, UK) (*see* **Note 2**).
4. Equipment for gel electrophoresis of products (e.g., CHEF DR II, DR III or CHEF Mapper, Bio-Rad).

2.5. Gel Staining and Documentation

1. Ethidium bromide: 10 mg/mL stock solution. Store at room temperature in a dark bottle. Ethidium bromide is a potent mutagen and should be handled with care.
2. Ultraviolet transilluminator (UVP Inc., Upland, CA).
3. Digital image capture system (e.g., Gel Doc 2000, Bio-Rad) or conventional camera and UV-sensitive film (Polaroid Ltd., Bedfordshire, UK).

3. Methods

To perform PFGE, specialized equipment is required: a gel tank with clamped electrodes, a chiller and pump, and a programmable power supply. As PFGE has evolved to become a routine procedure, a number of different PFGE systems have been developed, and several commercial pulsed-field units are currently available. The most popular system in use today is that using a contour-clamped homogeneous electric field (CHEF) that changes the

direction of the field electronically to reorient the DNA *(7,8)*. It does this by changing the polarity of a hexagonal electrode array in combination with a horizontal gel. Thus, DNA is reoriented at an oblique angle, generally 120°, which causes it to migrate in a zigzag manner through the gel. Ideally, the DNA should separate in straight lanes to simplify lane-to-lane comparisons, and the cell concentration should be the same in each lane. During electrophoresis it is possible to have mobility inversions in which larger DNA can move ahead of smaller DNA. Ramping, by which the reorientation pulse length is constantly increased during separation, minimizes inversions. This capability is included in most commercial instrumentation (e.g., Bio-Rad). Solutions should be made up in advance and made ready for use at the correct temperature well before they are required. For example, TE and TBE buffers, CSB, proteinase K stock solution, sarkosyl solution, and 2% molten agarose can all be pre-prepared. Wear gloves during most steps of the protocol to avoid nuclease contamination of the DNA samples from the operator's skin.

3.1. Preparation for Cell Culture and Lysis

1. Streak nutrient agar plates, TSA plates, or comparable medium with the cultures for testing and incubate at 37°C for 14–18 h. Usually, this is done overnight to achieve confluent growth.
2. Transfer cells directly from the plates into labeled microcentrifuge tubes containing 1.0 mL cold sterile CSB using a sterile 1.0-µL loop or moistened cotton swab. Resuspend cells by gentle spinning of the loop to disperse the cells evenly (s*ee* **Note 3**).
3. Cell suspensions need to be adjusted to give uniform concentrations of cells in each tube. This can be achieved by either adding additional cells or further dilution with CBS. Although each laboratory will need to establish its own concentration for the best results, depending on the equipment available, the following values provide a useful starting point:
   a. Spectrophotometer at 610-nm wavelength: OD (absorbance) of 1.35 ± 0.05.
   b. Turbidity meter (e.g., Gene-Trak photometer): 0.41 ± 0.03 units at $A_{450.}$
   c. McFarland standard density no. 5.
4. Equilibrate the 2% molten agarose (Megabase or SKG) to 55°C and pipet 500 µL per isolate into prewarmed microcentrifuge tubes in a heating block (s*ee* **Note 4**).
5. Mix 500 µL of cell suspension with proteinase K to give a final concentration of 1.0 mg/mL. For mixing we recommend the use of a 1,000-µL pipet and tips as smaller tips may cause DNA shearing.

6. Add 500 μL of the cell suspension/proteinase K mixture to a 500 μL aliquot of molten agarose and mix by gently pipeting up and down three or four times. Take care not to create air bubbles.
7. Immediately dispense the agarose/cell suspension/proteinase K mixture into prelabeled plug molds. There should be enough mixture to make four or five plugs. Again, do not allow air bubbles to form. Allow the plugs to solidify at room temperature for 15 min or for 5 min in a refrigerator at 4°C.
8. When set, transfer the plugs to prelabeled tubes or vessels (e.g., 5-mL bijoux) by gently pushing them from the mold. Each group of plugs (four or five) corresponding to a single sample may be lysed and washed together in the same vessel.
9. Calculate the amount of lysis buffer required for all the samples to be lysed and prepare it by adding proteinase K to a final concentration of 0.1 mg/mL
10. Add 2 mL lysis buffer to each tube or bijoux. It is important to ensure that the plugs are floating freely in the lysis buffer and have not become stuck to the sides of the vessel.
11. Incubate the tubes in a shaker water bath at 55°C for a minimum of 2 h. Ensure that the level of water in the water bath is higher than the level of lysis buffer in the vessels for adequate incubation (*see* **Note 5**).

3.2. Washing of PFGE Plugs

1. Remove the lysis vessels from the water bath and carefully discard the lysis buffer. This is done best using sterile wide-tipped plastic bulb pastettes. Take care not to damage the agarose plugs with the end of the pastette.
2. Washing the plugs several times to remove the lysis buffer and cell debris is an important part of the process that cannot be underestimated. Add 5–10 mL of prewarmed sterile reagent-grade water to each tube and shake the tubes vigorously for 10–15 min in a water bath at 55°C (*see* **Note 6**).
3. Carefully remove the water and repeat this step with another 5–10 mL of the prewarmed water. Again, shake for 10–15 min at 55°C.
4. Preheat sterile TE buffer to 55°C for the next wash steps. Repeat the same washing procedure three more times with TE buffer as previously with water (i.e. 3X 5–10 mL washes with TE buffer, each for 10–15 min at 55°C).
5. Plugs can be stored in TE buffer (2–8°C) prior to further application for several months to years without noticeable DNA degradation.

3.3. Restriction Digestion of DNA with XbaI

1. It is only necessary to digest a small slice of each plug with restriction enzyme, so the plugs require cutting to the correct size with a scalpel. The size and shape of the plug slice will depend on the comb that is to be used for making the wells in the gel. The teeth of the combs vary from approximately 5 to 10 mm, and plug slices are generally cut into slices between 3 and 5 mm wide (*see* **Note 7**). The slices are then placed into prelabeled microcentrifuge tubes (1.5 mL).
2. Allowing for 150 µL per plug to be digested, dilute an adequate volume of 10X reaction buffer H supplied with the enzyme 1:10 with sterile, ultrapure, nuclease-free water and add *Xba*I restriction enzyme to a final volume between 0.2 and 0.8 U/µL (*see* **Note 8**). As a general rule, if a higher volume of restriction enzyme is used, the incubation time may be reduced.
3. Add 150 µL of the restriction enzyme mixture to each microcentrifuge tube, ensuring that the plug slices are well covered and not stuck to the sides of the tube. The plug slices are incubated at 37°C for between 2 and 4 h. For example, if the final *Xba*I volume is 0.8 U/µL an incubation time of 2 h will be sufficient. It is also possible to incubate the plug slices at 37°C overnight using the lower volume of restriction enzyme.

3.4. Casting and Loading the Agarose Gel

1. The electrophoresis gel should be cast at least an hour before the restriction digestion process is completed. This will allow the agarose time to solidify fully prior to loading the plug slices. Gels should be poured on a level surface, ideally on a gel-leveling table.
2. Assemble a precleaned gel mold with its backing plate according to the manufacturer's instructions and decide on the size of comb to be used. For example, Bio-Rad supplies the following size gel molds for their CHEF systems, and the choice of mold will depend on the number of samples to be run:

   14×13 cm for 10 to 15 wells, 15-tooth comb

   14×21 cm for 15 to 20 wells, 20-tooth comb

   14×21 cm for more than 20 wells, 30-tooth comb (run widthways)

   The comb is placed in position with approximately 1–2 mm of space between the bottom of the teeth and the baseplate. Make sure the comb is sitting perpendicular to the base.
3. Using a pulsed-field-certified agarose and 0.5X TBE buffer, melt a suitable volume of agarose at 1.0% (w/v). The 14 × 21 cm casting mold will require 150 mL of molten agarose, while the 14 × 13 cm mold requires 100 mL. Fully melted and mixed agarose should be clear with no visible suspended particles.

Equilibrate the molten agarose in a water bath (50–60°C) before pouring (*see* **Note 9**).

4. The cooled gel is poured slowly and carefully into the casting mold, ensuring that there are no air bubbles trapped in the agarose. If present, remove any bubbles with a clean pipet tip immediately after the gel is poured. When the gel has solidified (30–45 min) remove the comb carefully, making sure that the wells are not damaged in the process.
5. Restricted plug slices are removed from the enzyme/buffer mixture and carefully inserted into the gel wells in a predetermined order. The plug slices can be manipulated using a small spatula so that they are gently pressed towards the front of the well, taking care to ensure that no air bubbles are present (*see* **Note 10**). A number of reference standards/molecular weight markers will need to be included in each run. A 15-well gel will require a minimum of three standards; for a 20-well gel use a minimum of four, and so forth (*see* **Note 2**). Place a standard in the first and last well of each gel with the other standards evenly distributed between the test samples.
6. Using a plastic bulb pastette, the wells are sealed with the small amount of melted 1% (w/v) agarose that was saved for this purpose, again taking care that no air bubbles are introduced.
7. Leave the gel to set for approximately 5–10 min before removing it from the casting mold. The gel should remain on the baseplate, and any excess agarose on the bottom or sides of the plate should be carefully removed with tissue.
8. Place the gel inside the gel frame within a precooled electrophoretic tank (*see* **subheading 3.5.2**) and close the cover of the tank.

3.5. Electrophoresis Preparation and Running Conditions

1. Prepare a volume of 0.5X TBE buffer and add this to the electrophoretic tank. The volume will depend on the model of tank used but will be approximately 2.0–2.5 L.
2. Switch on the variable-speed pump to circulate the buffer (70–80 units is approximately 1 L/min) and set the cooling/chiller module to 14°C. Remove any air bubbles in the buffer lines. This should be done at least 30 min before the gel is to be run to reach the required temperature. As DNA mobility depends on the separation temperature, the temperature must be kept constant during the run. Check that the electrophoresis unit is level and that buffer is circulating evenly through the system.
3. With the gel in place, set the conditions on the control module according to the manufacturer's instructions for programming and start the electrophoretic run. There is a multitude of running conditions to choose from that will vary with the organism tested and the degree of band separation required *(9–11)*.

Also there will be an element of variation according to the equipment used. However, as a starting point, the following well-published parameters ***(12–15)*** are suggested for *E. coli, Shigella* and *Salmonella* sp. (*see* **Note 11**).

Voltage: 6.0 V/cm (200 V)

Ramp: 2.0–64.0 s (for *Salmonella*), 2.2–54.2 s (for *E. coli, Shigella*)

Temperature: 14°C

Run time: 22 h (CHEF DR II), 20 h (CHEF DR III), 18 h (CHEF Mapper XA)

3.6. Gel Staining and Documentation of PFGE Profiles

1. Following completion of the electrophoresis run, the equipment is switched off, and the gel is carefully removed to a suitable container with a lid. Cover the gel in aqueous ethidium bromide (0.5 μg/mL) (*see* **Note 12**), close the lid and stain the gel for 10–15 min. Gentle agitation using an orbital shaker set at low speed will ensure even staining in all regions of the gel. Do not over- or understain the gel. The former will require extensive destaining to remove background interference, while the latter will leave less-concentrated bands too light to view or document.
2. After staining, the ethidium bromide is poured off, and the gel is destained for at least 60 min using distilled water. Ethidium bromide bound to the DNA will not be removed, but any excess of stain will rinse easily out of the agarose. This prevents unwanted background fluorescence in the gel.
3. PFGE profiles are visualised using an ultraviolet transilluminator and the image is captured either with a digital image capture system (e.g., Gel Doc 2000, Bio-Rad) or by conventional camera photography using a UV-sensitive film.
4. A sharp, clear image is critical for the accurate interpretation of results, so the aim is to optimize the image and remove artifacts, but with minimal manipulation, using any software. Images are usually saved in tagged image file format (TIFF). An example of the typical results produced is shown in **Fig. 1.** It is often useful to view a negative image together with the positive image when visualizing bands by eye.
5. Interpretation and analysis of PFGE profiles is best done using a software program such as BioNumerics software (Applied Maths, Belgium) or Molecular Analyst Fingerprinting Plus (Bio-Rad).
6. TIFF images are normalized by using the standards included in every gel. This allows gel-to-gel comparisons to be made with greater accuracy.

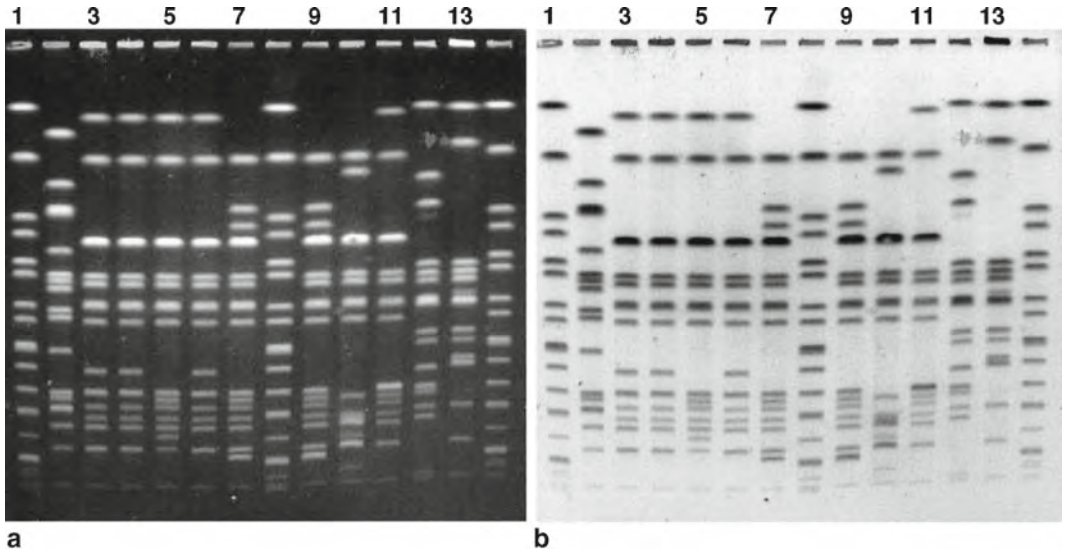

Fig. 1. PFGE profiles for various *Salmonella enterica* serovars. **(a)** positive image, **(b)** negative image. Lanes 1, 8, and 14, molecular weight marker (*S.* Braenderup, H9812, PulseNet, CDC, Atlanta); lane 2, *S.* Virchow; lanes 3–7 and 9–11, *S.* Bareilly; lanes 12 and 13, *S.* Newport.

7. Good-quality gel images are essential for the interpretation of PFGE profiles. This is especially the case to be able to match PFGE profiles to known profiles within a database library.
8. Each operator should apply a visual quality assurance check of the TIFFs before analysing the PFGE profiles. The following points should be noted:
   a. The overall clarity of the TIFF image should be good.
   b. The bands in the profiles should be sharp and distinct throughout the gel. It should be easy to view any doublets (double bands running closely together).
   c. The lines should all be straight without any significant curving on the outside lanes.
   d. The amount of DNA in the wells must be even. Under- or overloaded wells will produce lanes with lighter or darker bands that are difficult to interpret.
   e. The gel background should be free of spots and smears. Any debris present will interfere with analysis using a software program.
   f. There should be complete DNA restriction in all lanes. Partially restricted DNA produces faint shadow-like bands between more solid bands of the profiles.

g. There should be an adequate number of correctly placed standards to allow for normalizing the gel. The lowest band in the standard should be visible and have run to within 1–1.5 cm from the bottom of the gel.

4. Notes

1. It is important to make sure the best possible quality of water is used for making up reagents (e.g., use sterile ultrapure water for all reagents although it is permissible to use nonsterile ultrapure water for gels and electrophoresis running buffers and for destaining gels).
2. There are a number of commercially available molecular weight markers suitable for PFGE (e.g., lambda ladder pulsed-field gel marker). However, one of the best standards to use is a strain of *Salmonella* Braenderup (H9812) as used by the PulseNet networks (Centers for Disease Control and Prevention, Atlanta, GA) ***(16)***. It provides a stable fragment length marker over a wide range of evenly distributed fragment sizes.
3. Use several colonies from the plate to form a "rice grain-sized" pellet and store the microcentrifuge tubes on ice until a whole batch is completed.
4. Before cell lysis, *Listeria* requires a 10-min preincubation (37°C) with lysozyme to a final concentration of 1.0 mg/mL. In some protocols 1% SDS (w/v) is added to the molten agarose to help lysis, but we have not found this necessary with *Salmonella* preparations. As SDS can make the molten agarose viscous and difficult to pipet, we omit its use if possible.
5. It is also possible to incubate the tubes statically for a minimum of 4 h or alternatively overnight.
6. Although vigorous shaking is required for this part of the protocol, it is important to check periodically that the plugs are not being damaged by excessive agitation within the wash buffer. If a shaker water bath is not available, it is possible to wash plugs using prewarmed washes at ambient temperature using a standard benchtop shaker. However, it is best to increase the number of TE washes to six.
7. Cutting the plug can be performed within a sterile Petri dish with a piece of graph paper placed underneath to act as a size guide. The plug may be carefully removed from the TE buffer with a small spatula. This stage of the protocol requires some dexterity and skill. With practice the operator will gain experience

regarding whether the slices are cut horizontally or vertically to achieve the correct size.

8. For *E. coli, Shigella,* and *Salmonella* sp. *Xba*I is the restriction enzyme of first choice. It is, however, possible to use other enzymes (*Avr*II*/Bln*I*, Spe, Not*I) if added confirmation of a result is required (e.g., the final PFGE profiles of samples are indistinguishable from each other). Different organisms will require different restriction enzymes to achieve the best PFGE profiles (e.g., for *Listeria* use *Asc*I or *Apa*I; for *Campylobacter* use *Sma*I or *Kpn*I; etc.). Some protocols include a prerestriction incubation step of the plug slices in 1:10 dilution of 10X reaction buffer H at 37°C for 5–10 min, but this is optional.
9. As a small volume of melted 1% agarose will be needed to fill the wells to fix the plugs in position, it is useful to save a little of this mixture (5 mL) for this purpose. Keep it equilibrated in a water bath (50–60°C) until ready for use.
10. This process requires dexterity and practice so that the operator is consistent in technique and the resulting bands in each lane will be straight and sharp. It can also be tedious. An alternative method suggests loading the plug slices directly onto the bottom of the comb's teeth and air-drying them for 5–20 min after removing excess buffer with a tissue. The comb is only then placed into position in the gel mold, and the agarose, after cooling, is poured around the comb. It is a matter of personal preference as both methods serve equally well.
11. For *Listeria* use a run time between 20 and 22 h with a ramp of 4.0–40.01 s, and for *Campylobacter* use a run time between 18 and 20 h with a ramp of 6.76–38.35 s.
12. Ethidium bromide should be kept in a dark bottle as it is light sensitive. The solution can be reused several times before discarding it, but with each use it becomes depleted. As ethidium bromide is a mutagen and toxic, it should be handled and disposed of according to your laboratory's guidelines for hazardous compounds. Nitrile gloves are recommended throughout the gel-staining process.

Acknowledgment

I wish to thank Clare Maguire for providing the PFGE image used in **Fig. 1.**

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Chapter 7

Molecular Genotyping of Microbes by Multilocus PCR and Mass Spectrometry: A New Tool for Hospital Infection Control and Public Health Surveillance

David J. Ecker, Christian Massire, Lawrence B. Blyn, Steven A. Hofstadler, James C. Hannis, Mark W. Eshoo, Thomas A. Hall, and Rangarajan Sampath

Abstract

We describe a new technology for the molecular genotyping of microbes using a platform known commercially as the Ibis T5000. The technology couples multilocus polymerase chain reaction (PCR) to electrospray ionization/mass spectrometry (PCR/ESI-MS) and was developed to provide rapid, high-throughput, and precise digital analysis of either isolated colonies or original patient specimens on a platform suitable for use in hospital or reference diagnostic laboratories or public health settings. The PCR/ESI-MS method measures digital molecular signatures from microbes, enabling real-time epidemiological surveillance and outbreak investigation. This technology will facilitate understanding of the pathways by which infectious organisms spread and will enable appropriate interventions on a time frame not previously achievable.

Key words: Molecular genotyping, mass spectrometry, PCR/ESI-MS, base composition, Ibis T5000.

1. Introduction

Multilocus polymerase chain reaction (PCR) followed by electrospray ionization mass spectrometry (PCR/ESI-MS) is the analysis of PCR amplicons using ESI-MS. The technique was initially developed for the identification of microbes, including previously unknown or unculturable organisms, in original patient specimens or environmental surveillance samples in which multiple microbes may be present *(1–3)*.

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In brief, multiple pairs of primers are used to amplify carefully selected regions of pathogen genomes; the primer target sites are broadly conserved, but the amplified region carries information on the microbe's identity in its nucleotide base composition. Regions of this nature appear in the DNA that encodes ribosomal RNA and in housekeeping genes that encode essential proteins. Following PCR amplification, a fully automated ESI-MS analysis is performed. The mass spectrometer effectively weighs the PCR amplicons, or mixture of amplicons, with sufficient mass accuracy that the composition of A, G, C, and T can be deduced for each amplicon present. The base compositions are compared to a database of calculated base compositions derived from the sequences of known organisms to determine the identities of the microorganisms present. In the event that there is no match of the measured base composition with a sequence in the database, the nearest neighbor organism is identified. Thus, analysis by the PCR/ESI-MS method provides information that enables identification of a broad range of microbes in a sample without having to anticipate what microbes might be present. The identities of microbes in a mixed population are determined because the primers amplify the nucleic acids from all organisms in the sample simultaneously, and the mass spectrometer analyzes and reports on multiple peaks in the same spectrum.

2. High-Resolution Molecular Genotyping by Multilocus PCR and Mass Spectrometry

The Ibis T5000 technology was initially developed for broad bacterial and viral detection and identification; however, PCR/ESI-MS is also a very powerful tool for high-resolution molecular genotyping of microbes. Applications of the technology can be thought of in an hourglass model as illustrated in **Fig. 1**. The upper portion of the hourglass depicts identification of microbes, generally bacteria and viruses, present in an unknown sample at the species level as described. The utility of PCR/ESI-MS has been demonstrated for broad bacterial surveillance *(2)* and for identification of virus families, including coronaviruses *(4)*, influenza viruses *(5)*, adenoviruses *(6)*, alphaviruses *(7)*, and orthopoxviruses *(3)*. The bottom portion of the hourglass in **Fig. 1** refers to assays developed on the PCR/ESI-MS platform that are specific for a particular species; these assays reveal molecular details such as the presence of virulence factors, antibiotic or antiviral drug resistance, or high-resolution molecular signatures that distinguish closely related subspecies. These high-resolution molecular analyses require separate assays that investigate important questions unique to a particular microbe. For example, for *Staphylococcus aureus*, it is important to determine the presence

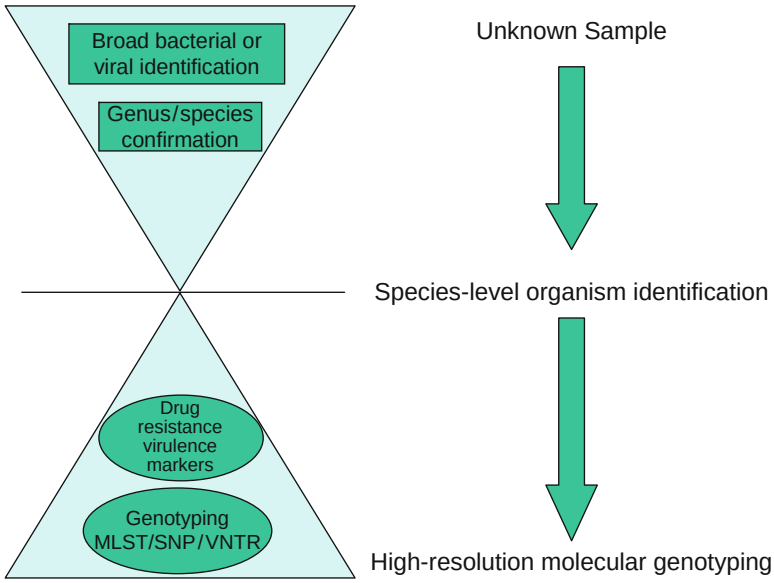

Fig. 1. Hourglass model for applications of PCR/ESI-MS. In broad surveillance mode (top of hourglass), the technology can be used to answer the question, Which organisms are in my sample? The pinch point is identification of the species, which is where most molecular methods are focused. The lower portion of the hourglass is the "drill-down" mode of PCR/ESI-MS. In this mode, species-specific primers yield high-resolution details that distinguish strain types and identify virulence and drug resistance markers. *(See Color Plates)*

or absence of certain virulence factors, mobile genetic elements, or mutations in housekeeping genes that mediate drug resistance. For understanding the genetic lineage of microbes, the PCR/ESI-MS method follows the general principles of multilocus sequence typing (MLST).

MLST is a high-resolution molecular tool for discriminating closely related bacterial subspecies *(8)* (*see* **Chapter 11** in this book). In this method, the data are digital and portable, facilitating comparison among laboratories worldwide. However, conventional MLST requires isolation of pure colonies of the target microbe followed by multiple PCR reactions and sequencing of each amplicon. While sequencing technology has become much more facile in recent years, it is still not practical to use conventional MLST in a clinical laboratory setting. Clinical and public health laboratories require simple, automated analytical methods that match their throughput needs and cost limitations. In contrast to conventional MLST, multilocus PCR/ESI-MS provides an automated, high-throughput alternative that approaches the resolution of sequence-based, conventional MLST and can be implemented in a clinical laboratory at very low per-sample costs.

The multilocus PCR/ESI-MS strategy is graphically depicted in **Fig. 2**. The same set of housekeeping genes used for conventional

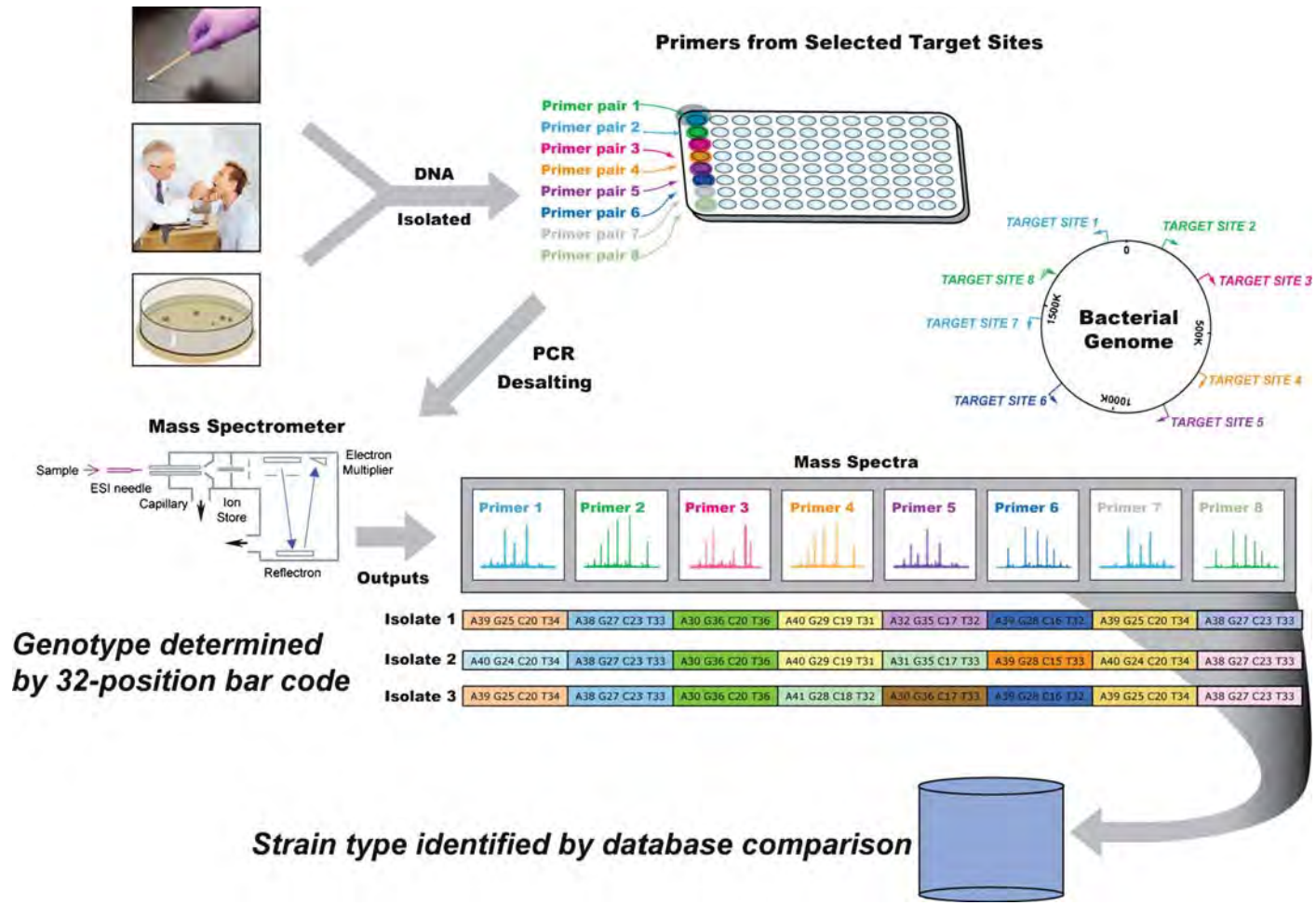

Fig. 2. Flow scheme for high-resolution genotyping of microbes by PCR/ESI-MS. A set of species-specific primer pairs are designed to distinguish strain types. Each primer pair generates a four-position bar code comprised of the AGCT count for each amplicon. Taken together, a set of eight primer pairs produces a 32-position bar code, which provides strain differentiation. The method works on isolated colonies or original patient or environmental samples. Mixed populations of microbes can be analyzed because multiple peaks in the mass spectrum can simultaneously be analyzed. *(See Color Plates)*

MLST are analyzed to identify the regions that contain the highest information content in their base compositions, and sets of primer pairs are designed to these regions. Typically, 100–150 nucleotide regions are selected for amplification. The information values of the amplicons are evaluated until an optimal set of primer pairs is identified. Each primer pair is assigned to a position in a 96-well plate such that a sample is amplified by eight pairs of primers and analyzed by MS. Each of the primer pairs produces an amplicon that results in a spectral signal and base composition, or four-position A, G, C, T signature (but since here amplicons are generally of constant length, each base composition signature actually contains only three independent variables). Base compositions from each of the eight primer sets result in a 24-dimensional digital signature that can be compared to calculated base composition signatures generated from an MLST database.

The ability to distinguish MLST alleles by PCR followed by MS is, at first glance, counterintuitively high. Molecular biologists generally think in terms of the sequence of the nucleotides as the signature of a microbe. But, while the potential number of distinct sequences within any given MLST locus is astronomical (4^x, where x is the number of nucleotides showing mutations), the number of actual, biologically relevant sequences is typically much more manageable: First, only a fraction (10–20%) of the positions within MLST loci show variation. Second, most of these sites do not display the full range of possible mutations, but merely transitions. Third, only a fraction of these sites is simultaneously mutated. Thus, only 50 to 100 alleles, differentiated by specific sets of mutations, are typically reported in MLST databases for a single locus.

This level of resolution can be approached by base composition analysis. Any single mutation that separates one allele from another can be identified by MS analysis since even a single-nucleotide substitution results in spectral signals that can be identified as distinct masses and compositions. There are 12 possible types of single mutations (A → G, A → C, A → T, G → A, G → C, G → T, C → A, C → G, C → T, T → A, T → G, and T → C), and all result in masses that are unique (*see* **Fig. 3**). As additional mutations occur, the resulting space of possible base compositions grows accordingly, following a third-degree polynomial expression (**Fig. 3**). Of course, not all possible base compositions are actually generated with a given set of alleles, and each allele does not necessarily generate its own distinct base composition. The most common way for two alleles to share the same base composition is to differ from each other by one of the six self-cancelling pairs of single-nucleotide polymorphisms (SNPs) (e.g., A → G and G → A or A → C and C → A). If three SNPs are involved, retrieving the same base composition involves one of the eight possible "triangular" mutation patterns (e.g., A → G, G → C,

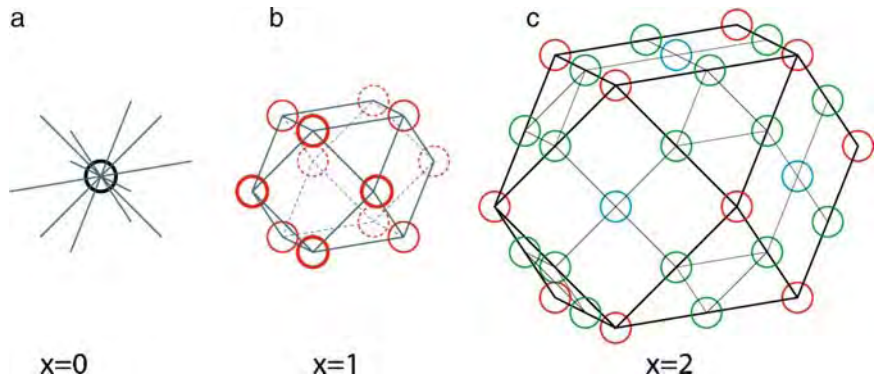

Fig. 3. Representation of the base composition space that is covered after x mutations. (**a**) The original base composition space (black sphere) can be affected by 12 distinct mutations (gray lines). (**b**) After one mutation, the 12 resulting base compositions define a hollow, cuboctahedron-shaped shell. Any of these 12 base composition spaces can be similarly affected by 12 additional mutations; in each case, only 1 mutation will revert back to the original base composition ($x = 0$), whereas 4 mutations would yield an adjacent base composition (within the same shell), and 7 mutations would yield base compositions located in the next $x = 2$ shell. (**c**) Base composition space for $x = 2$ mutations. For clarity, only the front-facing base compositions of the outer shell are represented. Equivalent positions are similarly colored. With each subsequent x_{th} mutation, an additional shell of $N(x) = 10x^2 + 2$ new, distinct base composition space is added. The total number of base compositions that can be reached after x mutations follows a third-degree polynomial progression: $N_{BC}(x) = (2x + 1)(5x(x + 1) + 3)/3$. *(See Color Plates)*

C → A), whereas with four SNPs "quadrangular" mutation patterns are possible (e.g., A → G, G → C, C → T, T → A). As is apparent in **Table 1**, the occurrence of such combinations decreases as the number of SNPs increases, meaning that base compositions naturally tend to be more diverse as the number of SNPs increases in the allele set. In practice, a typical PCR/ESI-MS amplicon of a MLST gene carries from two to six mutations, which is enough to observe a number of distinct base compositions in the same order of magnitude (about 70% on average) as the number of alleles that are distinguished by sequence within the same locus.

The practical utility of MS analysis of PCR amplicons to distinguish MLST alleles was determined by examination of multiple sequence alignments from housekeeping genes from *Acinetobacter baumannii, S. aureus, Pseudomonas aeruginosa,* and *Streptococcus pyogenes* (*see* **Fig. 4**). In all cases, a single primer pair targeted to a single allele excluded more than 60% of sequence types on average, and amplification of four loci resulted in elimination of more than 95% of all sequence types on average. Thus, by using six to eight primer pairs it is possible to resolve different isolates of these microbes at a level that is more than sufficient for establishing clonality in an outbreak investigation.

An important advantage of multilocus PCR/ESI-MS is that nucleic acid does not need to be isolated from pure colonies of the target microbe. Patient specimens have been successfully

Table 1
Fraction of Single-Nucleotide Polymorphisms (SNP) Combinations Silent in Mass Spectroscopic (MS) Analysis of Polymerase Chain Reaction Amplicons

Number of SNPs	Number of unique SNP combinations	Number of SNP combinations yielding no net base composition change	Fraction (%) of SNP combinations silent in MS analysis[a]
1	12	–	0.00
2	78	6	7.69
3	364	8	2.20
4	1,365	27	1.98
5	4,368	48	1.10
6	12,376	112	0.90
7	31,824	192	0.60
8	75,582	378	0.50
9	167,960	624	0.37
10	352,716	1,092	0.31

[a]Evolution of the fraction of SNP combinations silent by MS (fourth column) is given as a function of the number of SNPs (first column). Since the number N of unique SNP combinations (second column) is insensitive to the SNP order, it follows that the relation $N = (x + n - 1)!/x!/(n - 1)!$, where n is the number of possible single changes (12 here), and x is the total number of single changes made. The number of SNP combinations yielding no net change (third column) was determined empirically.

analyzed using this technology without culture *(2)*. As eliminating the culture step can save 1 or 2 d, multilocus PCR/ESI-MS can be used to track an epidemic on a time frame not previously achievable. Samples that contain more than one strain type in a mixture can also be analyzed because multiple amplicons are individually identified in the mass spectrum. The peak heights for each of the amplicons in the mixture can be used to determine the relative ratios of microbes in the sample, provided that the low abundance microbe represents at least 2–5% of the microbial population. The fact that some clinical samples have mixed populations of strain types is often missed when a culture step is used, as bias can be introduced by culture conditions, and multiple colonies from the same sample are not always analyzed.

For bacterial pathogens that have emerged in relatively recent history, the numbers of mutations found in housekeeping genes are limited, and genetic markers that evolve at faster evolutionary clock speeds are necessary to establish clonality. For these

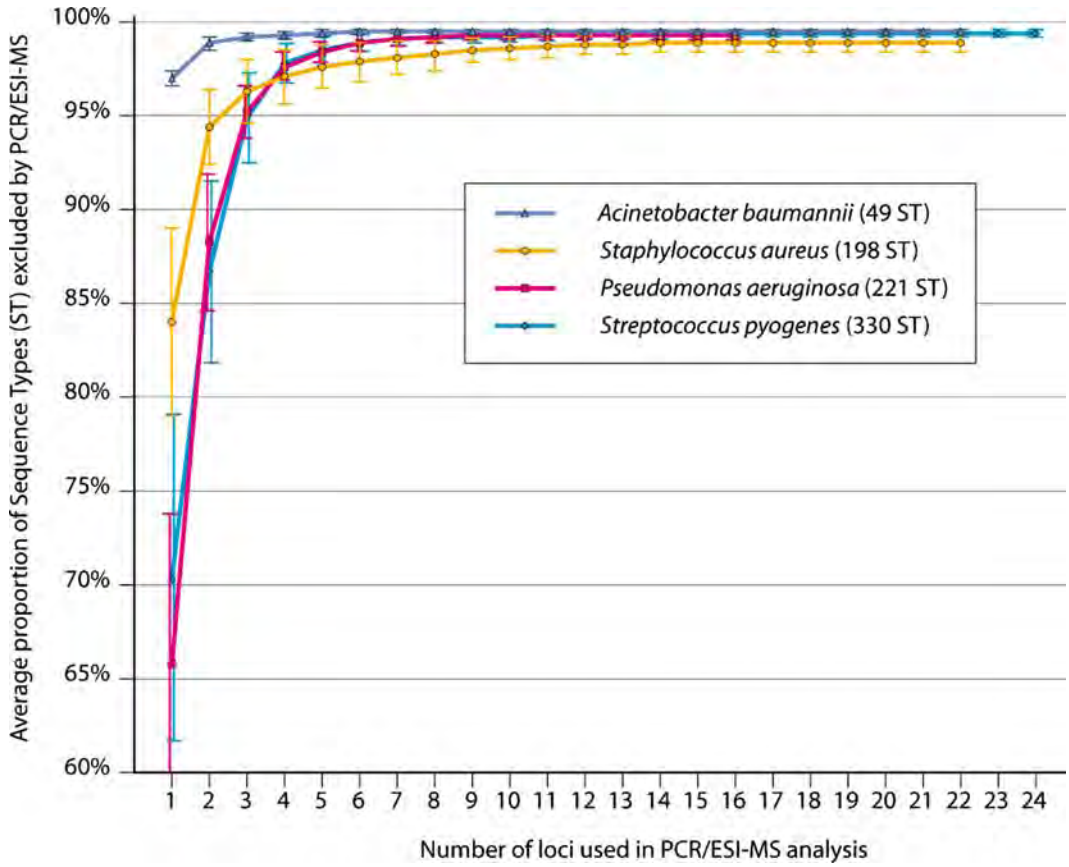

Fig. 4. Comparative resolution of PCR/ESI-MS typing schemes for four organisms. For each of the four organisms, a set of unique sequences types (STs) was first assembled using six or seven genes commonly used for MLST. These reference alignments were then used for the design of 16 to 24 primer pairs for PCR/ESI-MS analysis. The resolution provided by PCR/ESI-MS analysis was evaluated as follows: Starting with the primer pair providing the best sequence resolution, amplicon base compositions were determined for each of the sequence types. Comparison of these base composition signatures defined the number of sequences types that were incompatible with any particular type at this particular locus. The average proportion of sequence types excluded by PCR/ESI-MS with their corresponding standard deviations (vertical lines) was plotted versus the number of loci used in the analysis. This process was repeated, using base composition signatures extended by one additional locus at a time, to yield the full curves shown. *(See Color Plates)*

organisms, short repeated elements known as variable number of tandem repeats (VNTR) have proven to be useful markers ***(9)***. These elements vary in the number of repeats of short strings of nucleotides. Examples of organisms for which VNTR elements have been used to establish clonality are *Bacillus anthracis* ***(10)***, *Francisella tularensis* ***(9)***, and *Mycobacterium tuberculosis* ***(11)***. VNTR analysis can be conducted using PCR/ESI-MS simply by designing primers that bracket the VNTR. The base composition of the amplicon is used to precisely calculate the number of repeats as well as any single-nucleotide variations that may appear within the repeat, providing greater resolving power than the repeat count that is obtained from gel analysis. VNTR, SNP, and

MLST analyses can be combined into a single assay with PCR/ESI-MS, if simultaneous analysis of genetic biomarkers with a range of clock speeds is desired, simply by bracketing the appropriate target region on the microbial genome with PCR primers and assembling the primer set in 96-well plate configuration as shown in **Fig. 2**.

3. Examples of Applications of Multilocus PCR and Mass Spectrometry

3.1. Streptococcus pyogenes *Epidemic Analysis*

For high-resolution strain genotyping of *S. pyogenes*, a strategy was designed to generate strain-specific signatures like those provided by MLST *(2)*. Primer pairs were designed to the *S. pyogenes* MLST gene targets that correlate with the *emm* classification. To identify target regions that provided the highest resolution of species and least ambiguous *emm* classification by base composition analysis, we constructed an alignment of concatenated alleles of the seven MLST housekeeping genes from each of 212 previously *emm*-typed strains *(12)* and determined the number and location of the primer pairs that would maximize strain discrimination. An initial set of 24 primer pairs was selected that would amplify regions covering over 97% of the known nucleotide variations in the MLST sequencing targets. We then determined how much strain discrimination could be achieved from a smaller set of primers. Calculations showed that six pairs of primers allowed discrimination at the individual *emm*-type level of about 75% of all the *emm* types listed by Enright et al. *(12)*, while the remaining 25% clustered into groups of two or more *emm* types. This degree of resolution is sufficient for applications such as tracking the clonal expansion of a particular strain type during a specific epidemic.

We used this method to genotype *S. pyogenes* in patient samples taken at a military training camp during one of the most severe outbreaks of pneumonia associated with group A *Streptococcus* (GAS) in the United States since 1968 *(13)*. Throat swabs were taken from both healthy and hospitalized recruits and plated for selection of putative GAS colonies. A second set of 15 original patient specimens was taken during the height of this disease outbreak. The third set consisted of historical samples from disease outbreaks at this and other military training facilities during previous years. The fourth set of samples was collected from five geographically separated military facilities in the continental United States in the winter immediately following the severe outbreak.

Colonies isolated from GAS-selective media from all four collection periods were analyzed with the six GAS genotyping primers. The results of the base composition analysis with genotyping primer pairs for samples from all four collection periods

were compared to results from 5′-*emm* gene sequencing and the MLST gene sequencing methods in **Table 2**. When only these six primer pairs were used, some of the samples could not be resolved to a unique *emm* type. However, base composition analysis showed identification consistent with (either uniquely or as a member of a small set) 5′-*emm* gene sequencing or the MLST sequencing method. These data showed that the GAS genotypes found during the epidemic were remarkably homogeneous (*see* **Fig. 5**), as would be expected for a clonal expansion during an outbreak in which the same genotype was being passed from person to person. In contrast, surveillance samples taken at diverse military bases showed a heterogeneous pattern reflecting a normal disease season in the absence of a major outbreak. This study demonstrated the power of PCR/ESI-MS in a real epidemic setting.

3.2. Acinetobacter baumannii *Epidemic Analysis*

Acinetobacter baumannii is often associated with hospital-acquired infections, and *Acinetobacter* also has a history of association with war-wound infections. During the Vietnam War, *A. baumannii* was the most common gram-negative bacteria recovered from traumatic injuries to extremities *(14)*. This is because *Acinetobacter* naturally occurs in the soil. During blast injuries, wounds frequently become inoculated with soil organisms, leading to infections that later occur in the hospital. Over a 2-yr period from 2002 to 2004, military health officials identified 102 patients with blood cultures that grew *A. baumannii* from Landstuhl Regional Medical Center in Germany and from Walter Reed Army Medical Center (WRAMC) in the United States. In both facilities, the number of patients with *A. baumannii* bloodstream infections in 2003 and 2004 significantly exceeded those reported in previous years, suggesting nosocomial transmission.

Understanding the fundamental mechanisms underlying *Acinetobacter* infections, including the original sources of the infecting organisms, their clonality, and geographical spread, is important for the development of appropriate infection control measures. Genotyping allows investigation of clonal spread and can be used to identify the source of the original infection. We developed a high-throughput genotyping method for *Acinetobacter* using PCR/ESI-MS *(15)*. At the time the method was developed, there was no MLST database for *Acinetobacter,* so we used *Moraxella catarrhalis* (the most closely related organism that had an MLST database) as a model to select the housekeeping genes for sequencing of *A. baumannii* isolates and to identify regions diverse enough to distinguish between strains by PCR/ESI-MS. We sequenced regions of six housekeeping genes (*trpE*, *adk*, *efp*, *mutY*, *fumC*, *ppa*) from 267 *Acinetobacter* isolates and designed eight PCR primer target sites covering about 1,700 nucleotides overall.

Table 2
Base composition signatures for *Streptococcus pyogenes* and correlations with *emm* types

No. of instances	Location	Base compositions of target gene regions						Strain (emm-type)	
		murI	*mutS*	*xpt*	*yqiL*	*gki*	*gtr*	*Mass spectrometry*	*Gene sequencing*
75	MCRD San Diego[1]	A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A32 G35 C17 T32	A39 G28 C16 T32	3	3
2		A40 G24 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A31 G35 C17 T33	A39 G28 C15 T33	6	6
1		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A41 G28 C18 T32	A30 G36 C17 T33	A39 G28 C16 T32	28	28
6	NHRC San Diego–Archive[2]	A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A32 G35 C17 T32	A39 G28 C16 T32	3	3
3		A40 G24 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A30 G36 C20 T30	A39 G28 C15 T33	5, 58	5
6		A40 G24 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A31 G35 C17 T33	A39 G28 C15 T33	6	6
1		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A30 G36 C20 T30	A39 G28 C16 T32	11	11
3		A40 G24 C20 T34	A38 G26 C24 T33	A30 G36 C19 T37	A40 G29 C19 T31	A31 G35 C17 T33	A39 G28 C15 T33	12	12
1		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A31 G35 C17 T33	A38 G29 C15 T33	22	22
3		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A30 G36 C17 T33	A39 G28 C15 T33	25, 75	75
4		A40 G24 C20 T34	A38 G26 C24 T33	A30 G36 C20 T36	A41 G28 C19 T31	A30 G36 C18 T32	A39 G28 C15 T33	44/61, 82, 9	44/61
2		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C19 T37	A40 G29 C19 T31	A32 G35 C17 T32	A39 G28 C16 T32	53, 91	91
1	Ft. Leonard Wood	A39 G25 C20 T34	A38 G27 C24 T32	A30 G36 C20 T36	A40 G29 C19 T31	A30 G36 C17 T33	A39 G28 C15 T33	2	2
2		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A32 G35 C17 T32	A39 G28 C16 T32	3	3
1		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C19 T37	A41 G28 C19 T31	A31 G35 C17 T33	A39 G28 C15 T33	4	4
1		A40 G24 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A31 G35 C17 T33	A39 G28 C15 T33	6	6
11		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A30 G36 C17 T33	A39 G28 C15 T33	25 or 75	75
1		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C19 T37	A40 G29 C19 T31	A30 G36 C17 T33	A39 G28 C15 T33	25, 75, 33, 34, 4, 52, 84	75
1		A40 G24 C20 T34	A38 G26 C24 T33	A30 G36 C20 T36	A41 G28 C19 T31	A30 G36 C18 T32	A39 G28 C15 T33	44/61 or 82 or 9	44/61
2		A40 G24 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A30 G36 C20 T30	A39 G28 C15 T33	5 or 58	5
3	Ft. Sill	A40 G24 C20 T34	A38 G27 C23 T33	A30 G36 C19 T37	A40 G29 C19 T31	A30 G36 C18 T32	A39 G28 C15 T33	1	1
2		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A32 G35 C17 T32	A39 G28 C16 T32	3	3
1		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C19 T37	A41 G28 C19 T31	A31 G35 C17 T33	A39 G28 C15 T33	4	4
1		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A41 G28 C18 T32	A30 G36 C17 T33	A39 G28 C16 T32	28	28

(continued)

Table 2
(continued)

No. of instances	Location	Base compositions of target gene regions						Strain (emm-type)	
		murI	*mutS*	*xpt*	*yqiL*	*gki*	*gtr*	*Mass spectrometry*	*Gene sequencing*
1	Ft. Benning	A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A32 G35 C17 T32	A39 G28 C16 T32	3	3
1		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C19 T37	A41 G28 C19 T31	A31 G35 C17 T33	A39 G28 C15 T33	4	4
3		A40 G24 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A31 G35 C17 T33	A39 G28 C15 T33	6	6
1		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A30 G36 C20 T30	A39 G28 C16 T32	11	11
1		A40 G24 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A41 G28 C19 T31	A30 G36 C19 T31	A39 G28 C15 T33	13	94
1		A40 G24 C20 T34	A38 G26 C24 T33	A30 G36 C20 T36	A41 G28 C19 T31	A30 G36 C18 T32	A39 G28 C15 T33	44/61 or 82 or 9	82
1		A40 G24 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A40 G29 C19 T31	A30 G36 C20 T30	A39 G28 C15 T33	5 or 58	58
1		A39 G25 C20 T34	A38 G27 C23 T33	A30 G36 C20 T36	A41 G28 C19 T31	A30 G36 C18 T32	A39 G28 C15 T33	78 or 89	89

[1]MCRD: Marine Corps Recruit Depot.
[2]NHRC: Naval Health Research Center.

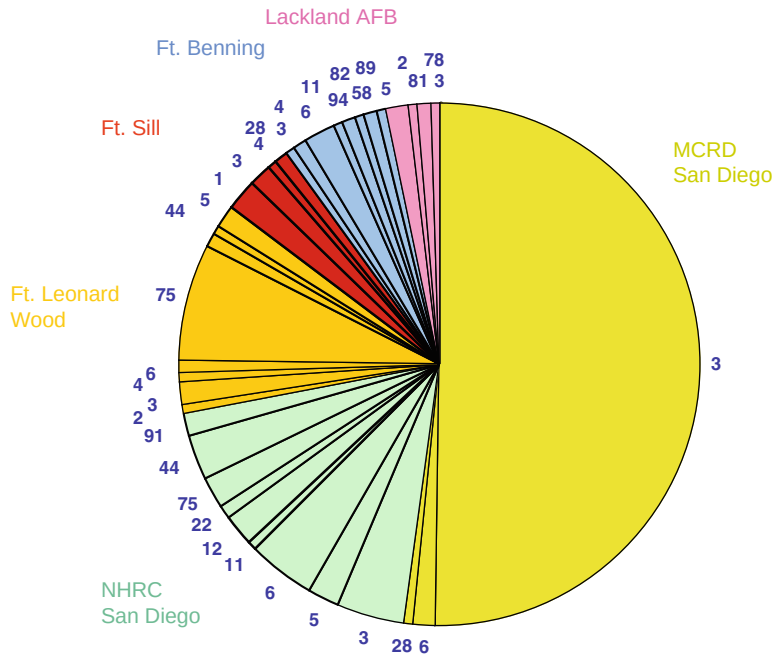

Fig. 5. Pie chart illustrating data given in Table 2. The area of each slice of pie is proportional to the number of instances of each *Streptococcus pyogenes emm* type. The colors indicate various military locations. MCRD: Marine Corps Recruit Depot; NHRC: Naval Health Research Center; AFB: Air Force Base. *(See Color Plates)*

Using this set of primers, isolates were analyzed from infected and colonized soldiers and civilians involved in an outbreak in the military health care system associated with the conflict in Iraq, from previously characterized outbreaks in European hospitals, and from culture collections. The goal of this study was to identify the reason for the increased nosocomial *Acinetobacter* infections observed during this period. Twenty-seven isolates from the outbreak in the military personnel were found to have genotypes representing different *Acinetobacter* species, including 8 representatives of *Acinetobacter* sp. 13TU and 13 representatives of *Acinetobacter* sp. 3. However, most of the isolates from the Iraqi conflict were *A. baumannii* (189 of 216 isolates). Among these, 111 isolates had genotypes identical or very similar to those associated with well-characterized *A. baumannii* isolates from European hospitals (**Table 3**). This observation suggested a second mode for the origin of *A. baumannii* infections: contamination with European strains that had developed multidrug resistance and properties that favored hospital transmission. Remarkably, isolates from WRAMC showed genotypes from all three major clones I, II, and III obtained from the European hospital collection *(16,17)*, suggesting that the U.S. service personnel were exposed to a diverse set of European strain types.

Table 3
Genotypes, Number of Isolates and Correlation with European Multidrug-Resistant *Acinetobacter* Clones

Organism	Genotype	Number of isolates	Clone type
A. baumannii	1, 10, 11, 47	65	II
	15, 16, 45, 46	24	I
	14	22	III
	(30 types)	78	
Acinetobacter sp. 13TU	(4 types)	8	
Acinetobacter sp. 3	(9 types)	13	
Other species	(6 types)	6	

A follow-up study was conducted by PCR/ESI-MS of *A. baumannii* isolates collected from wounded soldiers returning from the Iraqi conflict during 2006–2007 *(18)*. The distribution of genotypes obtained during this period was remarkably similar to those observed in samples collected during 2003–2004, suggesting a stable reservoir of strain types that continued to infect U.S. service personnel wounded in the war. This composition of genotypes was significantly different from the nosocomial strains identified at nonmilitary U.S. hospitals, dispelling the hypothesis that repatriated soldiers infected with *Acinetobacter* were having an impact on U.S. nonmilitary hospital infections.

3.3. Virus Identification and Genotyping

The PCR/ESI-MS technology is also useful for identifying viruses and for tracking the spread of viral infections through a population. Despite higher mutation rates and greater sequence variability than bacteria, conserved primer target sites can be identified that enable priming of entire genera or even complete viral families. RNA-dependent RNA polymerase is a housekeeping gene common to all RNA viruses that provides several target site opportunities for developing primers that amplify multiple species within a virus family. This strategy is powerful because a single PCR reaction analyzed by MS can be used to detect and identify tens to hundreds of related viral species. The inherently high mutation rate of viruses results in base composition differences that provide a high-resolution molecular signature of viral subtypes. Generally, at least two sets of primer pairs are targeted to different regions of the viral genome for each virus group, and potential misclassification is avoided because two regions taken

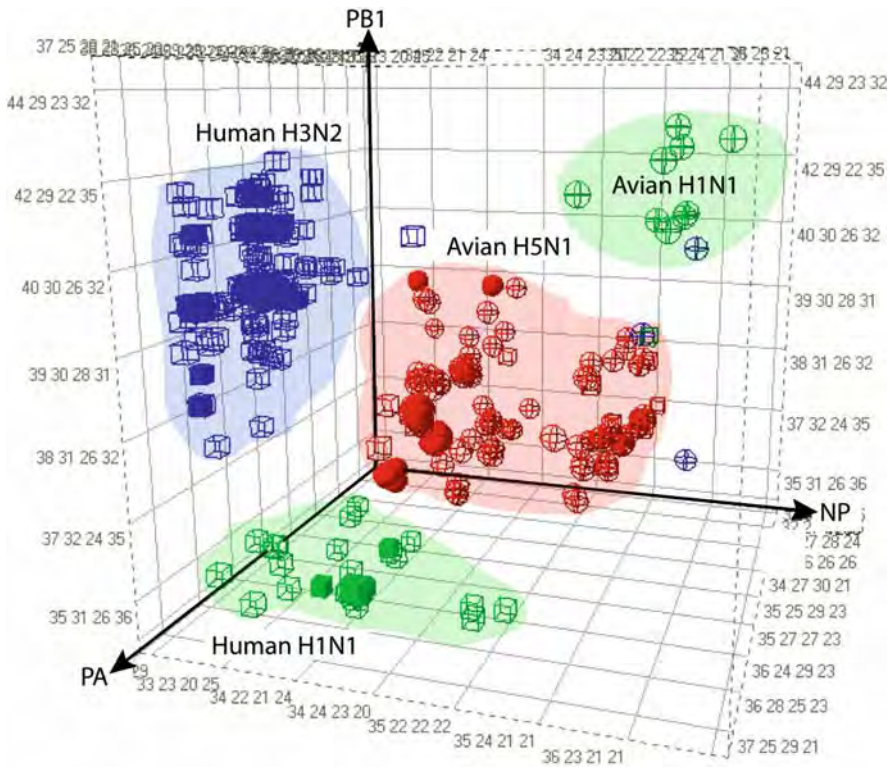

Fig. 6. Distribution of base compositions for influenza A viruses using three primer pairs. Hollow symbols represent calculated base compositions derived from sequences in GenBank, and solid symbols represent actual samples analyzed by PCR/ESI-MS. Red symbols, H5N1; green symbols, H1N1; blue symbols, H3N2. Cubes indicate human samples, and spheres indicate avian samples. *(See Color Plates)*

together provide unambiguous speciation and subtype determination. For example, we used PCR/ESI-MS to identify and subspeciate over 50 types of adenoviruses *(6)*. This strategy has also been used effectively for detection and strain typing of influenza viruses *(5)*, alphaviruses *(7)*, coronaviruses *(4)*, and orthopoxviruses *(3)*.

Base composition signatures provide a multidimensional fingerprint of the genomes of various viruses and can be used to determine clusters of related species/subtypes. One such representation (*see* **Fig. 6**) shows base composition data derived from the primer pairs targeted to PA, PB1, and NP gene segments of influenza A viruses. Human H3N2 and H1N1 viruses clustered independently from each other and from the avian/human H5N1 and H1N1 viruses. Thus, although mutations occur rapidly in viruses, base composition of certain regions can be used to cluster viruses into groups that are clearly distinguishable.

4. Conclusion

The Ibis T5000 PCR/ESI-MS technology couples PCR to ESI-MS and provides rapid, high-throughput, precise digital analysis of the microbes present in either isolated colonies or original patient specimens. The platform is suitable for use in hospital or reference diagnostic laboratories and other public health settings due to ease of use, high throughput, and affordability. The PCR/ESI-MS method measures digital molecular signatures from microbes, enabling real-time epidemiological surveillance and outbreak investigation. The method facilitates understanding of the pathways by which infectious organisms spread and enables appropriate interventions on a time frame not previously achievable.

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Chapter 8

Amplified Fragment Length Polymorphism Analysis

Norman K. Fry, Paul H.M. Savelkoul, and Paolo Visca

Abstract

Amplified fragment length polymorphism (AFLP) analysis is a universal polymerase chain reaction (PCR)-based DNA fingerprinting technique comprising three main stages: (i) digestion of genomic DNA with restriction endonucleases and ligation to double-stranded adaptors (each comprised of two oligonucleotides), thus creating restriction fragments with identical known adaptor sequences; (ii) specific amplification of a subset of these DNA fragments using primers (one labeled) targeting the adaptor sequences and additional selected bases within the unknown genomic DNA; and (iii) analysis of the patterns (usually automated). Differences or polymorphisms between samples are revealed by separation of the labeled fragments by electrophoresis (standard agarose, high-resolution denaturing acrylamide, or capillary gels). Comparison of banding patterns is typically achieved using dedicated fingerprinting analysis software. The advantages of AFLP analysis include the ability to use a universal protocol in combination with different restriction endonucleases and the choice of adding one or more selective nucleotides in the PCR primers to achieve optimal results relatively quickly without prior knowledge of DNA sequences from a large variety of (micro)organisms. The method also has the potential for high-throughput and local electronic database pattern storage with relatively low cost. Disadvantages include variation in the precision of sizing of fragments, leading to suboptimal reproducibility, particularly across different platforms.

Key words: AFLP, amplified fragment length polymorphism, DNA fingerprinting, genetic diversity.

1. Introduction

Many DNA fingerprinting techniques have been applied to microorganisms. The amplified fragment length polymorphism (AFLP) analysis method was originally developed to study inheritance markers in plants and patented in 1992 by Keygene N.V., Wageningen, The Netherlands (European patent application

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0534858A1). This method allowed the detection of DNA polymorphisms (AFLP markers) directly on gels without the requirement for Southern blotting and hybridization. In addition to its use in plants *(1)*, AFLP analysis has been applied to the fields of human genetics for tissue typing of individuals *(2)* and the fingerprinting of bacteria, fungi, and nematode genomes *(3–5)*. In this chapter we focus on the use of AFLP in bacterial fingerprinting, although a similar approach can be applied to other genomes. The name AFLP was originally chosen because of its similarity to the restriction fragment length polymorphism (RFLP) technique. Strictly, the term AFLP should not be used as an acronym because the technique reveals presence or absence of restriction fragments rather than length differences *(4)*. However, due to its widespread use as such in the literature we have used the terms AFLP and amplified fragment length polymorphism interchangeably here.

AFLP comprises the selective polymerase chain reaction (PCR) amplification of subsets of restriction fragments from a small amount of total genomic DNA digest. The three main steps, which are performed on purified DNA, are (i) restriction and ligation, (ii) selective amplification, and (iii) pattern analysis (*see* **Fig. 1**).

1.1. Restriction/ Ligation

Although methods using from one to four restriction endonucleases have been described *(3,4,6)*, typically for protocols with labeled products for which separation is achieved with high-resolution gels, two enzymes are used; one is described as a rare

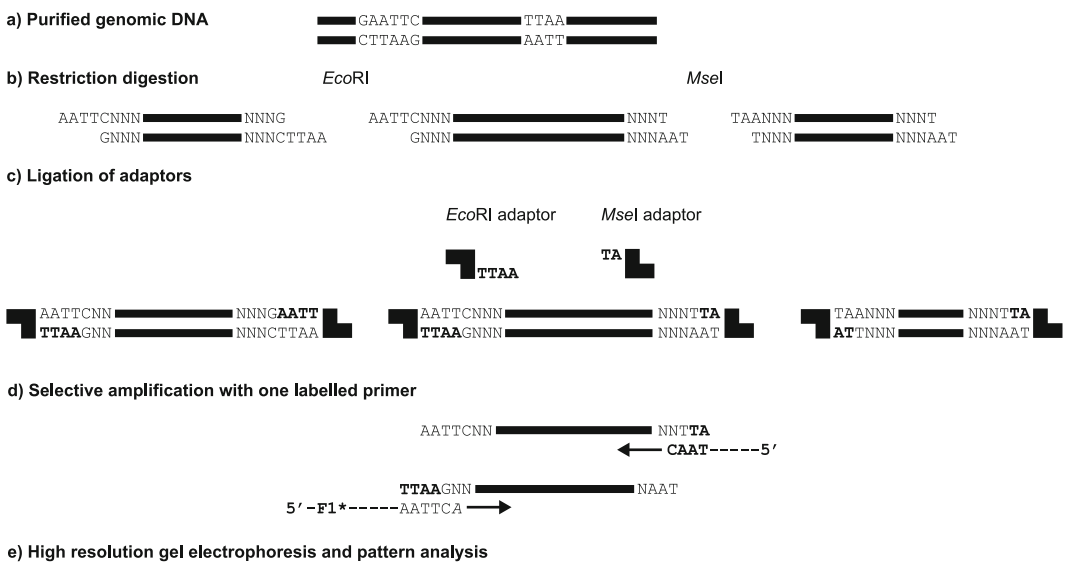

Fig. 1. Schematic representation of AFLP. Fl* fluorescent label.

cutter or of average cutting frequency, with a six-base recognition sequence (e.g., *Eco*RI, 5′-G↓AATTC-3′) and the other as a frequent cutter or with a higher cutting frequency, with a four-base recognition sequence (e.g., *Mse*I, 5′-T↓TAA-3′) *(7)*. The endonucleases *Eco*RI and *Mse*I are presented as examples in this chapter, but other enzymes can also be used. Isoschizomers can be used (e.g., *Tru*1I or *Tru*9I instead of *Mse*I), and for GC-rich genomes, the combination of *Pst*I and *Taq*I (recognition sequences 5′-CTGCA↓G-3′ and 5′-T↓CGA-3′, respectively) are recommended. Since the restriction/ligation reaction is performed in the low-salt T4 DNA ligation buffer, excess amounts of restriction enzymes (relative to the amount of target genomic DNA) are often used to compensate for the loss of enzyme activity (50–75%) under low-salt conditions.

1.2. Selective Amplification

1.2.1. AFLP Adaptors

AFLP adaptors have two components, a core sequence and an endonuclease-specific sequence. The endonuclease-specific sequence is designed to allow hybridization and subsequent ligation of the adaptor to the restriction fragments but does so without restoring the original restriction site. Each adaptor is comprised of two partially complementary oligonucleotides; the structures of the *Eco*RI and *Mse*I adaptors are shown in **Table 1**. Both primers contain one variant nucleotide, which abrogates the restriction site after ligation with the genomic DNA, preventing recutting. Both adaptors must be used at equimolar amounts in the restriction/ligation reaction. The ligated adaptors subsequently become universal target sites for the AFLP primers on all restricted/ligated DNA fragments.

Table 1
Structure of the *Eco*RI and *Mse*I Adaptors

EcoRI Adaptor	Genomic DNA	Oligonucleotide name
5′-CTCGTAGACTGCGTACC*	*aattc*$n_1n_2n_3n_4$	*Eco*RI–1
CATCTGACGCATGG*	TTAAG $n_1n_2n_3n_4$–5′	*Eco*RI–2
*Mse*I adaptor		
5′-GACGATGAGTCCTGAG*	*taa*$n_1n_2n_3n_4$	*Mse*I–1
TACTCAGGACTC*	ATT$n_1n_2n_3n_4$–5′	*Mse*I–2

*Change of original restriction site, which abrogates the restriction site after ligation with the genomic DNA (italicized), preventing recutting.

n = unknown genomic sequence.

1.2.2. AFLP Primers

AFLP primers have three components: a core sequence (CORE) that anneals with part of the adaptor, a restriction enzyme-specific sequence (ENZ) that anneals with the altered restriction enzyme recognition site sequence, and a selective extension (EXT). Variation in the number of selective nucleotides in EXT affects the resulting complexity of the patterns produced. The greater the number of selected nucleotides, the fewer the number of fragments produced. In theory, every selective nucleotide leads to a fourfold reduction in the number of AFLP fragments (assuming that the presence of the four possible nucleotides is equally distributed in the genome). Any of the four nucleotides can be used for the selective extension. Examples of *Eco*RI and *Mse*I AFLP primers with one selective nucleotide (C and A, respectively, from which the *Eco*RI-C and *Mse*I-A designations derive) are as follows:

	CORE	ENZ	EXT	
*Eco*RI-C	5′-GACTGCGTACC	AATTC	C	(only amplification when $n_1 = G$)
*Mse*I-A	5′-GATGAGTCCTGAG	TAA	A	(only amplification when $n_1 = T$)

Usually two oligonucleotide primers are used, one complementary to the six-base cutter (e.g., *Eco*RI) termini and one complementary to the *Mse*I termini. One of the primers can be labeled at the 5′-terminus, usually the primer for the rare-cutting enzyme (in this case the *Eco*RI primer).

1.3. Evolution of the Methodology

1.3.1. Basic Unlabeled (Nonfluorescent) AFLP

Valsangiacomo and colleagues were the first to apply this technique to the epidemiological study of *Legionella pneumophila* ***(3)***. This protocol used a single endonuclease (*Pst*I), with unlabeled AFLP primers, with detection of amplified fragments achieved by separation and staining on a standard agarose gel. The method performed well in a coded multicenter evaluation of genotypic methods for the epidemiological typing showing high epidemiological concordance ($E = 1.00$), although it did not give particularly high discrimination (0.89). Further studies determined that the intercenter reproducibility could be high ($R = 0.78–1.00$) using visual analysis ***(8)***. Following further standardization, types were designated on the basis of band sizes, and the method was adopted as an international standard for *L. pneumophila* genotyping by the European Working Group for Legionella Infections (EWGLI). However, while the method was robust and rapid, proficiency testing revealed that a significant proportion of laboratories could not correctly identify all isolates. This was usually due to data analysis issues rather than to the method itself. The method still remains in use for *L. pneumophila* typing by several reference laboratories, although there is now a DNA sequence-based scheme for the epidemiological typing of this organism ***(9–11)***. An example of the AFLP methodology is shown in **Fig. 2**. This single-endonuclease/agarose method has also been used for typing

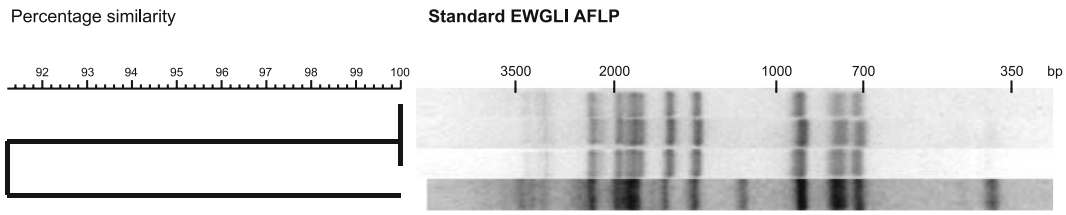

Fig. 2. Normalized patterns of *Legionella pneumophila* serogroup 1 isolates obtained following the standard EWGLI AFLP protocol (nonfluorescent). The top three isolates are epidemiologically related, and the lower one is unrelated.

of *Chlamydia pneumoniae* ***(12)*** and *Listeria monocytogenes* ***(13)***, and an up to four-endonuclease variation, designed to increase the discrimination above that obtained with one or two restriction endonucleases, has been applied to *Klebsiella pneumoniae* ***(6)***.

1.3.2. Radioisotopic AFLP

In the original method described by Keygene, the primers were labeled at the 5′-end using [γ-^{33}P]ATP (adenosine 5′-triphosphate) and T4 polynucleotide kinase ***(4)***. Following the PCR reactions, the labeled products were separated using denaturing polyacrylamide gels. The gels were then fixed and dried onto the glass plates and exposed to phosphoimage screens for 16 h; fingerprint patterns were visualized using a Fuji BAS-2000 phosphoimage analysis system (Fuji Photo Film Company Ltd., Japan). This method has the advantage that specific AFLP fragments can be isolated from the original polyacrylamide gel for further analysis (e.g., sequence determination).

1.3.3. Fluorescent AFLP

Later, nonradioisotopic labeling of primers was achieved using fluorescent labels, and fluorescent AFLP (fAFLP) offered a simpler, more rapid protocol that allowed analysis using an automated DNA sequencer ***(15,16)***. With the increasing availability of fluorescent (sequencing) platforms, primers can now be obtained ready labeled with a suitable fluorochrome such as Cy-5 (MWG Biotech) for the ALF Express DNA Sequencer (Amersham Pharmacia Biotech); D4-PA (Invitrogen) for detection on the Beckman CEQ 8000 DNA Analysis System (Beckman Coulter); or NED/FAM/JOE for the ABI 3100 Analysis System (Applied Biosystems).

An improvement in interrun reproducibility of fAFLP patterns was obtained by the switch from slab gel automated analysis systems to capillary gel analysis systems. In slab gels intrarun reproducibility is usually very high, but there is some gel-to-gel variation. However, the interrun reproducibility was significantly increased in capillary platforms ***(17)***. This has led to improved library database construction without run-to-run clustering artifacts. Due to this improvement, this technique has gained wide applicability in clinical typing of bacteria for infection control measurements and molecular epidemiology. An example

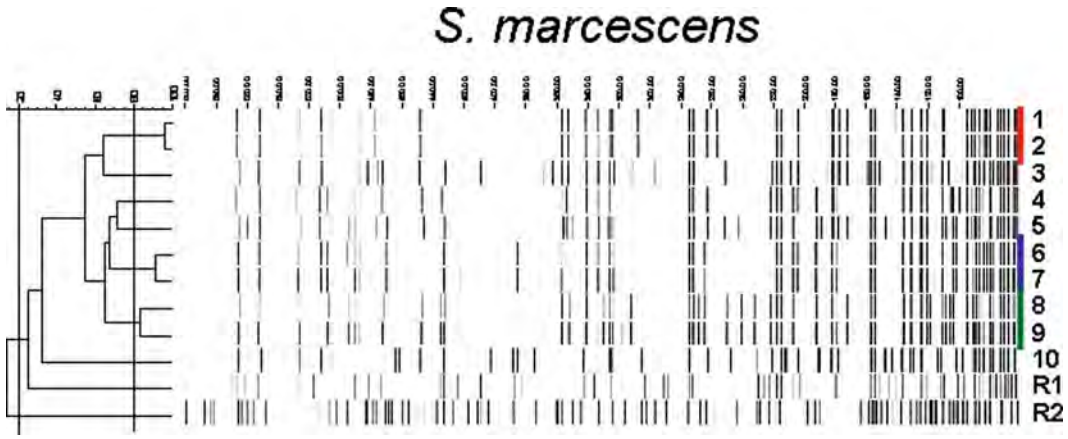

Fig. 3. Example of AFLP analysis of ten clinical isolates including two reference strains. Numbers 1 and 2, 6 and 7, and 8 and 9 are identical strains. All other strains are *Serratia marcescens* but independent isolates. R1, *S. marcescens* ATCC 13880^T; R2, *Serratia odorifera* ATCC 33077.

of AFLP patterns produced using the protocol described in **Subheading 3.2**. is shown in **Fig. 3**.

1.3.4. Commercial AFLP Kits

AFLP® is a proprietary technology of Keygene, and commercial developments led to the availability of AFLP kits for research applications from Invitrogen, Applied Biosystems, and LI-COR. AFLP kits for radioisotopic or chemiluminescent detection are available from Invitrogen, and LI-COR sells kits for detection on their automated infrared DNA analysis systems. AFLP kits are available for producing AFLP fingerprints of plants, microorganisms, yeast, and fungi; all kits are based on the standard *Eco*RI and *Mse*I digestion. A detailed protocol of the commercial AFLP kit for microorganisms is available from Invitrogen at http://tools.invitrogen.com/content/sfs/manuals/aflpmicroorganism_man.pdf. LI-COR also sells an AFLP kit for gene expression analysis experiments based on *Taq*I and *Mse*I digestion (*see* **Subheading 1.6**.). An example of AFLP fingerprints of four *Escherichia coli* strains and one *Legionella* strain using the Applied Biosystems kit is shown in **Fig. 4**.

The availability of fAFLP kits now means that as long as a laboratory has access to an automated DNA analysis platform, this methodology is now more accessible and requires less user input than the other methods and should be more reproducible. Thus, of the AFLP analysis methods available, analysis using commercial kits has effectively become a standard method for strain comparison. However, full details of the sequences of included adaptors/primers may be lacking, which can hinder comparative analyses. Some protocols provide an option to include a low-level selection or "preselective" amplification of the restriction fragments after the restriction/ligation step. As for all commercial kits, the

Fig. 4. An example of fAFLP fingerprints using the Applied Biosystems AFLP Microbial Fingerprinting protocol. The first 24 lanes show six samples each of four different *Escherichia coli* strains (each of the six samples represents a different growth phase of the organism). The final 11 lanes show different growth phases of a single strain of *Legionella pneumophila*. Note that the *E. coli* fingerprints are similar to each other and different from the *Legionella* fingerprint. Within a strain, all of the bands are reproducible. Image courtesy of Applied Biosystems.

instructions provided by the manufacturer should be followed carefully (*see* **Note 1**).

1.4. AFLP Fragment Length Predictor Programs

The increasing availability of complete genome sequences has led to the development of both commercial and freeware programs capable of predicting AFLP DNA fragment sizes. Typically, users can select or paste in a genome of interest, choose from a range of restriction endonucleases and selective nucleotides, and obtain predicted fragment sizes. Such programs (e.g., ALFIE, http://www.hpa-bioinfotools.org.uk/tools.html, or http://insilico.ehu.es/AFLP/ ***(14)*** can provide a useful starting point if the genome of the species of interest is available. A comparison of *in silico* predicted fragment sizes of the genomes of *E. coli* (the K-12 strain MG1655) and *Staphylococcus aureus* (strains Mu50 and N315) with those produced experimentally have been described by Arnold et al. ***(18)*** and Savelkoul et al. ***(17)***.

The use of *in silico* analysis and subsequent experimental testing offer the potential to make AFLP a very reliable and controlled technique. First, if the laboratory results match the *in silico* predicted

patterns, then it can be ensured that the quality of the procedure is optimal; second, the precise DNA segments on which the AFLP fragments are based can be known without sequencing. However, this assumes that the sequences used for the prediction contain no errors and that the genomes of target organisms do not differ significantly.

1.5. Advantages and Disadvantages of the Technique

The advantages of the AFLP technique are that it can potentially be used for DNA of any origin or complexity. Fingerprints can be produced without prior sequence knowledge using adaptor-specific primers, and the number of fragments detected can be adjusted by choosing either different restriction enzymes (e.g., depending on the GC content of a given organism) or selection of specific primer sets by adding selective nucleotides at the 3′ end. AFLP can enhance detection of polymorphisms in very closely related isolates or those with a small genome as long as sufficient variation exists in the genome. The method can also be used for both identification and typing of strains.

The disadvantages are that AFLP is a two- or three-stage process and takes at least 8 h to obtain results (depending on the number of strains). For data comparison, database storage dedicated equipment is required. Reproducibility appears platform dependent and is also affected by the skill and experience of the laboratory personnel.

1.6. Variations and Alternatives

In addition to its use in typing of strains, the AFLP method also has important research features. First, the method can be used in an alternative way to detect differences in RNA expression profiles. This so-called complementary DNA (cDNA)-AFLP is very applicable to eukaryotic genomes by using the A-tail on messenger RNA. However, besides protocols for eukaryotic microorganisms, those for bacteria have been described. In general, the principle is identical to the standard AFLP after cDNA synthesis with hexaprimers.

Another application is high-throughput AFLP (HT-AFLP). With this variation a substantial fraction of the genome can be screened by using several frequent cutting restriction enzymes and amplification of up to 64 primer combinations with all possible selective nucleotides. In this way small differences between genomes can be recognized based on numerous AFLP fragments. In addition to this last variation, AFLP can be carried out using radioisotopic labels with slab gels or the automated infrared LI-COR system (see (http://www.up.ac.za/academic/fabi/eucgenomics/euc_mapping/AFLP_protocol.pdf).

By using this approach specific marker bands can be isolated from the original gel, reamplified, cloned, and sequenced. In this way strain-specific sequences can be found and characterized.

2. Materials

2.1. Basic Unlabeled AFLP of Legionella pneumophila

2.1.1. DNA isolation

1. Buffered charcoal yeast extract (BCYE) agar (Oxoid, UK) plates.
2. Commercial DNA isolation kit, including a ribonuclease (RNase) step, capable of yielding high molecular size genomic DNA, such as the Nucleon BACC2 DNA Extraction kit (Nucleon Biosciences, UK) (*see* **Note 2**).

2.1.2. Restriction-Ligation Reaction

1. Adaptors: Oligonucleotides LG1, 5′-CTC GTA GAC TGC GTA CAT GCA, and LG2, 5′-TGT ACG CAG TCT AC (MWG Biotech) (*see* **Note 3**).
2. *Pst*I (Roche) (*see* **Note 4**).
3. T4 DNA ligase (Roche).
4. 10X ligation buffer: 660 m*M* Tris-HCl, pH 7.5, 50 m*M* magnesium chloride, 10 m*M* dithiothreitol, 10 m*M* ATP. Dilute at a 1:10 ratio with distilled water for 1X ligation buffer.
5. 2.5*M* ammonium acetate (Sigma).
6. 70% (v/v) ethanol.
7. Tris-EDTA (TE) buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.

2.1.3. Polymerase Chain Reaction

1. Nuclease-free water (Sigma).
2. *Taq* DNA polymerase (Life Technologies).
3. Selective primer (AFLP-PstI-G: 5′-GAC TGC GTA CAT GCA GG; MWG Biotech).
4. Mineral oil (Sigma).
5. DNA Engine (MJ Research).

2.1.4. Gel Electrophoresis

1. Agarose: 1.5% (w/v).
2. 1X TBE buffer: 0.089*M* Tris-borate, 1 m*M* EDTA.
3. Molecular size markers: GeneRuler DNA Ladder Mix (Fermentas Life Sciences).
4. Loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanoll FF, 15% Ficoll (type 400) in distilled water.
5. Ethidium bromide, 0.5 μg/mL (Sigma).
6. Digital imaging recorder.

2.1.5. Data Analysis

For data analysis, GelCompar or BioNumerics (Applied Maths) may be used.

2.2. Fluorescent AFLP (Nonkit)

2.2.1. DNA Isolation

1. QiaAmp DNA minikit (Qiagen, Hilden, Germany).
2. Proteinase K (Sigma).

2.2.2. DNA Quantification

1. Agarose (USB).
2. 1X TBE buffer: 0.089*M* Tris-borate, 1 m*M* EDTA.
3. λ DNA.
4. Ethidium bromide 0.5 μg/mL (Sigma).

2.2.3. Restriction-Ligation Reaction

1. Ligase buffer containing ATP (10 X New England Biolabs).
2. 0.5*M* NaCl.
3. Bovine serum albumin (BSA), 1 mg/mL (New England Biolabs).
4. Mse adaptor (Eurogentec), Msead 1: 5′-GAC GAT GAG TCC TGA G, Msead 2: 5′-TAC TCA GGA CTC ATC.
5. Eco adaptor (Eurogentec), Ecoad 1: 5′-CTC GTA GAC TGC GTA CC, Ecoad 2:5′-AAT TGG TAC GCA GTC TAC.
6. T4 DNA ligase (New England Biolabs).
7. *Mse*I (New England Biolabs).
8. *Eco*RI (New England Biolabs).
9. Distilled water.

2.2.4. Polymerase Chain Reaction

1. Primers: Eco-A (or Eco-0) (FAM) labeled 5′-GAC TGC GTA CCA ATT CA (50 ng/μL, Applied Biosystems) and Mse-C 5′-GAT GAG TCC TGA GTA AC (50 ng/μL, Eurogentec). The shaded nucleotide is the selective one.
2. 10 m*M* dNTP (deoxynucleotide 5′-triphosphate; Promega).
3. 10X PCR buffer (Applied Biosystems).
4. 25 m*M* $MgCl_2$ (Applied Biosystems).
5. Amplitaq *Taq* DNA polymerase (5 U/μL, Applied Biosystems).

2.2.5. Fragment Analysis

1. Hi-Di™ Formamide (Applied Biosystems).
2. Genescan 500 Rox.
3. ABI Prism 3100 automatic DNA sequencer.

3. Methods

3.1. Basic Unlabeled AFLP of Legionella pneumophila

Although the method described is somewhat labor intensive, the procedure was thoroughly evaluated in multicenter studies *(8)*.

3.1.1. DNA Isolation

1. *Legionella* strains are subcultured onto BCYE agar plates for 48–72 h at 37°C in a moist environment.
2. Harvest bacterial growth from the plates.
3. Prepare genomic DNA following the instructions for the Nucleon BACC2 DNA Extraction kit.

3.1.2. Restriction-Ligation Reaction

1. Perform the restriction-ligation reactions at 37°C for 2 h in a total volume of 20 μL. Each mix is composed of approximately 1 μg of genomic DNA, 200 ng of each adaptor-oligonucleotide (LG1 and LG2), 20 units of *Pst*I, 1 units of T4 DNA ligase, 1X ligation buffer.
2. Precipitate the tagged DNA fragments by using a final concentration of 2.5 *M* ammonium acetate in 100 μL and an equal volume of chilled (4°C) absolute ethanol. Incubate for 5 min at room temperature and centrifuge at 12,000*g* for 10 min at 4°C. Wash pellet once with 70% ethanol. Air-dry the precipitate and resuspend in 100 μL TE buffer.
3. Store resuspended DNA below –20°C.

3.1.3. Polymerase Chain Reaction

1. Immediately prior to the PCR reaction, prepare a dilution (typically 1:100) of the DNA in nuclease-free water (Sigma). Mix well and use 5 μL of this dilution as template DNA in the PCR reaction.
2. PCR is performed in a standard reaction mixture of 50 μL. Each reaction mix is comprised of template DNA (ca. 1 ng), 150 ng of each selective primer, 1 unit of *Taq* DNA polymerase, 20 m*M* Tris-HCl (pH 8.3), 100 m*M* KCl, 5 m*M* $MgCl_2$, 100 m*M* (each) deoxynucleoside triphosphate, and 0.02% (w/v) gelatin. Overlay with 1 drop mineral oil to prevent evaporation or the use heated-lid option, if available, without mineral oil.
3. Amplification is performed using the following parameters: 33 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 2.5 min.

3.1.4. Gel Electrophoresis

1. Amplified products (usually 5 μL of PCR mix) are separated by standard electrophoresis on 1.5% (w/v) agarose gels in 1X TBE at 100 V for 4 h (3.45 V/cm). Molecular size markers (GeneRuler DNA Ladder Mix) are used at 0.1 μg per millimeter lane width. To aid normalization of the gels each sample lane is adjacent to a marker lane.
2. Stain the gel with ethidium bromide for 30 min; rinse with distilled water.
3. Photograph or digitally record under UV transillumination.

3.1.5. Data Analysis

Data analysis is performed using GelCompar or BioNumerics (Applied Maths). Clustering is performed using the band-based option, and group analysis is performed with the Dice coefficient and the unweighted pair group method with averages (UPGMA) clustering method.

3.2. Fluorescent AFLP (Non-kit)

3.2.1. DNA Isolation

DNA isolation is carried out using the QiaAmp DNA minikit. Proteinase K treatment is not carried out for gram-positive bacteria. For yeast, proteinase K treatment is carried out for 2 h. Although not all yeast cells are lysed in this way, enough intact chromosomal DNA is obtained for AFLP analysis.

3.2.2. DNA Quantification

1. Load 5–10 µL DNA on agarose gel 1% (w/v) in 0.5X TBE buffer.
2. Load 5 µL quantification marker λ DNA 40 ng/µL, 20 ng/µL, 10 ng/µL, and 5 ng/µL. Estimate the amount of DNA by comparing the intensity of the DNA signal with the best fit of the known amount of λ DNA.

3.2.3. Restriction-Ligation Reaction

1. Prepare double-stranded adaptors by incubating the two adaptor primers Ecoad 1 (10 pmol/µL) and Ecoad 2 (10 pmol/µL) or Msead 1 (100 pmol/µL) and Msead 2 (100 pmol/µL) at 65°C for 5 min, slowly cooling to room temperature (e.g., in a PCR apparatus).
2. Add together the following: 5.00 µL target DNA (approx 10–50 ng), 1.00 µL Ligase buffer containing ATP, 1.00 µL NaCl (0.5*M*), 0.50 µL BSA (1 mg/mL), 0.20 µL Mse adaptor (50 pmol/µL), 0.20 µL Eco adaptor (5 pmol/µL), 0.20 µL T4 DNA ligase (80 units), 0.10 µL *Mse*I (1 unit), 0.05 µL *Eco*RI (1 unit), and 1.35 µL distilled water.
3. Incubate 3 h at 37°C.
4. Dilute 1:20 with 0.1X TE buffer.

3.2.4. Polymerase Chain Reaction

1. Add together the following: 5.00 µL target DNA, 0.20 µL dNTP, 1.00 µL 10X PCR buffer, 0.60 µL $MgCl_2$, 1.20 µL Mse primer, 0.40 µL Eco primer (FAM) labeled, 0.20 µL Amplitaq *Taq* DNA polymerase, and 1.40 µL distilled water.
2. Amplification is performed using the following parameters: hold 2 min at 72°C, then 30 s at 94°C, 30 s at 65°C; touchdown: first 12 cycles 0.7°C per cycle down, then 23 cycles at 56°C for 60 sec at 72°C, then hold 10 min at 72°C; finally, hold at 4°C forever.

3.2.5. Fragment Analysis and Interpretation

1. For the ABI Prism 3100 automatic DNA sequencer mix per sample: 2.5 µL PCR product, 22 µL Hi-Di-Formamide, and 0.5 µL Genescan 500 Rox in a special ABI plate (+septa).
2. Incubate 3 min at 95°C. Put directly on ice. Centrifuge briefly to remove air bubbles.
3. For interpretation, *see* **Note 5**.

4. Notes

1. As for all commercial kits the instructions provided by the manufacturer should be followed. See the following Web site for an example taken from the Applied Biosystems AFLP

protocol: http://www3.appliedbioa name=aaa systems.com/cms/groups/applied_markets_support/documents/general-documents/cms_040935.pd.

2. The AFLP protocol is a standard protocol that can be used for many bacteria and other organisms. One of the main practical pitfalls of the method is in obtaining purified DNA of the optimal quality and quantity. Although only a small amount of purified DNA is required for AFLP analysis, several protocols describe a requirement for more than this (approx 1 μg) for the initial restriction/ligation reaction. However, these relatively large amounts of starting material were based on the original procedures for large plant genomes (on the order of 100 Mb) and the first radioactive bacterial fingerprinting procedures. The current protocols for genomic analysis of bacterial genomes (on the order of 5 Mb) show that in practice 10–50 ng of high-quality total genomic DNA is sufficient for AFLP analysis ***(19)***. While more can be used, the presence of excessive amounts of DNA can result in suboptimal, less-reproducible AFLP patterns. This effect will increase when the purity of the DNA is unsatisfactory. This can be overcome by reducing the amount of bacteria included in the DNA isolation. In this way the DNA will reach a higher level of purify, and there will be a sufficient amount of purified DNA for the AFLP. The reason for this effect is due to the fact that too much DNA or suboptimal DNA will not result in complete restriction of the chromosomal DNA within the indicated digestion time. In addition, the quality of the DNA will be influenced by the characteristics of the bacterium. For example, bacteria producing abundant capsular material (e.g., *Pseudomonas*) should be used in even lower amounts after liquid subculture. Although a limited amount of DNA degradation can be tolerated, the best results will be obtained from template DNA of high molecular size. There are many suitable methods of DNA extraction, such as using hexadecyltrimethylammonium bromide and phenol-chloroform ***(20)*** or the guanidine-based isolation procedures described by Pitcher et al. ***(21)***, and many commercial kits. Generally, an RNase step is recommended to remove RNA prior to quantification of DNA using a spectrophotometer (A_{260} = 1 for 50 μg/mL and 40 μg/mL double- and single-stranded DNA, respectively) or dedicated instruments, such as the GeneQuant (GE Healthcare) or Nanodrop ND-1000 (LabTech International).
3. Standard oligonucleotide primers and adaptors can be readily obtained from a variety of commercial sources; fluorogenic-labeled primers for specific platforms are available from a more limited number of suppliers. In our experience, standard purification (i.e., salt free) of unlabeled oligonucleotides and

high-performance liquid chromatographic purification for labeled primers are sufficient. Standard storage conditions recommended by the manufacturers should be followed. Typically concentrated stock solutions are made and kept frozen (below –20°C), and small volumes of dilute working solutions prepared are kept at +4°C for up to 1 wk. The amount of exposure to light of fluorogenic primers should be limited by using darkened tubes for storage and minimizing handling time. Although primers can be stored for several years it is advisable not to use primers older than 1 yr in clinical applications.

4. The choice of restriction enzymes depends on the nature of the bacterium. The most often used combination for AFLP is *Eco*RI/*Mse*I. For agarose-based methods *Pst*I is the enzyme most often used as a single enzyme. Other enzyme combinations are used mainly based on the mole percentage guanine plus cytosine (mol% GC) content of the bacterium since this influences the amount of DNA fragments obtained. For instance, the *Pst*I-*Taq*I combination is better suited for GC-rich genomes. Reduction of too many fragments is accomplished by adding more selective nucleotides. In general, the amount of fragments in a standard AFLP amplification varies from 15 to 45 in labeled and 5 to 10 in unlabeled (agarose) AFLP patterns.
5. AFLP patterns can be interpreted for clinical purposes based on windows of similarity *(7)*. These windows have to be validated for each species. In general, there are three windows: I, identical strains (ca. 90–100% identity); II, different strains, identical species (ca. 35–90% homology); III, different species, identical genus (ca. < 35%). Level I is for strain typing, whereas level II is for species identification. The cutoff values for each window should be determined beforehand. For clinical conclusions infection control information concerning patients and clinical units should always be taken into account.

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Chapter 9

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) for the Genotyping of Bacterial Pathogens

Ibtissem Grissa, Gilles Vergnaud, and Christine Pourcel

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPRs) are DNA sequences composed of a succession of repeats (23- to 47-bp long) separated by unique sequences called spacers. Polymorphism can be observed in different strains of a species and may be used for genotyping. We describe protocols and bioinformatics tools that allow the identification of CRISPRs from sequenced genomes, their comparison, and their component determination (the direct repeats and the spacers). A schematic representation of the spacer organization can be produced, allowing an easy comparison between strains.

Key words: Bacteriophage, CRISPR, database, genotyping, phylogeny, spacer.

1. Introduction

Clustered regularly interspaced short palindromic repeats (CRISPRs) loci typically consist of the succession of 23- to 47-bp repeat elements, the direct repeats (DRs), separated by variable and non-repetitive sequences called spacers (*see* **Fig. 1**). A CRISPR generally possesses at one end a degenerated DR and at the other end a complete DR immediately followed by a sequence called the leader and acting as a promoter ***(1)***. In a single genome several CRISPRs with the same DRs can be found, but only one is associated with a group of four to six genes called *cas* (for CRISPR associated) ***(2)***. The CASS system (a CRISPR and several *cas* genes) has been identified in a broad range of prokaryotic species, almost all archaea, and 40% of bacteria. In the majority of cases the spacers, when identified, happen to be fragments of bacteriophages or

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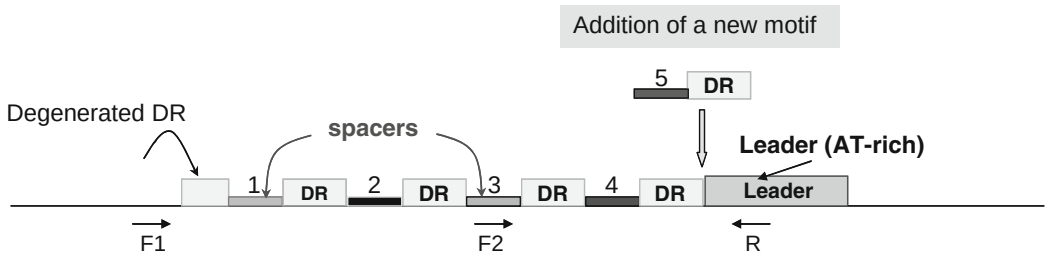


Fig. 1. Schematic representation of a CRISPR locus. The CRISPR is a succession of repeated sequences, the DRs, separated by unique sequences, the spacers. The first DR is often degenerated. Flanking the last DR is a sequence called the leader that acts as a promoter. In this figure the CRISPR is oriented such that the spacer numbered 1 is the oldest, whereas the newly added spacer is next to the leader.

plasmids ***(1,3)***. Different observations suggest that the CASS system constitutes a defense system against foreign sequences.

The CRISPR structure is continuously evolving, either through the addition of new motifs (a DR and a spacer) or by interstitial deletion of one or several motifs through recombination between two DRs. New motifs are added to the CRISPR in a polarized manner by duplicating the DR next to the leader and adding a new fragment of DNA ***(3,4)***. The currently sequenced CRISPR structures listed in CRISPRdb ***(5)*** show an important variability in the nature of DRs and the number of motifs (varying from 1 spacer to 276 in *Chloroflexus aurantiacus* J-10-fl).

Interestingly, within a particular species, comparative analysis of sequences between closely related strains revealed on the first hand a high degree of polymorphism from strain to strain and on the other hand an inheritable nature of spacers from parental strains. Developing a CRISPR typing scheme may thus be a good addition to classical typing techniques applicable for strain differentiation, epidemiological investigations, and phylogenetic reconstruction. We have tentatively used the polymorphism of one CRISPR to propose a phylogeny for *Yersinia pestis*, and we suggested that it could be also used to trace ancient bacterial DNA ***(3,6)***. To date, CRISPR typing has been used in a limited range of species: *Streptococcus pyogenes* ***(7)***, *Campylobacter jejuni* ***(8–10)***, *Yersinia pestis and Y. pseudotuberculosis* ***(3,6)***, *Thermotoga maritima* ***(11,12)***, *Corynebacterium diphtheriae* ***(13,14)***, *Streptococcus thermophilus* ***(15,16)***, and *Lactococcus casei* ***(17)***, but it has been mainly used in *Mycobacterium tuberculosis* ***(18)***. Indeed, the so called DR locus in *M. tuberculosis* is in fact a CRISPR element with which diversity inside the species is analyzed with the spoligotyping method (*see* **Chapter 10**). Spoligotyping only investigates the presence/absence of known spacers by hybridization and is well suited for a DR locus that is not acquiring new motifs (such as in *M. tuberculosis*) or when an extensive survey of the CRISPR diversity inside a species has been performed. In species with one or several rapidly evolving CRISPRs, polymerase chain

reaction (PCR) analysis and sequencing of these loci remain the best approach to investigate their diversity.

2. Materials

2.1. DNA Purification

Good-quality DNA should be available as CRISPRs may sometimes be large, and long-range PCR amplification is required.

1. The Qiagen DNeasy® tissue kit has been successfully used for different bacterial species.
2. The quality and concentration of DNA should be measured (e.g., using an ND-1000 spectrophotometer, NanoDrop®, Labtech, France).

2.2. PCR Amplification

1. Standard *Taq* polymerase (Qiagen, Roche, Promega, or Invitrogen).
2. The Qiagen kit provides the Q solution and corresponding buffer for amplification of GC-rich DNA. Alternatively, 0.5 *M* betain (Sigma) can be used in the PCR reaction.
3. dNTPs (deoxnucleotide 5′-triphosphates) (Eurogentec or MWG Biotech).
4. Reaction buffer is as recommended by the *Taq* polymerase manufacturer. The concentration of $MgCl_2$ in the reaction is 1.5 μ*M*.
5. Oligonucleotides are dissolved at 100 μ*M* in 10 m*M* Tris-HCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), pH 7.8.

2.3. Agarose Gel Electrophoresis

1. Standard molecular biology-grade agarose (from Invitrogen, Sigma, or Q-BIOgene).
2. Tris-borate EDTA (TBE) buffer: The 10X stock solution is 890 m*M* Tris-borate and 20 m*M* EDTA, pH 8.3 (Sigma).
3. The DNA size marker is the 100-bp ladder (from Bio-Rad, MBI Fermentas, or Euromedex).
4. Ethidium bromide stock solution, 10 mg/mL (Sigma).

2.4. Sequencing

1. PCR products are purified using the QIAquick PCR purification kit (Qiagen) or precipitated with a solution of PEG8000 20% (w/v), 2.5 *M* NaCl (*see* **Note 1**) ***(19)***.
2. Sequencing is performed using the primers used for PCR.

2.5. Data Analysis

The Web-based tools necessary for CRISPR identification and comparison are freely accessible at http://crispr.u-psud.fr/CRISPRcompar/. The output of the analysis consists of different Excel and text files.

3. Methods

The development of a CRISPR genotyping assay follows three main phases: the preprocessing phase, the typing assay *per se*, and the *in silico* postprocessing phase.

3.1. Preprocessing Phase

In the preprocessing phase, an *in silico* investigation is performed to find CRISPRs that could be potential typing markers. This is achieved by first checking whether the studied species harbors at least one CRISPR, using tools designed to identify these particular repeated sequences. It is thus necessary to have access to the genome sequence of at least one strain (even in an unassembled phase). When several CRISPRs are present in a single genome, they should be clearly differentiated, and primers should be designed to amplify specifically each locus. Since their relative positions in the genome may vary from strain to strain due to large-scale DNA rearrangements, CRISPR labels are assigned in each strain.

3.1.1. Checking for CRISPR Presence

1. Consult the CRISPRdb database (http://crispr.u-psud.fr/crispr/CRISPRdatabase.php?page=tax). Structures marked in pink correspond to confirmed CRISPRs and can be retained for further analyses (*see* **Note 2**).
2. Nonpublic sequenced genomes (or even contigs) can be submitted as a FASTA file to the CRISPRFinder program (http://crispr.u-psud.fr/Server/CRISPRfinder.php). The detected CRISPRs will be displayed either as confirmed or questionable, according to characteristics described by Grissa et al. *(20)* (*see* **Note 3**).

3.1.2. Interspecies Comparison of CRISPRs

When the genome of several strains of a given species has been sequenced, the CRISPRs, if any, can be classified and identified in each sequenced genome.

1. Go to the CRISPR Comparison Page (http://crispr.u-psud.fr/CRISPRcompar/), which analyses CRISPRs present in CRISPRdb.
2. Activate "Compare the CRISPRs of two or several genomes."
3. Select the strains to be compared either by browsing the strain taxonomy list or from the alphabetical list and click on the comparison button. The related CRISPRs will be labeled and displayed in a table. Each line corresponds to a CRISPR; the CRISPR ID, its position, and the number of spacers are given in the columns. An alignment of the flanking sequences is given when a locus is present in two strains.

4. In case that a locus is present in two strains, a link to the Spacer Dictionary Creator tools is available via the button "compare spacers." It is now possible to select the CRISPR to be typed according to the spacers' polymorphism and their number (*see* **Note 4**).

3.2. CRISPR Genotyping

It is necessary to get a rapid idea of a CRISPR's potential use as a genotyping tool as important variations have been observed between different species. Some CRISPRs may be present only in a subset of strains or they might show very little polymorphism. Indexing the marker's polymorphism is achieved by amplifying the CRISPR locus in a selected set of strains to check if the PCR products vary in size.

3.2.1. Assessing the CRISPR Polymorphism

1. Design PCR primers from the flanking sequences' alignment when several alleles of the same CRISPR are available. Otherwise, 20- to 30-bp long oligonucleotides can be picked in the flanking sequences at least 40 bp away from the first and last DR (**Fig. 1**, primers F1 and R) (*see* **Note 5**).
2. Alternatively it can be decided to analyze only the CRISPR portion that is growing by addition of new motifs. In this case a primer is chosen in the flanking region containing the leader and the other in one of the spacers (**Fig. 1**, primers F2 and R) (*see* **Note 6**).
3. Check the presence/absence of a CRISPR region and its polymorphism on a representative subset of 10–15 strains, including when possible a sequenced reference strain (*see* **Note 7**).

The following PCR conditions are routinely used: PCR reactions are performed in 15 μL of 1–5 ng of DNA, 1X reaction buffer, 1.5 m*M* $MgCl_2$, 3 U *Taq* DNA polymerase, 200 μ*M* of each dNTP, 0.3 μ*M* of each flanking primer. Amplification is performed using the following conditions: initial denaturation cycle for 5 min at 94°C, 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C to 60°C depending on the oligos, and elongation for 45 to 60 s at 72°C, plus a final elongation step for 10 min at 72°C. The PCR products are analyzed on a 2% (w/v) agarose gel in 0.5X TBE buffer, run at 8 V/cm. A variation in the PCR products' size reveals variation in the spacer content between strains, suggesting that this method is promising for strain discrimination of the studied bacterial species (*see* **Note 8**).

3.2.2. Setting a CRISPR Genotyping Assay

The CRISPR alleles of a larger collection of strains can now be analyzed by sequencing to get an idea of the spacer diversity and provide a catalogue of these spacers.

1. For sequencing of the amplicons, perform a PCR amplification in a total volume of 45 μL by multiplying all the reagents threefold.

2. To assess the efficiency of the PCR reaction, 2 μL of PCR products are run on a 2% agarose gel (*see* **Note 9**). Electrophoresis is performed in 0.5X TBE buffer run at 8 V/cm.
3. Purification of PCR products can be performed using dedicated kits. Alternatively, polyethylene glycol (PEG) precipitation can be performed (*see* **Note 10**). For this, the PCR reaction is transferred into a 1.5-mL Eppendorf tube, and 0.6 volume of PEG8000/NaCl solution is added. After 10 min at 37°C the tubes are centrifuged for 10 min at 12,148*g*. The PEG/NaCl is carefully removed by pipeting, avoiding the usually invisible DNA pellet, and 500 μL 80% ethanol is added. Centrifuge for 10 min at 12,148*g*, pour the ethanol, and dry the pellet.
4. Sequence the DNA using the primers used for PCR amplification. Use 10 to 20 ng purified PCR products in a sequencing reaction for a 100-bp sequence. Thus, to sequence a 500-bp PCR product, a 50- to 100-ng DNA must be used. PCR products and sequencing primers are sent for custom DNA sequencing to a specialized company (MWG Biotech, Germany, for example).

3.3. In Silico *Postprocessing Phase*

In the *in silico* postprocessing phase the spacers are identified from the sequenced alleles, annotated, compared to previously known spacers, and stored in a database also called a *dictionary*. Appropriate bioinformatics tools are available on the CRISPR Web service http://crispr.u-psud.fr/) to analyze the CRISPR sequencing data without any requirements in programming or computer skills ***(6)***. The site is called CRISPRtionary (*see* **Note 11**).

3.3.1. CRISPRtionary, the Spacer Dictionary Creator

1. Go to the corresponding page at http://crispr.u-psud.fr/CRISPRcompar/Dict/Dict.php.
2. Submit sequences in FASTA format, that is, for each sequence, the first line starts with a greater than sign ">" and contains a unique identifier per sequence. This is the sequence header, which must be in a single line. It is possible to put additional fields in the header separated by a pipeline "|"; these fields will be especially useful in the final output files.
3. If a catalogue of annotated spacers is already available in the literature or in a previous study in the laboratory, it is recommended to use this catalogue as a spacers' dictionary; that is, use an Excel file fulfilling the following properties (*see* **Note 12**):

The first row should contain column labels.

The first column should contain the spacer labels.

The second column may contain alternative labels or information about the spacers.

The third column should contain the spacer sequences.

The three first columns should not be empty and should not contain skipped rows.

4. If a previous Excel dictionary is used, check the appropriate sheet. If no dictionary is uploaded, press the "continue" button, and a file will be created.
5. The CRISPRFinder program is applied for each introduced sequence, separating DR and spacers. Several slightly different DR sequences might be proposed showing some nucleotide differences (especially with short arrays) or provided in the reverse complement orientation (when the CRISPR is present on the antisense strand) (**Fig. 2A**). Therefore, the user should select or introduce the appropriate DR sequence oriented such that the leader position is on the right. Short CRISPR sequences with degenerate DRs may not be displayed at this step, but they will be recovered in later stages.
6. After the DR selection, activate the "Find spacers" button. The CRISPR alleles are coded in a compact way by querying and updating the dictionary (**Fig. 2B,C**). When a spacer is already present in the dictionary, its code appears in the output, but when a new spacer is identified, it is numbered and added to the Excel file. The second column of the dictionary is also updated by indicating for each spacer the locus name to which it belongs and its occurrence order in this allele. Different loci names will be separated by an underscore "_" and orders by a colon ":"; for example, the spacer "f" of *Y. pestis* is the sixth spacer in the CO92 strain and is the third in the strain biovar Microtus str- 91001, so it will be coded in the second dictionary column as:"f_Yersinia_pestis_CO92:6_Yersinia_pestis_biovar_Microtus_str-_91001:3."

3.3.2. Collecting the Results of the Analysis

The results are displayed on the screen and are stored in a user-friendly database (downloadable Excel and text files) (*see* **Note 13**):

AnnotFasta: A text file representing the corresponding CRISPRs. Each motif (DR + spacer) is written on a separate line; the DR and spacer are separated by a tabulation and followed by the spacer label (*see* **Note 14**).

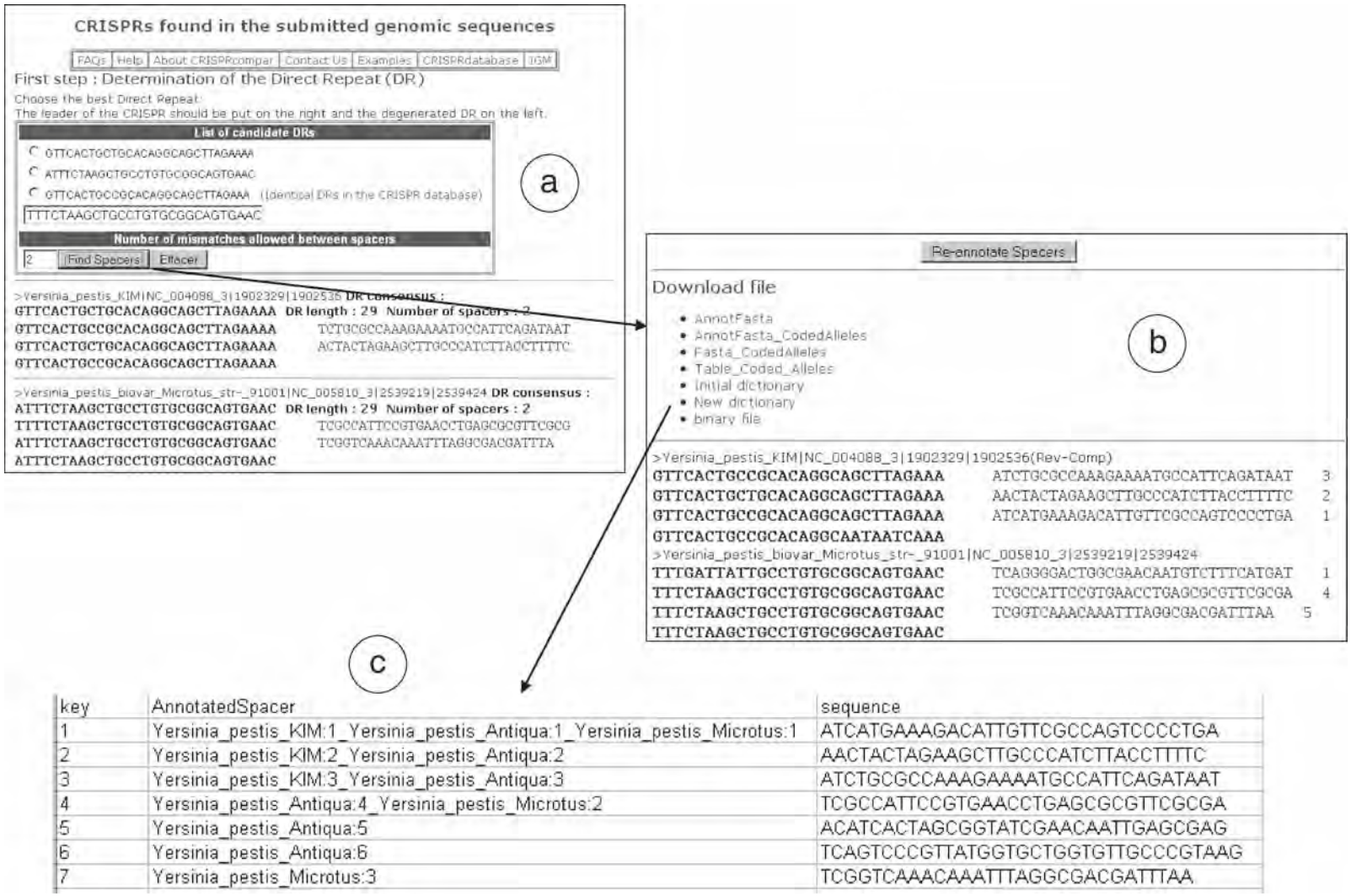
AnnotFasta_CodedAlleles: The same file as the previous one in addition to the spacer codes in the header separated by dots.

Fasta_CodedAlleles: The previous file represented in FASTA format.

Table_Coded_Alleles: Excel file representing one allele per row. The header information (separated by a pipeline in the submitted sequences) is presented in separate columns. The last column provides the spacer labels separated by dots.

Initial dictionary: The initial uploaded dictionary.

New dictionary: The updated dictionary.



key	AnnotatedSpacer	sequence
1	Yersinia_pestis_KIM:1_Yersinia_pestis_Antiqua:1_Yersinia_pestis_Microtus:1	ATCATGAAAGACATTGTTCGCCAGTCCCCTGA
2	Yersinia_pestis_KIM:2_Yersinia_pestis_Antiqua:2	AACTACTAGAAGCTTGCCCATCTTACCTTTTC
3	Yersinia_pestis_KIM:3_Yersinia_pestis_Antiqua:3	ATCTGCGCCAAAGAAAATGCCATTCAGATAAT
4	Yersinia_pestis_Antiqua:4_Yersinia_pestis_Microtus:2	TCGCCATTCCGTGAACCTGAGCGCGTTCGCGA
5	Yersinia_pestis_Antiqua:5	ACATCACTAGCGGTATCGAACAATTGAGCGAG
6	Yersinia_pestis_Antiqua:6	TCAGTCCCGTTATGGTGCTGGTGTTGCCCGTAAG
7	Yersinia_pestis_Microtus:3	TCGGTCAAACAAATTTAGGCGACGATTTAA

Fig. 2. Output of the spacer annotation and dictionary creator tools. Analysis of a CRISPR in three *Y. pestis* strains: (**a**) output using the CRISPRtionary tool in which several candidate DRs are proposed; (**b**) the annotated spacers and a list of files that can be downloaded; (**c**) a dictionary of annotated spacers and their position in the three strains.

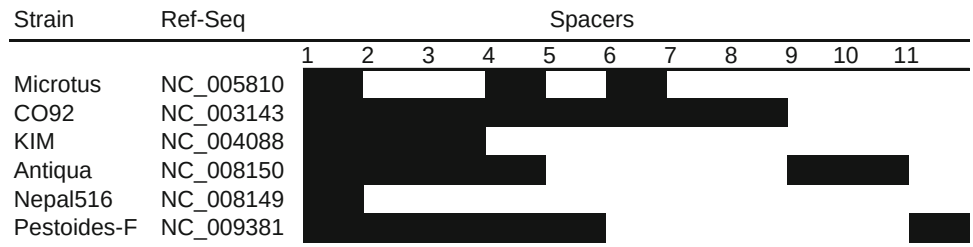


Fig. 3. Organization of the CRISPR locus in six *Y. pestis* strains. Using the binary file of the reannotated spacers, a schematic representation of the CRISPR is shown in which a black box indicates the presence of the spacer.

Binary file: Excel file in which columns represent the spacer labels and rows represent the queried alleles. For each CRISPR allele, a spacer will have a value 1 when it exists and 0 when it is absent. The binary file is especially interesting for providing a spoligotyping profile of the CRISPR and to visually illustrate the spacers' composition in the strains (**Fig. 3**).

3.3.3. Reannotating the Spacers

The obtained codes of newly added spacers to the dictionary are usually not ordered in a coherent way because the spacer labels are added according to the introduced alleles order.

1. To readjust them and obtain ordered numbers according to spacer acquisition by the CRISPR, use the "Re-annotate spacers" button. This will open the page http://crispr.u-psud.fr/cgi-bin/crispr/ReannotateSpacers.cgi.
2. Introduce a dictionary and the table-coded allele files or simply use the option "use previous files," and a new annotation of the spacers will be produced. In fact, the first spacer next to the degenerated DR will be coded "one," the next one "two," and so on (*see* **Note 15**).

4. Notes

1. This solution is very viscous. Cut the end of the pipet tip so that the section is wider, thus facilitating aspiration.
2. The smaller CRISPRs detected by CRISPRFinder consist of a leader and two DRs (a complete and a degenerated one) separated by a spacer. Large ones can contain more than 200 repeats. The presence of a CRISPR in a strain does not preclude its existence in all the members of the species.
3. Critical examination of the sequences must be performed as in some cases a confirmed CRISPR may in fact be a tandem repeat structure. In such "CRISPRs," the spacers will show a high degree of similarity.

4. It is preferable in a first step to avoid nonpolymorphic spacers and long CRISPRs (more than 60 spacers) because of technical constraints.
5. It is important for the spacer identification that the DRs on both ends of the CRISPR be included in the sequence.
6. Some bacterial species have rapidly evolving CRISPRs as a response to a quickly changing selection pressure associated with phage predation (such as bacterial strains present in food product and dietary supplements; ***15***). Hence, sequencing the extremity adjacent to the leader could be sufficient to differentiate and identify strains.
7. Carefully select the strain panel such that the control isolates belong to discernable genotypes (determined by another genotyping method, for example).
8. In some cases, PCR amplification fails with part of the tested strains due to either absence of the locus or high genetic divergence of the primer sequences.
9. Do not add the sample loading solution into the PCR tube as this might interfere with sequencing.
10. This protocol is very efficient for PCR products equal to or larger than 300 bp. It is rapid and cheap. It was described in **ref.** ***19***.
11. Now three sample dictionaries are available online with this tool: *Y. pestis* dictionary of 26 spacers ***(3)***, *C. jejuni* dictionary with 59 spacers ***(10)***, and *S. thermophilus* dictionary ***(15,21)*** with 328 spacers.
12. This is illustrated with a demonstrator based on the sequences of five *Y. pestis* genomes. An initial spacer catalogue was first created from the 26 published spacers, named using the alphabet from *a* to *z* ***(3)***.
13. The obtained files may be used to store the CRISPRs in a BioNumerics database (Applied Maths).
14. Rev-Comp option is added in the header when the DR orientation corresponds to the sequence on the antisense strand.
15. After observing the CRISPR diversity among the strains, the biologist may formulate hypotheses about the locus evolution. If a restricted number of spacers is present in all the strains with many internal absences, it may be postulated that the CRISPR locus evolves only by interstitial deletions. It is apparently the case for the *M. tuberculosis* CRISPR. Otherwise, when there is an important diversity of spacers next to the leader, it may be assumed that the CRISPR is still active and able to acquire new motifs.

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Chapter 10

Spoligotyping for Molecular Epidemiology of the *Mycobacterium tuberculosis* Complex

Jeffrey R. Driscoll

Abstract

Spacer oligonucleotide typing, or spoligotyping, is a rapid, polymerase chain reaction (PCR)-based method for genotyping strains of the *Mycobacterium tuberculosis* complex (MTB). Spoligotyping data can be represented in absolute terms (digitally), and the results can be readily shared among laboratories, thereby enabling the creation of large international databases. Since the spoligotype assay was standardized more than 10 yr ago, tens of thousands of isolates have been analyzed, giving a global picture of MTB strain diversity. The method is highly reproducible and has been developed into a high-throughput assay for large molecular epidemiology projects. In the United States, spoligotyping is employed on nearly all newly identified culture-positive cases of tuberculosis as part of a national genotyping program. The strengths of this method include its low cost, its digital data results, the good correlation of its results with other genetics markers, its fair level of overall differentiation of strains, its high-throughput capacity, and its ability to provide species information. However, the method's weaknesses include the inability of spoligotyping to differentiate well within large strain families such as the Beijing family, the potential for convergent evolution of patterns, the limited success in improving the assay through expansion, and the difficulty in obtaining the specialized membranes and instrumentation.

Key words: Epidemiology, genotyping, mycobacteria, spoligotyping, tuberculosis.

1. Introduction

DNA fingerprinting or genotyping of *Mycobacterium tuberculosis* complex (MTB) strains became a priority in the United States when in the early 1990s a staggering increase in cases of multidrug-resistant tuberculosis (TB) was observed in New York City *(1)*. Epidemiologists needed to know which cases were linked and where transmissions were occurring. They also needed to determine the size of the outbreak and to try to prevent further

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transmissions. The primary genotyping method available at the time, insertion element (IS) *6110*-based restriction fragment length polymorphism (RFLP) analysis *(2)*, provided excellent differentiation but required specialized software for analysis of the data as well as relatively long turnaround times for reporting of the results. Weeks or months could be required for the level of growth in culture necessary for performance of RFLP analysis. Data analysis required specialized matching software and expert interpretation for relating similar, but not identical, patterns.

Genotyping methods that could employ amplification of nucleic acids were assessed in efforts to develop an alternative to RFLP analysis. The first widely adopted polymerase chain reaction (PCR)-based method for genotyping was spacer oligonucleotide typing or spoligotyping. Kamerbeek et al. *(3)* described a reverse-hybridization protocol to assay for the presence or absence of 43 specific DNA spacer sequences in the direct repeat (DR) region that had been identified in the strains *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG (**Fig. 1**). The majority of the 43

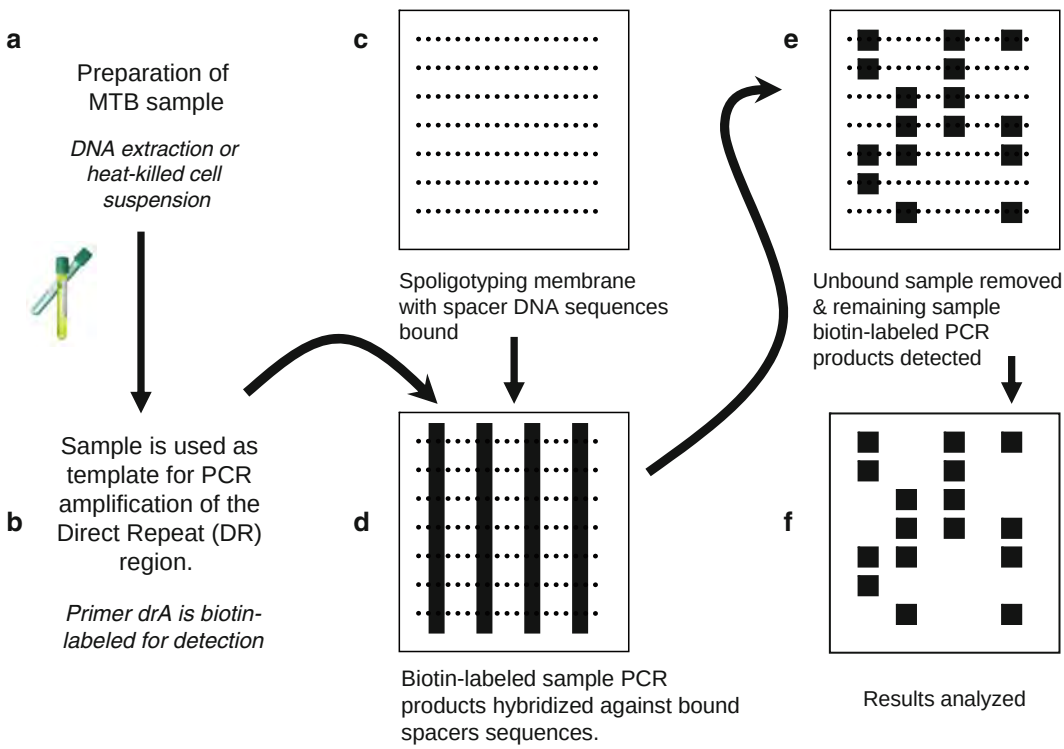


Fig. 1. Basis of the spoligotyping methodology. (**a**) A spoligotyping membrane. Dashed lines indicate the location of the bound polymorphic oligonucleotides, one corresponding to each of the 43 unique spacer sequences utilized in the assay. (**b**) The hybridization of the amplified samples (black bars) against each of the bound oligonuleotides. (**c**) The excess and nonspecifically bound sample is removed through a series of washes, and the remaining bound PCR products from the sample are detected. (**d**) A representation of the final results.

spacers were present in both H37Rv and BCG, but spacers 20, 21, and 33–36 were not present in H37Rv, and spacers 3, 9, 16, and 39–43 were missing in strains of BCG.

The DR region consists of a repeated 36-bp sequence interspersed with nonrepetitive 31- to 41-bp long DNA segments called spacer sequences *(4)*. Spoligotypes evolve through the loss of spacer sequences, presumably through homologous recombination of the DRs and excision of the recombined material during DNA replication. Spacer sequences can also appear to be lost through rearrangements by ISs like IS*6110*. Once spacers are lost, they are not regained, so the evolution is unidirectional. This unidirectional evolution through loss of spacers offers a clear model for evolution but also presents a challenge since a strain's spoligotype can evolve in such a way that it comes to resemble the signature associated with a different spoligotype family. The ability to encode spoligotyping data in a numerical format (**Fig. 2**) *(5)* immediately made the results readily shareable among laboratories and enabled the creation of an international database (SpolDB) *(6)*. This development allowed investigators to survey strain diversity and uncover global strain families, such as the Beijing and the Latin America Mediterranean (LAM) families.

Spoligotyping has been very successful in providing a tool for the rapid acquisition of MTB genotyping information and for the establishment of a global picture of MTB diversity *(6)*.

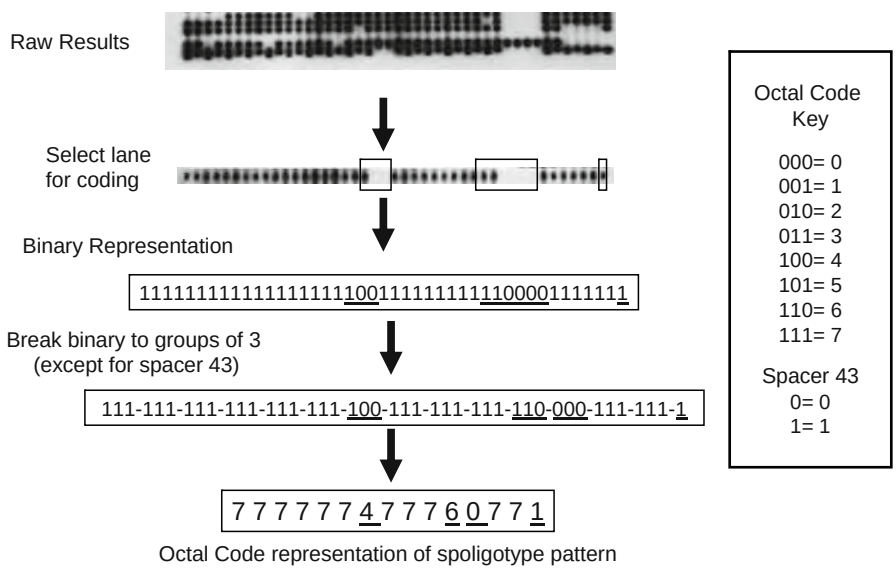


Fig. 2. Conversion of a raw spoligotype results to octal code representation *(5)*. The raw hybridization pattern is converted to a binary representation using 1's (indicating hybridization detected) and 0's (no hybridization detected). The binary string is separated into 14 groups of three, with spacer 43 remaining ungrouped. Each binary triplet is converted to the appropriate octal code designation (shown). The 15th digit of the octal is either 1 or 0 depending solely on the result for spacer 43.

However, the need still exists for supplemental genotyping information. The availability of multiple genotyping markers allows us to be able to "zoom in" to establish specific potential patient-to-patient transmissions and "zoom out" to examine regional and global trends in the spread of tuberculosis strains *(7)*. Alternative DNA fingerprinting methods may supplant spoligotyping in the future if more powerful markers are identified, widely adopted, and their patterns collected into large collaborative databases. For the present, the combination of spoligotype and mycobacterial interspersed repetitive unit (MIRU) data provides a good basis for molecular epidemiology *(8)*, although IS*6110*-based RFLP can still be required in a number of cases for optimal genetic cluster analysis *(9)*.

1.1. Species Identification Within the M. tuberculosis *Complex*

The MTB complex is made up of a group of closely related species: *Mycobacterium africanum*, *M. bovi*s, *Mycobacterium caprae*, *M. tuberculosis, Mycobacterium microti*, *Mycobacterium canettii*, and *Mycobacterium pinnipedii*. The presence or absence of certain spacer sequences acts as a signature for presumptive species identification *(10)*. For example, *M. bovis* isolates do not hybridize to spacers 39–43 but do generally hybridize to spacers 33–36 *(3)*. *Mycobacterium africanum* isolates do not hybridize to spacers 8, 9, and 39 but do generally hybridize to spacers 33–36. *Mycobacterium microti*, *M. canettii,* and *M. pinnipedii* have very different spoligotype patterns from the members of the MTB complex, which are more associated with human infections. These three species typically hybridize to few if any, in the case of M. Canettii of the traditional 43 spoligotyping spacers *(6,11)*.

1.2. Selective Versus Universal Genotyping

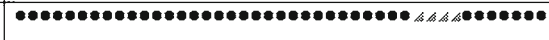
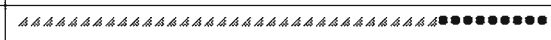
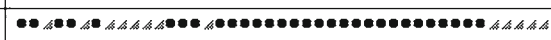
For a public health program, the choice between genotyping only certain MTB strains (selective genotyping) and genotyping every isolate (universal genotyping) comes down to cost issues and the capability to integrate the data to into program activities. The benefits of universal genotyping include earlier identification of false-positive MTB cultures (e.g., due to laboratory cross-contamination), discovery of unsuspected cases of MTB transmission (i.e., linking patients who had not previously been identified as contacts through conventional methods), confirmation of species identification within the MTB complex, and capability to generate a database to examine strain diversity in a particular region for monitoring program success in the control of tuberculosis *(1)*. Universal genotyping enables shorter turnaround times inasmuch as a method like spoligotyping can be performed as a routine activity in the laboratory workup of a patient's MTB strain *(12)*. Selective genotyping, in contrast, can entail requests for analysis of isolates weeks or months after the clinical mycobacteriology laboratory has received the specimen, and retrieval from archival storage may be difficult.

1.3. False Clustering Due to Commonality of Spoligotypes

In areas where particular genetic families are grossly dominant in a TB population, such as Beijing in East Asia *(13)*, spoligotyping without additional genotyping information is of limited value *(8,14)*. The Beijing spoligotype is highly stable, and variants are rarely observed.

Knowledge of the MTB strain diversity in an area is important in establishing the significance of genotyping matches for all fingerprinting methods, but this is especially true for spoligotyping *(15)*. **Table 1** lists the ten most commonly observed spoligotypes in SpolDB4 *(6)*. Generally, a finding that two patients match by one of these spoligotypes does not in of itself prove that the two strains are identical. Additional genotyping data acquired through MIRU or RFLP analysis is required in most cases to establish the significance of a typing match. However, in a well-characterized population, the appearance of two strains with a matching unique spoligotype pattern is likely to be significant, especially if other factors (**Fig. 3**) are present.

Table 1
Ten Most Frequently Observed Spoligotype Patterns in the Fourth International Spoligotype Database (SpolDB4)

Lineage	Spoligotype	Octal Code	SpolDB4 Frequency
T1		777777777760771	17.85
Beijing		000000000003771	11.28
BOV2		664073777777600	7.71
BOV1		676773777777600	6.68
Haarlem3		777777777720771	5.46
LAM9		777777607760771	5.37
Haarlem1		777777774020771	3.44
CAS1-Delhi		703777740003771	3.00
X2		777776777760601	2.98
EAI5		777777777413771	2.82

Source: From **ref. 6.**

For spoligotype hybridization patterns, a closed circle indicates hybridization observed at that spacer sequence, and a gray triangle indicates no hybridization. The spacers 1 through 43 are shown in sequence from left to right.

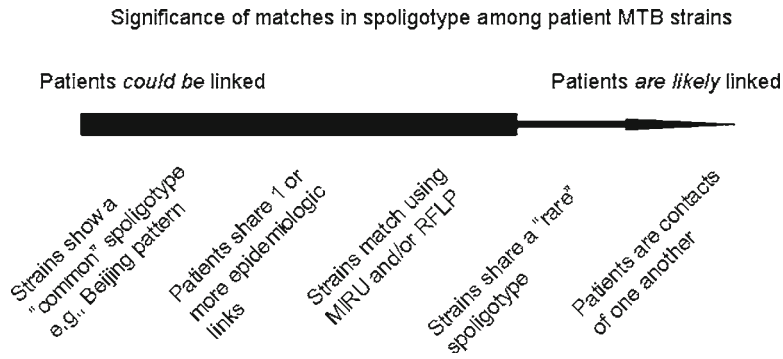


Fig. 3. Factors that aid in the assessment of the significance of a match between spoligotypes. Examples of genotyping and epidemiological data that are useful in deciding whether patients with matching spoligotypes are linked.

1.4. Application to Epidemiology

1.4.1. Application of Spoligotyping to Public Health Programs

With tuberculosis control programs incorporating genotyping data into their routine activities, the need to generate data as early as possible became important in order to direct contact investigations and to identify cases of false-positive cultures (e.g., laboratory cross-contamination) *(16)*. The New York City program was the first large-scale attempt to achieve this *(1)*. The largest genotyping program currently in operation is in the United States. It was developed by the Centers for Disease Control and Prevention, which has also developed a manual for implementation of genotyping data into routine tuberculosis control practice by state and local health departments *(17)*. The ability to obtain spoligotype results from early growth cultures, or even primary specimens, means a "real-time" approach to MTB genotyping is possible *(18)*. However, a single approach to the analysis of genotyping data for every program does not work. A program in an area with a low incidence of TB may find few matches among patient isolates, suggesting a low occurrence of recent transmissions *(19)*. In a high-incidence area a program will encounter a greater number of MTB strains with the more frequently observed spoligotypes (**Table 1**). This obscures the picture of recent transmission versus distant transmission *(1)*, thereby necessitating the use of additional genotyping assays, such as RFLP and MIRU *(18)*.

1.4.2. Identification of False-Positive Cultures of Tuberculosis

Laboratory cross-contamination of patient samples continues to be a problem that results in false diagnoses of tuberculosis. Among the most obvious examples have been laboratory cross-contamination events with the common laboratory strains H37Ra and H37Rv affecting determinations for multiple patient specimens *(17,20,21)*. H37Ra and H37Rv were derived from the same parent isolate, H37, collected in 1905. They share the

same spoligotype and have similar although not identical RFLP patterns *(21)*. To date, there have been no reports of a true patient isolate sharing the H37 spoligotype. Therefore, when a patient isolate is found to have a spoligotype matching H37, a laboratory cross-contamination event is likely to have occurred *(17)*. Cross-contamination of a patient's sample by a different patient's sample or mislabeling errors at the site of collection or the laboratory often requires further investigation, typically involving performing additional genotyping assays and review of patient clinical data *(17)*. It is important to remember that confirmation of a false-positive or cross-contaminated MTB specimen applies solely to the culture results; the diagnosis of tuberculosis in the patient is still made based on the entire clinical presentation.

2. Materials

2.1. Stock Buffers

1. 20X SSPE: 0.2*M* Na_2HPO_4, 3.6*M* $NaCl_2$, 20 m*M* ethylenediaminetetraacetic acid (EDTA), final pH should be 7.4–7.6, autoclaved and stable for 1 yr.
2. 0.5*M* EDTA, pH 8.0, autoclaved and stable for 1 yr.
3. 10% (w/v) sodium dodecyl sulfate (SDS), made fresh as required.

2.2. Polymerase Chain Reaction

Primers for DR region amplification: DRa (GGTTTTGGGTCTGACGAC, 5′ biotinylated) and DRb (CCGAGAGGGGACGGAAAC). Store reconstituted DRa and DRb and post-PCR products at 4°C (*see* **Note 1**).

2.3. Hybridization

1. Spoligotyping membrane (Ocimum Biosolutions Inc., formerly Isogen Biosciences B.V., Hyderabad, India).
2. MN45 miniblotter and support cushions (Immunetics, Inc., Boston, MA).
3. Rotating hybridization oven.

2.4. Detection

1. 500 U streptavidin-peroxidase conjugate (Roche Diagnostics, Indianapolis, IN), resuspended in 1 mL H_2O.
2. Enhanced chemiluminescence (ECL) detection reagents 1 and 2 (GE Healthcare Life Sciences, Piscataway, NJ).
3. X-ray film.
4. X-ray film developer.

3. Methods

The spoligotyping assay is currently performed by one of two methods. The most commonly employed method (**Fig. 1**) utilizes a nylon membrane to which 43 different oligonucleotides, corresponding to the 43 unique spacer sequences, have been individually bound *(3)*. A second method utilizes a high-throughput, multianalyte flow system (Luminex) *(22)*, permitting analysis of high numbers of strains without the need for membranes.

Detailed instructions on how to manufacture spoligotyping membranes have been previously published *(23)*. Commercially prepared spoligotyping membranes are commonly used (*see* **Note 2**). A wide variety of samples is suitable for the PCR reaction. Extracted DNA, heat-killed cell suspensions from growth medium, and even primary specimens have been successfully used as templates in PCR reactions *(3)*.

3.1. PCR Amplification of the DR Region

1. Prepare a 25-µL PCR reaction using 1–5 µL of cell suspension or 0.5–1 µL of extracted genomic DNA. A wide range of template concentrations seem suitable for DR region amplification.
2. Use the following PCR conditions: 3 min at 96°C, followed by 20 (extracted DNA) to 30 (cell suspension) cycles of 1 min at 96°C, 1 min at 55°C, and 30 s at 72°C, final extension of 5 min at 72°C (*see* **Note 3**).

3.2. Hybridization of PCR Samples to Spoligotyping Membrane

1. Add 150 µL of 2X SSPE/0.1% SDS to each tube containing the 20–25 µL post-PCR products (*see* **Note 4**).
2. Heat denature the diluted PCR products for 10 min at 100°C and cool on ice water for 2 min.
3. Wash the spoligotyping membrane for 5 min at 60°C in 2X SSPE/0.1% SDS.
4. Place the membrane and a support cushion into the miniblotter in such a way that the slots are oriented perpendicular to the line pattern of the applied oligonucleotides (*see* **Note 5**).
5. Fill the slots of the diluted PCR product (avoid air bubbles) and hybridize for 1 h at 60°C (*see* **Note 6**).

3.3. Posthybridization Steps

1. Following hybridization, remove the samples from the miniblotter by aspiration.
2. Wash the membrane twice in 2X SSPE/0.5% SDS for 10 min per wash at 60°C.
3. Place the membrane in a rolling bottle and allow it to cool to prevent inactivation of the peroxidase in the next step.

4. Add 2.5 μL of 500 U/mL streptavidin-peroxidase to 10 mL of 2X SSPE/0.5% SDS and add to roller bottle (*see* **Note 7**). Incubate the membrane in this solution for 45–60 min at 42°C with rotation (*see* **Note 8**).
5. Following this incubation, wash the membrane twice in 2X SSPE/0.5% SDS for 10 min per wash at 42°C.
6. Rinse the membrane twice with 2X SSPE for 5 min per wash at room temperature.

3.4. Chemiluminescent Detection

1. For chemiluminescent detection of hybridizing DNA, incubate the membrane for 1 min in 10 mL ECL detection reagent 1 mixed with 10 mL ECL detection reagent 2 at room temperature.
2. Briefly blot off excess ECL liquid, cover the membrane with plastic wrap, and expose to X-ray film for 2 min or longer.

3.5. Regeneration of the Membrane

The hybridized PCR product is dissociated from the membrane to regenerate the membrane for the next hybridization (*see* **Note 9**). A membrane can typically be regenerated for reuse at least 20 times.

1. Wash the membrane twice in 1% SDS at 80°C for 30 min.
2. Wash the membrane in 20 m*M* EDTA at room temperature for 15 min.
3. Store the membrane at 4°C sealed in a plastic bag containing 10 mL of 20 m*M* EDTA.

3.6. Data Analysis

3.6.1. Octal Code Nomenclature

The spoligotype patterns from X-ray film can either be read manually or scanned into a software package. For manual reads, it is recommended to have two people independently score the results for maximum accuracy. **Fig. 2** illustrates the process of assigning a 15-digit octal code *(5)* to a spoligotype result based on the pattern of hybridization. Spacers are grouped into triplets except for spacer 43. There is a number designation for each of the eight possible hybridization combinations for a group of three spacers as shown. The 15th digit of the octal code is either a 1 or a 0 based on the hybridization of spacer 43 alone.

3.6.2. Spoligotype Family Assignments

Once the octal code for a strain has been determined, the strain can then be assigned to a global strain family. Visual rules established as part of SpolDB *(6)* and an online software tool, SpotClust (http://www.rpi.edu/~bennek/EpiResearch) *(15)*, are available for aiding in the assignment of a spoligotype to one of the global strain families. The family assignment is useful for producing an overall picture of the strain diversity in a given population, for tracking changes in the TB population over time, and for comparing TB diversity between populations or areas. The criteria used to define spoligotype global families have been validated through comparison with MIRU data *(24)*.

The online version of the most recent international spoligotype database, SpolDB4, is called SIT VIT (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/). The user can enter a spoligotype octal or binary code to search whether that spoligotype has been previously reported. If it has, a shared type (ST) number is returned. That number can then be used to produce a map showing laboratories that have previously submitted that pattern. This information has the potential to be useful in deciphering the global origin or spread of a particular spoligotype.

3.7. Expansion of the Spoligotype Assay: Examination of Additional Spacer Sequences

Since the initial spoligotyping assay was based on a miniblotter with 43 usable sample chambers, the assay was limited to 43 different spacer sequences. Researchers have explored the potential of additional spacer sequences for "expanded" or "extended" spoligotyping *(25, 26)*. The hope was that screening for additional spacer sequences would improve the differentiability of the commonly observed spoligotypes. These expanded assays have not been standardized and are not commercially available, and they have unfortunately shown limited success in improving differentiation within the commonly observed patterns.

4. Notes

1. Never store biotinylated primers or biotinylated PCR products below 4°C.
2. Validate the proper manufacture of a new spoligotyping membrane on the first use by including a series of previously characterized strains.
3. To confirm PCR amplification of DR region, run a 5-µL aliquot from the reaction on a 1% miniagarose gel. A successful PCR reaction should appear as a ladder or smear of faint bands. If no PCR reactions can be observed, check oligonuleotide stocks for degradation/incorrect concentration.
4. To minimize handling of PCR products, use a 25-µL total volume PCR reaction in a 0.5-mL tube. The 150 µL of hybridization buffer may be directly added to the tube following amplification.
5. Do not reuse plastic support cushions in miniblotter.
6. Leakage into adjoining wells usually results from a dry membrane "wicking" sample into the adjoining well. Improper placement of the support cushion or membrane can also lead to this problem. Avoid wrinkling the membrane in the miniblotter. Ensure that the miniblotter is evenly tightened. Do not completely fill or overfill wells. Hybridization fluid may transfer to adjacent wells.

7. Discard stocks of strepavidin alkaline phosphatase 6 mo after rehydration.
8. Check hybridization temperature and the temperatures of the posthybridization wash buffers. Lowering the hybridization temperature and stringent washes from 60 to 55°C may help and does not add to any background problems or nonspecific hybridization.
9. With proper handling and storage, spoligotyping membranes can be reused 30 or more times.

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Chapter 11

Multilocus Sequence Typing

Ana Belén Ibarz Pavón and Martin C.J. Maiden

Abstract

Multilocus sequence typing (MLST) was first proposed in 1998 as a typing approach that enables the unambiguous characterization of bacterial isolates in a standardized, reproducible, and portable manner using the human pathogen *Neisseria meningitidis* as the exemplar organism. Since then, the approach has been applied to a large and growing number of organisms by public health laboratories and research institutions. MLST data, shared by investigators over the world via the Internet, have been successfully exploited in applications ranging from molecular epidemiological investigations to population biology and evolutionary analyses. This chapter describes the practical steps in the development and application of an MLST scheme and some of the common tools and techniques used to obtain the maximum benefit from the data. Considerations pertinent to the implementation of high-capacity MLST projects (i.e., those involving thousands of isolates) are discussed.

Key words: High-throughput sequencing, MLST, population genetics, sequence types.

1. Introduction

Multilocus sequence typing (MLST) *(1)* combined a number of technical and conceptual developments of the last two decades of the 20th century to provide a universal, portable, and precise means of typing bacteria *(1–3)*. The approach owed much to the pioneering technique of multilocus enzyme electrophoresis (MLEE) (*see* **Chapter 2**), from which it acquired its name *(4)*. A key conceptual development was the recognition that bacteria do not necessarily have a clonal population structure *(5,6)*, leading to the realization that patterns of genetic exchange among bacteria, and therefore of descent, could only be resolved by the analysis of nucleotide sequence data from multiple locations of the

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chromosome *(7)*. Developments in high-throughput nucleotide sequence determination and analysis permitted the generation of definitive genetic data from any locus on the chromosome of multiple isolates *(8)*. An advantage of nucleotide sequence data is that they can be disseminated via the Internet, particularly the World Wide Web *(9,10)*.

The first MLST scheme developed was for the human pathogen *Neisseria meningitidis (1)*, largely as a result of the leading role that studies of this organism had played in the development of the more sophisticated appreciation of bacterial population structure *(11–14)*. It is noteworthy that the success of this scheme was, to a great extent, due to its immediate acceptance by the wide community of researchers working on pathogenic *Neisseria*. This was due to the fact that the scheme was developed and promoted by a consortium of leading researchers in the fields of meningococcal epidemiology and population biology. Cooperation and collaboration continue to be cornerstones of successful MLST schemes.

MLST has since been applied to a number of different bacteria and eukaryotic organisms as a tool for the epidemiological analysis and surveillance of pathogens as well as to investigate their population structure and evolution. MLST has also been deployed in studies of the population structure of nonpathogenic bacteria *(2)*.

MLST provides a number of advantages over other typing approaches. First, it uses sequence data and can therefore detect changes at the DNA level that are not apparent by phenotypic approaches, such as serotyping, and by MLEE that uses the migration rate of proteins in starch gels. Second, it is a generic technique that can be readily reproduced and does not require access to specialized reagents or training. Third, modern methods of direct nucleotide sequencing, based on the polymerase chain reaction (PCR), do not require direct access to live bacterial isolates or high-quality genomic DNA. These techniques can be performed on killed cell suspensions, avoiding all the difficulties associated with the transport and manipulation of pathogens, or on clinical samples, such as the cerebrospinal fluid or blood of a patient undergoing antibiotic therapy, from which a live bacterial isolate might be difficult to obtain. Fourth, the data generated are fully portable among laboratories and can be shared throughout the world via the Internet. Finally, the Internet can also be used to disseminate MLST methods, providing standardization of approaches *(2)*.

This chapter describes the principles behind the development and application of an MLST scheme using the methods deployed in the *Neisseria* scheme as an example. In particular, the upscaling of MLST to enable the cost-effective typing of many hundreds or thousands of isolates is discussed. The general principles are applicable to essentially all bacteria, although the utility depends

on the diversity of the population under investigation and the question asked. The chapter concludes with an overview of some of the approaches available for the basic analysis of MLST data.

2. Materials

2.1. Isolate Collection

A representative sample of the population for which the scheme is to be developed (*see* **Note 1**).

2.2. Preparation of Killed Cell Suspensions

1. Freshly grown plates of bacterial cultures.
2. Boiling water bath.
3. Sterile phosphate-buffered saline (PBS).
4. 1.5- to 2.0-mL screw-capped microcentrifuge (Eppendorf) tubes (not double-walled or skirted tubes).
5. Sterile swabs/loops.

2.3. PCR Amplification of Gene Fragments

1. DNA template.
2. Forward and reverse primers.
3. DNA polymerase enzyme (*Taq* polymerase).
4. Deoxyribonucleoside 5′-triphosphates (dNTPs).
5. Buffer solution (supplied with the enzyme).
6. Magnesium chloride (supplied with the enzyme).
7. Microtiter plates resistant to high temperatures or 0.6-mL capacity microfuge tubes.
8. Thermocycler.

2.4. Gel electrophoresis

1. Agarose.
2. Ethidium bromide: 10 mg/mL stock solution.
3. Loading buffer.
4. TBE buffer: A 10X stock (0.89*M* Tris-HCl, 0.89*M* boric acid, 20 m*M* ethylenediaminetetraacetic acid [EDTA], pH 8.3).
5. Power source.
6. UV transilluminator.

2.5. PCR Product Purification

1. 1.5-mL microcentrifuge tubes.
2. Polyethylene glycol (PEG) 8000.
3. Sodium chloride.
4. Ethanol 70%.
5. Benchtop centrifuge.

2.6. Sequencing Reactions

1. Purified PCR products (DNA template).
2. Forward and reverse primers.
3. Sequencing kit containing DNA polymerase and labeled dNTPs.
4. Microtiter plates or 0.6-µL tubes.
5. Thermocycler.
6. DNA sequencer.

2.7. Purification of Sequencing Products

1. 1.5-mL microcentrifuge tubes.
2. 3*M* sodium acetate, pH 4.6.
3. Ethanol, 95% and 70%.
4. Benchtop centrifuge.

3. Methods

3.1. Killed Cell Suspensions

1. Heat the water bath until it boils.
2. Clearly label the screw-capped microcentrifuge tubes. Ensure that these labels will not come off during the heating step.
3. Dispense 0.5 mL of PBS in each microcentrifuge tube.
4. Make very thick suspensions of organisms by sweeping colonies from each culture plate using a swab or a loop and emulsifying in the PBS in the tubes.
5. Place the tubes in the boiling water bath and leave for 20 min.
6. Store the samples at –20°C. These samples are, in principle, killed and stable at room temperature. Once lack of viability has been confirmed, they can be handled in the laboratory and distributed as noninfectious material (*see* **Note 2**).

3.2. PCR Amplification (see Note 3)

1. Initialization: The reaction mix is heated to 94°C for 1 min to denature the DNA.
2. The following steps are repeated for 25–30 cycles:
   a. Denaturation at 94°C for 30 s.
   b. Primer annealing at 50–60°C for 30 s. This allows the primers to bind to the template DNA.
   c. Extension at 72°C. During this step, the *Taq* polymerase uses the dNTPs to synthesize a new DNA strand complementary to the template. The duration of this step depends on the length of the fragment that is to be amplified.

3. Final elongation at 72°C for 5–10 min to ensure that all the fragments are fully extended.
4. The reaction should be held at 4°C until removed from the thermocycler.

3.3. Agarose Gel Electrophoresis (see Note 4 and ref. 15)

1. Prepare a 1% (w/v) agarose gel by adding 1 g of agarose to 100 mL of TBE buffer.
2. Heat in a microwave until boiling.
3. Leave it to cool for 2–3 min.
4. Add 5 μL of ethidium bromide.
5. Insert the gel comb and wait until is solid.
6. Fill in the electrophoresis tank with TBE.
7. Insert the gel into the tank and remove the combs.
8. Mix 5 μL of PCR product with 2 μL of loading buffer.
9. Connect the gel tank to the power source.
10. Set the voltage to 140 V and leave it running for 15–20 min.
11. Visualize the gel using a UV transilluminator.

3.4. Purification of Amplicons (see Note 5)

1. Transfer the contents of each PCR tube into labeled 1.5-mL Eppendorf tubes. If microtiter plates are used, this step can be omitted.
2. Add 60 μL of 20% (w/v) PEG 8000, 2.5*M* sodium chloride to each tube and mix. Incubate for 30 min at room temperature.
3. Pellet the PCR products by spinning in a centrifuge at maximum speed for 15 min. For microtiter plates, spin for 1 h at 2,750*g*.
4. Discard the supernatant and wash the DNA pellet by adding 0.5 mL of 70% ethanol and spin at maximum speed for a further 5 min. For microtiter plates, add 150 μL of 70% ethanol and spin for 10 min at 2,750*g*. Repeat this step twice when using plates.
5. Discard the supernatant and dry pellets in the vacuum dryer. Microtiter plates can be dried by spinning upside down for 1 min at 500*g*.

3.5. Nucleotide Sequence Extension Reactions (see Note 6)

1. Mix the primer, template, and sequencing reagents in the optimized proportions.
2. Perform the extension reactions in a thermocycler, first conducting denaturation at 96°C for 1 min.

3. The following steps are repeated for 25 cycles: 96°C for 10 s, 50°C for 5 s, 60°C for 40 min.
4. Maintain the reaction at 4°C until removed from the thermocycler.

3.6. Purification of Sequencing Products

1. Transfer the contents of each PCR tube into labeled 1.5-mL Eppendorf tubes. If microtiter plates are used, this step can be omitted.
2. Add 2 μL of 3*M* sodium acetate and 50 μL of 95% ethanol to each tube and mix. Incubate for 45 min at room temperature.
3. Pellet the PCR products by spinning in a centrifuge at maximum speed for 15 min. For microtiter plates, spin for 1 h at 2,750*g*.
4. Discard the supernatant and wash the DNA pellet by adding 0.5 mL of 70% ethanol and spin at maximum speed for a further 5 min. For microtiter plates, add 150 μL of 70% ethanol and spin for 10 min at 2,750*g*.
5. Discard the supernatant and dry pellets in the vacuum dryer. Microtiter plates can be dried by spinning upside down for 1 min at 500*g*.
6. For separation and detection of extension products, *see* **Note 7**.

3.7. Data Management

3.7.1. Data Assembly

A variety of commercial and open source software packages are available for the assembly and editing of sequence chromatograms into compiled edited sequences, including the well-known Staden and GCG packages *(16,17)*. Specialist software for the compilation and analysis of MLST data is also available, for example, the START software package *(18)*. These packages allow many hundreds or even thousands of samples to be processed cost-effectively and rapidly. Inexpensive Linux-based software *(19)*, as well as commercial solutions, are available. The use of Internet-based databases and analytical tools designed for MLST analysis can automatically designate sequence type (ST) and clonal complex as well as facilitate storage and access to the data via Internet. This procedure is described in detail in **Chapter 21**.

The sequence type analysis and retrieval system (STARS) is specifically designed for the assembly of MLST data (http://www.cbrg.ox.ac.uk/~mchan/stars/). It uses PREGAP4 and GAP4 from the Staden package *(16)* to automatically assemble a large number of sequences, which can be retrieved and edited. For known alleles and STs, designation can be done directly from the STARS interface by interrogating an MLST database.

3.7.2. Data Storage

The maintenance of curated, Web-accessible databases is a key feature of MLST schemes. These databases act as dictionaries that allow bacterial isolates to be compared worldwide *(2)*. Database management is therefore central to the endeavor. The key part

of MLST databases is comprised of the allele sequences linked to MLST allele numbers for each locus and the definition of STs. In some cases, it may be appropriate to include information on higher-order organization of STs into clonal complexes or lineages in this database as is done with the *Neisseria* MLST Allelic Profile/ST Database. These data can then be linked to isolate databases that contain isolate specific information. It is important that there is a separation between the databases containing the allele and ST data and isolate data as many isolates will contain the same alleles or STs *(9)*.

3.8. Data Analysis

3.8.1. Analysis of MLST Data

The first question to be addressed with an MLST data set is whether the data conform to the clonal model of population structure. Clonal population structure is an inevitable consequence of asexual reproduction combined with diversity reduction events, such as periodic selection and sequential bottlenecks *(20)*. If an organism is clonal, then the analysis is greatly simplified as conventional phylogenetic trees can be employed. Clonality can be investigated by the congruence test *(21)*, which is based on the observation that, in a clonal population, the phylogenetic signal observed at different loci is the same or congruent *(22)*.

Most bacteria that have been analyzed by MLST are, however, nonclonal by the congruence test. For such organisms the clonal complex is a useful concept that groups genetically related organisms. Clonal complexes comprise groups of related STs that are likely to derive from a common ancestor. Currently, the designation of clonal complex is pragmatic and to an extent varies with different bacteria, but the important issue is that the grouping is consistent with what is known and understood about the genealogy of the organism. The BURST (based upon related sequences) algorithm is a rapid and effective algorithm that can be used to assign the central genotype of clonal complexes. The eBURST program *(23)* groups STs into groups according to user-defined criteria of a number of alleles in common to at least one other member of the group. The central genotype of a BURST group will be the one with the highest number of single-locus variants (SLVs). This will often coincide with the one most frequently isolated and therefore gives some biological meaning to the future designation of the clonal complex. The eBURST program and instructions can be found at http://eburst.mlst.net/. A number of clustering algorithms, such as the unweighted pair group method with arithmetic mean (UPGMA) *(24)* or split decomposition *(25)* can be used to cluster STs and reinforce the results obtained using eBURST.

3.8.2. Applying the Clone Complex Model

It is possible to rationalize the clonal complex structure of many bacteria in terms of the "epidemic clone model" *(5)* of bacterial population structure or modifications of it. Within this frame-

work, high prevalence of a single ST indicates the presence of a fast-spreading new clone from which variants are developing. In the absence of a formal means of defining such clones, it is necessary to implement a rational definition that will command support from the scientific community analyzing these bacteria. It is advisable to designate a committee of experts who ultimately decide on the management and nomenclature issues raised by the scheme.

3.9. High-Throughput MLST

One of the great advantages of MLST is its scalability from a single bacterial isolate to many hundreds or even thousands of samples. Upscaling of MLST is essential for large-scale studies and brings with it appreciable advantages in terms of reducing costs. Automation reduces staff input, and bulk purchase of reagents brings substantial cost savings. While automation brings substantial benefits, it does require substantial commitment and investment. During the setup process the various sections of the data production and analysis pipeline have to be analyzed and kept under review; potential bottlenecks can then be identified and handled. Any process is only as efficient and rapid as its least-efficient and slowest step. PCR and sequencing reactions can be automated by investing in a robotic platform that saves personnel time and minimizes error *(26,27)* or at least ensures that any error is deterministic rather than stochastic. A number of fast and reliable methods exist for the purification of amplification products that can be incorporated into the robotic platform, although consumables for these types of systems are often expensive. The PEG precipitation for PCR products and sodium acetate/ethanol precipitation for sequence reactions are highly cost-effective, but are time consuming and require investment in centrifugation equipment capable of sedimenting material in microtiter plates.

Optimization of the sequence reactions and the use of a centralized sequencing facility can further reduce costs as the use of reagents can be minimized, and costs can be further reduced by bulk purchase *(28)*. If automation is to be used, it is important to recognize that the processes are more akin to those found in industrial rather than conventional biological research organizations. Robotic equipment works most effectively when it is regularly used to perform highly repetitive operations. Once the equipment is working on a given application, a process that often requires appreciable investment of time and effort, temptation to further improve operation by minor modification should be resisted. Such attempts prevent the exploitation of the equipment efficiently and are at least as likely to degrade as to enhance the performance of the equipment.

3.10. Applications of MLST Data

3.10.1. Application to Public Health

Public health laboratories use MLST routinely for the characterization of clinical specimens *(29,30)*. For the meningococcus, for example, the information obtained has proven to be invaluable for the understanding and management of disease outbreaks *(31,32)*, epidemiological surveillance *(33,34)*, and the monitoring of public health interventions. Its application to clinical specimens has obvious implications for diagnosis and clinical management of cases caused by an organism that is notoriously difficult to isolate microbiologically from patients undergoing antibiotic therapy *(35–37)*.

3.10.2. Evolutionary and Population Genetic Analyses

MLST data have been used in a wide variety of applications, including evolutionary and population analysis of bacterial species, but to date they have been mostly used in molecular epidemiological studies of bacterial pathogens. Molecular epidemiology employs genetic techniques to characterize isolates of infectious agents or identify their presence and characteristics from clinical specimens. By this means their distribution and spread can be monitored, and if necessary, health interventions can be implemented. MLST has been applied to many bacteria, as recently reviewed *(2)*. MLST data can also be used to investigate the population structure of bacterial populations at different levels (e.g., temporal stratification or geographic distribution) as this can help to understand the transmission route of the infectious agent *(38)*. For this purpose, the analysis of molecular variance (AMOVA) *(39)* can be used to calculate the F statistic (F_{ST}) *(40)*, which measures the amount of genetic exchange that takes place among different groups of organisms. The Mantel test can be used to investigate the correlation between genetic and geographic distance, that is, whether isolates obtained from geographically close locations are more closely related to those found on more distant geographic areas *(38)*. Both tests can be easily implemented using the Arlequin software package *(39)*, which can be downloaded from http://lgb.unige.ch/arlequin/.

4. Notes

1. The isolates examined must be carefully chosen with a number of criteria in mind: They should represent the known genetic diversity of the population analyzed (which itself should be carefully defined); they should represent a variety of sources or environments from which the organism is often isolated; and they should be collected from a variety of geographic locations and an appropriate time frame.

2. The crucial step in this method is the rapid inactivation of cellular nucleases once the cells have been lysed.
3. In an MLST scheme, PCR conditions are ideally the same for all loci. This should be straightforward if primers are designed to have similar melting temperatures T_m and if the DNA fragments to be amplified are of similar lengths. Optimization is likely to be needed for novel primers.
4. Standard agarose gel electrophoresis can be employed to check that the amplification reactions have been successful and that amplicons of the expected size have been produced. It is recommended to check all the samples during the optimization period, but when the MLST scheme is fully developed and routinely applied on a large scale, only occasional verification is necessary *(**15**)*.
5. A variety of methods for purification are available, including many commercial kits. However, the purification method described here is an effective and inexpensive noncommercial method based on sodium chloride and PEG differential purification.
6. It is an absolute requirement for accurate sequence determination that sequence information from both DNA strands is used to compile the final sequence, so PCR reactions "forward" and "reverse" are required for each DNA molecule to be sequenced. The reactions are easily performed with proprietary kits that contain all of the necessary components, requiring only template DNA and specific primer to be added. Some local optimization is likely to be required for the primers and reagents used.
7. A variety of commercial instruments is available for the separation of extension reaction products, and a description of their operation is beyond the scope of this chapter. In most cases they are capillary based and generally operated by central sequencing facilities as they are high-cost assets that, to be cost-effective, have to be used on very large numbers of samples, usually representing a wide variety of applications. Although smaller-scale instruments suitable for the use by single laboratories are available, they are usually much more expensive to run. Commercial companies also offer sequencing services.

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Chapter 12

Multiple Locus Variable Number of Tandem Repeats Analysis

Gilles Vergnaud and Christine Pourcel

Abstract

Genotyping of bacteria through typing of loci containing a variable number of tandem repeats (VNTR) might become the gold standard for many pathogens. The development of genome sequencing has shown that such sequences were present in every species analyzed, and that polymorphism exists in at least a fraction of them. The length of these repetitions can vary from a single nucleotide to a few hundreds. This has implications for both the techniques used to measure the repeat number and the level of variability. In addition, tandem repeats can be part of coding regions or be intergenic and may play a direct role in the adaptation to the environment, thus having different observed evolution rates. For these reasons the choice of VNTR when setting a multiple-loci VNTR analysis (MLVA) assay is important. Although reasonable discrimination can be achieved with the typing of six to eight markers, in particular in species with high genomic diversity, it may be necessary to type 20 to 40 markers in monomorphic species or if an evolutionary meaningful assay is needed. Homoplasy (in the present context, two alleles containing the same repeat copy number in spite of a different history) is then compensated by the analysis of multiple markers. Finally, even if the underlying principles are relatively simple, quality standards must be implemented before this approach is widely accepted, and technology issues must be resolved to further lower the typing costs.

Key words: Database, genotyping, MLVA, MLVAbank, services on the Web, VNTR.

1. Introduction

The globalization of the world economy, with a dramatic increase of traveling of human beings and exchange of goods, is also as a consequence globalizing the spread of pathogens and associated infectious diseases. As a result, there is a growing requirement for tools enabling the real-time accountability and tracing of these

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pathogens. Such tools should satisfy a number of criteria. They should be "low cost" so that any new isolate can be routinely typed. They should be not too demanding from a technological point of view, so that partial typing, or first-level low-resolution typing, can be performed in any microbiology laboratory and not only in dedicated high-throughput facilities. The resulting data should open the way to the making of large-scale databases shared across the Internet, as well as small-scale databases for local surveillance within, for instance, a hospital setting. These requirements constitute one aspect of the new discipline called forensic microbiology.

Although many methods have been developed to investigate the epidemiology of pathogens, with some described in other chapters of this book, only a few qualify as potentially of use in this context. In the present chapter, we go through multiple loci variable number of tandem repeat (VNTR) analysis (MLVA), which for a number of reasons that we illustrate is currently considered as one of the most promising technologies regarding the epidemiology of microorganisms with relatively large genomes, such as bacteria.

It is now clear that, for many reasons, including biosafety, the appropriate typing technologies will target DNA. The resulting data need to be easily storable in a digital format so that eventually worldwide coverage of the pathogen diversity can be achieved. This excludes pattern-based technologies, such as randomly amplified polymorphic DNA (*see* Chapter 4), polymerase chain reaction (PCR) amplification of multiple interspersed repeated elements (*see* Chapter 5), pulsed-field gel electrophoresis (*see* Chapter 6), amplified fragment length polymorphism (*see* Chapter 8), and insertion element (IS) typing by Southern blot hybridization (*see* Chapter 14), for instance. In such techniques, patterns produced in different laboratories can be compared only if very strict quality standards are followed. The use of polymorphic tandem repeats to discriminate biological entities is not new and is not limited to microorganisms. We do not go through the history of tandem repeat analysis for which reviews exist (e.g., ***1, 2)***. The important feature of MLVA is that the analysis of a limited number of loci provides an overview of diversity within a bacterial species.

A number of aspects specific for tandem repeats analysis must be kept in mind. First, tandem repeat loci can be very variable in terms of mutation rates, with some loci having an extreme mutation rate while others are monomorphic. At present, this behavior cannot be predicted from the sequence itself and needs to be experimentally measured by eventually typing hundreds of strains, as was done previously for human forensics-related projects. The most highly polymorphic markers that usually result from a higher rate of mutation events will have a high homoplasy level.

Such markers are sometimes called "highly informative," which is ambiguous and not necessarily correct. On the contrary, an MLVA assay that would be based solely on such markers would probably be unable to cluster strains according to their true historical proximity, as illustrated previously with *Brucella (3)*.

2. Materials

2.1. DNA Purification

1. For some bacterial species, thermolysates can be prepared in water and stored at –20°C. Long-term stability needs to be evaluated for each species. In some instances, glass beads are used to break cells.
2. Purification kit such as Qiagen DNeasy® Tissue kit. DNAs are stored at –20°C.
3. ND-1000 spectrophotometer (NanoDrop®, Labtech, France).

2.2. PCR Amplification

1. Standard *Taq* polymerase (Roche, Promega, or Invitrogen) or *Pfu* polymerase when amplifying mononucleotide repeats (*see* **Note 1**).
2. The Qiagen kit provides the "Q solution" and corresponding buffer for amplification of GC-rich DNA. Alternatively, 1*M* betain (Sigma) can be used in the PCR reaction (*see* **Note 2**).
3. Fluorescent oligonucleotides to be used with the Beckman sequencer are from Sigma-Aldrich Proligo (www.proligo.com).
4. Deoxynucleotide 5′-triphosphates (dNTPs; Eurogentec, MWG Biotech, or Amersham).
5. Different thermocyclers (including PTC 200 DNA Engine and MyCycler, Bio-Rad) have been used efficiently.
6. PCR amplifications are done in microcap tubes (rows of 8 or 12 tubes) arranged in grids accommodating up to 96 tubes in a 96-well format compatible with multichannel electronic pipeting equipment (eight-channel Biohit dispensing range 0.1 to 10 µL, 1 to 20 µL).

2.3. Agarose Gel Electrophoresis

1. Standard agarose for gels up to 3% (w/v).
2. TBE electrophoresis buffer: A 5X TBE buffer (1.1*M* Tris-HCl, 900 m*M* boric acid, 25 m*M* ethylenediaminetetraacetic acid [EDTA], pH 8.3) is prepared as a stock solution and is used at 0.5X concentration for migration. Buffer solution can be used for up to three or four runs.
3. Metaphor (FMC Bioproducts-Cambrex) is used for 4% (w/v) gel, either pure or mixed 1:1 with standard agarose.

4. 100-bp ladder or 20-bp ladder from Bio-Rad, MBI Fermentas, or Euromedex (*see* **Note 3**).
5. Electrophoresis chambers compatible with 20- to 24-cm wide gels by 20 to 40 cm long, with 40 to 50 wells, and spacing compatible with multichannel pipeting (*see* **Note 4**).
6. 10X loading buffer: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 50% (v/v) glycerol, 50% (v/v) TE. TE is a 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0 solution.
7. Ethidium bromide: purchase as 10 mg/mL aqueous stock solution; can be stored at 4°C for years. Use at 0.25 μg/mL final concentration in 0.5X TBE buffer.

2.4. Band Size Determination

The band size can be determined using dedicated software (e.g., Quantity One v. 4.2.1 of Bio-Rad) or the BioNumerics software (Applied Maths).

3. Methods

3.1. Running an MLVA Assay

A list of bacteria for which VNTR markers have been identified and tested on collections of strains is shown in **Table 1**. In some cases these assays are very preliminary since only a small number of strains have been analyzed, and much remains to be done to definitely select an informative VNTR panel and to measure their relative value. In other cases, such as for *Mycobacterium tuberculosis*, many data are already available, and the assay can be considered more reliable. However, no consensus has yet been adopted in the corresponding scientific community (*see* **Note 5**).

If an MLVA assay is available in the literature, genotyping will consist of *(1)* preparing DNA samples; *(2)* VNTR amplification and estimation of repeat number; and *(3)* data analysis and storage.

3.1.1. The DNA Samples

Very good results have been obtained from thermolysates for *M. tuberculosis* or *Legionella pneumophila*. In some instances, MLVA typing has even been done on crude biological samples with a sufficient bacterial load *(4)*. In other cases, and for some species such as *Pseudomonas aeruginosa*, a purification step of the DNA is mandatory to get reliable PCR amplifications *(5)*.

3.1.2. PCR Amplification

1. Perform the PCR reactions in a total volume of 15 μL, containing 1–5 ng of DNA, 1X reaction buffer, 1.5 m*M* $MgCl_2$, 1 unit of *Taq* DNA polymerase, 200 μ*M* of each dNTP, 0.3 μ*M* of each flanking primer.
2. Use the following conditions for amplification: Initial denaturation cycle for 5 min at 94°C, 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55 to 60°C depending on

Table 1
Published MLVA Schemes

Bacteria	VNTR loci[a]	Repeat sizes (bp)	No. of isolates	Method	References
Bacillus anthracis	8 24 (18)	2–36 9–78	426 32	Sequencing gel Agarose	*(22)* *(9)*
Bartonella henselae	5	45–146	44	Agarose	*(23)*
Bordetella pertussis	6	5–15	198	Sequencing gel	*(24)*
Borrelia sp.	10	2–21	41	Sequencing gel	*(25)*
Brucella sp.	8 18	8 6–134	22 236	Capillary electrophoresis Agarose	*(3)* *(26)*
Burkholderia pseudomallei	32	6–15	213	Capillary electrophoresis	*(12)*
Candida albicans	3	4	100	Sequencing gel	*(27)*
Candida glabrata	6	2–3	127	Capillary electrophoresis	*(28)*
Clostridium difficile	7 7	3–8 6–17	86 86	Capillary electrophoresis Sequencing	*(29)* *(30)*
Clostridium perfringens	5	6–21	112	Agarose	*(31)*
Coxiella burnetii	17 7	6–126 6–21	42	Agarose Capillary electrophoresis	*(32)* *(33)*
Enterococcus faecalis	7	141–393	83	Agarose	*(34)*
Enterococcus faecium	6	121–279	392	Agarose	*(35)*
Escherichia coli O157	7 7	6–18 6–30	81 73	Sequencing Capillary electrophoresis	*(36)* *(37)*
E. coli, Shigella	7	6–39	72	Capillary electrophoresis	*(38)*
Francisella tularensis	6 25	2–21 2–23	56 192	Sequencing gel Sequencing gel	*(39)* *(40)*
Hemophilus influenzae	5	3–6	20	Agarose	*(41)*
Lactobacillus casei	9	6–24	63	Capillary electrophoresis	*(42)*
Legionella pneumophila	6 13	18–45 7–125	78 99	Agarose Agarose	*(43)* *(16)*
Leptospira interrogans	7 6	34–77 36–138	51 39	Agarose Agarose	*(44)* *(45)*
Leptospira interrogans kirschneri	5	34–77	243	Agarose	*(46)*
Listeria monocytogenes	6	9–18	25	Agarose	*(47)*
Mycobacterium avium	6 5	53 20–70	73 50	Agarose Agarose	*(48)* *(49)*
Mycobacterium leprae	5 9	2–3 1–27	12 4	Sequencing Sequencing gel	*(50)* *(51)*

(continued)

Table 1 (continued)

Bacteria	VNTR loci[a]	Repeat sizes (bp)	No. of isolates	Method	References
Mycobacterium tuberculosis	7	15–79	25	Agarose	*(52)*
	12 (10)	53	31	Agarose	*(53)*
	6	69	100	Agarose	*(54)*
	21 (8)	9–58	90	Agarose	*(21)*
Mycobacterium ulcerans	13	53–71	29	Agarose	*(55)*
M. ulcerans and M. marinum	7	53	66	Agilent	*(56)*
Mycoplasma mycoides	3	12–75	39	Agarose	*(57)*
Neisseria meningitidis	12	4–30	100	Capillary electrophoresis	*(58)*
	12	4–21	92	Capillary electrophoresis	*(59)*
Pseudomonas aeruginosa	7	6–115	89	Agarose	*(60)*
	15	6–129	163	Agarose/capillary electrophoresis	*(5)*
Salmonella typhimurium/ typhi	8	6–189	102	Capillary electrophoresis	*(61)*
	10 (7)	3–20	99	Agarose	*(62)*
Staphylococcus aureus	7	48–159	16	Agarose	*(63)*
Staphylococcus aureus	5	9–81	34	Agarose[b]	*(64)*
	8	9–560	200	Capillary electrophoresis[b]	*(65)*
Streptococcus pneumoniae	16	12–60	56	Agarose	*(66)*
Streptococcus uberis	7	13–208	88	Agarose	*(67)*
Salmonella enterica	10	6–232	50	Agarose	*(68)*
Shigella sonnei	26	6–168	536	Capillary electrophoresis	*(69)*
Theileria parva (protozoa)	60	2–21	20	Agar/spreadex	*(70)*
Xylella fastidiosa	7	7–9	27	Agarose	*(71)*
Yersinia pestis	25	9–60	3 + 180	Agarose	*(9,72)*
	42 (26)	1–45	24 + 156	Sequencing gel	*(73,74)*

[a]In parentheses are indicated the number of VNTR not previously published.
[b]Not a bona fide MLVA assay since the five loci used are coamplified to produce a multiband pattern, and no analysis is done on a locus-by-locus basis.

the primers, and elongation for 45 to 60 s at 72°C followed by a final elongation step for 10 min at 72°C.

3.1.3. Agarose Gel Electrophoresis

The length of PCR products can be estimated by different approaches. Importantly, the required accuracy is directly related to the repeat unit size of the loci to be used. Obviously, if the markers used for MLVA typing have a repeat unit size of more

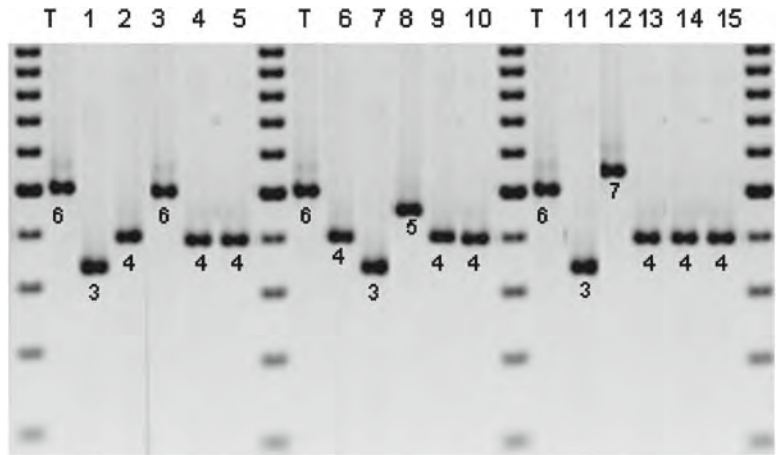


Fig. 1. Agarose gel electrophoresis of VNTR amplicons. PCR products of the *M. tuberculosis* Mtub 39 VNTR (58-bp repeat) on the reference strain H37Rv (T) and 15 isolates. The size marker is loaded every six samples.

than 50 bp (as is the case for some important pathogens such as *M. tuberculosis)*, it is not necessary to achieve a precision of plus or minus 1 bp (**Fig. 1**). In theory, a precision just below ±50% of the repeat unit size is sufficient. Accordingly, different allele-calling methods are used. The most frequently used method, at least in the assay development phase, is the ordinary agarose gel. The approximately ±2% imprecision of this approach allows the typing of loci with 6-bp repeat units if the allele size does not exceed 150–200 bp (*see* **Note 6**).

1. Add 2 µL of 10X loading buffer to the PCR products. Load 2 to 3 µL of PCR products in 2 to 3% (w/v) agarose gels made of standard agarose for repeats 9 bp and larger. (for the analysis of smaller repeats, 4% (w/v) agarose gels, comprising 2% Metaphor plus 2% regular agarose can be used) (*see* **Note 7**).
2. Perform the electrophoresis in 20- to 24-cm wide gels made in 0.5X TBE buffer, run at 8 V/cm. For each group of five isolates, include a reference strain. To ensure an adequate size assignment of the PCR products, reference size markers are run every six samples (**Fig. 1**). DNA size markers routinely used are the 100-bp ladder or 20-bp ladder (for repeats 8 bp and smaller).
3. Stain the gels after the run in 0.5–1.0 µg/mL ethidium bromide for 15 to 30 min. Then, rinse the gel with water and photograph under ultraviolet illumination (*see* **Note 8**).

3.1.4. Agarose Gel Image Analysis

The PCR product sizes can be estimated from the digital image of the gel using dedicated software usually provided with the camera. First, the position of the cursor relative to the DNA band is adjusted to achieve optimum size matching with the reference strain used as internal control, and then the cursor is similarly positioned for all the other strains run in the same gel. Size assign-

ment is confirmed by visual inspection of gels and comparative analysis of strains for each marker. For repeats of 12 bp and more, visual estimation of the band size is possible with the help of a chart in which all the known alleles are indicated (*see* **Note 9**).

3.1.5. Capillary Gel Electrophoresis

Apart from the regular agarose gel, the equipment most often used is capillary electrophoresis apparatus, in particular equipment initially developed for DNA sequencing purposes. The precision and reproducibility achieved by such machines (routinely ±0.5 bp in a 80- to 650-bp range) enables the typing of very short repeat units, although the typing of long mononucleotides or even dinucleotide repeat tracts can be technically demanding ***(6)***. Also the size estimates provided, deduced by comparison with a size standard, can be wrong by a few basepairs in a very reproducible way. This has been illustrated by different studies ***(7,8)***. The discrepancy for a given machine and for each locus and allele must be measured experimentally. These machines require the use of fluorescent primers. Because the underlying technology was developed for DNA sequencing purposes, at least four different colors are available, one of which must be used for the DNA size standard. This availability of different colors makes it possible to pool different PCR products to analyze multiple loci in the same run (*see* **Note10**) and consequently reduce the costs.

Other capillary electrophoresis machines specifically developed for measuring the length of DNA fragments (rather than sequencing) and that do not require the use of fluorescent primers are also very promising (including Agilent 2100, Caliper Labchip90, Qiagen QIAxcel), in spite of a slightly lower precision ***(7,8)***.

3.1.6. Nomenclature and Description of MLVA Profiles

The repeat length and number of repetitions are conveniently determined in sequenced genomes using the Microbial Tandem Repeats Database (http://minisatellites.u-psud.fr) ***(9,10)***.

1. Check that amplification of DNA from the strain from which the genome has been sequenced produces amplicons of the expected size.
2. Estimate the number of repeats in new alleles by subtracting the invariable flanking region from the amplicon size, then dividing by the repeat unit length as determined for the reference strain. For example, if the size of the PCR amplification product for a 45-bp VNTR is 205 bp in the sequenced strain for two repeat units, it implies that the number of repeats found in amplicons of size x produced with the same primers is $((x - 205)/45 + 2)$ repeat units.
3. The null designation is given when no amplification is repeatedly observed at a given locus (*see* **Note 11**).

3.1.7. Verification of Unexpected Allele Size

Intermediate-size alleles may result from intermediate-size repeat units or from small deletions in the flanking sequence. Sequencing

of any such allele is necessary to analyze the origin of the unexpected size.

1. The full-length sequences of selected PCR products are determined on both strands following DNA purification by the QIAquick PCR purification kit (Qiagen) or by polyethylene glycol (PEG) precipitation as described in **ref.** *11*. Data obtained with forward- and reverse-sequencing primers are combined, and sequences are manually aligned.
2. The alleles are reported as half size to indicate the existence of abnormal alleles.

3.1.8. Data Storage and Analysis

Ideally, data should be stored in databases together with all known information on the strain (i.e., phenotypic and biochemical characteristics, origin, clinical or environmental information, etc.).

1. For each isolate, enter the VNTR size in the form of a digit corresponding to the number of repeats into an Excel file.
2. Import the data matrix into data-mining tools or into more conventional biology-oriented clustering methods. The currently preferred method to measure the distance between two strains is simple counting of the number of markers at which the two strains differ divided by the total number of markers and expressed as a percentage (*see* **Note 12**).
3. When the amount of data is sufficient, it becomes possible to precisely estimate the mutation rate at each VNTR and the relative frequency of single and multiple repeat unit gains and losses at each locus ***(12–14)***. Using this knowledge, it is possible to define individual distance coefficients (*see* **Note 13**).
4. Once a good-quality data set has been produced, it is desirable to make it accessible via the Internet (*see* **Note 14**). At least three research groups (*see* **Note 15**) have developed sites that make possible the hosting of databases produced by other groups. For instance, using the MLVAbank at http://mlva.u-psud.fr, MLVA typers can create their own account to manage personal databases without control from the hosting institution. Databases can be made public, or be shared within a community, with different rights. Different panels of markers can be selected by users to take into account local usages. This hosting facility may be seen as a repository for MLVA data. It is not curated by the hosting institution in an approach that is reminiscent of DNA sequence repositories such as Genbank and European Molecular Biology Laboratory (EMBL).

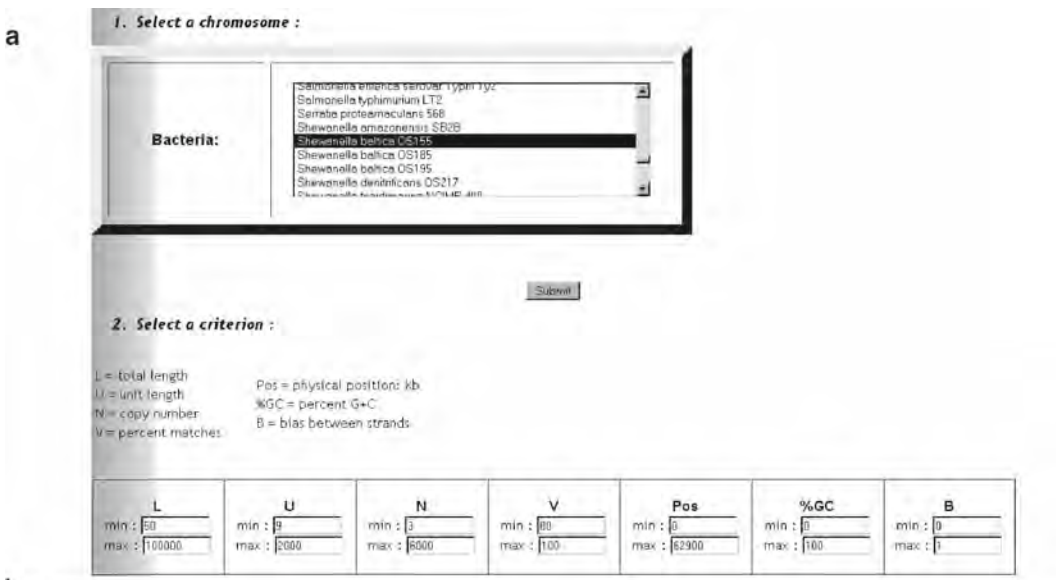
3.2. Developing a New MLVA Assay

Once a bacterial genome has been sequenced (fully or partially), it is possible to search for tandem repeats to test the potential use of MLVA for genotyping of the species. The availability of two or

more strains of the species highly facilitates the search for polymorphic markers. Web-based tools have been developed to facilitate the first steps in setting a new MLVA scheme (*see* **Note 16**).

3.2.1. Identification of Tandem repeats from a Single Genome

1. Go to the Microorganisms Tandem Repeat Database (http://minisatellites.u-psud.fr/) developed by Denoeud and Vergnaud ***(10)*** and follow the link "The Microorganisms Tandem Repeats Database" and then "bacteria". Select the strain to be searched and choose the parameters that will define the tandem repeats of interest. For a first assay, it is interesting to select repeats of 9 bp and longer, repeated at least three times and showing an 80% internal conservation. When repeats fulfilling these criteria exist, they are displayed in a table as shown on **Fig. 2**.
2. Click on the alignment link to see the repeat sequence and a 500-bp sequence flanking it on both sides.



Physical position (kb)	Contig name	Unit length	Copy number	Total length	Percent matches	%C	%G	%T	%A	GC bias	Sequence	TR of the family
4	Shewanella_baltica_OS155	9	13.8	123	80%	6%	38%	11%	43%	0.72	alignment / linear seq	Group
12	Shewanella_baltica_OS155	261	12.1	3161	88%	30%	24%	23%	21%	0.11	alignment / linear seq	Group
29	Shewanella_baltica_OS155	24	3.0	73	100%	8%	24%	42%	24%	0.49	alignment / linear seq	Group
31	Shewanella_baltica_OS155	9	5.9	53	93%	22%	9%	24%	44%	0.41	alignment / linear seq	Group
102	Shewanella_baltica_OS155	18	7.8	140	91%	17%	44%	12%	25%	0.44	alignment / linear seq	Group
623	Shewanella_baltica_OS155	118	3.7	436	98%	20%	20%	28%	29%	0	alignment / linear seq	Group

Fig. 2. The tandem repeats database. Snapshots of the Web-based database query and of the output. (**a**) The strain of interest is selected as well as the characteristics of the VNTRs to be searched. (**b**) The repeats are displayed in a table showing the position on the genome, the repeat size, and copy number as well as other information on its composition. The sequence can be retrieved using the link in the rightmost column.

3. Select primers in sequences flanking the VNTR, at least 40 bp away from the first and last repeats (*see* **Note 17**), in such a way that the same annealing temperature can be used for all the PCRs. This is particularly important if multiplexing is to be used.

3.2.2. Identification of VNTR by Comparison of Two or More Genomes

When the genomes of two strains of a given species have been sequenced, it is possible to compare the size of repeated sequences to select those possessing a different number of repeats and therefore representing good VNTR candidates.

1. Access the strain comparison page (from http://minisatellites.u-psud.fr/, follow the link "The Microorganisms Tandem Repeats Database" and then "strain comparison page") and select the bacteria to be searched.
2. Choose the tandem repeats characteristics.
3. A table will show repeats that present polymorphism between the two strains.

3.2.3. The Choice of a Panel of Markers

The excessive use of microsatellites (2- to 8-bp repeat units), some of which tend to be unstable, as reported in several studies, may cause difficulties because of their especially high homoplasy levels ***(3)***. In addition, they necessitate the use of sequencing gels or methods with equivalent resolution, which are usually not routinely used in the bacteriology laboratory.

3.2.4. Criteria for Evaluation of MLVA

Standard efficacy criteria of a new MLVA scheme, including typeability (*T*), reproducibility (*R*), stability (*S*), epidemiological concordance (*E*), and discriminatory power (expressed as the Hunter-Gaston diversity index [*HGDI*]), are determined as reported elsewhere ***(15,16)***.

The polymorphism index of individual or combined VNTR loci can be calculated using a selected panel of strains and *HGDI* ***(17)***, an application of the Simpson' s index of diversity ***(18)***. Although very useful to compare the discriminatory power of assays, it does not measure the relevance of the discrimination that is achieved by a given marker or combination of markers (*see* **Note 18**).

3.3. Reviewing MLVA Reports

MLVA typing is still an emerging domain, and the quality of MLVA reports is unequal. In particular, some reports neglect basic rules, some of which are specific for MLVA. To accelerate the development of MLVA, we propose here a checklist for reviewers of MLVA articles:

Check that the proposed markers are indeed new markers and have not been previously described under other names.

Check that the MLVA typing data are made accessible and that the allele-calling convention is clearly described by giving the repeat unit size and repeat copy number in the first genome

sequenced for the corresponding species. Encourage the deposition of data into one of the existing MLVA data repositories.

Check that the assay is an MLVA assay and not a pattern-based assay in which multiple loci are simultaneously revealed, but not analyzed to the point of deducing the repeat copy number at each locus individually.

3.4. Future Prospects

In spite of very promising progress and significant advantages, at least in theory, MLVA is not replacing existing technologies, such as pulsed-field gel electrophoresis, as fast as could have been expected. There are several reasons for this. One is the lack of standardization and reference databases. In this area, funding bodies have a major role to play by stimulating and supporting the actors involved to agree on international standards. Another reason is the lack of demand for large-scale molecular epidemiological tools. The United States are one exception illustrating this fact: a large unified market has led to requirements for genotyping systems covering the whole country. No such requirements exist in Europe, where national-scale approaches are still the rule in practice. Usually, a single national reference laboratory will organize molecular typing, and in this context, pattern-based approaches can be used in spite of their limitations (limited possibilities of interlaboratory exchanges). Still another reason for the slow emergence of MLVA is its relative cost as it requires multiple PCR amplifications. Once reference typing panels have been defined, it is hoped that multiplexing technologies will be developed to permit genotyping of a strain using (ideally) a single PCR amplification.

4. Notes

1. From a technical point of view, it is convenient to classify tandem repeats among three classes: the "minisatellites" with repeat units longer than 9 bp, the microsatellites with repeat units in the 2- to 8-bp range, and the homopolymeric repeats (often called Single Nucleotide Repeats or SNRs). The larger repeat unit loci can usually be typed on a wide range of DNA fragment-sizing equipment. The microsatellites will usually require more sophisticated equipment, and the mononucleotide repeats with 1-bp variations require specific protocols, including the use of different polymerases, such as *Pfu (6)*.
2. The simple addition of betain is very effective in terms of priming specificity. It may help resolve multiple-band amplifications. To have its full effect, the use of a special PCR buffer is recommended *(19)*.

3. Size marker: Select size markers containing similar amounts of DNA in each band so that the fluorescence intensity of each band is similar.
4. A voltage of 8–10 V/cm is usually applied (i.e., if the distance between the electrodes is 30 cm, voltage is 250–300 V). If a cover is necessary, it may be important to check for buffer temperature and avoid overheating by recirculating and cooling the buffer. If the two electrodes are identical (i.e., platinum electrodes), it is recommended to revert the migration polarity every five runs to avoid running distortions.
5. VNTR panel consensus: Special effort should be put to reach a consensus on which panel of VNTR to use for a given MLVA assay. This is being achieved for *Brucella*, for which a set of different panels with increasing discriminatory power has been defined.
6. Shorter repeat units or larger size ranges can be resolved using higher resolution, usually with precast gels, such as Biorex.
7. Gel quality: Special attention must be given to the quality of the gel as this will allow precise size assignment. It is recommended to pour the gel at a temperature between 60 and 65°C.
8. Ethidium bromide staining: It is mandatory to stain the gel after migration, especially when looking at small-size PCR product as the ethidium bromide in the gel will migrate backward.
9. See, for instance, http://mlva.u-psud.fr/BRUCELLA/spip.php?article93.
10. The development of this equipment was driven by sequencing, and DNA fragment size measurement is only a secondary application. With the advent of new sequencing technologies, it remains to be seen whether this "DNA fragment-sizing" market will be sufficient to maintain these machines or if other approaches will be needed. A number of alternative equipment, such as the ones developed or distributed for instance by QIAGEN, Caliper, and Agilent, might then replace capillary apparatus aimed at DNA sequencing.
11. In some instances, an inappropriate initial numbering may result in the calling of "zero-repeat" alleles as more strains are investigated *(20)*. This is because very often a tandem repeat locus does not contain an integer number of repeats but rather contains a partial repeat at one end. If alleles at such a locus with, for instance, 1.5, 2.5, 3.5 repeat units are called 1, 2, 3 rather than 2, 3, 4, an allele containing 0.5 repeats will be coded 0. Eventually, normalizing bodies will be needed to avoid such ambiguities.
12. This is a very crude similarity measure that gives the same weight to all markers. It also considers that alleles that differ by one repeat unit are not evolutionarily closer than alleles that differ by many repeat units. The two assumptions are often wrong,

but in spite of this, the resulting clustering analyses make sense. This is because the use of multiple markers compensates for variable homoplasy levels at individual markers.

13. *Brucella* MLVA data, for instance, are already analyzed by dividing the markers into three different sets, or panels, and giving a different weight to each panel.
14. An MLVA data set was made accessible in an interactive way for the first time in 2002 *(21)*. Since then, a few other databases dedicated to one or a few pathogens have been put on line.
15. See http://mlva.u-psud.fr; http://www.mlva.eu; http://www.pasteur.fr/mlva.
16. The development of MLVA usually comprises three phases. In the first phase, polymorphic markers are identified. Usually, a few publications cover this first step. In the second phase, more typing data are produced, and the characteristics of individual markers are refined. Consensus marker panels progressively emerge. In the third phase, typing databases are produced, and consensus panels are agreed on.
17. In this way, representative alleles can more easily be sequenced using the same primers. Otherwise, if the primers are located too close from the tandem repeat start, sequencing data will often miss the first basepairs of the tandem repeat.
18. Eventually, it should make sense to consider that two strains that differ at one highly variable marker are more similar than two strains that differ at a moderately variable marker. More sophisticated distance coefficients can be developed once many strains have been typed.

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Chapter 13

Comparison of Molecular Typing Methods Applied to *Clostridium difficile*

Ed J. Kuijper, Renate J. van den Berg, and Jon S. Brazier

Abstract

Since the 1980s the epidemiology of *Clostridium difficile* infection (CDI) has been investigated by the application of many different typing or fingerprinting methods. To study the epidemiology of CDI, a typing method with a high discriminatory power, typeability, and reproducibility is required. Molecular typing methods are generally regarded as having advantages over phenotypic methods in terms of the stability of genomic markers and providing greater levels of typeability. A growing number of molecular methods have been applied to *C. difficile*. For the early and rapid detection of outbreak situations, methods such as restriction enzyme analysis, arbitrary primed polymerase chain reaction (PCR), and PCR ribotyping are commonly used. For long-term epidemiology, multilocus sequence typing, multilocus variable number of tandem repeats analysis, and amplified fragment length polymorphism are of interest. Currently, the PCR-ribotyping method and the library of PCR ribotypes in Cardiff are the benchmarks to which most typing studies around the world are compared. Multilocus variable number of tandem repeats analysis is the most discriminative typing method and will contribute significantly to our understanding of the epidemiology of this important nosocomial pathogen.

Key words: *Clostridium difficile*, MLVA, PCR ribotyping, REA, subtyping.

1. Introduction

Since the recognition of *Clostridium difficile* as the causative agent of pseudomembranous colitis in 1978, this anaerobic spore-forming bacterium has emerged as an important enteropathogen. Pathogenic *C. difficile* organisms release toxins that ultimately mediate diarrhea and colitis. Colonic injury and inflammation result from the production of two protein toxins: enterotoxin A (TcdA; 308,000 M_r) and cytotoxin B (TcdB; 270,000 M_r). Genes for the

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Table 1
Characteristics of Genotyping Methods

Method	Target	Discriminatory power	Typeability	Reproducibility	Performance	Interpretation	Costs	Interlaboratory exchange
Plasmid profiling	Extrachromosomal plasmid	–	–	+	±	±	+	+
REA	Whole genome, restriction	+	+	±	±	–	+	–
RFLP	Whole genome, restriction	–	+	±	±	±	+	–
AP-PCR/RAPD	Whole genome, random PCR primers	++	+	±	+	+	+	–
PCR ribotyping	16S–23S intergenic spacer region	++	+	+++	+++	++	+	+++
PFGE	Whole genome, restriction	+++	±	+++	±	±	+++	±
Toxinotyping	Toxin A and B genes	+	+	+++	++	+	+	++
fliC PCR-RFLP	Flagellin gene	±	+	++	++	++	+	++
slpA PCR-RFLP	S-layer precursor gene	±	+	++	++	++	+	++
AFLP	Whole genome, restriction	++	+	++	±	+	++	±
MLST	Seven housekeeping and ten virulence-associated genes	++	+	+++	++	+++	++	+++
MLVA	Whole genome, tandem repeats	+++	+	+++	++	+++	++	+++

REA; restriction enzyme analysis, RFLP; restriction fragment length polymorphism, AP-PCR; arbitrary primed PCR, RAPD; random amplified polymorphic DNA, PFGE; pulsed-field gel electrophoresis, AFLP; amplified fragment length polymorphism, MLST; multilocus sequence typing, MLVA; multilocus VNTR analysis

binary toxin are located outside the pathogenicity locus (PaLoc), but the role of this toxin is unclear *(1)*. The illness associated with *C. difficile* (*C. difficile* infection, CDI) ranges from mild diarrhea to life-threatening colitis.

To study the epidemiology of CDI, a typing method with a high discriminatory power, typeability, and reproducibility is required. Typing methods are also used to determine the role of the environment and patient-to-patient transmission in the cause of infection and for the investigation of outbreaks. The recurrence rate of *C. difficile*-associated disease is around 15–20%, and typing methods can be applied to distinguish recurrences in relapse, due to the same strain, or reinfection, due to a new strain *(1)*.

Typing methods can be classified in two large categories, consisting of phenotypic and genotypic methods. Phenotypic methods differentiate on the basis of products of gene expression, whereas genotyping methods analyze the genetic profile of the strains. Molecular typing methods are generally regarded as having advantages over phenotypic methods in terms of the stability of genomic markers and providing greater levels of typeability. A growing number of molecular methods have been applied to *C. difficile*, and these are described here (*see* **Table 1** for an overview).

2. Traditional Molecular Typing Methods for *C. difficile*

2.1. Plasmid profiling

Plasmid profiling was the first genotypic typing method applied to *C. difficile (2)*. The fact that not all *C. difficile* strains contained these extrachromosomal elements made the typeability of this method very low. In addition, strains may lose or acquire plasmids and thereby change plasmid profile *(2–4)*.

2.2. Restriction Enzyme Analysis and Restriction Fragment Length Polymorphism

Restriction enzyme, or endonuclease, analysis (REA) uses the whole genomic DNA. This DNA is digested by rare-cutter restriction enzymes, resulting in restriction fragments readable by polyacrylamide gel electrophoresis (PAGE) or agarose gel electrophoresis. The first applied REA was described by Kuijper et al. using *Hin*dIII and *Xba*I for restriction and agarose gels for analysis of the fragments *(5)*. They found that the strains detected in two patients were indistinguishable from four samples from the hospital environment, thereby showing the applicability of this method for typing *C. difficile*. They also found that the method was stable after subculturing. Another study described the use of *Cfo*I as the restriction enzyme; however, *Hin*dIII is still mostly used *(6,7)*. REA has been used as the standard typing method in North America because of its high discriminatory power and stability *(7)*, but the interpretation of REA banding patterns is

subjective, and comparative analysis of isolates has to be performed on the same gel. REA is a highly discriminatory and reproducible method; it is, however, a technically demanding procedure and very labor intensive, especially for analyzing the complex banding patterns of large numbers of isolates. For these reasons, REA data are difficult to exchange between laboratories, which is becoming an increasingly important factor for evaluating typing methods.

Restriction fragment length polymorphism (RFLP) is an alternative method that involves initial REA digestion and gel electrophoresis followed by Southern blotting with selected labeled nucleic acid probes to highlight specific restriction site heterogeneity. The difference between REA and RFLP is very small, and the designations are used interchangeably in different studies. The first description of RFLP was by Bowman et al.; restriction enzyme (*Hin*dIII) digestion was followed by gel electrophoresis and subsequent Southern blot transfer and hybridization with labeled *Escherichia coli* ribosomal RNA (rRNA) probes ***(8)***. In comparison with sodium dodecyl sulfate PAGE, immunoblotting, and REA, RFLP with an eubacterial 16S rRNA probe provided simpler patterns and yielded good discrimination ***(9)***. Another study compared the RFLP with enhanced chemiluminescence to REA, both using the *Hin*dIII enzyme for restriction ***(10)***. REA was found far more discriminatory than RFLP (34 versus 6 types among 116 isolates). REA and RFLP methods have now generally been superseded by methods based on amplification of selected targets using the polymerase chain reaction (PCR).

2.3. Arbitrary Primed PCR and Random Amplified Polymorphic DNA

Arbitrary primed PCR (AP-PCR) and random amplified polymorphic DNA (RAPD) are two methods based on PCR amplification (*see* **Chapter 4**). The primers do not have a known homology to the target sequence; subsequently, a low annealing temperature is applied. The difference between AP-PCR and RAPD is the application of a single primer versus the use of two short primers, respectively. The first described AP-PCR used six different arbitrary primers of 10–11 bp and detected six different patterns among six isolates ***(11)***.

In an outbreak among eight acquired immunodeficiency syndrome (AIDS) patients, the AP-PCR was applied using one arbitrary primer of 10 bp, differing only one nucleotide from one of the primers used by McMillin et al. ***(12)***. Among the eight isolates, seven revealed an identical AP-PCR pattern, whereas four reference strains could be discriminated from each other and the outbreak isolates. The authors concluded that the AP-PCR is simple, rapid, and discriminative for typing *C. difficile*.

Another outbreak was investigated with nearly similar arbitrary primers as in the first two studies, but a lack of reproducibility of the AP-PCR was found ***(13)***. Compared to a phenotypic method such as immunoblotting, AP-PCR resulted in better typeability

(14), and good correlation was found between AP-PCR and REA data *(15–17)*.

AP-PCR usually results in 3–12 bands between 450 and 1,300 bp, which can simply be analyzed on agarose gels. The method is cost-effective but is extremely sensitive to PCR conditions. Therefore, AP-PCR has low reproducibility, and it is difficult to establish interlaboratory comparison with this method *(18)*.

RAPD was first applied on *C. difficile* by Barbut et al. *(19)*. RAPD commonly uses two oligonucleotide primers that are short in length (ca. 10 bp) and of arbitrary sequence. Barbut et al. evaluated a RAPD method using two 10-bp primers in an investigation of CDI in AIDS patients. An identical profile was in 15 of 25 isolates, indicating a common source. RAPD compared well with pulsed-field gel electrophoresis (PFGE); while easier to perform, the results are more difficult to analyze, however *(20)*. The applicability of RAPD in the analysis of relapses versus reinfection in patients infected with the human immunodeficiency virus was shown by Alonso et al. *(21)*. Relapses were detected in 64% of patients, whereas 32% had a reinfection, and 4% had both a relapse and a reinfection *(21)*.

2.4. PCR Ribotyping

PCR ribotyping uses specific primers complimentary to sites within the RNA operons and was first applied to *C. difficile* by Gurtler, who targeted the amplification process at the spacer regions between the 16S and 23S rRNA genes *(22)*. *Clostridium difficile* was shown to possess multiple copies of the rRNA genes, which varied not only in number between strains but also in size between different copies on the same genome *(22,23)*. The method developed by Gurtler and Mayall, using radiolabeling and a long-running PAGE, detected 14 PCR ribotypes among 24 strains. The approach was simplified by Cartwright et al., who applied PCR ribotyping to 102 isolates obtained from 73 symptomatic patients *(24)*. A total of 41 types was recognized, and five of six outbreak isolates were identical *(24)*. Using the same primers as Gürtler, the PCR fragments could be separated by straightforward agarose gel electrophoresis instead of denaturing PAGE gels. The banding patterns were not affected by the quantity of DNA used in the reaction (a problem associated with AP-PCR and RAPD methods), the PCR ribotype marker was stable, and its expression was reproducible. In a comparison with the other PCR-based typing method AP-PCR, PCR ribotyping was very discriminatory and showed an agreement of 83% with PFGE, compared to 60% and 44% for AP-PCR *(25)*.

To obtain smaller fragments for better analysis on agarose gels, new primers, closer to the spacer region, were designed by O'Neill et al. in 1996 *(26)*. The amplicons, ranging from 250 to 600 bp in length, could be separated by straightforward agarose gel electrophoresis. This approach was adapted for routine use

after simplifying the method for DNA extraction *(26)*. The discriminatory power of this PCR ribotyping was compared to Delmee's serogroups, and different banding patterns were demonstrated for each of the 19 serogroups described at that time. Using these primers, at least 116 types could be discriminated within an isolate collection including nontoxinogenic and environmental strains *(27)*.

This method has since been used routinely by the U.K. Anaerobe Reference Laboratory in Cardiff, which has provided a *C. difficile* typing service for the United Kingdom since 1995. From nearly 10,000 isolates from all sources examined, a library that currently consists of over 160 distinct PCR ribotypes has been constructed. The nomenclature of types designated by this method is a three-figure numeral, and the status of this PCR ribotype library was published in 1996 when 116 types (types 001 to 116) were recognized *(27)*.

Bidet et al. further optimized PCR ribotyping using new, more specific primers based on known sequences of the 16S and 23S genes of *C. difficile (28)*. Although the method by Bidet shows better separation of bands, a large library has not yet been established as is the case with the O'Neill method, which is used worldwide *(26–29)*.

PCR ribotyping has proved a robust genotyping method, being stable and reproducible *(24,25,29–31)*. Results can be used for interlaboratory comparison and for the generation of libraries. PCR ribotyping is currently the preferred typing method in our laboratories.

2.5. Toxinotyping

Toxinotyping involves the detection of polymorphisms in the toxin A and B genes and surrounding regulatory genes, an area of the genome known collectively as the *pathogenicity locus* (PaLoc). Six regions of the PaLoc (A1–A3 and B1–B3) are amplified and digested by restriction enzymes, like in REA *(32)*. B1 and A3 are considered the most variable and are therefore good markers for detecting most toxinotypes *(33)*. Until now, 26 toxinotypes (0–X, XIa, XIb, XII–XXIV) can be discriminated among *C. difficile* strains *(32–34)*; http://www.mf.uni-mb.si/mikro/tox). Toxinotyping has been compared to serogrouping and PCR ribotyping, and a good correlation was found. Some toxinotypes are strictly associated with certain serogroups (e.g., toxinotype VIII is always seen in serogroup F strains). However, toxinotyping could further distinguish subgroups within the serogroups *(32)*. A specific PCR ribotype was usually associated with similar patterns of the toxin genes, but both methods are able to subtype each other, making toxinotyping a good addition to typing schemes *(33)*.

Barbut et al. applied the toxinotyping method on toxin A variant strains that represented 2.7% of diarrheal cases in adults

and children. Two variant types were identified by PCR of fragment A3; one type was related to toxinotype VII, due to a deletion of 600 bp in fragment A3, whereas the other type was related to toxinotype XIV, with an insertion of about 200 bp *(35)*. In a study of 153 clinical isolates from an American hospital, 11.1% of strains belonged to toxinotypes other than toxinotype 0. An additional toxin, the binary toxin, was found only in nine strains, all of which were variant toxinotypes *(36)*.

The reproducibility of the method is 100%, and the discriminatory power is good, although, for example, PFGE and PCR ribotyping show more discrimination between strains. The most important advantage of this typing method is that a clear view of the toxin status of *C. difficile* strains can be acquired.

3. Recently Developed Typing Methods for *C. difficile*

3.1. flic*C* Typing

An alternative PCR target for typing purposes is the flagellin gene *flicC*, described by Tasteyre et al. *(37)*. In a study of 47 isolates belonging to 11 different serogroups, three profiles could be recognized. When the method was expanded with RFLP analysis, nine different RFLP patterns were recognized. Although nonflagellated strains were included, they did contain the *fli*C gene. In a study with nine toxin A–/B+ strains, only three strains showed flagella. However, all nine strains belonged to the same type using *fliC* PCR-RFLP *(38)*.

3.2. slp*A* Typing

Another gene studied for typing is the *slp*A gene, encoding an S-layer precursor protein of *C. difficile*. Seven S types have been recognized, of which one type accounted for 73% of the clinical cases and 93% of the environmental cases *(39)*. Thirty-two strains belonging to ten serogroups were used for PCR-RFLP and sequencing analysis of the variable region. This RFLP-sequence combination led to sequences identical within a given serogroup and differences between serogroups and was therefore thought of as an alternative typing method for *C. difficile (40)*. The *slpA* genotyping by PCR-RFLP was subsequently tested on Japanese outbreak strains and resulted in three subtypes. The method was also applied directly on fecal samples, and results were in complete agreement with the cultured strains from these samples *(41)*. Typing of *slp*A is considered a reproducible method with the advantage of interlaboratory data exchange. The s*lp*A typing of strains of 14 different PCR ribotypes identified 9 groups; PCR ribotypes showed completely identical *slp*A sequence in two cases and 1- to 3-bp differences within other groups *(42)*.

3.3. Amplified Fragment Length Polymorphism

Amplified fragment length polymorphism (AFLP) has also been applied as a typing method for *C. difficile* ***(43)***. The AFLP method uses restriction, ligation, and selective amplification on the whole genome. Differentiation can be made due to variation per type in restriction site mutations, mutations in the sequences adjacent to the restriction sites and complementary to the selective primer extensions, and insertions and deletions within the amplified fragments. While the reproducibility of AFLP was similar to PFGE, Klaassen et al. showed that the typeability of AFLP was better, especially for isolates for which some DNA degradation had occurred. In addition, AFLP was found to be faster and easier to perform on small quantities of DNA ***(43)***. Analysis of 30 clinical isolates encompassing all known sero(sub)groups and of 30 PCR ribotype 017 toxin A–/B+ isolates from various countries showed that the discriminatory power of AFLP was similar to that of PFGE ***(44)***.

3.4. Multilocus Sequence Typing

Multilocus sequence typing (MLST) has also been tested as a typing method for *C. difficile*. MLST consists of DNA sequence analysis of housekeeping genes after PCR amplification and is mostly used to study genetic relationships and population structures ***(45)***. MLST developed for *C. difficile* includes seven housekeeping genes. Among 72 isolates from various origins, 62 PCR ribotypes and 34 sequence types (STs) could be discriminated. In a dendrogram representing the relationships between the STs, three divergent lineages could be recognized, of which one strictly contained toxin A–/B+ strains ***(45)***. The method was further expanded by the inclusion of ten virulence-associated genes, among which were *fli*C, *slp*A, *tcd*A, *tcd*B, and *tcd*D ***(46)***. A total of 29 isolates from various origins and representing 22 STs selected from the lineages found in their first study were investigated. The polymorphisms detected in the virulence-associated genes were comparable to those of the housekeeping genes. However, *cwp66* and *slpA* appeared highly polymorphic, although only 11 and 16 alleles could be detected, respectively. Again, toxin A–/B+ strains belonged to a homogeneous lineage, and a fourth lineage could be characterized in contrast to the method based on only housekeeping genes ***(46)***. No association was found between the STs and the clinical presentation or the source of the isolates ***(45,46)***. It was concluded that MLST with the virulence-associated genes included was more discriminatory than the housekeeping genes alone, although this could depend on the genes chosen. The main advantage of the method is the yield of unambiguous sequence data. No comparisons with other techniques have been described to date.

3.5. Multilocus Variable Number of Tandem Repeats Analysis

The analysis of the sequenced human and bacterial genomes revealed a high percentage of DNA that consisted of a variable number of tandem repeats (VNTR). The repeats vary in size,

location, complexity, and repeat mode and can occur clustered in one genomic area or dispersed throughout the entire genome. These repeat arrays can be targets for genomic events, such as DNA polymerase slippage and recombination. It is the polymorphic property of the VNTRs that led to the application in identification and typing of bacteria. Multilocus VNTR analysis (MLVA) has already been tested successfully on a number of bacterial species due to its high reproducibility, high discriminatory power, and typeability ***(47)***. The availability of the complete sequence of the *C. difficile* genome of strain 630 (http://www.sanger.ac.uk/Projects/C_difficile/;***48)*** provided the opportunity to identify these short tandem repeats.

The MLVA developed by Marsh et al. uses automated sequence detection and subsequent manual determination of the number of tandem repeats per locus ***(49)***. Seven short tandem repeat loci were amplified from 40 isolates from two different sources, and REA was tested on every strain as well. The stability was good, although differences of one repeat could arise. This MLVA clustered outbreak strains of the same REA type and discriminated different REA types from each other.

For a faster and easier application of the MLVA for *C. difficile*, a new method was developed using smaller short tandem repeats (2–9 bp) to facilitate automated fragment analysis with multicolored capillary electrophoresis instead of sequencing ***(50)***. This MLVA technique was compared to PCR ribotyping and tested on a set of 56 reference strains encompassing 31 serogroups and 25 toxinotypes. In addition, clinical isolates were included from outbreaks in different countries due to the new emerging type 027 and the toxin A–/B+ strain PCR ribotype 017. Of seven VNTR, four were identical to those used in the study of Marsh et al. ***(49)***. MLVA was highly (100%) reproducible with an excellent stability of all seven loci. All tested PCR ribotypes could be recognized, including the seven subtypes of 001. In contrast to PCR ribotyping, MLVA was able to discriminate strains belonging to serogroups A7 from A11, A9 from A10, A8 from S1, H from K, and A14 from S4. Toxin A–/B+ strains could be recognized as eight country-specific clusters. All strains with 100% similarity belonged to country-specific clusters. Interestingly, toxin A–/B+ strain could be differentiated from all other types using the combination of two markers. PCR ribotype 027 strains from several outbreaks in the Netherlands were clustered in 14 different groups using MLVA; the clusters were mostly hospital specific. All strains were completely identical to each other with the combination of three markers with 10, 4, and 2 repeats, respectively. Only the U.K. strain showed six repeats for a marker instead of ten, indicating a possible difference between type 027 strains from specific countries.

The utility of MLVA and PFGE to identify clusters of CDI was tested among 91 isolates of PCR ribotype 027 (NAP1, for North

American pulsed-field type 1) from nine hospitals in England *(51)*. PFGE discriminated between ribotype 027 strains at greater than 98% similarity, identifying five pulsovars (I to V) with 1 to 53 isolates each. MLVA was markedly more discriminatory, identifying 23 types with 1 to 15 isolates (>71% similarity). MLVA was far superior to PFGE for analyzing clusters of CDI both within and between institutions.

In a study using isolates from laboratories in Canada, the Netherlands, the United Kingdom, and the United States, seven *C. difficile* typing techniques were compared: MLVA, AFLP, *slpA* sequence typing, PCR ribotyping, REA, MLST, and PFGE *(52)*. All 42 isolates were typeable by all techniques, but only REA and MLVA showed sufficient discrimination to distinguish strains from different outbreaks *(52)*. MLVA has also been applied to study local outbreaks of clindamycin-resistant *C. difficile* PCR ribotype 027 strains *(53,54)*.

4. Conclusions

All typing methods have certain advantages and disadvantages, but their ultimate contribution to knowledge is dictated by their performance according to the criteria listed by Struelens: typeability, reproducibility, stability, discriminatory power, and epidemiological concordance. It should also have technical advantages, such as ease of performance, relative low cost, and high throughput. In due course, as new methods come and go, one method will probably emerge as the most suitable. Currently, the PCR-ribotyping method and library of PCR ribotypes in Cardiff are the benchmark to which most typing studies around the world are compared, and more important, they have probably contributed most to our current knowledge of the global epidemiology of *C. difficile*. Undoubtedly, further advances in molecular subtyping methods will add even further to our understanding of the epidemiology this important nosocomial pathogen.

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Chapter 14

Genotyping of *Mycobacterium tuberculosis* Clinical Isolates Using IS*6110*-Based Restriction Fragment Length Polymorphism Analysis

Pablo Bifani, Natalia Kurepina, Barun Mathema, Xiao-Ming Wang, and Barry Kreiswirth

Abstract

A number of phylogenetic studies of *Mycobacterium tuberculosis* have suggested a highly clonal population structure. Despite the extreme homogeneity of *M. tuberculosis* strains, the genome is punctuated by a number of polymorphic regions that give rise to sufficient diversity, thus forming the basis for molecular epidemiologic studies of tuberculosis. As such, insertion sequence (IS) *6110*, which is unique to members of the *M. tuberculosis* complex and is present in variable numbers and in discrete genomic locales among strains, has been extensively used in molecular epidemiologic studies. Genotyping, using IS*6110*-based restriction fragment length polymorphism (RFLP), was standardized by the international community, and this has facilitated inter- and intralaboratory comparison, thereby serving as a model system for subspeciation of *M. tuberculosis*. When IS*6110*-based RFLP was used in conjunction with conventional epidemiologic data, its utility was realized. In this chapter, we discuss the basic methodology for conducting IS*6110*-based RFLP and analyzing the resulting hybridization profiles.

Key words: IS*6110* insertion sequence, molecular epidemiology, *Mycobacterium tuberculosis*, Southern blot hybridization.

1. Introduction

Tuberculosis (TB) remains one of the world's most prevalent infectious diseases, accounting for 9 million new cases and 1.7 million deaths in 2006 alone *(1)*. The problem is exacerbated by the growing number of individuals with TB coinfected with the human immunodeficiency virus, as well as by cases of multidrug-resistant

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TB (MDR-TB) and extensively drug-resistant TB (XDR-TB). A report by the World Health Organization estimated 458,000 individuals with MDR-TB globally *(2)*, underlining the urgent need to control this disease. Although efforts are being made in search of new antimycobacterial compounds, various therapies, and vaccine development, history has proven that the most efficacious means of controlling infections relies on the implementation of adequate public health measures and improvement of basic living conditions.

Thus, to improve TB control efforts, a more thorough understanding of TB epidemiology is essential. This can be achieved by elucidating transmission dynamics, the contribution of recent versus reactive disease, as well as the nature and extent of drug resistance among studied populations. Due to the intrinsic characteristics of TB's etiologic agent, *Mycobacterium tuberculosis*, that is, slow growth (~24 h doubling time), a long latency period, and airborne route of infection, some key epidemiologic questions remain elusive. Recently, however, our understanding of TB epidemiology has benefited extensively from the integration of molecular techniques with conventional epidemiologic data; which is known as *molecular epidemiology*. This is providing greater resolution to address previously unanswered questions relevant to TB control and prevention. With increased access to genomic information, a number of techniques have been developed to genotype or fingerprint *M. tuberculosis*. In some cases, these techniques have been implemented on a large number of clinical isolates from diverse geographic and epidemiologic sources, thereby rendering *M. tuberculosis* genotyping a model system in the nascent field of molecular epidemiology. Here we discuss some of the characteristics and applications of insertion sequence (IS) *6110*-based fingerprinting of *M. tuberculosis* isolates for molecular epidemiological studies. The specific protocols for *M. tuberculosis* strain genotyping may vary according to the resources available and on approval by the institutional biosafety committees of the local or national laboratories.

1.1. Characteristics of the M. tuberculosis IS6110

Mycobacterium tuberculosis is a member of group of closely related species, collectively known as the *M. tuberculosis* complex (MTBC), which is comprised of seven members (*M. tuberculosis* sensu stricto, *Mycobacterium africanum, Mycobacterium bovis, Mycobacterium canetti, Mycobacterium microti, Mycobacterium caprae,* and *Mycobacterium pinnipedii*). Among other unique features, such as extreme genetic homogeneity and wide host-specific ranges, the MTBC bears a unique IS, IS*6110*. IS*6110* is a transposable element that is a member of the IS3 family of ISs or mobile elements (*3*).

Briefly, IS*6110* is a 1,355-bp long fragment that encodes four enzymes required for its own transposition and insertion and is

flanked by imperfect 28-bp inverted repeats *(4)*. On insertion, a 3- to 4-bp target duplication is generated, and loss is usually accompanied by deletions (genomic scars) of the flanking regions. IS*6110* may transpose into functional genes or regulatory sequences and hence can alter gene expression and subsequently the protein profile, thus in some cases altering the phenotype *(5)*. IS*6110* is found in virtually all *M. tuberculosis* strains (**Fig. 1**), with some bearing up to 24 copies, although there exist strains, albeit rarely, with no IS*6110* elements. The factors that drive IS*6110* transposition have not yet been fully understood, but transposition has been shown not to be dependent on sequence variation of the element itself as IS*6110* is highly conserved throughout (*6*).

The observed diversity in IS*6110* copy number and genomic position between unrelated strains of *M. tuberculosis* can be utilized to examine microevolutionary processes. In addition, phylogenetic studies using synonymous single-nucleotide polymorphisms

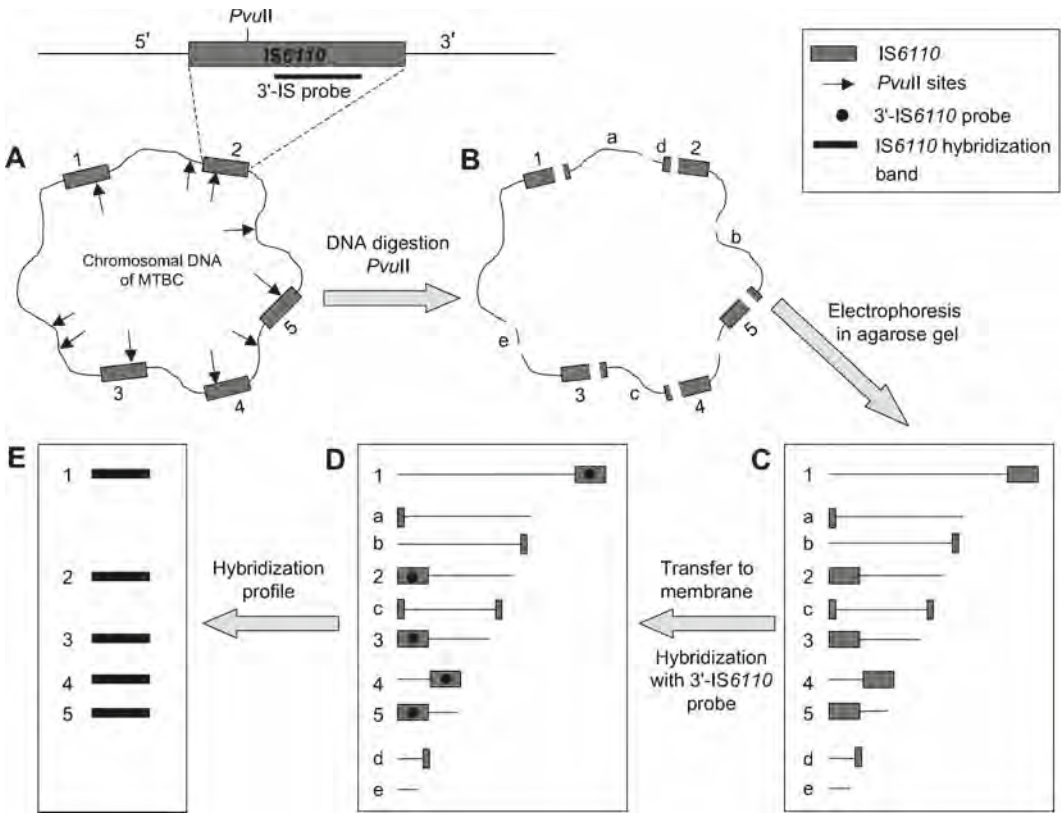

Fig. 1. The schema of the principal procedures in IS*6110*-based RFLP genotyping. (**A**) Localization of five IS*6110* copies in the *M. tuberculosis* genome (positions and orientation of IS may be different in different clinical isolates); (**B**) *Pvu*II digest of chromosomal DNA; (**C**) electrophoresis in agarose gel distributes DNA fragments according to their molecular weight (size); (**D**) the presence of IS*6110* 3′-specific arm in chromosomal DNA fragments; (**E**) bands revealed after hybridization with IS*6110*-specific probe.

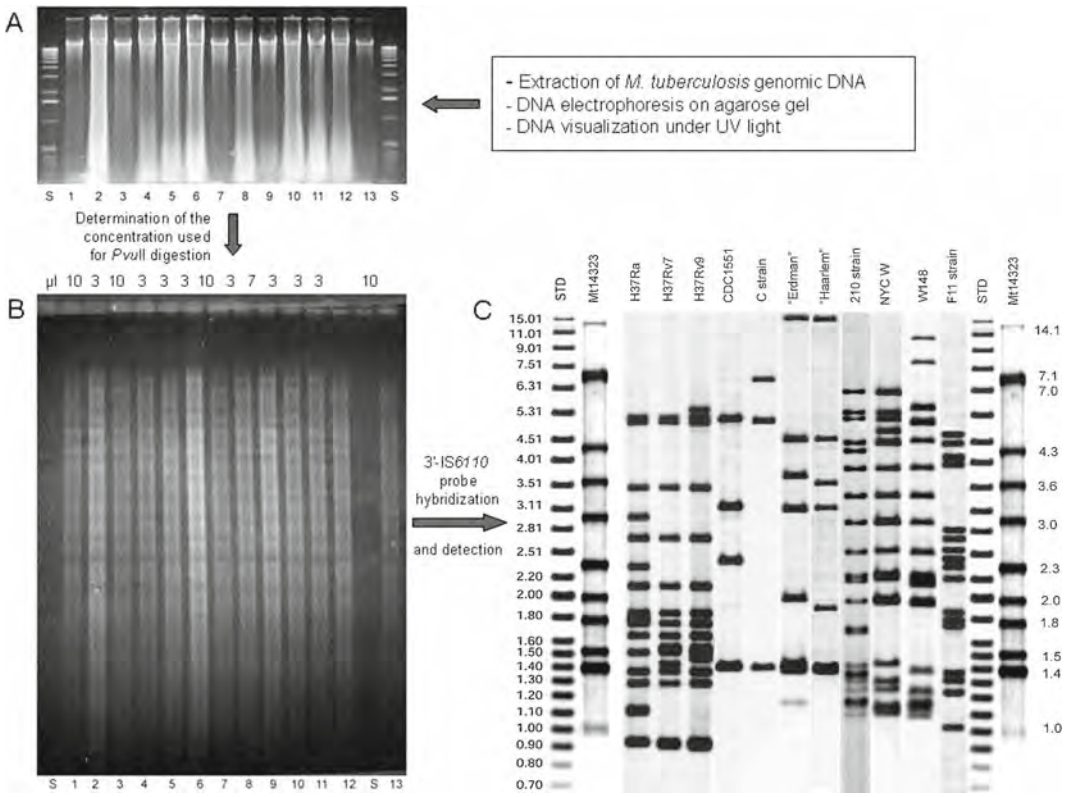

Fig. 2. Example of hybridization blot in different stages of DNA analysis. (**A**) Electrophoresis of total chromosomal DNA, 5 µL of each sample loaded. Calculation of the amount of chromosomal DNA needed for hybridization (bottom: sample number; under: volume of DNA required). (**B**) Electrophoresis of *Pvu*II-digested chromosomal DNA, stained with ethidium bromide. (**C**) Blot after overnight hybridization with IS*6110*-specific probe and detection procedures.

(sSNPs) have shown that IS*6110* copy number and location are similar within discrete lineages (or clades), underlying evolutionary significance, as illustrated in **Fig. 2**. Not surprisingly, isolates with distinct IS*6110* profiles seem to aggregate socially and therefore track to geographic locales where they may be endemic.

*1.2. IS*6110*-Based Restriction Fragment Length Polymorphisms*

One key aspect underlying the success of molecular epidemiologic studies of *M. tuberculosis* is the implementation and adoption of a standardized protocol for IS*6110*-Southern blot hybridization. This standardized protocol allowed for inter- and intralaboratory comparative analysis and set a precedent for other bacterial genotyping systems. The standardization involved (i) the use of restriction endonuclease *Pvu*II for *M. tuberculosis* genomic DNA digestion, yielding a smear of *Pvu*II-flanked fragments; (ii) the selection of the 3′ (right-side) fragment of IS*6110* as a

hybridization probe; and (iii) a standardized molecular weight marker and technical recommendations for conducting IS*6110*-based restriction fragment length polymorphism (RFLP) *(7)*. Briefly, as shown in **Fig. 3**, *Pvu*II-restricted fragments of chromosomal DNA are separated by electrophoresis, blotted onto a nitrocellulose membrane and hybridized with the 3′-IS*6110* fragment as a hybridization probe (the IS*6110* elements contain one asymmetrical *Pvu*II restriction site with 3′-arm 900-bp long). The DNA fragment serving as the IS*6110*-specific probe can be generated by polymerase chain reaction (PCR) using *M. tuberculosis* chromosomal DNA. Alternatively, an *Escherichia coli* plasmid, such as pUC18, containing the cloned 3′-IS*6110* fragment as the target DNA for amplification can be used. This plasmid containing a 312-bp 3′-IS*6110* fragment (pUCIS) can be requested from Dr. Kurepina at PHRI (Newark, NJ). The probe can be labeled using radioactive ^{32}P or with a chemiluminescence kit (ECL, GE Healthcare, UK; GE Healthcare Life Sciences, Piscataway, NJ). Following hybridization and detection, the number of bands (= number of IS*6110* copies per genome) and patterns of bands on

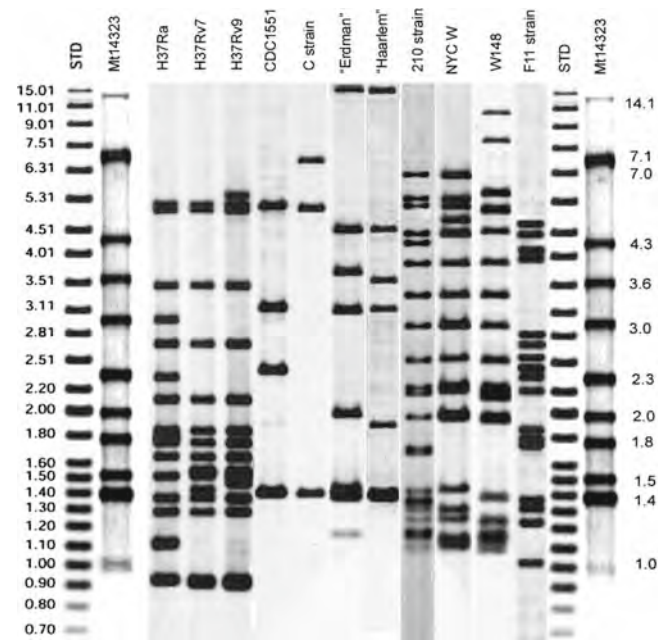

Fig. 3. IS*6110*-based RFLP images of the most known and annotated clinical and laboratory *M. tuberculosis* strains. STD, standards, developed by the U.S. Centers for Disease Control and Prevention (CDC); Mt14323, strain used as standard for *M. tuberculosis* comparison. H37Ra, H37Rv7, and H37Rv9 are laboratory strains; CDC1551, C strain, "Erdman" and "Haarlem" (strains completely sequenced); W strain, W148, and F11 (sequencing in progress).

the blot (size of *Pvu*II-flanked chromosomal fragment containing the right-side IS*6110* band) form the fingerprint of the strain.

1.3. Applications of *IS*6110 *RFLP in TB Epidemiology*

The main objective of molecular epidemiology is to associate particular epidemiologic data with strain relatedness, for example, to identify chains of transmission. In general, IS*6110* RFLP analysis has been a highly valuable tool when analyzing populations of *M. tuberculosis* isolates. The stability of IS*6110* has proven to be sufficient to identify the same strains spreading from one patient to another, thus, implicating transmission. Yet, IS*6110* is sufficiently mobile to show diversity within a given population. The two criteria, stability and diversification over the time (biological clock), are fulfilled by IS*6110,* making it a suitable epidemiological marker. The exception lies in *M. tuberculosis* isolates comprising fewer than six copies of IS*6110*; thus, by convention, it has been agreed that RFLP pattern discrimination is reliable for samples possessing six or more IS*6110* copies. This limitation does not represent a problem in cosmopolitan cities, where strain diversity is extensive. However, in some geographical regions, such as southern India, isolates carrying one or two insertions are predominant, hence rendering this analytical tool futile.

IS*6110* RFLP has proven to be highly significant in the analysis of samples harboring multiple IS*6110* copy number isolates. For example, the W-Beijing strains (with more than eight IS*6110* elements) that are predominant in China, Southeast Asia, and Eurasia can be sufficiently discriminated by IS*6110* RFLP analysis. The relatedness and frequency of certain strain types in the populations can be determined, as is the case with other groups of related strains (e.g., Harleem strains).

1.4. Combining *IS*6110 with Other Molecular Techniques for Specific Strain Identification

In general, it can be assumed that isolates with more than five IS*6110* copies displaying different IS*6110* RFLP profiles may be cases of reactive disease, while clusters (i.e., identical IS*6110* profiles) may represent a transmission event. Isolates with fewer than six bands need to be analyzed by other unrelated molecular techniques to draw proper inference on chains of transmission. Other molecular markers can be spoligotyping, mycobacterial interspersed repetitive unit variable number tandem repeat (MIRU-VNTR) analysis, deligotyping, SNPs, or any particular characteristics of the isolates in question, including unique sequences, duplications, deletions, insertions, or drug resistance phenotypes ***(8)***. In addition, coupling IS*6110* fingerprinting with other molecular markers can be useful in identifying clonal strains and substrain families or following the microevolution of a given strain in a population. Such specific markers have been used to determine the clonal nature of the W-Beijing strain family ***(9)***, to identify substrain families of epidemiologically related W-Beijing phenotypes ***(10–12)***, and to follow the microevolution of isolates with

few IS*6110* elements *(13)*. Deletion of spacers within the direct repeat locus (*see* **Chapter 10**) can be in some cases associated with an IS*6110* band shift, as shown in **Fig. 4** for strains BW90 and BW900 or strain W14 (*12*).

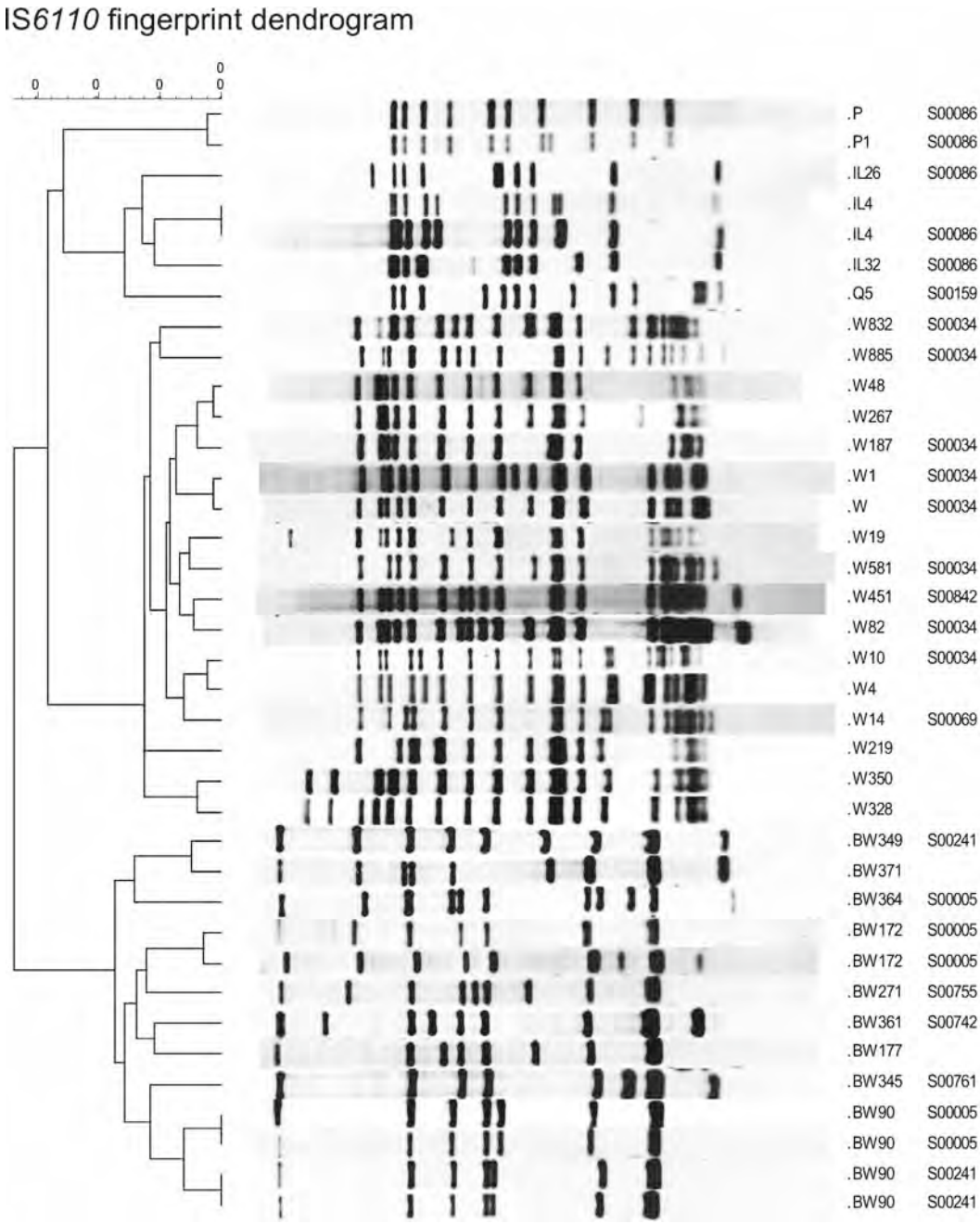

Fig. 4. Dendrogram of clinical *M. tuberculosis* strains (Bionumerics, Applied Math, Belgium). These three groups of isolates correspond to the spoligofamilies identified by spoligotyping technique (*see* **Chapter 10**). Strains W and W1 differ by one band and were part of the same outbreak in New York City in the 1990s.

2. Materials

2.1. Media for *M. tuberculosis* Culture

1. Preferred choice is Lowenstein Jensen (LJ) (Difco or homemade) slants.
2. Alternatively, use Middlebrook 7H11 agar (Difco). Dissolve 21 g in 900 mL deionized water, supplement with 10 mL of 50% glycerol (sterile stock 50/50 glycerol/deionized water). Melt the agar at 100°C and autoclave at 121°C for 15 min. Cool to 55°C and add 100 mL of prewarmed (37°C) Middlebrook OADC supplement (Difco). Mix and pour plates.

2.2. DNA Isolation

1. Proteinase K (Boehringer Mannheim): Stock aliquots of 10 mg/mL at –20°C.
2. Lysozyme (Boehringer Mannheim): Stock aliquots of 10 mg/mL at –20°C.
3. Ribonuclease (RNase) A (Boehringer Mannheim).
4. 10X TE buffer: 100 m*M* Tris-HCL, pH 8.0, and 10 m*M* ethylenediaminetetraacetic acid (EDTA) in distilled water. For 1X TE, dilute in a ratio of 1:10 with distilled water.
5. 10% sodium dodecyl sulfate (SDS): Dissolve 10 g of SDS in 100 mL distilled water at 65°C.
6. 10% *N*-acetyl-N_3-trimethyl ammonium bromide (CTAB) (Merck) in 0.7*M* NaCl: Slowly add 10 g of CTAB to 4.1 g NaCl in 100 mL distilled water at 65°C. Adjust volume to 100 mL.
7. Chloroform/isoamyl alcohol (24:1): Mix 24 volumes of chloroform with 1 volume of isoamyl alcohol.
8. 5*M* NaCl: Dissolve 29.2 g NaCl (Fisher) in 100 mL distilled water.
9. 70% ethanol.
10. Water bath (80°C) and thermomixer (up to 60°C).
11. Centrifuge for 1.5-mL Eppendorf tubes.
12. Speed Vac (Savant Speed Vac Systems, GMI Inc., MN).

2.3. Electrophoresis

1. Electrophoretic-grade agarose (FMC).
2. 10X TBE buffer: Dissolve 108 g Tris-base and 55 g boric acid in 700 mL distilled water and 40 mL of 0.5*M* EDTA at pH 8.0. Adjust to 1 L and autoclave. For use, dilute to 1X in distilled water.
3. Horizontal electrophoretic chamber, 20 cm in length. Bio-Rad DNA Sub Cell chamber for membranes 20 × 15 cm (Bio-Rad Laboratories).
4. Electrical power supply (e.g., EC-103, E-C Apparatus Corp., Pittsburg, PA; EB103, Fisher Biotech, Pittsburgh, PA).

5. Loading dye: 5 mL 10X TBE, 25 mL glycerol, 15 mL H_2O, 5 mL 1% (w/v) double dye (1% bromophenol blue and xylene cyanol in H_2O).
6. DNA fragment size standards. Several size standards have been used. The most commonly used standard is Mt14323, which consists of chromosomal DNA isolated from clinical strain 14323, digested with *Pvu*II and loaded on each agarose gel (*see* **Note 1**).

2.4. Vacuum Blotting

1. Hybond-N+ (Nytron) membrane 20 × 15 cm (Amersham Biosciences, UK) or Zeta-Probe Blotting membranes (Bio-Rad Laboratories, USA).
2. 20X SSC stock solution: 3*M* NaCl, 0.3*M* Na_3-citrate, pH 7.0. Dilute 10X SSC in a ratio of 1:2 with distilled water.
3. 1*M* HCl (hydrogen chloride): Dilute 85.5 mL of concentrated HCl in 914.5 mL distilled water. For 0.25*M* HCl, dilute 1*M* HCl in a ratio of 1:4 with distilled water or 5 mL of concentrated HCl diluted to 500 mL in distilled water.
4. 4*M* NaOH (sodium hydroxide): Dissolve 160 g NaOH in 800 mL distilled water. Adjust to 1 L. For 0.4*M* NaOH, dilute 4*M* NaOH in a ratio of 1:10 with distilled water.
5. Soak I: 0.5*M* NaOH and 1.5*M* NaCl (dissolve 60 g NaOH and 262.98 g NaCl in 3 L of water).
6. Soak II: 0.5*M* Tris–HCl and 1.5*M* NaCl (dissolve 125.2 g Tris and 175.33 g NaCl in 2 L of water, adjust to pH 7.2 with 80 mL of HCl).
7. Vacuum blotter (VacuGene XL, Farmacia) (*see* **Note 2**).
8. UV crosslinker (FB-UV XL-100, Fisher Scientific, Fisher Biotech).

2.5. Preparation of Probe from Genomic DNA or Plasmid

1. The primers RIS3, 5′-CGTCGAACGGCTGATGACCA; 6110-R, 5′-GGCGGGTCCAGATGGCTTGC are used for the amplification of the IS*6110* probe from chromosomal *M. tuberculosis* DNA. T7 universal primers can be used for the amplification of the IS*6110* fragment from pUCIS.
2. A 50 µL reaction mixture should contain 10 pmol of each primer, 1 ng of genomic DNA, 200 µ*M* deoxyribonucleoside triphosphates, 1X PCR buffer (pH 8.3), 1.5 m*M* $MgCl_2$, and 1–2 units of *Taq* polymerase. The PCR conditions include 95°C at 4 min for initial denaturation followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s followed by a single 2-min extension at 72°C.

2.6. Hybridization, Washing, and Detection

1. Enhanced Chemiluminescense Direct Nucleic Acid Labeling and Detection Kit (ECL kit, Amersham). The ECL kit includes labeling reagent, glutaraldehyde, double-distilled water for probe dilution, hybridization buffer, and blocking agent.

2. Pro-Blot hybridization oven (Labnet International Inc.) and roller bottles (Robbins Scientific or Labnet International Inc.).
3. Primary wash buffer: Mix 360 g urea and 4 g SDS (or 20 mL of 20% SDS) in 25 mL 20X SSC; adjust to 1 L with distilled water.
4. Secondary wash buffer: 2X SSC. Dilute 20X SSC stock solution at a ratio of 1:10 with distilled water.
5. Hyperfilm™ MP (Amersham Bioscience, UK) or Blue Lite Autorad Film 8 × 10 in. (ISC BioExpress, USA) or any appropriate sensitive films.
6. Film developing: Any appropriate equipment for X-ray film developing (alternatively, use manually mixed reagents for developing and fixation).

2.7. Data Analysis

1. Scanner.
2. Sun Sparc5 Workstation (Sun Microsystems) with Whole Band Analyzer software, version 3.4 (BioImage) ***(13)***.
3. BioNumerics (Applied Math, Belgium, latest version 5.1), GelCompare (Applied Math, Belgium). Instructions are included in manual, or for more details *see* **ref.*14***.

3. Methods

3.1. Bacterial Cultures

1. Incubate LJ cultures for 5–8 wk at 37°C with caps slightly opened.
2. Collect bacteria before the color of LJ media turns yellow.
3. For 7H11 (7H10) agar cultures, place Petri dishes in ziplock bags to avoid drying (*see* **Note 3**).

3.2. Mycobacterium tuberculosis DNA Isolation Procedure and Quantification

1. Collect a loop-full of *M. tuberculosis* colonies from LJ slant or Middlebrook 7H11 agar plate (*see* **Note 4**) and suspend in 500 µL H_2O in a 1.5-mL Eppendorf tube.
2. For heat kill, incubate tubes immersed in a water bath for 30 min at 80°C.
3. Samples can be frozen at this stage at –20 or –70°C and stored, if necessary.
4. Place sample tubes on Eppendorf thermomixer adjusted to 60°C. Add 70 µL 10% SDS and 50 µL proteinase K (stock solution); mix for 1 h at 60°C in low mode with shaking.
5. Preheat 5*M* NaCl and 10% CTAB to 60°C.
6. While the samples are still at 60°C, add 100 µL 5*M* NaCl. Mix thoroughly by inverting by hand.

7. Add 100 µL 10% CTAB; mix thoroughly by inverting by hand.
8. Incubate further for 15 min at 60°C in the thermomixer. Freeze for 15 min at –70°C. Samples can be stored at that stage if necessary.
9. Defrost samples at 60°C in the thermomixer.
10. Add 700 µL chloroform/isoamyl alcohol (24:1); invert carefully by hand 20–25 times (do not shake). A white homogeneous solution should appear.
11. Centrifuge for 10 min at 16,000*g*.
12. Transfer the upper (aqueous) phase (~700 µL) to a new tube with 500 µL cold isopropanol; mix by tilting the tube up and down several times. DNA precipitate may be visible at this point.
13. Set at –20°C for at least 30 min or at 4°C overnight.
14. Centrifuge for 10 min at 16,000*g*.
15. Decant and wash the pellet with 70% ethanol. Centrifuge for 5–10 min at 16,000*g*.
16. Decant, dry in Speed Vac centrifuge for less than 10 min at low drying rate.
17. Add 55–100 µL H_2O (or 1X TE) depending on the size of DNA pellet. Run 5 µL on a 1% (w/v) agarose gel in TBE buffer for 1 h at approx 100 V with a 1-kb DNA ladder; stain gel in ethidium bromide solution for a few hours for better results.
18. The quantification of isolated chromosomal DNA can be achieved by visual evaluation of DNA concentration loaded on the gel (for example, *see* **Fig. 5**).

3.3. DNA Digestion and Electrophoresis

1. *Pvu*II restriction of genomic DNA: Add 2.5 µL buffer and 1.5 µL *Pvu*II to 21 µL of a total volume of DNA and water. Incubate in water bath for 4 h at 37°C.
2. Prepare 200–250 mL 1% agarose in 1X TBE buffer and pour gel (15 × 20 cm; 20-tooth comb for 18 samples and two flanking standards).
3. Following restriction, add 5 µL of loading dye to each sample, mix, and load gel. Load molecular weight markers or standards (STDs) in lanes 1 and 19 (if a second gel is run the same day, load STD in lanes 1 and 18 on the second gel to avoid confusion).
4. Run at 90 V until dye front has run approx 1 cm into the gel, then run overnight at 36 V at room temperature.
5. Stop gel when the dye front nears the end of the gel (approximately 16 h) and stain gel with ethidium bromide. Photograph the stained gel.

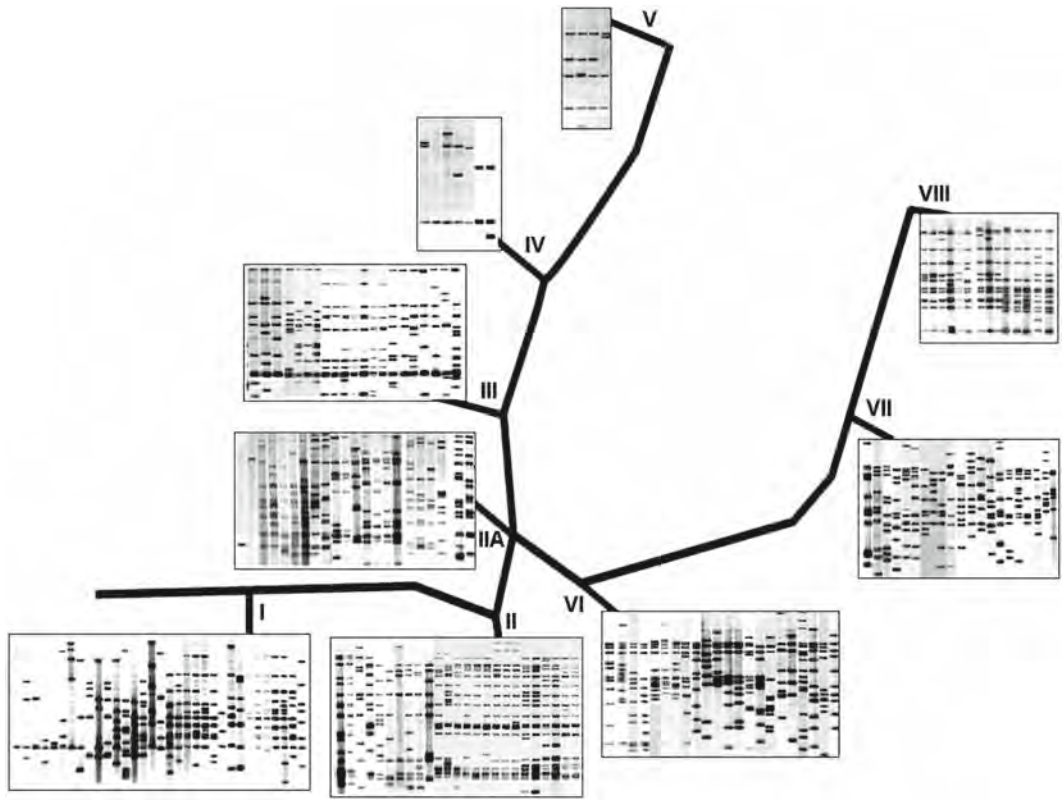

Fig. 5. Representative IS*6110* profiles superimposed on single-nucleotide polymorphism (SNP)-derived phylogenetic framework of *M. tuberculosis*. Based on SNP analysis of *M. tuberculosis* clinical isolates (including 1,743 strains from the Public Health Research Institute Tuberculosis Center strain collection), a phylogenetic tree with the nine clusters of *M. tuberculosis* isolates was used to illustrate common IS*6110* profiles. Some IS*6110* patterns are characteristic of given genetic cluster groups. (Adapted from **ref. *8***.)

3.4. Southern Blot

1. Rinse the Hybond-N+ membrane in water, followed by submersion in 10X SSC for 5 min.
2. Place the membrane on a porous support, cover with plastic mask, clamp unit, and place gel on top of the membrane. The size of the gel should be 0.5–1 cm larger than the window in the mask to create a vacuum in the transfer unit.
3. Attach to vacuum and adjust the suction unit to pull 50 mbar.
4. Flood the gel with 0.25*M* HCl for 20 min. Remove the fluid by vacuum aspiration using a pipet.
5. Flood the gel with soak I buffer for 20 min. Remove the fluid by vacuum aspiration using a pipet.
6. Flood the gel with soak II buffer for 20 min. Remove the fluid by vacuum aspiration using a pipet.
7. Flood the gel with 10X SSC for 1.5 h. Remove the fluid by vacuum aspiration using a pipet.

8. Without turning off the vacuum, remove the gel from the vacuum blotter, transfer the membrane to paper towels, and let the membrane air-dry for 10 min.
9. Irradiate the membrane twice to crosslink DNA fragments to membrane.

3.5. Hybridization

1. Prehybridize the membrane in 30 mL of hybridization buffer (ECL kit buffer with blocking agent, prepared in advance, as recommended by the manufacturer), rotating at approx 2.3 rcf and 42°C for 30 min in roller tube in the hybridization oven.
2. For each membrane, combine 10 µL of IS*6110*-specific probe and 10 µL of the provided water in an Eppendorf tube (*see* **Note 5**). Boil for 5 min in water and then place on ice for 10 min. Add an equal volume of labeling reagent (20 µL) and glutaraldehyde (ECL kit) (20 µL) (*see* **Note 6**), total volume of 60 µL per membrane. Incubate at 37°C in a water bath for 10–15 min.
3. Remove hybridization buffer from the tube, add the labeled probe into the buffer, and return to the tube containing the membrane.
4. Hybridize the membrane overnight, rotating at 3.3 rcf and 40°C.

3.6. Washing the Membrane After Hybridization

1. Remove the hybridization buffer, which can be reused once without adding additional probe if frozen at –20°C. Rinse the membrane in the roller bottle with approx 40 mL of primary wash buffer; discard the solution.
2. Add fresh primary wash buffer and set rotation of roller bottle for 30 min at approx 2.3 rcf at 40°C; discard the solution.
3. Rinse membrane with secondary wash buffer (2X SSC); discard solution. Add approx 30 mL secondary buffer and rotate the bottle for 10 min.
4. Remove the membrane from the bottle using 2X SSC buffer and soak in 2X SSC in a wide container on a shaking platform for 5–10 min.
5. Discard solution and repeat soaking as in **step 4** (the membrane should not be incubated in secondary wash buffer for more than 30 min).

3.7. Detection of Chemoluminescent-Labeled DNA Fragments

1. Mix 10 mL of solution 1 with 10 mL of solution 2 (ECL detection kit). This amount is sufficient for one membrane; when detecting more than one membrane, increase the total volume to 15 + 15 mL.
2. Incubate each membrane for 1 min at room temperature, carefully rotating the container. At this stage it is not required to work in darkness.
3. Wrap the membrane in plastic wrap, remove additional liquid with paper towels, and in a dark room place a film in the cassette (appropriate for X-ray-sensitive films).

4. Expose for approximately 10 min, replace film in dark room, and determine length of next exposure according to intensity of hybridization bands on the first film; increase time of exposure if bands are weak, and decrease exposure if bands are very dark. The time of exposure varies from 1 to 30 min, depending on the amount of chromosomal DNA loaded, quality of the hybridization probe, and ECL kit.

3.8. Rehybridization of the Membrane with Other Probes

The membrane used for IS*6110* RFLP can be reused for hybridization with other probes, such as the 5′-IS*6110* fragment or any other region of interest (*see* **refs.** *9* and *12* for examples). When the ECL kit is used, no additional stripping procedure is required. Simply expose the membrane to daylight for 20–24 h and prepare a new labeled probe as indicated. Prehybridization is not needed for the second probe. The same membrane can be rehybridized with different DNA probes up to six times without losing the quality of images.

3.9. Digitalization of Image

All software programs available for RFLP image analysis need the hybridization blot scanned and transformed into TIFF or JPEG file formats. The details of the RFLP analysis procedure are described by the software manufacturers and are not the subject of this chapter. In general, the image of the new isolate is compared to previously analyzed images in the collection, and the level of strain similarity (typically expressed as a percentage) is determined using statistical methods. Two images are considered identical if the number of hybridization bands and their location on the blots match 100%. Strains with (n + 1) hybridization bands may represent related strains, possibly from the same transmission chain, but further analysis using different biomarkers might be required for confirmation. Strains that differ by more than one hybridization band or strains showing a shift of some bands on the blot (different molecular weight of fragments) may still represent isolates from the same strain families but with a lower index of similarity. **Figure 4** represents selected images of three *M. tuberculosis* families: Haarlem, W-Beijing, and Latin America Mediterranean (LAM). Identical strains with IS*6110* copy number equal to or less than six require additional analyses (e.g., spoligotyping or MIRU-VNTR analysis).

4. Notes

1. The advantages of Mt14323 are easy amplification, wide size range of *Pvu*II-fragments (from 0.9 to 14 kb), and ability for direct interlaboratory strain comparisons (**Fig. 1**, lane 2).

The Centers for Disease Control and Prevention have developed a standard set of cloned IS*6110*-3′-fragments, ranging in size from 0.7 to 15 kb with 1-kb increments (**Fig. 1**, lane 1). This IS*6110* standard is used by all participating laboratories of the National Tuberculosis Genotyping and Surveillance Network, USA.

2. Alternative equipment for the capillary transfer can be used.
3. Fresh cultures of clinical *M. tuberculosis* isolates provide the best results for IS*6110*-based RFLP genotyping. LJ media are preferable for culture of *M. tuberculosis* strains as chromosomal DNA is less degraded, and hybridization bands are more distinguishable.
4. Use of prewetted cotton swabs makes this procedure more effective.
5. Depending on the probe concentration, 15 μL of the probe may be combined with 5 μL H_2O.
6. Thoroughly mix labeling reagent and DNA prior to addition of the glutaraldehyde.

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Chapter 15

spa Typing for Epidemiological Surveillance of *Staphylococcus aureus*

Marie Hallin, Alexander W. Friedrich, and Marc J. Struelens

Abstract

The *spa* typing method is based on sequencing of the polymorphic X region of the protein A gene (*spa*), present in all strains of *Staphylococcus aureus*. The X region is constituted of a variable number of 24-bp repeats flanked by well-conserved regions. This single-locus sequence-based typing method combines a number of technical advantages, such as rapidity, reproducibility, and portability. Moreover, due to its repeat structure, the *spa* locus simultaneously indexes micro- and macrovariations, enabling the use of *spa* typing in both local and global epidemiological studies. These studies are facilitated by the establishment of standardized *spa* type nomenclature and Internet shared databases.

Key words: Epidemiology; methicillin resistance; phylogeny; *S. aureus*; sequence analysis; software; staphylococcal protein A; typing methods.

1. Introduction

1.1. Typing Methods Available for Staphylococcus aureus

Staphylococcus aureus is a leading human pathogen responsible for a wide range of diseases, from superficial skin infections to life-threatening conditions, such as bacteremia, endocarditis, pneumonia, or toxic shock syndrome (*1*). Since the early 1960s, when they first emerged (*2*), strains of *S. aureus* resistant to methicillin and other β-lactams (MRSA) have spread worldwide and caused outbreaks in the hospital setting as well as in the community, thereby becoming a major public health threat (*3*). During the last decades, diverse typing methods, first phenotypic, then genotypic,

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have been used for monitoring *S. aureus* spread. Among these, pulsed-field gel electrophoresis (PFGE) of genomic macrorestriction fragments is considered the gold-standard method (***4***). However, PFGE is a technically demanding and labor-intensive method. Moreover, its interpretation leaves room to subjectivity (***5***), and interlaboratory results comparison remains difficult and subject to strict adherence to standardized protocols and interpretation criteria (***6–8***).

Multilocus sequence typing (MLST), based on the sequence polymorphism of approx 500-bp long fragments of seven housekeeping genes was designed to study the *S. aureus* population genetic structure. This technique, applied to large *S. aureus* strain collections, revealed that the population structure is essentially clonal, and that the large majority of epidemic MRSA clones belong to a few phylogenetically distinct lineages or clonal complexes (CCs) (***9***).

MLST has also proved to be adequate for long-term global epidemiology and the study of recent evolution of *S. aureus* (***9,10***). However, MLST typing remains too expensive and labor intensive for its application to outbreak investigations and routine surveillance (***10,11***).

In recent years, more focused sequence-based methods have been developed to provide fast, unambiguous, and exportable typing data. Among these, the sequence determination of the polymorphic X region of *spa* gene, called *spa* typing, is gaining favor as a reliable tool for typing *S. aureus*. Frenay et al. were, in 1994, the first team to target the polymorphic X region of *spa* gene as an epidemiological marker. At that time, the X region was amplified and its size estimated by electrophoresis (***12***). In 1996, the same team improved the technique by performing sequence analysis of the X region (***13***). Since then, many studies have evaluated the usefulness of this technique for diverse epidemiological purposes and confirmed its ease of use and speed. Initially, two limitations hampered use of *spa* typing for surveillance: the lack of software capable of identifying and clustering repeat units and profiles and the lack of consensus nomenclature allowing interlaboratory exchange of results. These limits have been recently overcome, making *spa* typing a prime alternative to PFGE for typing *S. aureus*. In this chapter, we outline the biological basis of *spa* locus polymorphism and performance of *spa* sequence typing and describe the methods of analysis and data interpretation as well as international *spa* typing networks.

1.2. Structural Specificity of the spa Gene Hypervariable X Region

Protein A is a cell-wall component bound to the peptidoglycan of *S. aureus* by its COOH-terminal part. It interacts with the Fc-fragment of immunoglobulins by its NH_2-terminal part and thereby inhibits phagocytosis by polymorphonuclear leucocytes. The *spa* gene is composed of an N-terminal region encoding four

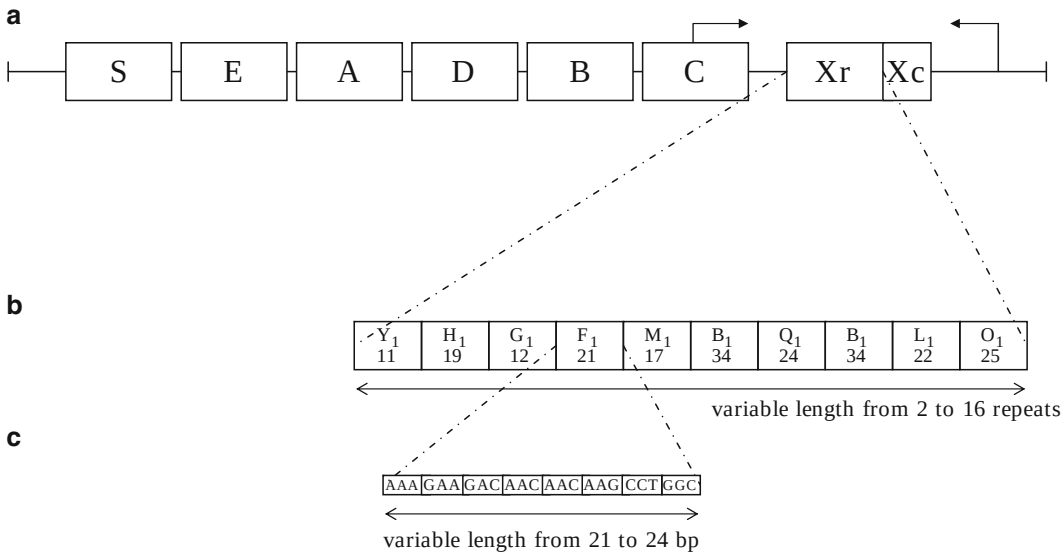

Fig. 1. (**a**) Schematic map of the *spa* gene. (Adapted from refs. *18, 20, 41*.) S is a signal sequence; A to D are IgG-binding domains; X is the C-terminal part, divided in two regions, the VNTR region (Xr) and a constant region coding for cell wall attachment (Xc). Arrows indicate the primers' localization. (**b**) The repeat structure of the Xr region. The *spa* type illustrated is t008 (Ridom-Harmsen et al. nomenclature) or YHGFMBQBLO (Kreiswirth nomenclature). (**c**) The DNA sequence of the *spa* repeat 21 (Ridom) or -F_1 (Kreiswrirth) repeat.

to five homologous immunoglobulin G (IgG) binding units, while its C-terminal sequence, called the X region, exhibits a variable number of short (24-bp) repeated units flanked by well-conserved regions (**Fig. 1**) (*14*).

The origin of the so-called variable number of tandem repeats (VNTR) structure of the X region is explained by the slipped strand mispairing model. Basically, illegitimate basepairing due to stretches and loops in short repeated unit motifs occurs during DNA replication, leading the DNA polymerase to delete or insert repeat units (*15*).

This VNTR structure is specific to neither the *spa* gene nor *S. aureus*. These hypervariable regions have been identified in many bacterial species and are frequently used as epidemiological markers. For example, the *spa* gene is one of the targets used by several multilocus VNTR analysis (MLVA) typing systems developed for *S. aureus*, together with *sdrC, D*, and *E, sspA, ClfA* and *B*, *cna*, or *fnBP* genes (*16*).

1.3. spa Typing Nomenclature

Two *spa* type designations have been used in the last years, one developed by Harmsen et al. (*17*) and one developed by Kreiswirth et al. (*18*). The latter has been changed recently, and comparison between the old designation and the new designation as well as a comparison between the Harmsen and the Kreiswirth nomenclature is only possible via computerized tools. The general

approach is, however, similar for both nomenclatures: Each repeat identified is associated to a code (numerical for the Harmsen et al. and alphanumerical for the Kreiswirth et al. nomenclature). Each *n*-long repeat profile corresponds to an *n*-long code constituted by the succession of the repeat's codes, as illustrated in **Fig. 1B**. In addition, in the Harmsen et al. nomenclature, a "type" number preceded by the letter t is then assigned to every distinct repeat profile (*see* **Note 1**). Due to its broader international use, we focus in the following section on the Harmsen et al. (Ridom) spa type designation.

1.4. spa *Typing Performance and Application Field*

As a sequence-based method, *spa* typing possesses many obvious advantages, such as rapidity, ease of use, suitability for computerized analysis, storage, and (ex)portability of results (***19***).

1.4.1. Typeability, Reproducibility, and Stability

Typeability is virtually 100%, although mutations in the flanking conserved regions of the X region, used for primer design, have occasionally been described (***20***), leading to amplification problems. However, a limitation of the clustering analysis may occasionally occur with isolates presenting short repeat profiles. Indeed, the epidemiological information contained by these profiles may be insufficient to permit reliable clustering (*see* **Note 2**).

Reproducibility is excellent (100%) both intra- and interlaboratory (***18,21***). The *spa* typing has been proved stable in vitro (***18***) and to a lesser extent in vivo (***13***). However, the longitudinal study of ten persistent infections of cystic fibrosis (CF) patients by a single *S. aureus* clone (as determined by PFGE) demonstrated occasional mutational events (deletions, point mutation, or duplication of *spa* repeats) in 10% of the sequential isolates studied (***22***). This finding could also be explained by the existence of closely related co-colonizing isolates, as described later by the same team (***23***), and cannot be generalized given the peculiarity of the ecological niche and selective pressure present in the airways of CF patients.

1.4.2. Epidemiological Concordance and Discriminatory Power

The use of *spa* typing for outbreak investigation was validated by Shopsin et al. (***18***) on a well-documented strain collection containing 29 isolates belonging to four distinct outbreaks. Discriminatory power of *spa* typing, evaluated on several large strain collections, was found to be similar to PFGE, with a Simpson Index of diversity ranging from 0.97 to 0.98 (***4,20,24***).

1.4.3. Concordance with Gold Standard Methods and Phylogenetic Inference

Besides a high discriminatory power, *spa* typing is usually in good concordance with PFGE (using the Tenover criteria (***25***)), either at the type level (from 96% to 98%) (***20,24***) or between clusters (93%, using BURP (based upon repeat patterns) algorithm; *see* **Subheading 3.4.2.)** (***4,24,26***). Furthermore, concordance with MLST and BURST (based upon related sequences) clustering also proved to be very high (97% to 99%) (***4,24***).

This ability of *spa* typing to combine a high discriminatory power with a high concordance with MLST as a single-locus marker resides in its repeat composition and organization. Point mutations (occurring at a low rate and subject to purifying selection) permit a reliable lineage assignment, while additions or deletions of repeats (fast-occurring) index intralineage variations, enabling the use of *spa* typing for both long- and short-term epidemiology (***19***).

However, when assuming that an isolate belongs to an MLST lineage based on its *spa* type, one should be aware of possible misclassification problems, as identified for a few lineages by several authors (***4,24,27,28***). Indeed, strains belonging to distant MLST CCs can present identical or similar *spa* profiles and cluster (using the BURP algorithm) together in a unique *spa* group. This still unexplained phenomenon could possibly be in certain cases due to recombination events involving the *spa* locus (***27***). Other cases can be caused by large chromosomal replacement encompassing the *spa* gene, such as described by Robinson and Enright for ST239 and ST34 (***28***).

2. Materials

The materials listed are only suggestions as many excellent alternatives exist.

2.1. DNA Extraction

1. Columbia agar plates with 5% sheep blood (Biomérieux, Marcy l'Etoile, France).
2. Lysostaphin solution (1 mg/mL) (AMBI Products LLC, New York).
3. Proteinase K solution (1 mg/mL) (Sigma).
4. DNase (deoxyribonuclease)-free water.
5. Tris-HCl, 0.1*M*, pH 8.0 solution.
6. Water baths or heater blocks (37, 60, 100°C).
7. Vortex.

2.2. Polymerase Chain Reaction (PCR)

1. AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA).
2. Deoxynucleotide 5′-triphosphates (dNTPs; Promega, Madison, WI).
3. High-performance liquid chromatographic-cleaned primers (spa-1113f, spa-1514r; for complete sequence, *see* **Subheading 3.2.**) (MWG-Biotech, Ebersberg, Germany).
4. PCR buffer II (Applied Biosystems).

5. End-point thermocycler: GeneAmp PCR System 9700 (Applied Biosystems).
6. Elution spin column: Kit Quantum prep PCR Kleen Spin Columns (Bio-Rad).

2.3. DNA Sequencing

1. ABI Prism BigDye Terminator V3.1 Sequencing Kit (Applied Biosystems).
2. ABI prism 4100 sequencing machine (Applied Biosystems).
3. Microplates: MultiScreen HV, clear plate 45UL (Millipore, Billerica, MA).
4. Sephadex G 50 (Amersham Biosciences, Freiburg, Germany).
5. Microplate centrifuge, up to 2100 g, rotor 11123 (Sigma).

2.4. Data Analysis

1. StaphType software (Ridom GmbH, Würzburg, Germany) or Bionumerics (Applied Maths, Ghent, Belgium).
2. Internet connection.

3. Methods

3.1. DNA Extraction

Multiple extraction protocols ranging from a simple boiling step (*29*) to commercial tissue or blood extraction kits (*27*) or glass bead mechanical lysis have been described to be suitable for *spa* typing (*see* **Note 3**). A rapid extraction protocol can be used as follows:

1. Suspend one colony of *S. aureus* cultured for 24 h on Columbia agar with 5% sheep blood in 45 µL of DNase-free water and 5 µL of lysostaphin (1 mg/mL), vortex, and incubate for 10 min at 37°C.
2. Add 45µL of DNase-free water, 5 µL of proteinase K 2 mg/mL and 150 µL of Tris-HCl 0.1*M,* pH 8.0; vortex and incubate 10 min at 60°C and then 5 min at 100°C.
3. Of this lysate, 5 µL is used as the DNA template in the PCR reaction.

3.2. DNA Amplification

Several pairs of primers have been described, numbered from the forward strand of *S. aureus* DNA (GenBank accession no. J01786), for example:

spa-1113f [1092–1113] (5′-TAA AGA CGA TCC TTC GGT GAG C) and spa-1514r [1534–1514] (5′-CAG CAG TAG TGC CGT TTG CT-3′) (*30*).

spa-1095f [1095–1113] (5′- AGA CGA TCC TTC GGT GAG C) and spa-1517r [1517–1496] (5′-GCT TTT GCA ATG TCA TTT ACT G-3′) (*17,18*).

The DNA amplification protocol (recommended by the Ridom software supplier, www.ridom.de) is the following:

1. Add genomic staphylococcal DNA in a PCR mixture to achieve 50 µL of final volume containing 1.25 units of *Taq* polymerase, 1.5 m*M* $MgCl_2$, 200 µ*M* dNTPs, 0.2 µ*M* of each primer (spa-1113f, spa-1514r) and 5 µL of 10X PCR buffer.
2. Cycling conditions consist of an initial denaturation step of 5 min at 80°C, followed by 35 cycles of 45 s of denaturation at 94°C, 45 s of annealing at 60°C, 90 s of extension at 72°C, and a final extension step of 10 min at 72°C (*see* **Notes 4–6**).
3. The PCR product is then purified, either by an enzymatic method or by elution spin column, and can be stored at 4°C.

3.3. DNA Sequencing

1. Use the following reaction mix: 20 to 30 ng of amplified and purified DNA in a final reaction volume of 10 µL containing 2 µL of premix and 1 µL of buffer from the kit and 0.5 m*M* of each primer (use the same primers as for the amplification step).
2. Amplification parameters are the following: An initial denaturation step of 2 min at 96°C, followed by 25 cycles of 30 s of denaturation at 96°C, 15 s of annealing at 50°C, and 60 s of extension at 60°C.
3. The products are then purified and concentrated prior to sequencing, either by ethanol precipitation or by commercial elution spin columns (Dye-ex, Quiagen, Hilden, Germany) or microplates loaded with Sephadex G 50.

3.4. Data Interpretation

3.4.1. spa *Type Assignment*

The VNTR structure of the *spa* locus makes the traditional sequence alignment (using the substitutions, insertions, and deletions [indel] of a single-position model) improper to accurately identify *spa* repeat units and assess how these units are organized. Several programs, both "in-house" and commercial, have been described and can be successfully used for this purpose (***17,18,31***). Among those, the StaphType software (Ridom) is the most widely used. Other bioinformatic tools, such as Bionumerics (Applied Maths) also allow *spa* analysis and *spa* type designation using the Ridom nomenclature. Submissions from the Bionumerics software to the *spa* server will be possible in the near future via the online SeqNet gatekeeper interface (www.seqnet.org; *see* **Subheading 3.4.3.**). Both software include internal quality control systems that ensure that extrapolation of repeat and repeat organization are made on sequence data (chromatograms) of sufficient quality (*see* **Note 7**).

3.4.2. *Grouping of Related* spa *Types*

Until recently, the only way to cluster or classify *spa* types was to visually estimate the similarity of their repeat profiles. This method, although feasible with limited size strain collections and found to concordantly classify strains as compared with MLST

results (*20,29,32,33*), is difficult to apply to large collections. The BURP algorithm is an automated algorithm—implemented in the StaphType software—that can cluster (*spa*-CC) *spa* types (*17,34*). Repeat duplication and excision are taken into account (in addition to single-position substitution and indel events) when the relatedness of different *spa* types is calculated. A "cost" accounting for the "steps" of evolution between each examined pair of *spa* types is calculated, whereas the algorithm tries to minimize these steps (parsimony assumption) (*34*). BURP offers two user-defined parameters that influence clustering: exclusion of *spa* types that are shorter than *x* repeats and the maximum number of costs *y* for clustering *spa* types into the same group. Using these parameters, short *spa* types (presenting limited evolutionary information) can be excluded from further analysis (*see* **Note 2**), and maximum costs can be adapted to the size of the strain collection studied and the question asked in terms of space and time evolutionary scale (small versus large outbreak investigation versus long-term epidemiological surveillance studies) (*see* **Note 8**).

3.4.3. The StaphType Software

The StaphType software combines three modules: a sequence editor, a database, and a report generator. For each *spa* sequence downloaded by the software, epidemiological information concerning the isolate typed can be recorded in the database module.

3.4.3.1. Sequence Analysis and spa *Type Designation*

The sequence analysis starts with the download of both forward and reverse sequences files (FASTA format or preferably ABI and SCF chromatograms). The software searches then automatically for the 5′ and 3′ signature sequences (conserved flanking regions), constructs a consensus sequence, and detects the *spa* repeats succession (*17*). In case of already known *spa* repeat succession, the *spa* type designation is automatically downloaded from the *spa* server Web site. New *spa* repeats and *spa* types detected by one laboratory using the StaphType software are automatically given a preliminary name in the local database (e.g., txAA or txAB). The laboratory typing data can then be synchronized via the Internet; the new sequences are then matched automatically with *spa* types found by other participants. If the repeat succession is revealed as new, a new type number is assigned for all future detection of this *spa* repeat profile, ensuring a continuously updated common nomenclature. Type numbers are assigned by the order of submission; no relatedness can be deduced from the closeness of two t-numbers. If the repeat succession has already been described and synchronized by another laboratory, the preliminary name in the local database of the inquiring laboratory is automatically changed to the preexisting denomination.

3.4.3.2. *Automated Quality Control of* spa *Typing Data*

As the *spa* server receives more than 1,000 submissions per month, a major goal is the maintenance of excellence of the data

quality. Therefore, the curator of the SeqNet.org database (*see* **Subheading 3.4.5.**) has set up rules for procedure and internal and external quality control schemes:

1. An internal quality control system is integrated in the StaphType software: To each downloaded sequence is attached a quality index, which corresponds to a sequence error probability. The *spa* typing sequences with low reliability cannot be synchronized via the server and are rejected by the SeqNet curator (*see* **Note 9**).
2. The external quality control consists of the performance of a certification for all new SeqNet.org members and a regular proficiency test, based on known ring trials. This external quality certificate is sent out to the laboratory when capacities for high-quality sequence typing has been established. During the certification process, the curators assist the new SeqNet.org aspirants in the development of sequence capacity, often accompanied by a 3-d stay at the sequencing facilities of the coordinators or participating in one of the hands-on laboratory workshops.

3.4.3.3. Data Ownership

Data ownership on the *spa* server is ruled by the SeqNet.org initiative, which curates the data for all submitters. It is important to mention that all data on the *spa* server are strictly incrementally synchronized. This means that all synchronized data, after passing quality control and assignment of the *spa* type, are stored with a single laboratory identifier. Every submitter using direct submission can choose which epidemiological data should be shared on the Web site (s*ee* **Note 10**). International study groups or regional and national networks can opt for not making visible their data submission on the public home page as long as wished by the interested group. In this way, intellectual data property of each single submitter is protected.

3.4.4. The Central spa Server

The Harmsen et al. nomenclature for the designation of *spa* types has been made universally accessible by establishing the central *spa* server. It allows the automated quality control of submitted sequence data, and the central synchronization rends the submitted data publicly available on the online Web site (www.SpaServer.ridom.de). Users of other *spa* analyzing software tools than StaphType are able to synchronize with the *spa* server via an online uploading interface while fulfilling all given quality criteria checked by the SeqNet.org curators. Until now, agreements between SeqNet.org and two developers of *spa* analyzing software (Ridom at www.ridom.de and Applied Maths at www.applied-maths.com) have been achieved. SeqNet.org will serve as gatekeeper for quality for the synchronization of *spa* sequences from submitters using one of the *spa* analyzing tools.

3.4.5. The SeqNet.org spa Typing Network

The central *spa* server (which has been developed by Ridom) is curated by the SeqNet.org initiative (***35***) on behalf of all users. SeqNet.org currently is an initiative of 45 laboratories from 25 European countries (1 laboratory from Lebanon) founded in 2004 at the University of Münster in Germany (http://www.SeqNet.org). Its main objective is to establish a European network of excellence for sequence-based typing of microbial pathogens, having its main focus on *S. aureus*. SeqNet.org comprises a large number of national reference laboratories as well as university and some veterinary laboratories. The principle goal of SeqNet.org is to create unambiguous, electronic, portable, easily comparable typing data of excellent quality for local infection control and national and European surveillance of sentinel microorganisms, such as MRSA.

Currently, parallel to the SeqNet.org laboratories, more than 140 other submitting laboratories have synchronized their *spa* types with the database. Although, the *spa* database in its current form essentially is used as a *spa* type dictionary, ensuring a common nomenclature, providing molecular typing data in real time, and maintaining typing data quality, its data entries on frequencies of *spa* types and country of submission can already provide valuable information regarding geographical dissemination and occurrence of the *spa* types by country (***36***).

Furthermore, the *spa* server can be used by regional, national, or international public health or research networks to filter *spa* data from the network's participating laboratories, hospitals, and medical practices. Important examples are the Dutch-German cross-border networks EUREGIO MRSA-net Twente/Münsterland (***37,38***) and the MRSA network of the EUREGIO Maas-Rhein (***39***) In both cases, *spa* typing ensures not only the intrahospital but also the cross-border comparability and Euregional data ownership of the typing data.

4. Notes

1. A translating tool from one nomenclature to the other can be downloaded from the *spa* server Web site (www. SpaServer.ridom.de).
2. Isolates presenting short *spa* profile, although technically typable, should be excluded from clustering analysis. Five has been proposed (***34***) as the minimum number of repeats necessary to infer relatedness.
3. An initial "staphylococcal-specific" lysis step using lysostaphin (24,27) is however recommended to ensure sufficient bacterial lysis.

4. Several other amplification protocols have been described and proved to be efficient (***18,40***).
5. Visualization of the amplified DNA by conventional electrophoresis in 1 or 2% agarose gel is recommended prior to the sequencing step. The average amplified product size should be between 300 and 600 bp but varies following the number of *spa* repeats.
6. For isolates that are nontypable using the primers cited, SeqNet.org recommends using the following primers (A. Mellmann, personal communication 2007).
   (a) spa-239f (5′-ACTAGGTGTAGGTATTGCATCTGT-3′)
   (b) spa-1717r (5′-TCCAGCTAATAACGCTGCACCTAA-3′)
   (c) spa-1084f (5′-ACAACGTAACGGCTTCATCC-3′)
   (d) spa-1618r (5′-TTAGCATCTGCATGGTTTGC-3′)

   However, 1 of 1,000 isolates remains nontypable. The reason might be that some *S. aureus* isolates present large deletions in the *spa* gene that can affect the primer binding sites.
7. Manual editing of *spa* sequence data should be avoided as much as possible because it can easily lead to misidentification of repeats and subsequently to the attribution of an incorrect *spa* type.
8. This parameter is by default set to four by the Ridom software. However, this was calibrated to suit long-term evolution characterization (i.e., maximal concordance with MLST data) (***34***). For small outbreak investigation, this parameter can be lowered.
9. If the 5′/3′ signatures are found, the repeats are correct, and the sequence is traced correctly, the reliability value given is 100 (good). If 5′/3′ signatures are found, the repeat succession contains no low-quality basis, and there is a consensus of traces, then the reliability value for quality given is 110 (very good) (***12***). Last, if the criteria for 110 are fulfilled and there are fewer than five editing steps of the sequence, the reliability value for quality given is 120 (excellent). In the case that the 5′/3′ signatures are not correctly found, signature positions are shifted, or base quality is low, the reliability values are between 90 and 40 (sufficient) or between 30 and 0 (poor) (***33***). Each *spa* typing sequence with a reliability value lower than 100 cannot be synchronized via the server and is rejected by the SeqNet curators.
10. The submitter is also able to withdraw his or her data at any time by resynchronizing with the server and indicating the deletion of its submission. In such case, only the *spa* type and the information on the sequence quality will remain on the server.

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Chapter 16

Sequencing of Viral Genes

Carol Holm-Hansen and Kirsti Vainio

Abstract

The use of molecular techniques in epidemiology gives a better understanding of viral transmission and diversity, and helps to define and characterizc outbreaks. By elucidating transmission patterns and defining outbreak parameters, appropriate preventive measures can be implemented in a timely fashion. Previously, the understanding of viral classification and phylogeny was difficult due to the challenges inherent in studying viruses. Automated cycle sequencing that uses fluorescently labeled nucleotides has revolutionized epidemiological studies of viruses at the molecular level. Sequencing of viral genes enables the identification and characterization of viruses, and sequence data are essential when investigating the etiology, dissemination, and transmission of viral infections as well as for disease surveillance and prevention. The present chapter focuses on the use of sequence analyses in epidemiological investigations.

Key words: Automated cycle sequencing, BigDye Terminator chemistry, PCR amplicon, phylogenetic analysis, virus diversity.

1. Introduction

The field of molecular epidemiology has emerged from the integration of molecular data into traditional epidemiologic research. Molecular techniques enable the characterization and comparison of different virus strains at the genomic level and are important tools for investigating the epidemiology of viral infections, at an individual or global level, and in retrospective investigations or surveillance. Sequence data have made it possible to distinguish and characterize local outbreaks, to detect dispersed international outbreaks, and to identify transmission chains by tracing the source of environmentally transmitted viruses (*1*) or

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the dissemination routes of person-to-person (*2*) and zoonotic viral infections (*3*). Sequence data have also enabled the study of the origin and relatedness of viral strains, the detection of new strains and variants, and the emergence of drug resistance across strain generations. In addition, monitoring the diversity of viral agents in clinical virology contributes to better diagnostics, treatment, and prophylaxis (*4*). Combined molecular and epidemiological data have been used to improve prevention and control of infectious viral diseases and thus provide clear benefits for public health. Some examples for which sequence data have been used to complement epidemiological investigations are given below.

The application of sequence data has been essential for the surveillance of measles in the World Health Organization (WHO) European region. While the WHO European Region has targeted the elimination of measles by 2010, this goal may not be fully realized. Several measles genotypes imported from other continents have caused prolonged circulation and large outbreaks among unvaccinated and highly mobile communities in several European countries. In Norway, with only a few cases of measles per year, molecular typing has shown that all cases have been associated with import from endemic regions or specific reservoirs (*5–7*). As shown in **Fig. 1**, all sequences obtained from measles cases in Norway were identical to virus types circulating in countries where the patients came from or had recently traveled.

Combined sequence and epidemiological data have also given information on transmission, dissemination, and drift of norovirus strains in Europe (DIVINE-NET: http://www.eufoodborneviruses.co.uk/). In addition, sequence data have been used for the rapid detection of international norovirus outbreaks (e.g., caused by raspberries or on cruise ships) and the emergence of new global variants within the dominating genotype (*8,9*). In outbreak investigation, sequence data have been the most important tool used to link norovirus cases.

Viral genomes exist in different forms: DNA or RNA, single or double stranded, segmented or not segmented, plus or minus polarity, circular or linear. Viral genomes vary both in size (from 3,200 nucleotides to 1.2 million basepairs) and in complexity. Despite this diversity, a single method can be used to characterize and compare viral genes. Nucleotide sequencing provides the best differentiation between viral strains, and the polymerase chain reaction (PCR) is the favored technique for generating templates for nucleotide sequencing. The choice of PCR primers is crucial for generating the correct PCR product (gene or region) to be sequenced. Sequencing regions with high variability will usually yield the most appropriate information when closely related strains are to be compared, whereas more conserved regions should be sequenced when comparing distantly related strains. The genes coding for viral structural proteins are usually the most

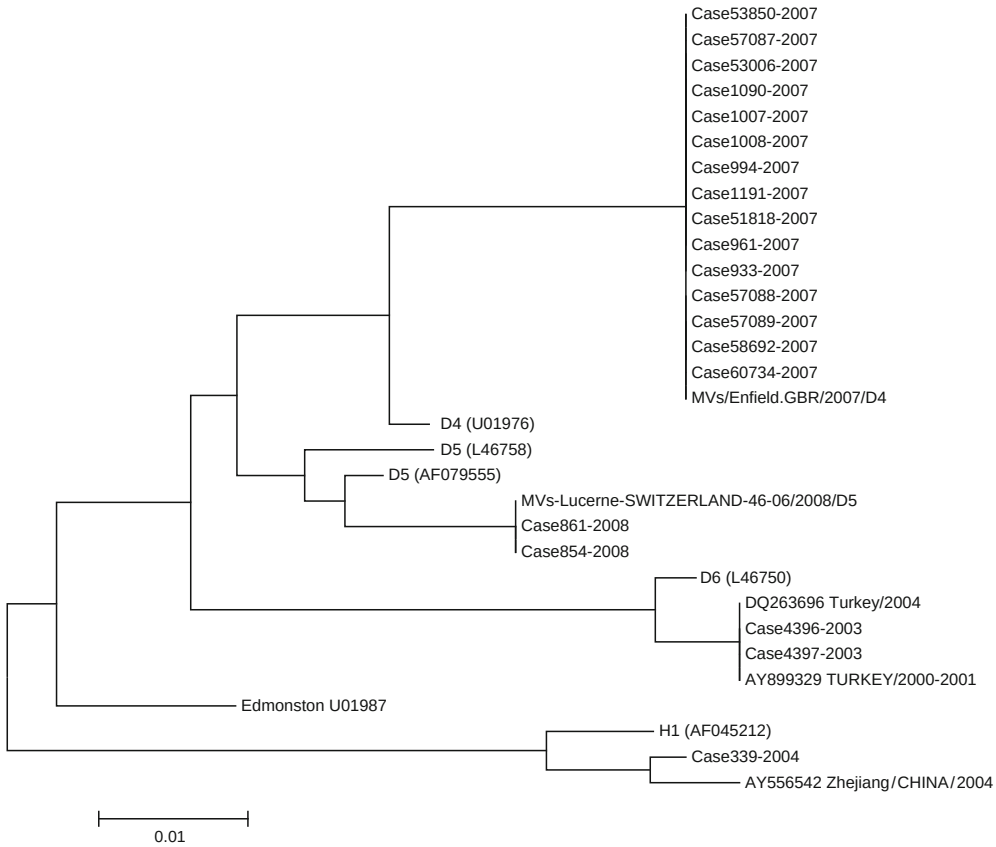

Fig. 1. Relationship between measles viruses from Norwegian cases and measles viruses detected in regions/countries the patients came from or had recently visited. The analysis was based on the N-gene sequence, and Norwegian cases were identical within each cluster. The Norwegian cases have been given names according to the year of isolation. The genotypes from Norwegian cases belonged to H1, D4, D5, and D6. The phylogenetic tree includes WHO Measles Reference Strains (2001) available on the EMBL database.

variable. Sequencing of variable genomic regions can be used to differentiate viruses into types, groups, subtypes, and strains, for example, human immunodeficiency virus (HIV) 1 and HIV-2; HIV-1 groups M, N, and O; the nine genetically distinct subtypes of HIV-1 group M; and various strains known as circulating or unique recombinant forms. It is also important to sequence regions that have been investigated in previous studies to have at disposition comparable data from a large number of other strains in nucleic acid databases.

The first step in nucleotide sequencing is template preparation by extraction of nucleic acids from samples likely to contain virus. There are many commercially available kits for manual viral nucleic acid extraction, as well as instruments that perform the extraction process automatically. In the case of DNA viruses the next step is PCR, whereas in the case of RNA viruses the genome has to be

reverse transcribed from RNA to complementary DNA (cDNA) before the PCR step.

DNA sequencing methods were developed in the mid-1970s. The two original DNA sequencing techniques are, however, very different in principle. In the enzymatic or dideoxy chain termination method of Sanger (*10*), a new DNA strand is synthesized from a template using a DNA polymerase, while in the chemical degradation method of Maxam and Gilbert (*11*) the original DNA is degraded. The dideoxy chain termination method is currently the most widely used technique for sequencing of viral genes and in the past decade has become an invaluable tool for molecular epidemiological investigations. In addition, the concepts of PCR technology have been utilized to enable the sequencing reaction to be cycled. A "cycled" dideoxy chain termination method, now known as cycle sequencing (*12*), forms the basis of sequencing reactions used in automated DNA sequencers. Automated methods have revolutionized the use of DNA sequences in molecular epidemiological investigations. Viral gene sequences are used to illustrate phylogenetic relationships visualized as "family trees" between viruses that elucidate viral evolution and possible routes of dissemination. The rapid evolution of viral genes is a significant advantage for studying relatedness and enables virus lineages to be differentiated even within an outbreak.

The purpose of this chapter is to provide a detailed protocol for the direct nucleic acid sequencing of PCR products generated from viral genes. Recommendations for protocols addressing the extraction of nucleic acids from sample material containing virus, reverse transcription of RNA genomes, and nucleic acid amplification by PCR are also included in this chapter. The method described is optimized for the ABI Prism® 310 Genetic Analyzer or ABI 3730 DNA Analyzer. While there are several other commercially available automated DNA sequencers, the biochemistry is common for all: dideoxy chain termination cycle sequencing using fluorescently labeled terminators.

2. Materials

2.1. Extraction of Viral Nucleic Acids

1. Any kits for isolation of viral nucleic acids may be used, and the kits include all required reagents and spin columns for isolation, for example, QIAamp Viral RNA Mini Kit for manual RNA isolation, QIAamp DNA Mini Kit for manual DNA isolation (Qiagen, Hilden, Germany) and MagNa Pure LC Total Nucleic Acid Isolation Kit for automated isolation of viral RNA or DNA (Roche, Mannheim, Germany).
2. MagNA Pure LC Instrument for automated nucleic acid isolation and equipment: tubes, trays, and pipets (Roche).

3. Microcentrifuge (e.g., Eppendorf centrifuge 5415D) for 0.5-mL microcentrifuge tubes.
4. Microcentrifuge tubes (e.g., Eppendorf polypropylene 0.5 mL with locking caps and Microamp PCR 0.2-mL tubes).
5. Automatic pipets capable of dispensing 0.5–20 μL and 10–100 μL.

2.2. Polymerase Chain Reaction and Template Preparation

The isolated nucleic acid can be used in PCR or reverse-transcriptase (RT)-PCR on different instruments/platforms and on standard thermal block cyclers.

1. Any kits for amplification of nucleic acids may be used, and the kits include all required reagents for amplification, such as, OneStep RT-PCR Kit for amplification of RNA viruses, Taq PCR Core Kit for amplification of DNA viruses, or QuantiTect Virus Kits for amplification of both RNA and DNA viruses (Qiagen).
2. Thermal cyclers (GeneAmp® 9700 or 2700, Perkin Elmer 2400, or Eppendorf Master Gradient) for cycle-sequencing reactions.
3. A temperature-cycling incubator capable of executing two consecutive programs over a temperature range of 45–95°C with an incubator chamber for 0.5-mL microcentrifuge tubes.
4. ExoSAP-IT® for PCR product cleanup; store at –20°C (USB Corp., OH). Eventually, use QIAquick-spin PCR purification kit (Qiagen).
5. Microcentrifuge (e.g., Eppendorf centrifuge 5415D) for 0.5-mL microcentrifuge tubes.
6. Microcentrifuge tubes (e.g., Eppendorf polypropylene 0.5 mL with locking caps and Microamp PCR 0.2-mL tubes).
7. Automatic pipets capable of dispensing 0.5–20 μL and 10–100 μL.

2.3. Cycle-Sequencing Reaction

1. Automatic sequencers with capillary electrophoresis technology: ABI Prism 310 Genetic Analyzer or Applied Biosystems 3730 DNA Analyzer (Applied Biosystems Inc., Foster City, CA) suitable for fluorescent sequencing using BigDye® Terminator chemistry and any primer.
2. Applied Biosystems 3730 DNA Analyzer equipment: 3130 and 3100 Series Plate Base 96-Wells, 3130 and 3100 series Plate Retainer 96-Wells, and plate Septa 96-Wells.
3. ABI Prism 310 Genetic Analyzer equipment: tubes and caps.
4. BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems. The kit provides the required reagents for the sequencing reaction in a premixed, ready-to-use format. Single- or double-stranded DNA, PCR fragments, and large templates may be sequenced.
5. Sequencing primers. Any primer may be used. The PCR primer may also be used as the sequencing primer, although at a different concentration.

6. 3*M* sodium acetate, pH 4.6.
7. Ethanol, molecular biology grade and 70%.
8. 125 m*M* ethylenediaminetetraacetic acid (EDTA), pH 8.0.
9. Heater blocks or water baths capable of temperatures up to 95°C.
10. Hi-Di formamide, genetic analysis grade (Applied Biosystems).
11. POP7™ Performance Optimized Polymer and 10X buffer with EDTA (Applied Biosystems).

3. Methods

PCR is currently the favored technique for generating templates for sequencing of viral genes. This chapter therefore focuses on sequencing PCR amplicons. A few suggestions for isolation of viral RNA and DNA as well as preparation of PCR template are provided. For detailed procedures on nucleic acid purification and PCR template preparation, refer to manufacturers' protocols that are continuously improved and updated.

3.1. Extraction of Viral Nucleic Acids

Viruses contain either RNA or DNA genomes. There are several commercially available manual nucleic acid isolation kits for the isolation of viral RNA, viral DNA, or total viral nucleic acids. Many of the kits may be used for isolation of viral nucleic acids from a variety of specimens (serum, plasma, whole blood, urine, cerebrospinal fluid, cell culture supernatant, stool, tissue, cell-free body fluid). There are also several kits for isolation of viral nucleic acids with automated nucleic acid extractors (e.g., ManNA Pure, Roche). Any manual or automated protocol should work well. In our laboratory we use Qiagen kits (e.g., QIAamp Viral RNA Mini Kit and QIAamp DNA Mini Kit) for manual nucleic acid isolation and MagNA Pure LC Total Nucleic Acid Isolation Kit for automated nucleic acid isolation from clinical specimens. These kits provide nucleic acid free of PCR inhibitors.

3.2. PCR Template Preparation

There are several kits for PCR amplification of isolated nucleic acids, and the PCR amplicons are used as templates for the sequencing reaction. The isolated DNA from DNA viruses may be amplified directly (e.g., Taq PCR Core Kit, Qiagen), whereas isolated RNA from RNA viruses must be reverse transcribed to cDNA before the PCR amplification step. One-step RT-PCR kits (e.g., OneStep RT-PCR Kit, Qiagen) provide a fast and successful alternative to performing a separate reverse-transcriptase and PCR reaction. Any primers may be used with these kits (specific primers, random primers, etc.).

3.3. PCR Product (Template) Purification

The most important factor for obtaining good sequence data with dye terminators is a clean, unique target with a single binding site for the primer (*see* **Notes 1–4**). There are many protocols for purification of PCR products prior to sequencing, and any protocol to remove deoxynucleotide 5′-triphosphates (dNTPs) and primers should work.

ExoSAP-IT (shrimp alkaline phosphatase [SAP] and exonuclease I [Exo I]) purification is a simple, fast, and efficient method for the purification of PCR products for sequencing. The ExoSAP-IT degrades nucleotides and single-stranded DNA (primers) and is particularly useful when limiting concentrations of primers and nucleotides cannot be used in PCR.

1. Set up a reaction with ExoSAP-IT (one tube for each reaction/template) by mixing 7 μL of PCR product and 2 μL of ExoSAP-IT. Vortex briefly.
2. Run on thermocycler as follows: One cycle at 37°C for 15 min, 80°C for 15 min, and 4°C indefinitely.
3. The samples may be kept at 4–8°C until cycle sequencing is performed. Long-term storage should be at –20°C.
4. PCR product purification with QIAquick PCR Purification kit (Qiagen) is also compatible with BigDye chemistry. This method is suitable for PCR fragments ranging from 100 to 1,000 bp. See the manufacturer's manual for a detailed protocol.

3.4. Cycle-Sequencing Reaction

Cycle sequencing using BigDye Terminator chemistry provides reproducible results for sequencing of PCR fragments (*see* **Notes 1–5**). The method is quick, convenient, and commonly used. It is important to sequence the template in both directions to minimize sequencing errors and to include a control DNA template in each set of sequencing reactions. Control DNA is included in the kit.

The BigDye Terminator protocol recommends the use of 8 μL of the Big Dye v1.1 Terminator Ready Reaction mix in a final reaction volume of 20 μL. Good results are, however, obtained with half of the recommended volume when purified PCR fragments are sequenced.

1. For each reaction (total volume 10 μL), add the following reagents to a separate tube:
   a. 4 μL Big Dye v1.1 Terminator Ready Reaction mix.
   b. 1 μL 3.2 μ*M* primer (final concentration 3.2 pmol).
   c. 1–5 μL template (2–10 ng purified PCR product/μL).
   d. 0–4 μL distilled H_2O.
2. Mix well, spin briefly, and run on thermocycler as follows:

   Step 1: 96°C 1 min.

   Step 2: 96°C 10 s.

Step 3: 50°C 5 s.

Step 4: 60°C 1 min.

Step 5: Repeat steps 2–4, 25 times.

Step 6: 4°C, indefinitely.

3. Hold at 4°C until ready to purify.

3.5. Purification of Extension Products

The unincorporated dye terminators must be removed prior to capillary electrophoresis (*see* **Note 6**). The ethanol/EDTA/sodium acetate precipitation protocol works well and is known to be suitable for generating clean sequences using BigDye Terminator v1.1 Cycle Sequencing Kits. Other methods may also be used.

1. For each sequencing reaction, prepare a separate 1.5-mL microcentrifuge tube with the following:
   a. 1 µL 3*M* sodium acetate, pH 4.6.
   b. 25 µL 96% ethanol.
   c. 1 µL 125 m*M* EDTA, pH 8.0.
   d. 10 µL sequencing product.
2. Mix briefly.
3. Keep on ice for 10 min.
4. Spin in a microcentrifuge at maximum speed for 15–30 min at 4°C.
5. Carefully remove the supernatant completely with a pipet and discard.
6. Add 80 µL 70% ethanol to the pellet.
7. Keep at room temperature for 2–3 min (may be stored overnight at –20°C).
8. Remove the supernatant completely.
9. Dry the pellet on a thermocycler (or heat block) with caps open for 3 min at 60°C. It is important that the pellet is completely dried. The dried pellet may be stored at –20°C until being prepared for automatic sequencing.
10. Dissolve the dried pellet in 15 µL Hi-Di formamide immediately before preparing the samples for automatic sequencing. Samples with Hi-Di formamide should not be kept at room temperature for more than 2–3 d. Long-term storage should be at –20°C.
11. Heat the tubes with closed caps for 2 min at 94°C on a thermocycler (heat block).
12. Remove the tubes immediately and place on ice for 1–2 min.
13. Spin the tubes in a microcentrifuge at maximum speed for 2 s.
14. Load the samples for capillary gel electrophoresis as follows:

a. ABI Prism 310 Genetic Analyzer: Load 15 μL of reconstituted sample to the tubes belonging to the instrument.

b. ABI 3730 DNA Analyzer: Dilute each reconstituted sample 10X in Hi-Di formamide. Load 10 μL of this 1:10 dilution to microtiter plate wells belonging to the instrument.

15. All of the empty wells in the microtiter plate must be filled with Hi-Di formamide.

3.6. Capillary Gel Electrophoresis and Data Collection

Prepare the automatic sequencer according to the manual provided with the equipment, and use POP7™ polymer and 10X buffer with EDTA from Applied Biosystems to fill the capillaries. After preparation, load the samples (tubes or tray), set up the data collection software, and start the sequencer according to the manual. Make sure that the thin capillary is aligned properly with the sample in the tray/tube to take up the entire DNA sample to be sequenced. The sequencing products are subsequently separated and read by the automatic sequencers (*see* **Notes 6–8**).

The BigDye Terminator chemistry uses four different fluorescent labels, allowing the reaction to be analyzed in a single lane. Briefly, the fluorescently labeled DNA fragments (the terminated extension products) are separated and identified using capillary gel electrophoresis. A laser at the end of the capillary excites the ddNTP (dedioxynucleotides) dyes, causing the incorporated labeled dideoxynucleotides to illuminate different colors. The colors are analyzed by the computer, and the user is provided with a chromatogram and the suggested DNA sequence.

3.7. Sequence Analysis and Assessment

Automatic sequencers compile and deliver a computer file with a chromatogram and a suggested sequence. In most cases the chromatograms and sequences delivered by the sequencer should be carefully interpreted and manually edited before using the sequence data. The automatic analysis of the chromatogram peaks may be incorrect, especially early in the sequence (the first 40–50 bases from the primer binding sites) or at the end when the resolution of large fragments is not optimal (*see* **Notes 7–9**). There are several programs for comparing and editing sequences (e.g., Sequencher 4.5 and Bioedit). The edited sequences should then be compared with sequences from different databases (EMBL or GenBank) to gain as much information about the sequences as possible. The databases are available through the Internet, and information may be obtained using different search tools (e.g., FASTA and BLAST).

3.8. Phylogenetic Analysis

Phylogenetic analysis provides important molecular epidemiological information. The aim of phylogenetic analysis is to compare sequences, analyze gene families, and estimate evolutionary relationships.

The result of molecular phylogenetic analysis is visualized as a phylogenetic "tree" or dendrogram. DNA or RNA of closely related organisms usually exhibits a high degree of sequence similarity. Molecular phylogeny uses such sequence heterogeneity to build a "relationship tree" that illustrates the probable evolution of various organisms. There are numerous software programs that estimate phylogenies, and many are free of charge via the Internet.

The quality of phylogenetic analysis largely depends on the quality of the sequence alignment. Interactive software programs generate both multiple-sequence alignments (e.g., ClustalW, Pileup) and phylogenetic trees. The MEGA (Molecular Evolutionary Genetics Analysis) 4.5 software is a user-friendly interactive program that constructs multiple alignments and phylogenetic trees. For example, when using MEGA to generate a phylogenetic tree, sequences are automatically aligned using ClustalW.

4. Notes

1. Template concentration and purity are the two most common causes of poor or no sequence data. The amount of template used in a sequencing reaction can affect the quality of sequence data, and there is a "threshold" amount that must be used to generate any sequence data. The recommended amount of PCR product is 1–3 ng for 100- to 200-bp fragments, 3–10 ng for 200- to 500-bp fragments, and 2–20 ng for 500- to 1,000-bp fragments *(13)*. In general, the optimal concentration of template may be determined by multiplying the length of the template in kilobases by 25 ng. Too much DNA may cause premature termination of signal. This occurs when the dNTPs in the cycle-sequencing reaction are distributed among too many extending chains. The dNTPs will be depleted early in the reaction and thus yield an excessive amount of short fragments. Quantification of template may be determined by gel electrophoresis. Fluorescent sequencing is very sensitive to certain contaminants in the DNA sample, including dNTPs, primers, and salts. It is critical to remove excess PCR primers from the sequencing reaction. PCR primers will act as sequencing primers and lead to extra bands that correspond to the complementary strands from opposite orientations. Thus, incomplete removal of PCR primers prior to sequencing may yield ambiguous results that are visualized as sequences with numerous double peaks at single positions. Excess dNTPs will disturb the specific ratios of dNTPs/ddNTPs in the sequencing reaction.
2. It is imperative to ensure that the PCR product to be sequenced is the correct fragment. Multiple PCR products in a single

sequencing reaction will yield ambiguous sequences. Visualizing PCR products on an agarose gel will give a good indication of the quality of the product. In the case of multiple products (bands), gel purification of the desired product is necessary. Gel separation of PCR products may, however, be difficult if the products are similar in size (e.g., amplifying related DNA). In this case, optimization of the PCR reaction may be necessary, or new PCR primers may need to be designed to use a more specific priming site. Restriction sites in PCR fragments may also be used to identify the correct product bands. Alternatively, nested primers may be employed to reamplify the desired product. Nested primers will verify the identity of the product and simultaneously eliminate any unwanted products.

3. PCR primers that are used as sequencing primers must be suitable for the cycle sequencing conditions. While inefficient primers are sometimes acceptable for PCR, the same primers may fail in sequencing (which is a linear amplification). The melting temperature T_m of sequencing primers should be between 50 and 60°C. In addition, the primers must not form primer-dimers as this will deplete the availability of primers needed for the sequencing reaction. Sequencing primers should be 18–24 nucleotides in length and approximately 50% in GC content *(14)*. High GC content or long primers may increase the formation of secondary structures that influence the melting temperature. It is preferable to choose primers with a low melting temperature.
4. Removal of components that may inhibit the sequencing reaction is necessary. Nuclease contamination in a template preparation, as well as repeatedly thawing/freezing samples, can degrade DNA over time. High concentrations of impure DNA may also contain a larger proportion of contaminants (excess primers, dNTPs, salts) that may reduce the quality of the DNA sequence generated. Generally, reisolation and purification of the template DNA are necessary to obtain good DNA sequences. It is wise to limit the time and intensity of UV illumination to a minimum when extracting PCR products from gels to reduce DNA degradation. Prepare fresh stocks of commonly used reagents, such as buffers, using high-quality distilled water.
5. Several factors may result in early termination of sequence data throughout the sequencing reaction, including template concentration, deoxyribonuclease (DNase) contamination, and secondary structures. Secondary structures that do not melt during cycle sequencing can cause premature termination of sequences. Addition of DNA denaturants (e.g., formamide or dimethyl sulfoxide [DMSO]) to the sequencing reaction may

reduce early termination. Denaturants may melt duplex formation and enable optimal polymerase activity. Changing the cycle-sequencing parameters to include a higher denaturation temperature (98 vs. 96°C) and eliminating the 50°C annealing step may be useful. The 60°C cycle will in this case function as both the annealing and extension steps. High salt concentrations may also result in premature termination. Sequencing the opposite strand will sometimes yield better results.

6. Purification of sequencing reactions is important to gain good sequence data. Purification using ethanol/EDTA/sodium acetate precipitation is recommended when a good signal from the first base is required (*see* the BigDye kit manual). However, it is important to use the correct ethanol concentration ***(14)***. Too high ethanol concentrations will result in precipitation of residual terminators along with the sequencing products, whereas too low concentrations will result in no signal due to a failed reaction. Ethanol precipitation also removes excess salts. Ethanol contamination may also occur when the sample is insufficiently dried after precipitation and may inhibit sequencing reactions.
7. Multiple peaks under the primary sequence peak and many "N"s within the sequence may indicate the presence of two nucleotides at the same position (polymorphisms), high background, or the presence of multiple products. High background may also be a problem if contaminated reagents are used for either template preparation or sequencing reactions. When background poses a problem, it is necessary to view the average signal strength and edit the sequences manually.
8. Low signal strength may be the result of too little or degraded DNA or primer, inhibitory components, contaminated reagents, or poor primer binding. A correct primer concentration and annealing temperature are critical.
9. Difficulties with the sequencer may be due to improper capillary filling when fresh polymer is being pumped through the array. See the manual for guidelines addressing instrument-related problems.

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Chapter 17

Full Sequencing of Viral Genomes: Practical Strategies Used for the Amplification and Characterization of Foot-and-Mouth Disease Virus

Eleanor M. Cottam, Jemma Wadsworth, Nick J. Knowles, and Donald P. King

Abstract

Nucleic acid sequencing is now commonplace in most research and diagnostic virology laboratories. The data generated can be used to compare novel strains with other viruses and allow the genetic basis of important phenotypic characteristics, such as antigenic determinants, to be elucidated. Furthermore, virus sequence data can also be used to address more fundamental questions relating to the evolution of viruses. Recent advances in laboratory methodologies allow rapid sequencing of virus genomes. For the first time, this opens up the potential for using genome sequencing to reconstruct virus transmission trees with extremely high resolution and to quickly reveal and identify the origin of unresolved transmission events within discrete infection clusters. Using foot-and-mouth disease virus as an example, this chapter describes strategies that can be successfully used to amplify and sequence the full genomes of RNA viruses. Practical considerations for protocol design and optimization are discussed, with particular emphasis on the software programs used to assemble large contigs and analyze the sequence data for high-resolution epidemiology.

Key words: Complete genome, foot-and-mouth disease virus, nucleotide sequence, virus.

1. Introduction

During the past 15 years, a number of incremental improvements have been made to methods used to generate nucleotide sequence data. The principle underpinning the mostly widely used sequencing approaches is based on the dideoxynucleotide

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chain-termination method initially devised by Fred Sanger in the 1970s (*1*). The throughput and robustness of these methods have been improved by the use of fluorescent dyes and capillary separation technologies, such that the routine assembly of large fragments of genomic DNA (>10 kb) is now achievable by many modestly equipped laboratories. For the large part, protocols developed to sequence large fragments of nucleic acid can also be adapted to characterize the genomes of RNA viruses, which typically are 15 kb or less. Full-genome sequences of viruses can be used to address fundamental questions relating to evolution, identification of critical antigenic determinants, and viral molecular epidemiology. Although sequencing small numbers of some viral genomes can be straightforward, specific protocols and work flows are required to effectively manage projects that aim to characterize the molecular epidemiology of viral transmission.

Using foot-and-mouth disease virus (FMDV) as an example, this chapter describes strategies that can be successfully used to amplify and sequence the complete genomes of RNA viruses. Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed livestock (cattle, sheep, pigs, goats, and water buffalo). The causative agent is a virus belonging to the genus *Aphthovirus* (family: Picornaviridae) that exists as seven antigenically distinct serotypes, each comprising numerous and constantly evolving variants (*2*). The genome of FMDV is approximately 8,300 nucleotides in length. It comprises a polyadenylated positive-sense RNA that encodes a single polyprotein, which is posttranslationally cleaved into constituent capsid proteins and nonstructural proteins involved in viral replication.

In common with most other RNA viruses, the enzyme (RNA-dependent RNA polymerase) responsible for replication of the FMDV genome has poor fidelity, such that changes to the nucleotide sequence frequently occur and are inherited to progeny viruses. This rapid evolution rate of FMDV allows virus transmission trees to be reconstructed with extremely high resolution, opening up the possibility of using these data to retrospectively reveal and identify the origin of unresolved transmission events (*3,4*). In addition to forensic molecular epidemiology, full-genome sequence data have also recently contributed to our understanding of a number of aspects of FMDV evolution, including (i) evolutionary rates (5); (ii) sites and importance of recombination (*6,7*); (iii) identification of ordered RNA structures (*8*); and (iv) contribution and significance of the quasi-species phenomenon to evolution (*9*). Sequence data from a wide variety of FMDV isolates also play an important role in the reiterative design of oligonucleotide primers used for molecular assays for routine diagnostic use in reference laboratories (for pan-reactive and serotype-specific detection and strain characterization).

1.1. Amplification Strategies: Design and Targeting of Polymerase Chain Reaction Primers

The extent of the run length obtained by capillary sequencers places a limit on the maximum distance between oligonucleotide primers (either in the polymerase chain reaction [PCR] amplification or cycle sequencing setup stages). In contrast to DNA targets, which are relatively stable, researchers who study RNA viruses, such as picornaviruses, are familiar with the plasticity of viral genomes. This high variability poses particular challenges for the design of pan-reactive oligonucleotide primers to reliably amplify complete viral complementary DNAs (cDNAs). For viruses such as FMDV, the existence of multiple serotypes (whose nucleotide sequences may vary by as much as 50% in some genome regions) can further complicate the identification of suitable target sequences.

As a consequence, the two extremes of the sequencing strategies used for FMDV are illustrated in **Fig. 1** (*see* **Fig. 1a,b**) and shown by representative agarose gels in **Fig. 2**. In both of these

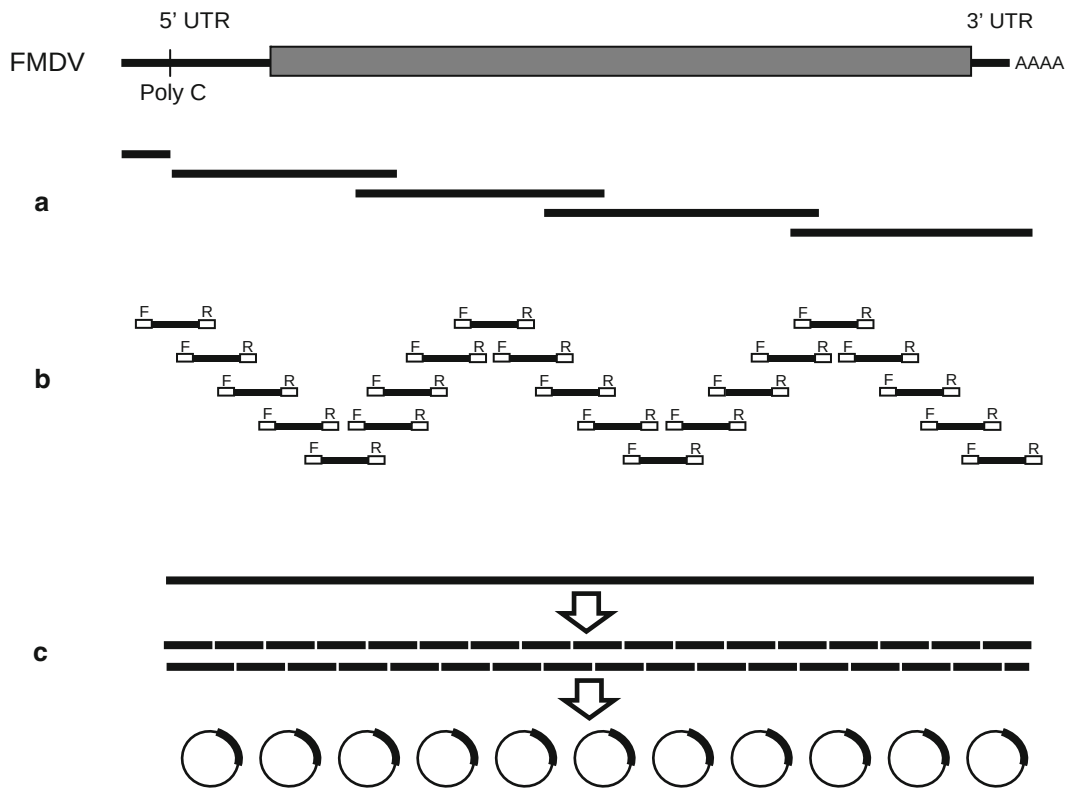

Fig. 1. Outline of RT-PCR strategies that have been used to amplify the complete genome sequences of FMDV. (**a**) Long overlapping products (~3 kb) are generated by PCR using full-length cDNA as a template. Sequences are obtained using a panel of specific sequencing primers (*see* **ref. 3**). (**b**) Short products (~700 bp) are generated using FMDV-specific primers, which also incorporate regions (labelled F and R) targeted by the sequencing primers (*10*). (**c**) Long-range RT-PCR is used to amplify a product comprising the complete L fragment of FMDV. This may either be sequenced using many specific primers (*11*) or can be fragmented by restriction digest and cloned into a bacterial plasmid vector (pilot studies using this method have been undertaken by IAH in collaboration with the Wellcome Trust Sanger Institute, Cambridge).

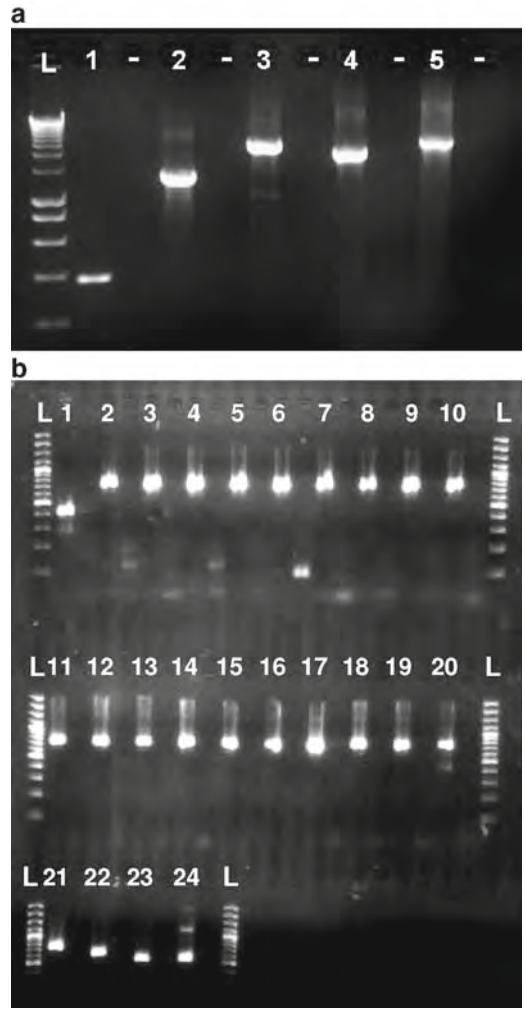

Fig. 2. Amplification of full-FMDV genomes by RT-PCR. Agarose gel electrophoresis shows RT-PCR fragments representing amplification of the entire FMDV genome. (**a**) amplification of O/UKG/2001 genome using 5 RT-PCR fragments and (**b**) amplification of O/UKG/2007 genomes using 24 RT-PCR fragments.

approaches (adopted for the characterization of FMD outbreaks in the United Kingdom in 2001 and 2007), a large number of specific primers were required. Furthermore, these oligonucleotides are specific for defined lineages of FMDV, limiting their use for study of other genotypes of FMDV. Other FMD laboratories have used similar approaches also requiring large numbers of primers (*11–15*). Additional protocols, such as rapid amplification of cDNA ends (RACE), can be used to generate sequence data for the terminal ends of the genome and regions close to the poly (C) tract of FMDV. To reduce the complexity of the cycle-sequencing reactions, recognition sequences for universal sequencing primers (such as M13) can be incorporated into the 5′ ends of the primers used for PCR (*see* **Fig. 1a**).

Alternative approaches, such as shotgun cloning (for example, **Fig. 1c**) are also being considered for full-genome sequencing. Initially, these use long-range PCR to amplify large fragments of the virus genome (possibly even encompassing entire genomic sequences). These PCR products are subsequently fragmented and cloned into plasmid vectors prior to sequencing and reconstruction of the viral sequence. Since this approach uses only two viral-specific primers (which can be targeted to highly conserved regions) and is not reliant on internal virus-specific primers, this method may provide a more suitable approach that has a broader sensitivity to different viral variants. However, these methods need to balance the advantages in diagnostic sensitivity that are gained from using a smaller number of primers with the drawback of lower analytical sensitivity that may arise from amplifying large PCR products (in comparison to shorter fragments).

1.2. Overview of Approaches Used for FMDV Sequencing

In this chapter, a guide protocol that has been successfully used to sequence FMDV is presented. Although some of the finer details are specific to FMDV, the general approaches described are broadly applicable to other RNA viruses. Indeed, similar methods have been described recently to characterize the genomes of other viruses that infect humans, livestock, and plants (*16–23*).

2. Materials

2.1. RNA Extraction

1. 0.04*M* phosphate-buffered saline: 35 m*M* Na_2HPO_4, 5.7 m*M* KH_2PO_4, pH 7.6. Store at room temperature.
2. Sterile sand (Fine Sifted, BDH). Small aliquots (~3 g) are prepared and autoclaved prior to use. Store at room temperature.
3. Sterile pestle and mortar (Fisher); autoclave prior to use.
4. TRIzol Reagent (Invitrogen). Store at +2 to 8°C. This solution contains phenol and guanidine isothiocyanate; care should be taken to minimize skin contact and inhalation.
5. Chloroform (AnalaR Grade, BDH) (toxic and probable carcinogen; care should be taken to minimize inhalation and ingestion).
6. 0.2*M* glycogen (Roche).
7. Isopropanol (propan-2-ol) (AnalaR Grade, BDH).
8. Ethanol (AnalaR Grade, BDH). Store at +2 to 8°C.
9. Nuclease-free water (deoxyribonuclease [DNase] and ribonuclease [RNase] free) (Invitrogen). Store at room temperature.

2.2. Reverse Transcription and PCR Amplification

1. Random hexamers (Promega). Store at –20°C.
2. Deoxynucleotide 5′-triphosphate (dNTP) mixture (Promega). The dNTP mix is a premixed solution containing sodium salts of dATP (deoxyadenosine 5′-triphosphate), dCTP (deoxycytosine 5′-triphosphate), dGTP (deoxyguanosine 5′-triphosphate), and dTTP (deoxythymidine 5′-triphosphate), each at 10 m*M* in water. Store at –20°C.
3. Oligonucleotide primers (Sigma-Aldrich). Complete list of primers used for PCR amplification of FMDV are described elsewhere (***2,3,10***).
4. Reverse transcription kit: SuperScript™ III RT (Invitrogen). Enzyme is supplied with a vial of 5X first-strand buffer (250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$) and a vial of 100 m*M* dithiothreitol (DTT). Store at –20°C.
5. RNaseOUT (Invitrogen); store at –20°C.
6. GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare).

2.3. PCR Cleanup Prior to Setting Up Sequencing Reactions

1. Agarose (UltraPure™, Invitrogen). Store at room temperature.
2. Tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer (National Diagnostics). 10X solution: When diluted, the 1X solution contains 89 m*M* Tris-base, 89 m*M* boric acid (pH 8.3), and 2 m*M* Na_2EDTA. Store at room temperature.
3. Ethidium bromide (UltraPure). Of a 10 mg/mL stock solution, added 2 µL to 100 mL gels to visualize PCR bands. Ethidium bromide is a potent mutagen. Therefore, care should be taken to minimize exposure and to ensure correct disposal of material (solutions and gels) containing ethidium bromide. Store at room temperature.
4. 6X loading buffer for samples to be tested by agarose gel electrophoresis (Invitrogen).
5. DNA standards, if required (Invitrogen).

3. Methods

3.1. RNA Extraction (see Notes 1 and 2)

1. Using sterile sand and a pestle and mortar, prepare a 10% (w/v) suspension of the tissue sample in phosphate-buffered saline. Liquid samples (such as serum) can be processed straight to step 3. Depending on application and nature of the sample to be tested (*see* **Note 3**), alternative RNA extraction protocols can also be used (such as commercially available silica-based spin columns).
2. Centrifuge at 300g for 10 min.

3. Add 200 µL of the sample supernatant to 1 mL of TRIzol reagent in a microfuge tube (*see* **Note 4**).
4. Add 240 µL of chloroform directly to the tube.
5. Mix the tube by inversion and centrifuge for 15 min at 10,000g at 2–8°C.
6. Transfer the top phase to a fresh microfuge tube and add 1µL of 0.2M glycogen.
7. Add an equivalent volume of isopropanol.
8. Mix the tube by inversion and centrifuge for 15 min at 10,000g at 2–8 °C.
9. Carefully wash the pellet (containing the RNA) with ice-cold 70% ethanol and recentrifuge for 15 min (10,000g at 2–8 °C).
10. Air-dry the pellet and resuspend RNA in nuclease-free water.

3.2. Reverse Transcription and PCR Amplification

1. Prepare primer mix (9 µL) containing 30 pmol reverse primer [5′-GGC GGC CGC TTT TTT TTT TTT TTT-3′], 50 ng random hexamers, and 30 nmol of each dNTP (3 µL of a 10 m*M* solution).
2. Add to 12 µL prepared RNA.
3. Denature the RNA by incubating the RNA/primer mix at 70°C for 3 min and place on ice for 3 min.
4. Add 17 µL of reverse-transcription (RT) mix containing 8 µL first-strand buffer, 2 µL 0.1 mM DTT, 2 µL RNaseOUT, 5 µL nuclease-free water.
5. Add 2 µL Superscript III Reverse Transcriptase.
6. Incubate at 42°C for 1–4 h followed by 85°C for 5 min. A specific PCR amplifying the 5′ end of the genome can be used to test that complete first-strand cDNAs have been generated.
7. Cleanup cDNA using GFX PCR DNA and Gel Band Purification kit according to manufacturers' instructions and elute in 50 µL. This step removes unincorporated primers and dNTPs from the RT reaction.
8. Set up a PCR master mix in a clean room using the primer sets required for amplification of the genomic fragments.
9. Add 2.5 µL cDNA to each reaction in a separate area away from the PCR clean room (*see* **Note 5**).
10. Run thermocycling program (as described in refs. **2**, **3**, **10**; *see* **Note 6**).

3.3. PCR Cleanup Prior to Setting Up Sequencing Reactions

1. Run 2 µL of PCR product on 1.2% (w/v) agarose gel at 105 V for 30 min to check reaction has worked.
2. Clean up cDNA using GFX PCR DNA and Gel Band Purification kit according to manufacturers' instructions.

3. Quantify DNA concentration in purified PCR product. This can be done using a spectrophotometer (e.g., Nanodrop, Thermo Fisher Scientific) or by agarose gel electrophoresis using DNA standards (*see* **Subheading 2.3**.).
4. Dilute products to give appropriate concentrations for sequencing.
5. Prepare sequencing reaction using diluted PCR product.

3.4. Analysis of Sequence Data

Sequencing viral genomes can quickly accumulate a large amount of data (*see* **Note 7**). Software programs (such as Lasergene, http://www.dnastar.com/) can be used to simplify the alignment of individual sequences and to rapidly assemble large contigs. The minimum criterion for acceptance of a final sequence is that each nucleotide position should be determined by sequencing reactions in either direction (forward and reverse).

Currently, the genetic evolution and relationships of viruses are studied by analyzing their genetic sequence data by phylogenetic methods. Phylogenetic trees are constructed and used to deduce the genetic relatedness of the viruses. There are different methods for constructing phylogenetic trees; the first approach developed was the maximum parsimony methodology, but more recently maximum likelihood (*24*) and Bayesian methods (*25*) are the preferred techniques for tree construction. Other methods based on distance matrixes, such as neighbor-joining (*26*) or unweighted pair-group method with arithmetic mean (UPGMA) (*27*), which calculate genetic distance from multiple sequence alignments, are simpler to implement but do not invoke an evolutionary model.

Maximum parsimony determines the most parsimonious tree requiring the least evolutionary steps. This method is simple and as such makes very few assumptions about the evolutionary process. However, certain features of genetic evolution of organisms present problems when using this method of tree construction. First, inaccuracies can occur as a result of the existence of homoplasy. Homoplasy describes processes, such as convergent evolution, by which a single mutation can occur twice on independent branches of a tree. Hence, it implies that two sequences sharing a mutation were not necessarily derived from a common ancestor that also contained this mutation. Another hurdle to overcome is back-mutation, by which a mutation reverts to its original genotype. This can cause the specific sequence to appear more ancestral than is necessarily the case. A further drawback to the method of maximum parsimony is that it takes no account of the rate at which mutations arise and the varying probabilities of different mutations occurring (i.e., transversions vs. transitions).

For these reasons, the parametric method of maximum likelihood is usually preferred as it provides the most probable tree that suits a specific determined evolutionary model. Providing that the model employed is a reasonable approximation of the evolutionary processes that gave rise to the observed genetic data,

this analysis is potentially more powerful than other methods. The evolutionary model may include a large number of parameters accounting for differences in the probabilities of various character states, differences in the occurrence of particular substitutions/mutations, and differences in the probabilities of change among characters. With the sophisticated models such as the Hasegawa-Kishino-Yano (HKY) model (*28*) and the general time reversible (GTR) model (*29*), an improved idea of phylogeny is achieved, although fitting an incorrect model can give incorrect results. The suitability of models can be tested using a program such as model test (*30*).

Maximum likelihood estimation of tree phylogeny is generally preferable to maximum parsimony because it is statistically consistent with a better statistical foundation, and it allows complex modeling of evolutionary processes. However, the maximum likelihood method has a computing limitation for large numbers of sequences. To infer statistical confidence in either maximum parsimony or maximum likelihood, constructed phylogenies bootstrap analyses (*31*) are performed. A further method to infer phylogenies is that of Bayesian inference, which generates a posterior distribution for a parameter based on the prior for that parameter and the likelihood of the data (represented by the sequence alignment). In other words, whereas maximum likelihood analysis investigates the probability of the observed data given a specific evolutionary model, Bayesian inference looks at the probability that a model is correct given the observed data set. With the availability of Markov chain Monte Carlo methods (*32*), Bayesian inference can be a preferred choice for tree estimation because it can be faster than maximum likelihood, and no bootstrapping is required as the posterior probabilities determine the statistical confidence in the tree.

Although in the majority of incidences maximum likelihood or Bayesian inference is preferable for tree construction, in certain situations maximum parsimony can be a viable alternative. When studying closely related sequences over a short time period the likelihood of back-mutation is relatively low, and hence maximum parsimony tree construction is likely to give an accurate estimation of tree phylogeny. Phylogenetic analysis of virus sequences is often performed with the aim of tracing specific virus history, and in these cases the method of statistical parsimony can be used. The distances depicted by parsimony trees represent the actual number of differences between sequences, whereas for a maximum likelihood tree the probability of change is shown (**Fig. 3**).

Often when studying viruses, closely related sequences are being investigated, with a focus on the accumulation of changes, and in this case a simpler representation of the raw data as depicted by parsimony is desirable. The TCS statistical parsimony program (*34*) can position sequences internally on a branch, which assists in

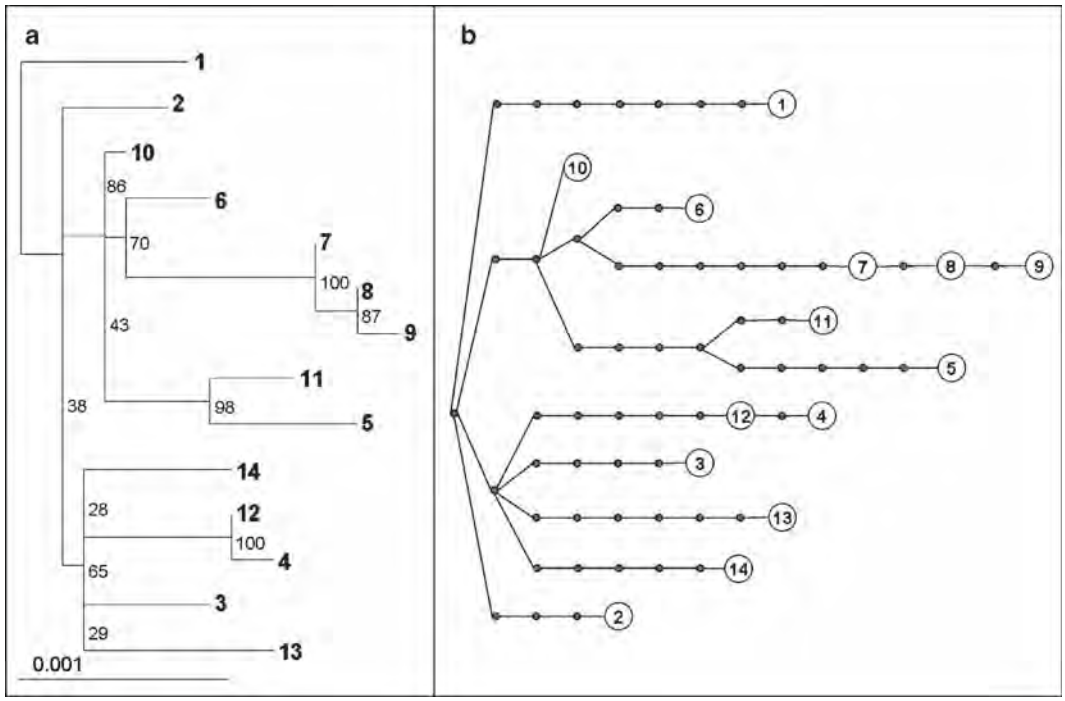

Fig. 3. Phylogenetic analysis of FMDV genomes from the 2001 outbreak in the United Kingdom. (**a**) Maximum likelihood phylogenetic tree representing 14 FMDV complete genomes rooted to sequence "1," constructed using PhyloWIN95 (*33*), incorporating the HKY model of nucleotide substitution with gamma distributed rate heterogeneity. Bootstrap values from 1,000 replicates are shown. (**b**) Statistical parsimony representation of the same 14 complete FMDV sequences, constructed using TCS (Version 1.21; ***34***). Each line represents a nucleotide substitution and each dot a putative ancestor virus.

depicting directly ancestral sequences (*see* **Fig. 3b**). Although the statistical parsimony trees drawn by TCS are not bootstrapped, if the data comprise the complete genome sequences of the sampled viruses, then the tree is as accurate and as representative as it can be: It is not sensitive to the choice of a single arbitrary locus because there are no further genetic data retrievable. A useful Web site that lists available phylogenetic programs for analyzing sequence data is http://evolution.genetics.washington.edu/phylip/software.html.

3.5. Future Technologies

Newer technologies are currently being developed that offer the potential to eliminate the use of capillary electrophoresis and even greater throughput. Resequencing microarrays have been developed and used to determine the sequence of the severe acute respiratory syndrome (SARS) coronavirus (***35,36***). However, development of specific arrays is heavily resource dependent and currently likely to be deployed only in niche markets. Of the newer technologies, sequential ligation systems (SOLiD), solid-phase primer amplification (Solexa), and bead-and-well-based pyrosequencing methods (such as the 454 platform) have the

capacity to generate reads of 4–20 Mb in a single run. Although this might be considered excessive for characterization of individual viral genomes, these approaches may allow infrequent mutations within a viral population to be detected. Thus, these methods may be ideal for dissecting the genetic variability within viral populations.

4. Notes

1. In addition to ensuring that all solutions used for RNA extraction are RNase free, pipets and work surfaces should be cleaned using 10% bleach followed by DNAzap (Ambion) prior to and between each sample processed.
2. A logical work flow for processing the samples for sequencing projects is highly recommended (*see* **Fig. 4** for an example). This is particularly important for high-resolution molecular epidemiological studies since the discrimination of samples may be dependent on the accurate determination of only a

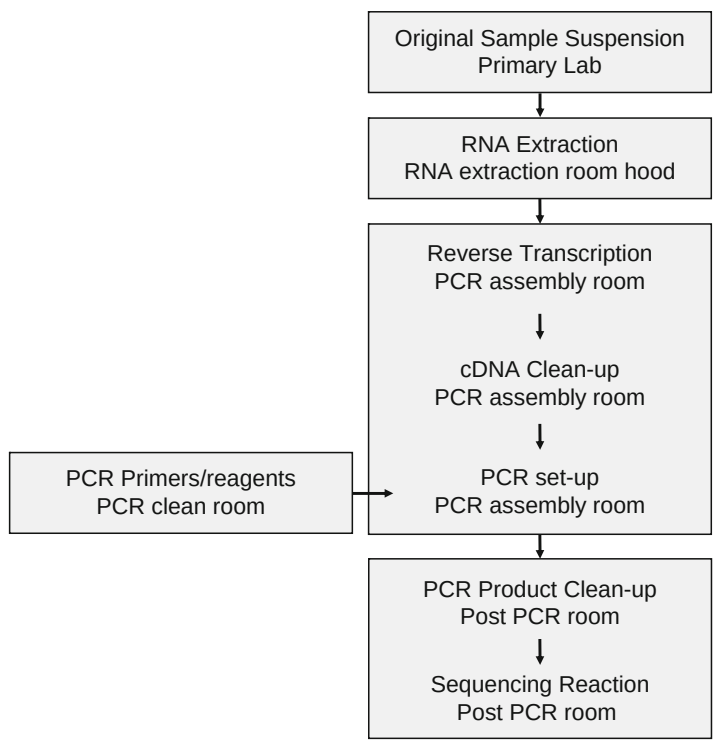

Fig. 4. Physical separation of the laboratory activities: outline of the separate steps required for the amplification and sequencing of FMDV genomes.

few nucleotide differences in the complete genome length *(3)*. Therefore, it is important that care is taken to minimize cross-contamination between samples (particularly post-PCR products). If possible, samples should be processed independently (including suitable negative control material), and the study should be organized to attempt to maximize the differences between successive samples tested.

3. A variety of sample types (including blood, tissues, esophageal-pharyngeal fluid, and cell culture supernatant) can be tested; however, it is usually preferable to test primary material (such as clinical samples) since it is possible that cell culture passage or molecular cloning of viruses can introduce nucleotide changes that can influence the interpretation of results.
4. Once placed in TRIzol reagent, samples can be stored for extended periods (at a wide range of temperatures, –70 to +4°C).
5. The requirement to perform a high number of downstream sequencing reactions may necessitate that a relatively large volume of PCR product is generated requiring pooling of RNA, cDNA, or post-PCR products. An additional practical consideration is the fidelity of the DNA polymerase used for the PCR amplification step; if possible proofreading enzymes that are widely available should be used.
6. In common with other long PCR methods, the parameters of the protocol used for amplification of viral genomes should be optimized prior to routine use. Steps to be considered include the components of the RT or PCR mixes and the cycling times used for amplification. In initial experiments, a PCR targeting a fragment of the 5′ end of the genome can be used to confirm that full-length cDNA has been produced in the RT reaction.
7. In general, these methods provide an accurate estimation of the viral consensus sequence. However, it is important to recognize that this sequence will be a composite of the component variability that, to a greater or lesser extent, may be present. In spite of concerns that it is theoretically possible that the sequence generated will not represent an actual virus species present in the sample, studies with FMDV indicated that the majority of molecular clones have identical sequences to the consensus *(37)*. Testing of duplicate samples can generate identical results *(4)*, demonstrating that these methods are accurate, and as long as the viral concentrations are relatively high, consensus sequences obtained will mask any individual proofreading errors that might arise due to low fidelity of reverse transcriptase and polymerase enzymes. These aspects relating to accurate determination of the sequences of specific viral genomes (rather than consensus sequences) will be of particular concern in studies

that aim to characterize the genetic population structure within samples (i.e., the quasi-species nature of a virus). New technologies and approaches (*see* **Subheading 3.5**.) may be utilized to address these important questions that underpin our understanding of viral evolution.

Acknowledgments

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Chapter 18

Bacterial Genome Sequencing

Hervé Tettelin and Tamara Feldblyum

Abstract

For over 30 yr, the Sanger method has been the standard for DNA sequencing. Instruments have been developed and improved over time to increase throughput, but they always relied on the same technology. Today, we are facing a revolution in DNA sequencing with many drastically different platforms that have become or will soon become available on the market. We review a number of sequencing technologies and provide examples of applications. We also discuss the impact genomics and new DNA sequencing approaches have had on various fields of biological research.

Key words: Bacteria, diversity, genome, pathogen, sequencing technology.

1. Introduction

1.1. The Advent of Whole-Genome Shotgun Sequencing

Until the completion of the genome sequence of *Haemophilus influenzae* Rd *(1)* by the whole-genome shotgun approach, genome sequencing projects relied on the availability of a physical map of the organism of interest *(2)*. Such a map is a set of partially overlapping clones from a genomic library, usually with large inserts like cosmids (ca. 40 kb) or bacterial artificial chromosomes (BACs) (ca. 100 kb), which are ordered along the genome. The construction of physical maps typically involves fingerprinting of a large number of clones and assembly of the map based on fingerprint overlaps. Once the map is assembled, a subset of individual clones tiling the genome is selected and used for sequencing. Although physical maps or derivatives thereof are still currently used to increase the accuracy of eukaryotic genome sequencing projects that involve complex repeats, most of the whole-genome

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sequencing now is conducted using the shotgun approach. This technique involves the sequencing of a large number of clones chosen randomly from a whole-genome library. Such clones typically have small-to-medium-size inserts (2–10 kb) and are often sequenced from both ends of the insert (paired ends) to provide scaffolding of repeats. Random sequences are then aligned and assembled together into contigs to reconstruct the structure of the genome. Resulting assemblies typically consist of a number of contigs separated by gaps in which sequence information is missing, most often due to complex repeats that are hard to assemble, DNA regions that are difficult to sequence (e.g., secondary structures), or fragments missing from the genomic library (unclonable regions). Gap closure is usually laborious because it inevitably tackles the most difficult regions of the genome to sequence, and it involves a variety of molecular biology techniques, depending on the case at hand *(3)*.

Whether a whole bacterial genome sequence obtained is complete (gap free) or a draft (a set of contigs separated by gaps), the genes it harbors can be systematically predicted using tools such as Glimmer *(4)* or GeneMark *(5)*. The function of the proteins encoded by those genes can be predicted in 60 to 90% of the cases by homology to characterized proteins available in the ever-growing public databases. Predicted coding regions are typically searched against public databases with BlastP *(6)* and assigned an annotation as well as a functional role category *(7)* or Gene Ontology (GO) terms *(8)*. To further enhance function assignment, the proteins are also searched against databases of hidden Markov models (HMMs) built on protein family/superfamily multiple-sequence alignments *(9,10)*. In addition to *ab* initio prediction of coding regions, comparative genomics is used to drive and improve annotation as well as to make it more homogeneous across strains or species. These steps are just some of the basic aspects of a full-blown genome annotation pipeline whose description is beyond the scope of this chapter.

The genomics field is now increasingly turning towards metagenomics that applies genomics techniques to the study of complex communities of microbial organisms directly in their natural environments and without the need of laboratory isolation and cultivation. It aims to capture the total microbial gene diversity in an environment, shedding light on the biological processes found there. Examples of metagenomics projects include environmental studies as those of seawater *(11)* or soil *(12)*, and medical applications, such as the human microbiome project *(13)*.

1.2. Completed and Ongoing Bacterial Genome Projects

As of February 2009, the Genomes Online Database (GOLD; www.genomesonline.org) reports 792 complete published bacterial genomes and another 2,392 ongoing bacterial genome

projects. These include all the major human pathogens, a growing number of other pathogens, and bacteria of environmental and industrial relevance. Such a flood of genomics data requires the design and access to databases that enable interrogation of the information in a biological context. Some databases like the Comprehensive Microbial Resource (cmr.jcvi.org) aim at providing comparative power across a comprehensive list of completely sequenced species. Other databases target a subset of species like the Bioinformatics Resource Centers (www.brc-central.org). The Bioinformatics Links Directory (bioinformatics.ca/links_directory) features a long list of links to molecular resources, tools, and databases *(14)*. This directory provides an excellent starting point for users to get acquainted with the most useful and powerful publicly available tools for genomic data mining and analysis.

2. Sequencing Technologies

2.1. Existing Technologies

Ever since whole-genome shotgun sequencing became the standard, the intrinsic sequencing approach did not change until recently. Although several generations of improved automated sequencers were developed and the speed of shotgun sequencing increased 25 times between 1986, when the first automated DNA sequencer (Applied Biosystems model 370A) was commercialized, and 2005, when the AB 3730xl capillary sequencer dominated the laboratories, the steps in the high-throughput shotgun sequencing process did not evolve. Genomic DNA was fragmented into pieces of 2–40 kb and genomic libraries constructed by cloning the fragments into plasmid or fosmid vectors and transforming the constructs into *Escherichia coli* for replication and propagation. The plasmid or fosmid DNA was then isolated and used as the sequencing template for dideoxy-mediated chain termination sequencing reactions. The dideoxy-mediated chain termination sequencing chemistry has been the standard in the field since its discovery and publication in 1975 by Fred Sanger *(15,16)*.

The way DNA sequence information is generated was revolutionized in 2005 by 454 Life Sciences with the release of a sequencing platform, the Genome Sequencer 20 (www.roche-applied-science.com) that is based on totally different chemistry and technology *(17)* (*see* **Table 1** for a summary of the sequencing technologies described in this section). It does not require cloning of the shotgun fragments and therefore eliminates the cloning bias for fragments that were unstable or could not be propagated in *E. coli*.

The 454 sequencing sample preparation steps include DNA fragmentation, end repair, capture of the fragments on beads,

Table 1
Characteristics of Sequencing Technologies

Sequencer	Sequencing chemistry	Sample preparation	Estimated throughput	Read length (basepairs)	Accuracy base read (consensus)
ABI 3730xl DNA Analyzer	Dideoxy termination	8 d	0.08 Mb/run 1 Mb/d	800–1,000	>99%
454-Roche Genome Sequencer FLX (Titanium)	Sequencing by synthesis, Pyrosequencing	5 d	400–600 Mb/run 10 h	400	>99.5% (99.99% at ~20X coverage)
Illumina-Solexa 1G Genome Analyzer$_{II}$ System	Sequencing by synthesis, Reversible terminators	1 d	2–15 Gb/run 2–8 days	35–75	98–99 (99.99% at > 3X coverage)
ABI SOLiD 2	Stepwise ligation	2–4 d	16 Gb/run 6–8 days	35	99.94% (99.999% at 15X coverage)
Helicos BioSciences Heli-Scope Single Molecule Sequencer	Sequencing by synthesis	1 day	21–28 Gb/run 8 days	30–35	>95% [99.995% at >20X coverage]

Note: This table provides information on the technology, throughput, read length, and accuracy of platforms on the market as of December 2008 (described in **Subheading 2.1.**).

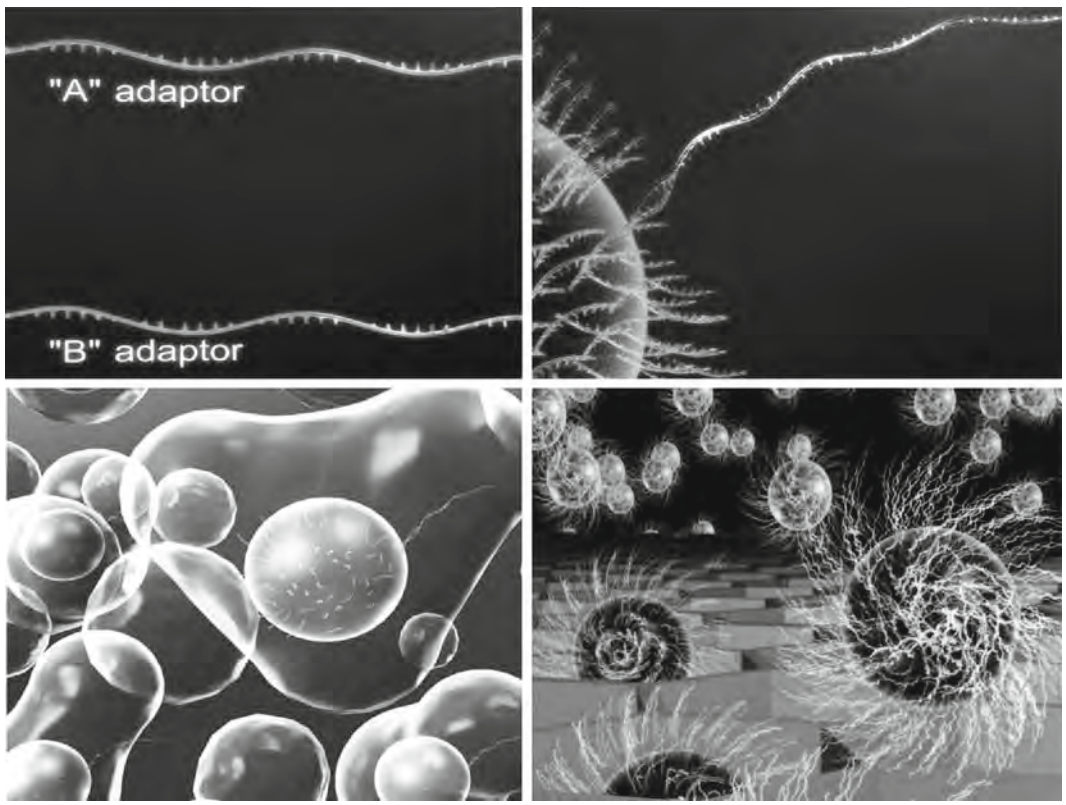

Fig. 1. The 454 sequencing steps: ligation of adapters, capture of DNA fragments on beads, and clonal amplification by PCR in emulsion microreactors.

polymerase chain reaction (PCR) clonal amplification of the captured fragments in aqueous-oil emulsion microreactors, breaking of the microreactors, and enrichment for beads with amplified DNA (**Fig. 1**). The beads are then loaded into wells on a picotiter plate that is placed on the surface of a charge-coupled device (CCD). The sequencing is performed by synthesis using a modified pyrosequencing *(18)* procedure on solid support (**Fig. 2**). Nucleotides are sequentially passed through the flow chamber, and complementary nucleotides are incorporated in the wells containing the template-carrying beads. Inorganic pyrophosphate and photons are generated during the synthesis, and the signal from the individual wells is captured by the CCD, enabling reading of the template sequence.

The initial read length for each template was about 100 bases, which is much shorter than the average 800 bases read length routinely achieved by Sanger sequencing. It was improved to an average of 250 bases with the introduction of a second-generation 454 FLX sequencer and has reached over 400 bases for a total of 1–2 Gb in 24 hours with further improved consumables and software upgrades (Titanium series). Paired-end

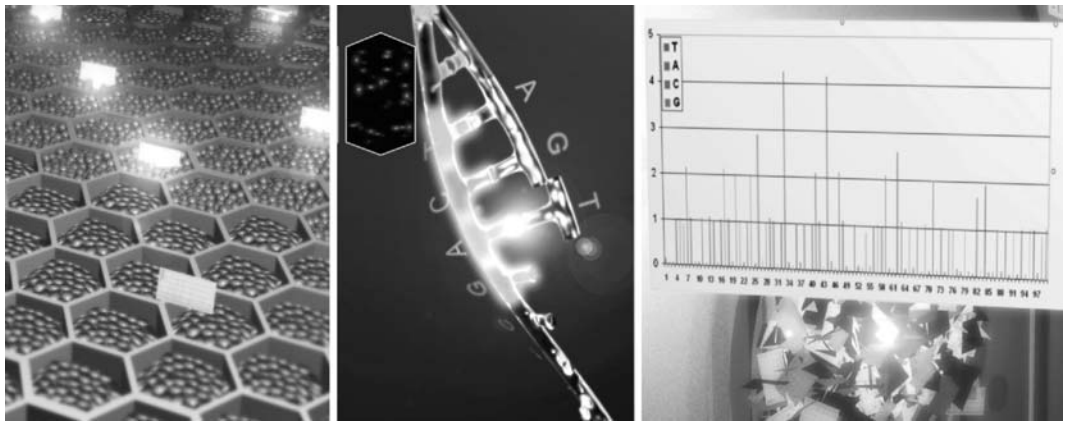

Fig. 2. The 454 sequencing steps: loading of picotiter plate, pyrosequencing, and reading of the flowgram.

sequencing protocol that generates 110 base-pair tags separated by 3000 bp genomic distance and that mimics the Sanger double-strand sequencing was released in 2008. This approach will improve the *de novo* sequence assembly and may overcome one of the drawbacks of the technology, namely, the inability to correctly assemble large repeats. The other main drawback relates to homopolymeric tracts, especially in monomers longer than three or four bases, for which the quantitation of the signal is not always accurate because all the bases in the tract are added in one nucleotide flow (e.g., six A's in a row) and generate a single signal of higher intensity. This problem is partially overcome by the generation of a much deeper sequence coverage of each base than is traditionally achieved with Sanger sequencing. Each base is sequenced approx 30 times, instead of only 5–8 times, in a single 454 run for an average bacterial genome.

Another popular sequencing instrument is the Illumina Genome Analyzer (www.illumina.com), released in June 2006. It employs the Solexa technology that is based on sequencing by synthesis, reversible terminator chemistry, and clonal single-molecule array technology. A DNA sample of 100 ng/μg is fragmented, and approximately 40 million random DNA fragments are immobilized on the optically transparent planar surface of a flow cell at a density of up to 10 million/cm^2. The fragments are amplified (solid-phase amplification) to create close to 1,000 local copies of each fragment. All four fluorescently labeled modified nucleotides possessing the reverse termination property and a polymerase are added to the array for sequencing. A laser light of a specific wavelength for each base excites the label on the incorporated nucleotides, and fluorescence is detected by a CCD. The fluorophore and the reversible terminator are then removed, and the cycle of incorporation, detection, and identification is repeated. A sequencing run generates up to 1 Gb

in random short sequence reads of 30–50 bases, producing an evenly distributed high coverage of reads that can be aligned with a reference genome sequence. The scalable nature of this platform allows for a paired-end run on a single flow cell generating up to 2 billion bases in a 6-day run. The presence of all four reversible-terminator deoxynucleotide 5′-triphosphates (dNTPs) during each sequencing cycle minimizes the incorporation bias and increases the accuracy of base calls. The quality of each base call is determined independently; therefore, sequencing through homopolymeric regions poses no additional challenge.

The ABI SOLiD system, which is based on sequencing by sequential ligation, was released by Applied Biosystems in October 2007 (www.appliedbiosystems.com). The technology combines massively parallel clonally amplified sample preparation, sequencing by ligation, and fluorescently tagged nucleotide detection. Up to 10 μg of starting material is fragmented, the 60–90 base DNA pieces are linked to magnetic beads and clonally amplified in emulsion PCR microreactors (similar to the 454 protocol). The beads containing amplified DNA are covalently bound to glass slides and loaded onto the SOLiD Analyzer. The sequence of the fragments is generated by ligation with four-color dye-labeled probes. Every fourth and fifth base is interrogated; the probe with exact matching bases ligates to the bound fragment, the color signal is detected and recorded at the fifth base, and the fluorescent tag is cleaved. After seven cycles of ligation, the original primer is stripped from the template, and a new primer offset by one base is hybridized to begin the next series of ligations. Sequences of the first 25–35 bases are determined after five rounds of primer resets. The technology is also developed for paired-end sequencing across a range of library insert sizes from 0.6 to 10 kb. Interrogating two adjacent bases simultaneously during the sequencing process improves the base call accuracy and may allow distinction between sequencing errors and single-nucleotide polymorphisms (SNPs). Currently the SOLiD Analyzer generates about 2 Gb of data per run for a fragmented library and about 4 Gb for a paired-end library sequenced on two slides.

The HeliScope instrument from Helicos Biosciences (www.helicosbio.com) is the first commercialized single-molecule sequencing platform that does not require DNA amplification. The technology is based on the work of Steven Quake ***(19)***, who developed a novel surface chemistry that anchored DNA molecules to microchannels and demonstrated the proof of principle that the sequence of a template can be revealed by sequential addition of one type of labeled nucleotide and DNA polymerase. The Helicos sequencing by synthesis is performed in two flow cells in which single-stranded DNA molecules are captured on a

chemically treated surface at a density of 100 million/cm^2. The DNA polymerase and fluorescently labeled bases are sequentially added, and if the base is complementary to the template, it is incorporated into the synthesized strand. Unincorporated bases are removed, the positions of the fluorescent bases are captured, and the fluorescent tag is cleaved. While a base is added in one cell the image is captured in the other cell. The first-generation HeliScope systems released in early 2008 produce 25–55 base reads with <5% raw error rate for a total capacity of the instrument estimated to be about 1–3 Gb/d. The instrument has the potential to generate up to 10^9 bases per hour with future improvements in chemistries and the flow cell. Single-molecule sequencing without DNA template amplification eliminates the risk of introducing errors during the amplification step but increases the probability of a wrong base call with a single-molecule template because of base misincorporations by the polymerase. This technology has the potential to considerably reduce the sequencing costs because of its massively parallel nature and minimal use of reagents if the data generated prove to be highly accurate.

2.2. Combination of Sequencing Technologies

The combination of different technologies produces the most complete genome sequences. Disadvantages of one platform are complemented by the ability to resolve these features with a different technology. For example, sequences that cannot be propagated in *E. coli* are not represented in the cloned libraries, resulting in sequence gaps in shotgun assemblies. The steps of cloning and propagation in the bacterial host are absent in the 454 sample preparation work flow. The fragmented genomic DNA is amplified on beads in emulsion PCR; therefore no host biases affect the composition of the amplified library. On the other hand, the pyrosequencing chemistry utilized on the 454 platform exhibits limited capability in accurately resolving homopolymers longer than three or four bases, whereas the Sanger dideoxy-terminator sequencing produces the accurate number of consecutively repeated bases. The hybrid assembly of shotgun reads generated by these two platforms results in an accurate and almost complete microbial genome sequence, reducing, and in some cases eliminating, the need for manual genome finishing ***(20)***. The 454 platform is also an effective tool for sequencing through areas of secondary structures (hard stops) that are often prevalent in genomes with high G + C content (>60% G + C).

The important factors are not only the total bases produced by a sequencer, but also the read length obtained from each sequenced fragment. It is especially significant in the case of *de novo* sequencing and assembly of the shotgun reads. Addition of a large number of relatively short pyrosequencing reads did not result in significant reduction in gaps in genomes containing

a large number of repetitive areas or in the number of physical (unlinked) gaps *(20)*. The use of paired ends is therefore necessary in this kind of genome.

McCutcheon and Moran *(21)* demonstrated that a complete and accurate genome sequence of *Sulcia muelleri* can be obtained by combining two of the new higher-throughput technologies. They mapped short accurate reads (33 bases) from a partial Solexa Genome Analyzer run onto assembled 454 data. In doing so, they successfully corrected the 454 sequencing errors in the homopolymeric regions and eliminated the majority of the frameshifts in coding regions.

2.3. Future Technologies

In addition to the sequencing platforms released in the last 2–3 years, numerous other companies and academic groups continue their quest for a technology that will yield a $1,000 human genome *(22)* and bring sequencing closer to clinical applications.

Intelligent Bio-Systems (www.intelligentbiosystems.com) is developing a Pinpoint sequencer with predicted capacity of several gigabasepairs per day. It is based on sequencing by synthesis of PCR-amplified DNA fragments captured on a high-density glass chip. The modified nucleotides, containing an end cap and labeled with a base-specific removable fluorescent dye, are incorporated during the DNA strand synthesis. The array is scanned; the fluorescent label on the terminal base is detected, recorded, and then cleaved along with the end cap.

The VisiGen Biotechnologies sequencing chemistry (www.visigenbio.com) is based on real-time single-molecule fluorescence detection in a massive parallel array. The DNA polymerase is modified with a fluorescent donor, and each nucleotide is color coded with an acceptor fluorescent tag. During the extension reaction, when a nucleotide is incorporated into the growing DNA strand, energy transfers from the polymerase to the nucleotide (fluorescence resonance energy transfer, FRET), and a base-specific signal is emitted and detected in real time. The goal of VisiGen is to achieve a sequencing rate of 1 Mb/s per machine and generate read lengths of about 1,000 bases.

Mobious (www.mobious.com) is combining biological molecular systems and artificial nanostructures to create novel single-molecule sequencing and array-based sequencing-by-synthesis platforms. Mobious's approach circumvents the use of labeled nucleotides and secondary enzymes. Known as polykinetic sequencing, it takes advantage of the selective mechanisms occurring during the polymerase reaction, discriminating between the time the DNA polymerase takes to add a complementary base to a growing strand and the time it takes to reject a noncomplementary base. By labeling the polymerase with a fluorescent tag

and adding a single nucleotide at a time, the sequence is derived by measuring the amount of time the polymerase attaches to the growing strand.

NABsys (www.nabsys.com) in partnership with Brown University is developing the hybridization-assisted nanopore sequencing (HANS) platform. The genomic DNA is fragmented in 100-kb pieces and made single stranded. Oligonucleotides representing all possible permutations of six bases are attached to the fragments one at a time. The fragments with annealed oligonucleotides are then passed through nanopores, and the current flow through the pore is recorded. A drop in current indicates the presence of an oligonucleotide. The profiles of 6-mer positions along each fragment are then assembled into the 6-mer map of the entire genome. Finally, the profiles of each possible 6-mer are aligned, and the whole-genome sequence is derived.

Pacific Biosciences (www.pacificbiosciences.com) is exploring a different approach to sequencing a single native DNA molecule in real time. The SMRT™ sequencer utilizes a zero-mode waveguide based on Harold Craighead's and Stephen Turner's work at Cornell University *(23,24)*. The zero-mode waveguides are nanometer-scale holes in a very thin metal film in which a single DNA polymerase molecule is captured. The sequence is determined during the DNA replication in real time as the DNA polymerase adds nucleotides, each tagged with a different fluorescent dye, to the growing complementary DNA (cDNA) strand. Because light can penetrate only a very short distance past the hole, the imaging equipment illuminates just the base added. The sensor can detect only that spotlight, but not all other free-floating fluorescent bases. The current reported throughput of the SMRT system is about 10 kb/s or 36 Mb/h, but projected improvements could bring the sequencing speed to 100 Gb/h. If the promise of this technology is realized, the human genome could be sequenced in about 4 min for less than $1,000.

The goal of Reveo (www.reveo.com/vision), developers of the OmniMoRA (omni molecular recognizer application), is to sequence a human genome in less than 1 min. They rely on physical electrooptic methods and nanotechnology rather than the traditional chemical methods. Sequencing is performed by scanning stretched immobilized single-strand DNA using an array of nano-knife-edge blades as detectors. Accelerated electrons excite the bases, which vibrate with specific frequencies, and the molecular vibration characteristics are measured and recorded for each of the nucleotides. The same device could sequence amino acids in a protein. It has the potential to achieve improvements over existing sequencing instruments that may lead to 100% error-free reads and 100% coverage of the human genome in minutes for pennies per genome.

3. Applications

Until future technologies become readily available, the increase in sequencing throughput will come with shorter read lengths or other varying drawbacks. Although the combination of different technologies in a single project can alleviate some of the drawbacks and improve sequencing results (*see* **Subheading 2.2**), newer technologies can be used individually in applications that go beyond genome *de novo* sequencing.

The most obvious use of a very large number of short reads is to align them onto a reference complete genome to identify differences, in particular small local ones like SNPs. The ability to reliably distinguish valid SNPs from sequencing errors depends on the average quality of the reads generated by the technology as well as its throughput and ability to perform several runs easily, which directly correlates to the average number of individual reads that will span any given SNP.

If paired ends are combined with very high-throughput sequencing technologies, the rapid mapping of an extremely large number of sequence pairs onto a reference genome enables the identification and characterization of rearrangements, such as deletions, insertions, and inversions, that happened between the pairs. For instance, the 454 technology was used to detect and map more than 1,000 structural variations, 3 kb or larger, between two humans using the human genome as a reference *(25)*.

The higher throughput of new sequencing platforms also positions them favorably for the detection of rare entities or events. For instance, 454 sequencing was used to generate expressed sequence tags (ESTs) from RNA captured by laser microdissection from the shoot apical meristem of maize. In a single run, over 25,000 genes were tagged, and 30% of the 454 ESTs did not align with ESTs previously generated for this tissue. The 454 ESTs included rare transcripts that had not yet been captured *(26)*. Sequencing cDNA from RNA samples using these technologies also provides for accurate determination of the relative abundance of individual RNAs within the sample simply by comparing the relative abundance of sequences obtained for each gene/transcript. A study indicated that this approach combines the high-throughput advantage of serial analysis of gene expression (SAGE) with the mapping accuracy of EST sequencing that provides longer reads than the short SAGE tags *(27)*. The study also determined that short and long transcripts (<80 bases or >300–400 bases, respectively) are underrepresented in 454 sequencing reads. However, the limitation with longer transcripts can easily be overcome by shearing the starting material, for instance, by nebulization. Nevertheless, in another study 454

was used in conjunction with Solexa to detect small regulatory RNAs (20–30 bases), including microRNAs *(28)*. It is foreseeable that new sequencing technologies will make microarray-based comparative genomic hybridizations (CGHs) and transcriptional profiling obsolete in the near future given their higher accuracy than microarray hybridization results and broader dynamic range of detection. The Solexa technology has also been used for ChIP-Seq *(29)* to study protein-DNA interactions, for instance, to characterize promoter-binding sites; the identification of DNA methylation patterns in Arabidospsis *(30)*; and the mapping of nucleosome positions in humans *(31)*. The list of applications is as diverse as it is long, and it will continue to grow as additional technologies come online.

It should be noted that the use of these new technologies comes with significant challenges that are sometimes overlooked by interested users. First, the sheer throughput of the new platforms generates amounts of data that can rapidly become extremely difficult to handle, from the very basic aspect of storage on digital media to the need for robust software applications to process and analyze the data. Second, the disparity in sequencing accuracy, read length, and read type (e.g., Sanger electropherograms vs. 454 flowgrams) renders the assembly of reads from individual platforms or combinations thereof quite challenging. Tools are currently being developed to address this issue *(32)*, and it is hoped that they will enable users to select technologies that best suit their needs and generate reliable assemblies for further analysis.

4. Impact of Whole-Genome Sequencing

The availability of genome sequence information and tools to mine it has revolutionized the way researchers in many fields design their experiments. Molecular biology is probably the most affected discipline as manipulation of genes has become so powerful with the knowledge of their sequence. Despite the large number of species sequenced, genomics continues to unravel genes that had not been seen before and whose function is unknown. One of the goals of the genomic art is to point experts toward a set of new genes that are of interest to their research. For instance, the context of these genes—operons, distribution across species or strains thereof (comparative genomics), or phylogenetic trees—can help gain insights into their potential role and open avenues for research. Subsequently, the identification of novel gene functions will lead to new research applications.

Genomics has shed light on many aspects of evolution *(33)*: genome reduction as seen in the case of obligate intracellular bacteria; genome plasticity (rearrangements, mobile elements); gene duplication and diversification of protein function; lateral gene transfer and acquisition of new functions; adaptation to environments; virulence; and so on. It has also had an impact on industrial processes, bioremediation, and biotransformation, as well as medicine with the accelerated development of vaccines (*see* **Subheading 5**.), drugs, and diagnostics. Epidemiology is of course intimately connected to genomics. The latter provides a whole-genome perspective to the classifications derived from the subsets of markers measured by molecular epidemiology techniques. On the other hand, epidemiology is excellent at identifying strains that should be selected for whole-genome sequencing to encompass and thoroughly characterize the breadth of diversity at hand.

Many new disciplines have also emerged or significantly expanded in the postgenomic era, including

1. Functional genomics that tackles the function of genes at the whole-genome level. Transcriptomics, using microarrays or cDNA sequencing techniques, identifies the transcriptional level of the entire gene repertoire under various conditions. For example, Grifantini et al. identified the genes expressed on interaction of *Neisseria meningitidis* with epithelial cells *(34)*. Proteomics achieves a similar goal but by looking at the protein level rather than the messenger RNA (mRNA) level. For instance, Pieper et al. studied the resistance of *Staphylococcus aureus* to vancomycin using comparative proteomics approaches *(35)*. Metabolomics tackles yet another level of the cell biology: the profile of all metabolites, the small molecules that are the end product of specific cellular processes *(36)*. Interactomics investigates the protein-protein interactions within the bacterial cell or between the bacterial proteins and their host, for instance, using two-hybrid techniques *(37)*. The difficult task of reconciling the knowledge gained from all these approaches belongs to the rising field of systems biology, in which studies are conducted at the level of whole cells or communities *(38)*.
2. Synthetic biology: Given the sequence of an entire genome, it is possible to synthesize genes *de novo*, usually starting from long synthetic oligonucleotides that are assembled together sequentially. A common application is the optimization of codon usage within a gene of interest for more efficient heterologous expression, for instance, for the production of a specific compound. More recently, investigators have attempted to identify the minimal genome, the smallest set of genes that enables life, and synthesized the minimal genome of *Mycoplasma genitalium (39,40)*.

3. Structural genomics: Obtaining the three-dimensional structure of proteins is the ultimate step toward characterizing their function and understanding their interaction with their environment. Comparative genomics facilitates the comprehensive identification of protein families representative of particular protein functions. Ongoing projects aim at systematically crystallizing a representative member of each of those families and deciphering their three-dimensional structure ***(41)***. Given the high degree of diversity among some protein families, subsequent efforts will likely be aimed at crystallizing multiple members of the more diverse families to shed light into the evolution of function.

5. Reverse Vaccinology and Bacterial Diversity

One of the main goals of sequencing the genome of many strains and species of bacterial pathogens is to identify novel tools to help combat disease. Reverse vaccinology ***(42)*** makes use of genomic sequence information to identify novel and better-suited protein candidates for vaccine development. Knowing the genome provides access to all the proteins it encodes and an understanding of their diversity, thus enabling a more informed selection of vaccine candidates.

Reverse vaccinology was pioneered in 2000 on serogroup B *Neisseria meningitidis* ***(43,44)***. Based on the genomic data, all proteins predicted to be surface exposed and therefore likely to be accessible to antibodies were identified *in silico*. Criteria for prediction included proteins known to carry out functions at the surface of the cell; exclusion of proteins known to be cytoplasmic; exclusion of proteins likely to be embedded in the cell's membrane and inaccessible to antibodies; and amino acid motifs characteristic of targeting to the membrane (signal peptides), anchoring in the lipid bilayer (lipoproteins), anchoring in the outer membrane of gram-negative bacteria or the cell wall of gram-positive bacteria, and interaction with host proteins or structures (e.g., integrin-binding domain) ***(45)***. This resulted in a list of approx 600 genes that were systematically cloned in *E. coli* for expression of recombinant proteins. About 350 proteins that were successfully expressed and purified were used for characterization of their antigenicity and accessibility on the cell surface. Of these, 85 were positive in one or more of the following assays: Western blot (specificity), flow cytometry or immunoprecipitation (accessibility), and bactericidal activity (ability of the antisera to kill the bacteria in vitro when combined with human complement) ***(46)***. The last is a good indicator that the antigen is a promising vaccine candidate. The seven best candidates that satisfied all criteria were selected

and sequenced across a panel of diverse strains of *N. meningitidis* representing all serotypes and spanning the phylogeny of the species ***(44)***. Five of the seven candidates were completely conserved across the entire panel of strains. Thus, for the first time in decades of classical vaccinology, five extremely strong vaccine candidates likely to confer general protection against serogroup B strains of *N. meningitidis* were identified. These were combined and tested in infant rats challenged intraperitoneally with lethal doses of *N. meningitidis*. The cocktail, when formulated with adjuvants suitable for human use, conferred protection in rats against 90% of a panel of 85 *N. meningitidis* strains representative of the global population diversity ***(47)***. The cocktail is currently being tested in human clinical trials ***(47)***.

Since then, the reverse vaccinology approach has been applied to numerous microbial species. Another striking example of its importance is the case of group B *Streptococcus (Streptococcus agalactiae)*. The first genome sequence of this species did not provide antigens able to confer general protection against the diversity of strains encountered in the clinic. The generation of eight complete genome sequences encompassing the major disease-causing serotypes indicated that *S. agalactiae* is a very diverse species. Indeed, each new genome sequence provided an average of 33 new genes, and mathematical extrapolation of the trend indicated that a very large number of genomes would have to be sequenced before the entire gene repertoire of group *B Streptococcus* could be determined ***(48,49)***. This led to the concept of the bacterial pan-genome, composed of the core genome: the genes present in all sequenced strains and the dispensable genome made of genes present in a subset of the strains. The latter contributes to the diversity of the species and provides the tools for evolution and adaptation. In the case of *S. agalactiae*, the pan-genome is described as open, meaning that the size of the pan-genome is undetermined and is likely to be very large. Other species, such as *Bacillus anthracis*, exhibit a closed pan-genome because only four genome sequences are sufficient to describe their entire gene repertoire ***(49)***. The pan-genome concept has deep implications for the diversity of the species and the discovery of vaccine candidates. In the case of *S. agalactiae*, a cocktail of four antigens, only one of which was part of the core genome, had to be used to confer broad protection ***(50)***.

Reverse vaccinology is only one example of the power of bacterial genome sequencing in the modern era of genomics. In the near future, a remarkably large number of bacterial species will have one or several genome sequences available (complete or draft), including unculturable species, thanks to metagenomics and other approaches. This wealth of data will continue to alter the way we conduct research and warrants an exciting future in our respective fields.

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Chapter 19

DNA Microarray for Molecular Epidemiology of *Salmonella*

Stephan Huehn and Burkhard Malorny

Abstract

Salmonellosis is a common infection estimated to affect 3 billion people and to cause 200,000 deaths every year. Infections can appear as enteric fever, gastroenteritis, bacteremia, or extraintestinal focal infection. The course of the disease depends on a variety of factors, including infective dose, immune status of the host, and the genetic background of both the host and the pathogen. It has been recognized that certain *Salmonella* types play a major role in the epidemiology of *Salmonella*. Here we describe a DNA microarray comprised of 282 sixty-mer oligonucleotide probes to study the epidemiology of *Salmonella enterica* subsp. *enterica* isolates at the genotypic level. The probes detect targets encoding genes associated with pathogenicity, antibiotic resistance, fimbriae, prophages, flagella (H antigens), lipopolysaccharides (O antigens), plasmids, insertion sequence elements, and metabolism. The probes are printed on glass slides, and whole-genomic fluorescence-labeled *Salmonella* DNA is hybridized to the substrate. For quality assurance, a number of controls are included on the microarray.

Key words: Characterization, epidemiology, microarray, *Salmonella* spp, typing.

1. Introduction

Salmonella is a major zoonotic food-borne pathogen that causes outbreaks and sporadic cases of gastroenteritis worldwide in humans *(1)*. Currently, two species are recognized in the genus *Salmonella*: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* has been subdivided into six subspecies: *S. enterica* subsp. *enterica* (designated subspecies I), *S. enterica* subsp. *salamae* (subspecies II), *S. enterica* subsp. *arizonae* (subspecies IIIa), *S. enterica* subsp. *diarizonae* (subspecies IIIb), *S. enterica*

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subsp. *houtenae* (subspecies IV), and *S. enterica* subsp. *indica* (subspecies VI). Subspecies I strains are usually isolated from humans and warm-blooded animals, while the other subspecies are usually recovered from cold-blooded animals and the environment. According to the Kauffmann-White scheme, subspecies are further divided into serotypes, which are widely used as an epidemiological standardized typing method. Serotyping is based on the antigenic variability of the lipopolysaccharide moieties (O antigens), flagellar proteins (H1 and H2 antigens), and capsular polysaccharides (Vi antigens).

In general, *Salmonella enterica* from human infections can be subdivided into two groups: the enteric fever (typhoidal) group and the nontyphoidal salmonellae, which typically cause gastroenteritis but occasionally, under certain conditions, can cause invasive disease. Mainly, five serotypes are involved in enteric fever: Typhi, Paratyphi A, Paratyphi B, Paratyphi C and related serotypes (Choleraesusis), and Sendai *(2)*. The other approx 2,500 known serotypes belong to the nontyphoidal salmonellae. Although rare, nontyphoidal salmonellae can cause systemic disease, typically when the host's defense is compromised. Specific nontyphoidal serotypes appear to be associated with rather high ratios of invasiveness compared to other serotypes, for example, Dublin, Heidelberg, Brandenburg, and Virchow *(3)*. *Salmonella* Enteritidis and *S.* Typhimurium are the most epidemiological important serotypes because they are responsible for more than 80% of all human infections worldwide *(4)*.

A number of virulence factors and virulence mechanisms have been identified in *Salmonella*. Among those are the type III secretion system, the lipopolysaccharide, and intracellular survival and pathogenicity islands (SPIs) that play a major role in the pathogenicity and epidemiology of *Salmonella*. The various *Salmonella* genomes contain horizontally acquired genetic elements that might play a role in infection, host adaptation, disease development, and spread of antibiotic resistance determinants. Lateral gene transfer is a major contributor to *Salmonella* evolution *(5)*.

A recently identified region that is associated with enhanced virulence is the *Salmonella* Genomic Island 1 (SGI 1), encoding multidrug resistance. SGI 1 is a chromosomally encoded gene cluster, with a size of 43 kb, originally found in a Canadian *S.* Typhimurium phage type DT104 isolate. Recently, SGI 1 was also detected in other, epidemic *Salmonella* serotypes (e.g., Agona, Albany, Newport, Paratyphi dT+) *(6)*. DT104 is associated with enhanced virulence and multidrug resistance. SGI 1 apparently spreads horizontally and represents a public health concern in regard to the future treatment of *Salmonella* infections.

We describe here a protocol to produce a DNA microarray comprised of 282 oligonucleotide probes to study the epidemiology of *Salmonella*. Furthermore, a protocol for the hybridization

and an example to analyze the results are provided. With this DNA microarray it is possible to analyze the presence or absence of a defined gene set of a few hundred specific *Salmonella* target sequences within one experiment (*see* **Appendix**).

2. Materials

All buffers and double-distilled water must be sterilized by either autoclaving or filtration.

2.1. Salmonella *DNA Purification*

1. Microcentrifuge (Eppendorf, Hamburg, Germany).
2. Vortex mixer.
3. Thermal block (thermomixer 5436, Eppendorf) or water bath, capable of being heated to 95°C.
4. DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany).
5. Proteinase K (>600 mAU/mL) solution (Qiagen).
6. Ribonuclease (RNase) A (100 mg/mL) (Qiagen).

2.2. Microarray Production

1. Microarrayer (QArray Mini, Genetix, UK).
2. Thermal block.
3. 384-well polypropylene microarray plate with cover (e.g., Genetix, UK).
4. Pretreated glass slides (CodeLink Activated Slides, GE Healthcare).
5. Desiccator.
6. Slide holder tube (AdvaTube, Advalytix, Munich, Germany).
7. C6 amino-linked oligonucleotides, synthesis scale 0.04 µ*M*, 100 pmol/µL concentration (Metabion, Munich, Germany).
8. 2X print buffer: 300 m*M* sodium phosphate. Dissolve 0.41 g NaH_2PO_4, 3.79 g Na_2HPO_4 in 100 mL distilled water, adjust to pH 8.5.
9. Blocking solution: 100 m*M* Tris-HCl, 50 m*M* ethanolamine, pH 9.0 (*see* **Note 1**).
10. Glass chamber with slide rack (e.g., staining dish with tray, Schiefferdecker type, Duran, Mainz, Germany).

2.3. DNA Labeling and Purification

1. Thermal block or water bath capable of being heated to 95°C.
2. Water bath.
3. Microcentrifuge.
4. Concentrator.

5. Exo-Klenow fragment (5 U/μL, Klenow fragment of DNA polymerase I, GE Healthcare).
6. 10X nucleotide mix with 5-aminohexylacrylamido-dCTP in 10 m*M* Tris-HCl and 1 m*M* ethylenediaminetetraacetic acid (EDTA), pH 8.0 (Bio Prime Plus Array CGH Genomic Labelling System, Invitrogen).
7. Panomer 9 solution covalently labeled at 5′-end with fluorophore AlexaFluor 555 or AlexaFluor 647 (Bio Prime Plus Array CGH Genomic Labelling System, Invitrogen).
8. Reaction buffer 2.5X: 125 m*M* Tris-HCl, 12.5 m*M* $MgCl_2$, pH 7.5.
9. Stop buffer: 0.5*M* EDTA, adjust to pH 8.0.
10. Binding buffer B2: 40% (v/v) 2-propanol, supplied with Labelling System (Bio Prime Plus Array CGH Genomic Labelling System, Invitrogen).
11. Washing buffer W1: 80% (v/v) ethanol, supplied with Labelling System (Bio Prime Plus Array CGH Genomic Labelling System, Invitrogen).
12. Elution buffer E1: 10 m*M* Tris-HCl, pH 8.5.
13. DNA purification spin columns with collection tubes (Bio Prime Plus Array CGH Genomic Labelling System, Invitrogen).
14. 1.5-mL amber reaction tubes (Eppendorf).

2.4. Hybridization

1. Hybridization chamber (sciHYBCHAMBER, Scienion, Berlin, Germany).
2. BfR hybridization buffer: 400 μL formamide, 100 μL Denhardt's solution (Fluka), 100 μL 10% (w/v) sodium dodecyl sulfate (SDS), 150 μL 20X SSC (3*M* NaCl, 0.3*M* sodium citrate, pH 7.0), 250 μL 20% (w/v) dextrane sulfate.
3. Water bath heated to 42°C.
4. Lifter slips (MSeries 22 × 26.5 mm Erie Scientific Company, Portsmouth, NH).
5. Wash solution I (1X SSC + 0.3% w/v SDS): 150 m*M* NaCl, 15 m*M* sodium citrate, and 1 m*M* SDS in double-distilled water.
6. Wash solution II (0.2X SSC): 30 m*M* NaCl, 3.0 m*M* sodium citrate in double-distilled water.
7. Wash solution III (0.05X SSC): 7.5 m*M* NaCl, 0.75 m*M* sodium citrate in double-distilled water.

2.5. Scanning and Data Analysis

1. Two-color laser scanner (excitation by 532 and 635 nm), including analysis software (e.g., GenePix 4000B scanner, Gene Pix Pro 6.0 software, Axon Instruments, CA).

2. Table calculation software (e.g., Microsoft Excel, Redmond, WA).
3. Visualization and analysis software (e.g., BioNumerics 5.0, Applied Maths, Ghent, Belgium).

3. Methods

The microarray protocol described consists of the following steps: (1) *Salmonella* DNA purification from pure cultures; (2) production of the microarray; (3) genomic DNA labeling; (4) microarray hybridization of labeled genomic DNA; and (5) scanning and data analysis.

3.1. Salmonella *DNA Purification*

For the purification of *Salmonella* DNA, the DNeasy Blood and Tissue Kit from Qiagen is used.

1. Transfer 1.6 mL *Salmonella* overnight culture (16–18 h incubated at 37°C) into a clean 2.0-mL reaction tube. Centrifuge at 10,000*g* for 4 min (*see* **Note 2**).
2. Discard the supernatant carefully.
3. Resuspend the pellet completely in 180 µL ATL buffer by vortexing.
4. Add 25 µL proteinase K; mix by vortexing briefly.
5. Incubate the suspension at 56°C for 3 h using a thermomixer at 750 rpm.
6. Centrifuge the tube for 10 s.
7. Let the tube cool at room temperature to approx 40°C.
8. Add 5 µL RNase A (100 mg/mL), mix by vortexing, and incubate for 5 min at room temperature.
9. Mix by vortexing for 15 s. Add 210 µL AL buffer and mix thoroughly by vortexing.
10. Add 210 µL 96–100% ethanol and mix by vortexing immediately to yield a homogeneous solution.
11. Pipet the mixture into the DNeasy Mini spin column placed in a 2-mL collection tube.
12. Centrifuge at 10,000*g* for 1 min. Discard the collection tube and place the column in a new collection tube.
13. Add 500 µL AW1 buffer and centrifuge at 10,000*g* for 1 min. Discard the flowthrough and collection tube and place the column in a new collection tube.
14. Add 500 µL AW2 buffer and centrifuge at 10,000*g* for 1 min.

15. To dry the DNeasy membrane, centrifuge for 3 min at 17,500*g*. Discard the flowthrough and the collection tube.
16. Place the DNeasy Mini spin column in a 1.5-mL microcentrifuge tube and pipet 50 µL AE directly onto the DNeasy membrane.
17. Incubate at room temperature for 5 min, then centrifuge at 10,000*g* for 1 min to elute DNA.
18. Repeat elution step and centrifuge at 17,500*g* for 2 min.
19. Store the DNA at 4°C until fluorescence labeling.

3.2. DNA Microarray Production

The source plate should be prepared in a room free of *Salmonella* DNA (*see* **Note 2**).

3.2.1. Preparation of the Source Plate

1. Dilute 6 mL of print buffer with 2.4 mL double-distilled water. Fill the 384-well microarray plate with 21 µL per well using a multichannel pipet. Add 9 µL of each 100 m*M* oligonucleotide probe using a multichannel pipet (*see* **Note 3** and **Appendix**).
2. Add 9 µL of double-distilled water in the wells that do not contain oligonucleotide probes.

3.2.2. Print Process

Here we describe the printing conditions and application of a QArray Mini microarrayer (Genetix) for the array production. For other microarrayers, the printing conditions should be adapted.

1. Place the slides onto the slide holder. The activated surface must be placed on top (*see* **Note 4**).
2. Place eight pins in the print head and adjust the head for approx 0.5-mm inking depth. The source inking order is set by rows.
3. For slide design, select 8-pins/7-fields order and arraying by fields. For field layout, use fields 1, 2, 5, and 6 (*see* **Note 5** and **Fig. 1a**).
4. Set the pattern dimension to 160 µm estimated spot size, row count 6, column count 8, row pitch 750 µm, column pitch 500 µm.
5. Set the number of blots required before printing on the sample slides to 5 and the blot pitch to 650 µm.
6. Set the washing program between oligonucleotide inking to 3,000 ms washing using distilled water, 500 ms waiting. Repeat washing step six times. The final step is 3,000 ms washing, 35,000 ms drying with compressed air, and 5,000 ms waiting (*see* **Note 6**).

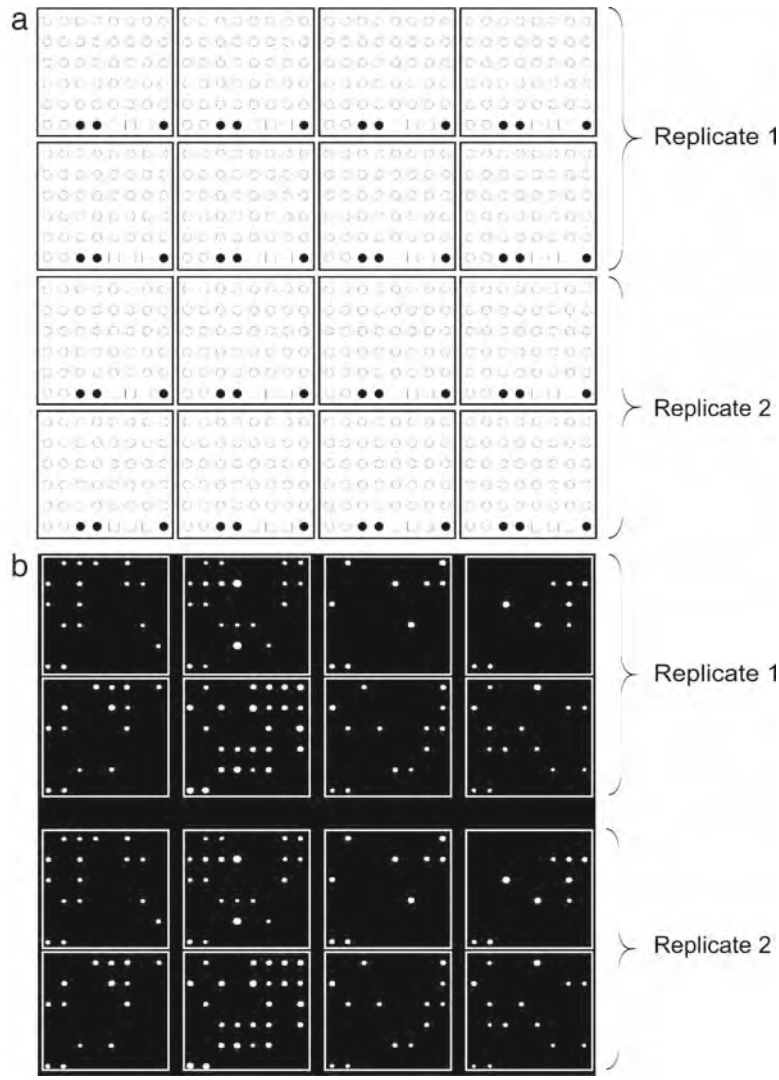

Fig. 1. *Salmonella* DNA microarray. (**a**) Scheme of the array showing the segmentation into 16 blocks. All blocks contain a control row including positive control spots (diamond), print buffer (contamination) control spots (black circle), and negative control spots (square). The oligonucleotide probes are positioned within the upper 5 rows of each block (white circle). Each probe is printed as replicate. (**b**) Hybridized *Salmonella* DNA labeled with AlexaFluor 555. A gray spot indicates the presence of a target sequence within the genome.

7. Print the slides using 24 stamps per inking. Set the stamp time to 10 ms and inking time to 2 s.
8. After printing, place the slides immediately in a sealed chamber (e.g., glass chamber with slide rack) containing saturated sodium chloride solution at the bottom. Close the

chamber with a lid and seal the chamber with parafilm. Keep the chamber overnight at room temperature.

3.2.3. Postcoupling Processing

1. Block residual reactive groups using preheated blocking solution at 50°C for 30 min with gentle shaking in a sealed chamber.
2. Rinse the slides in distilled water and afterward in postprint washing solution.
3. Wash the slides using preheated postprint washing solution at 50°C for 30 min with gentle shaking in a sealed chamber.
4. Rinse the slides in distilled water.
5. Wash the slides using preheated distilled water at 50°C for 30 min with gentle shaking in a sealed chamber.
6. Place four slides each in a slide holder tube and spin the tube in a centrifuge for 3 min at 5,000*g*.
7. Store the slides until use at room temperature, protected from light and humidity.

3.3. Labeling of Genomic DNA

For the labeling of the genomic *Salmonella* DNA, the BioPrime Plus Array CGH Genomic Labeling System is used. General requirements for the labeling according to standard laboratory praxis have to be considered (*see* **Notes 2** and **7**).

3.3.1. DNA Labeling

1. Pipet approx 4 μg *Salmonella* genomic DNA in a maximum volume of 24 μL in an amber 1.5-mL microcentrifuge tube and adjust the volume to 24 μL with sterile water (*see* **Note 8**).
2. Add 20 μL fluorophore-random oligonucleotide mix (Panomer 9 resuspended in reaction buffer), mix by vortexing gently, and briefly centrifuge to the collect the contents.
3. Incubate the tube at 95°C for 10 min, immediately cool on ice, protected from light, for 5 min.
4. On ice, add 5 μL 10X fluorophore nucleotide mix with AlexaFluor 555-aha-dCTP or AlexaFluor 647-aha-dCTP and 1.5 μL exo-Klenow fragment (*see* **Note 9**). Mix gently briefly by vortexing and centrifuge to collect the contents.
5. Incubate the tube at 37°C for 3.5 h in a water bath protected from light.
6. Add 5 μL stop buffer to the tube and place on ice.

3.3.2. Purification of Labeled DNA

1. Add 200 μL binding buffer B2 to the labeled DNA and briefly mix by vortexing.

2. Load the sample onto the PureLink Spin Column placed in a 2-mL collection tube.
3. Centrifuge at 10,000*g* for 1 min. Discard the flowthrough and place the column in a new collection tube.
4. Add 650 µL of wash buffer W1 onto the column.
5. Centrifuge at 10,000*g* for 1 min. Discard the flowthrough and place the column in a new collection tube.
6. Centrifuge the column at 17,500*g* for 3 min to remove any residual wash buffer.
7. Place the column in a new sterile amber 1.5-mL collection tube.
8. Add 55 µL elution buffer E1 to the center of the column and incubate at room temperature for 5 min.
9. Centrifuge the column at 17,500*g* for 2 min. The flowthrough contains the purified labeled DNA.
10. Dry the eluate in a vacuum concentrator at 60°C for 25 min.

3.4. Microarray Hybridization of the Labeled DNA

Protect the labeled DNA from light as much as possible at all steps (*see* **Note 2**).

3.4.1. Hybridization

1. Place the slide containing the printed probes into the hybridization chamber. Place one lifter slip per array field onto the slide. Fill 30 µL of sterile water into humidity wells and prewarm the closed chamber at 42°C for 10 min.
2. Add 30 µL of hybridization buffer to the labeled and dried DNA and resuspend the DNA by careful pipeting. Avoid air bubbles.
3. Incubate the sample at 95°C for 2 min.
4. Centrifuge briefly to collect the content and to avoid liquid at the tube walls.
5. Open the prepared hybridization chamber.
6. Load the sample carefully under the lifter slip. Avoid air bubbles.
7. Close the hybridization chamber and incubate at 42°C for approx 18 h in a water bath.

3.4.2. Washing

1. After incubation, open the hybridization chamber and remove the slide from the chamber with tweezers. Immediately rinse

the slides in 300 mL wash solution I, preheated at 34°C, to remove the lifter slips.

2. Place the slide in a slide holder and wash it at 34°C for 3 min in wash solution I with gentle shaking.
3. Rinse the slide with wash solution II, preheated at 34°C.
4. Wash the slide at 34°C for 3 min in wash solution II with gentle shaking.
5. Rinse the slide with wash solution III, preheated at 34°C.
6. Wash the slide at 34°C for 3 min in wash solution III with gentle shaking.
7. Rinse the slide in sterile water at room temperature.
8. Place the slide in a holder tube and centrifuge the tube at 5,000*g* for 3 min.
9. Store the slides until scanning, dry and protected from light, at room temperature.

3.5. Scanning and Data Analysis

1. Prescan the slide using a microarray scanner according to the manufacturer's instructions. Select the green laser light channel for the DNA, if labeled with AlexaFluor 555 and the red laser light for the DNA, if labeled with AlexaFluor 647. Adjust the PMT gain (*see* **Note 10**).
2. Define the array field and perform a full scan with high resolution (10 μm pixel resolution).
3. Save the scanned array in TIFF format (*see* **Fig. 1b**).
4. Automatically align signals for identifying and analyzing individual features using a GAL (GenePix Array List) file (*see* **Note 11**) and quantify feature intensities using the GenePix Pro software. Subtract the local background intensity from each feature intensity. Save the raw feature intensities as a text file and import the file in a table calculation program (e.g., Excel) for normalization.

3.5.1. Normalization

1. Calculate the signal intensity average of the two positive control spots (ttrC probe) of each block. Calculate a ratio between the ttrC intensity and each feature intensity of the corresponding block. Based on the ratio the presence, absence, or uncertainty of the target will be defined. Using CodeLink Activated slides the cutoff ratio is set to 0.25. A normalized ratio over 0.25 is considered as target sequence presence. A normalized ratio between 0.25 and 0.15 is considered an uncertain result. In this case, an individual decision has to be made (*see* **Note 12**). A normalized ratio below 0.15 is considered as target sequence absent.

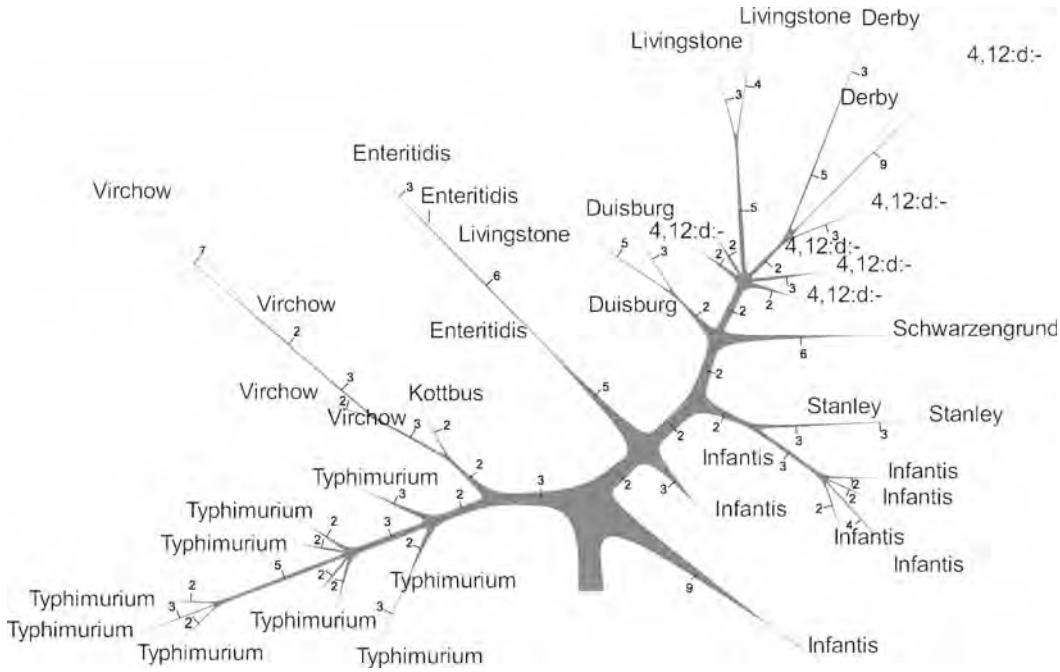

Fig. 2. Maximum parsimony tree calculated by BioNumerics 5.1. The tree exemplarily shows the differences of various *Salmonella* isolates tested based on 75 pathogenicity genes. Strains belonging to a certain serovar cluster together. The small numbers indicate the number of genes different between the branches.

2. Import a table indicating the presence or absence of each individual target from one strain to BioNumerics 5.1. Select certain target marker groups (e.g., pathogenicity) and perform a maximum parsimony tree to visualize the differences in the gene set between the strains tested (*see* **Fig. 2**).

4. Notes

1. Prepare the solution without adding ethanolamine; adjust to pH 9. Add ethanolamine directly before use.
2. Use pipet tips with filters only. Carefully avoid cross-contamination of the samples. The reagents, especially the enzymes, should be kept on ice during pipeting. To avoid fluids on the reaction tube wall, spin the tubes shortly in a microcentrifuge before use.

3. To be able to use multichannel pipets, order oligonucleotide probes in a 96-deep well microtiter plate.
4. The print chamber and slide surfaces should be free of dust. The humidity should be between 25 and 50%.
5. One field is divided into eight blocks (eight columns, six rows) representing a full set of probes (see Fig. 1a). All fields have the same probe assignment. Consequently, one slide contains two arrays; fields 1 and 2 form the first array, fields 5 and 6 the second array.
6. The compressed air should be totally clean. Avoid propellants and oil aerosols. Especially, propellants influence the surface tension, which influences the spot size and may generate extremely large spots.
7. After adding the fluorophores carefully protect the solutions from light for the whole process. Especially, UV light will bleach and lower the signal intensity. Use amber reaction tubes.
8. At least 4 μg of genomic DNA should be used, but not more than 10 μg.
9. Generally, AlexaFluor 555-aha-dCTP can be used for labeling. We have observed that this fluorophore labels DNA more efficiently, resulting in stronger signal intensities, than using AlexaFluor 647-aha-dCTP. There is no difference in the specificity of the fluorophores.
10. Positive control spots (ttrC probe) should be used for defining optimal signal intensity. Pixels with intensities out of the range (e.g., over 65,000 raw intensity) shall be strongly avoided since it is not an accurate measurement of the pixel intensity.
11. A GAL (GenePix Array List) file defines an array of blocks to match the size and positioning of printed features and to apply substance names to the features. The file can be usually generated by the microarrayer software.
12. The signal intensity depends on the DNA quality, the hybridization reaction, as well as the labeling reaction. Moreover, the cutoff value may differ between different chips with different surfaces and fluorophores.

Acknowledgments

We thank Cornelia Bunge-Croissant and Ernst Junker for technical assistance.

Table 1
Appendix. DNA Microarray Probe Sequences and Their Specific Characteristics

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
X01385	aac(3)-IV	Resistance	Aminoglycoside-3″-acetyltransferase, encoding GEN resistance	GACACGATGCCAACACGACGCTGCATCTT-GCCGAGTTGATGGCAAAGGTTCCCTATG	57
AJ310480	aacC1	Resistance	Aminoglycoside-3″-acetyltransferase, encoding GEN resistance	CTTATGTGATCTACGTGCAAGCAGATTACGGT-GACGATCCCGCAGTGGCTCTCTATA	57
S68058	aacC2,3	Resistance	Aminoglycoside-3″-acetyltransferase, encoding GEN resistance	GAAGAAACGGTGAAGTCGCCTGGAAAACGGCAT-CAGAATACGATTCAAACGGCATTC	57
AJ009820	aadA1a	Resistance	Aminoglycoside-3-adenyltransferase, encoding STR/SPE resistance	GAAGTGGTGATCGCCGAAGTATCGACTCAAC-TATCAGAGGTAGTTGGCGTCATCGAG	57
AF261825	aadA2,3,8	Resistance	Aminoglycoside-3-adenyltransferase, encoding STR/SPE resistance	AAATTTCGAACCAACTATCAGAGGT-GCTAAGCGTCATTGAGCGCCATCTGGAATCAA	57
AF169041	aadA5,4	Resistance	Aminoglycoside-3-adenyltransferase, encoding STR/SPE resistance	GTTCTTGCTCTTGCTCGCATTTGGTACAGCGCT-TCAACTGGTCTCATTGCTCCTAAG	57
AF078527	aadB	Resistance	Aminoglycoside-2″-adenyltransferase, encoding GEN resistance	CATGGAGGAGTTGGACTATGGATTCTTAGCGGA-GATCGGGGATGAGTTACTTGACTG	57
AE008792	abe_B	Serotyping	CDP-abequose synthase: Serogroup B	ACCTTCATATACTGAGTATCAAGTTGGAACTGGT-GCTGGGGTAAGTTTGAAAGATTTTCTGGT	63
X61917	abe_C2-C3	Serotyping	CDP-abequose synthase: Serogroup C_2-C_3	TGCATTAAAGCGTCCTATAACCGAGCCAACGAT-TATCAATACCTTGATTGAATGGTTGA	59
NC_003197	acrF	Resistance	Acridine resistance protein F	GACATCTCTGACTATGTCGCCTCTAACATTAAG-GATTCTATCAGCCGTCTGAATGGT	57
U43280	agfA	Fimbrial	Thin aggregative fimbriae precursor	GAACTGACTCAGAATGGTTTCAGAAATAAT-GCCACCATCGACCAGTGGAACGCTAAA	57

(continued)

Table 1 (continued)

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
AF024666	aphA1-Iab	Resistance	Aminoglycoside-3″-phosphotransferase, encoding KAN resistance	TTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGAC	57
DQ177329	armA	Resistance	16S rRNA methylase, extended-spectrum β-lactamase- resistant	AAAGTCTTTATCTGGAAAGGAGAAGGGAATGGAAGAGAATTACCAGCTATGGTTTGAATCTTT	63
AF013573	avrA	Pathogenicity	SP1- encoded protein; inhibits the key pro-inflammatory	ACCGAAGCATTGACCTGTATTGTTGAGCGTCTGGAAAGTGAAATTATAGATGGCAGC	57
AE008835	barA	Pathogenicity	Sensory histidine kinase	GCGGCTCGACCTTCTGGTTTCATATTAATCTTGATCTTAACCCAAATGTCATTATTGACGGGC	63
AE008694	bcfC	Fimbrial	Fimbrial usher, bovine colonization factor	GCACAGTCAGGAACCAATTTACAGCTTATGGGCTATCGCTATTCAACCTCGGGCTTT	57
AJ238349	$bla_{oxa1\ like}$	Resistance	Extended- spectrum β-lactamase, encoding AMP resistance	TTCTCTGGAGATAAAGAAAGAAACAACGGATTAACAGAAGCATGGCTCGAAAGTAGCT	58
AJ238349	$bla_{oxa1\ like}$	Resistance	Extended- spectrum β-lactamase, encoding AMP resistance	CCCAAAGGAATGGAGATCTGGAACAGCAATCATACACCAAAGACGTGGATGCAATTT	57
AF153200	bla_{pse1}	Resistance	Extended- spectrum β-lactamase, encoding AMP resistance	AGTATTACAGCAGTTGTGTGGAGTGAGCATCAAGCCCCAATTATTGTGAGCATCTATCT	59
AF153200	bla_{pse1}	Resistance	Extended- spectrum β-lactamase, encoding AMP resistance	GCAAGTTGAACAAGACGTTAAGGCAATTGAAGTTTCTCTTTCTGCTCGTATAGGTGTTTCCG	62
AF309824	$bla_{tem\text{-}1\ like}$	Resistance	Extended- spectrum β-lactamase, encoding AMP resistance	TAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGG	57
AF309824	$bla_{tem\text{-}1\ like}$	Resistance	Extended- spectrum β-lactamase, encoding AMP resistance	AGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATC	57

AY123253	catA1	Resistance	Chloramphenicol acetyl transferase, encoding CHL resistance	ACATATATTCGCAAGATGTGGCGTGTTACGGT-GAAAACCTGGCCTATTTCCCTAAAG	57
AL627271	cdtB	Pathogenicity	Cytolethal distending toxin, secreted protein	GGAATCTTCAGGGCTCTTCAGCATCTACA-GAAAGTAAATGGAATGTCAATGTCAGAC	57
M64556	cmlA1like	Resistance	Chloramphenicol exporter, encoding CHL resistance	AAATATGGGCTTTGCAGTCCGTGTTAGGCTT-TATTGCTCCAATGTGGCTAGTGGGTATT	59
X77455	cmy-1 (bla fox-1)	Resistance	CMY-type extended- spectrum β-lactamase, encoding AMP resistance	GAGTTCAGAAGAGCTAAGAAGTTGCTTGAGG-TACTGGGTTGCATTGATAATAGTCATG	58
U77414	cmy-2 like	Resistance	CMY-type extended- spectrum β-lactamase: AMP resistance	CTATTCCGGGTATGGCCGTTGCCGTTATCTAC-CAGGGAAAACCCTATTATTTCACCT	57
NC_003197	copR	Resistance	SPI 5, Ccopper resistance protein	CAGGAACATTATTCATTGATTATTCTTGATATTAT-GCTGCCGGGGCTTGATGGATGG	57
AE016840	csgA	Fimbrial	Major curlin subunit precursor	TAGGCCAGGGTGCGGATAACAGTACTATT-GAACTGACTCAGAATGGTTTCAGAAACAATG	60
AE008724	cstA	Metabolism	Carbon starvation protein	ATGAATAAATCAGGGAAATACCTCGTCT-GGACAGCGCTCTCAGTATTGGGTGCGTTT	57
X92507	ctx-M2	Resistance	CTX-M2 extended- spectrum β-lactamase: AMP resistance	TATAGCGACAATACTGCCATGAATAAGCTGATT-GCCCATCTGGGTGGTCCCGATAAA	57
AY341107	cutF	Metabolism	Putative copper homeostasis protein	CAGCCTGTACGCTATTTGCATTGATAGGGTG-TAATAACCGTGCGGAAGTTGACGCCC	57
AY103456	dfrA1 like	Resistance	Dihydrofolate reductase, encoding TMP resistance	ACCCAACCGAAAGTATGCGGTCGTAACACGT-TCAAGTTTTACATCTGACAATGAGAAC	58
AF175203	dfrA12	Resistance	Dihydrofolate reductase, encoding TMP resistance	CGGCAAGCCTCTACCGAACCGTCACACATTGG-TAATCTCACGCCAAGCTAACTACCG	57
AJ313522	dfrA14	Resistance	Dihydrofolate reductase, encoding TMP resistance	GGTCGTTACCCGCTCAGGTTGGACATCAAAT-GATGACAATGTAGTTGTATTTCAGTC	57

(continued)

Table 1 (continued)

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
AF220757	dfrA17,7	Resistance	Dihydrofolate reductase, encoding TMP resistance	TCTTCCAAATCGCAAATATGCAGTAGTGTCAAA-GAACGGAATTTCAAGCTCAAATGAA	58
AE008878	dgoA	Metabolism	Galactonate dehydratase	GAAAATAACTCACATCACCACGTACCGTTTAC-CTCCACGTTGGATGTTCCTGAAAATCG	59
AE008723	entF	Pathogenicity	Enterobactin synthetase, component F (non-ribosomal peptide synthetase)	CGCTATTTGGCCCGGTGCTCAACATAAAAGT-GTTTGATTATCATCTGGATCTTCCTGG	58
NC_003197	envR	Pathogenicity	Transcriptional repressor, envelope Rregulator for envCD, acrEF	ACTTATTCAGGATAGGCTTACGGGATGCT-GGAATGATAATCCTTTACAGGATCTACG	57
Y14067	fhuA_Spa	Pathogenicity	Outer membrane protein receptor	GTTACGACTGGGCCGATCAAGAGTCTCTTAAC-CGCACTACTGGCATCACATCTAAAC	57
AE008703	fhuA_STM	Pathogenicity	Outer membrane protein receptor	GTTGACAACGAGCGTTTACAGAATTTCAGCGTA-GATACACAACTGGAAAGTAAATTCGC	59
AE008721	fimA	Fimbrial	Major type 1 subunit fimbrin (pilin)	GACAATAGCACTACCGCAACCGGCGTCGGGATT-GAGATTCTTGATAATACCTCTTCA	57
AE008787	fliC/fljB	Serotyping	Filament structural protein, detects all H antigens	CACAAGTCATTAATACAAACAGCCTGTCGCTGTT-GACCCAGAATAACCTGAACAAATCCC	60
AY649698	fliC_b	Serotyping	Filament structural protein, detects b antigen	ACAGTTACTGAAAACCAAATTGTAGACGCTGTTA-CACCGACGCCAGTTGATACAGTC	57
AL627272	fliC_d	Serotyping	Filament structural protein, detects d antigen	AACCAAATTGCTGAAGTAACAAAAGAGGGTGTT-GATACGACCACAGTTGCGGCTCAA	57
AJ292284	fliC_e,h	Serotyping	Filament structural protein, detects e,h antigen	CTTGAAGCCGGTGGCAAGTACTATGCTGCAAC-CTATGACGAAGGTACAGGTAAAATC	57
AJ292278	fliC_e,n,x-e,n,z15	Serotyping	Filament structural protein, detects e,n,x and e,n,z_{15} antigens	CCCAACTAAATCTACTGTTACAGGTGATACCGCT-GTTACTAAGGTACAGGTTAATGCTCCT	61

M84980	fliC_g,x	Serotyping	Filament structural protein, detects g complex- associated antigens	GTACCGCTGAAGCCAAAGCGATAGCTGGT-GCCATTAAAGGTGGTAAGGAAGGAGATA	57
AE008787	fliC_i	Serotyping	Filament structural protein, detects i antigen	GGTCTTGGTGGTACTGACCAGAAAATTGAT-GGCGATTTAAAATTTGATGATACGACTGGA	60
U06201	flic_m,t	Serotyping	Filament structural protein, detects m,t antigen	CAACTCAGGGGCGGTAGTAACTGACACCACT-GCTCCAACTGTTCCTGATAAAGTATA	57
X04505	fliC_r	Serotyping	Filament structural protein, detects r antigen	AAGTCACTTTAACTGGCACACCAACAGGAC-CAATTACTGCTGGCTTCCCTTCAACTG	57
AY434692	fliC_z10	Serotyping	Filament structural protein, detects z10 antigen	AAACTGCTGGAATTACTGGTGCTACAT-TAAAAGCTGGTATTACTGGTACAACGACA-GAAACCG	63
AY649736	fliC_z4,z23	Serotyping	Filament structural protein, detects z4,z23 antigen	AAATTAGATGTGACTAAAGGAATCGCAACCACTG-TAAGCTCTGGAGCCTCGGTAGTT	57
AE008826	fljA	Serotyping	Repressor of phase-1 flagellin	GTGTGAGGACATCCAATGGCAATCATAT-GCAAGTTATGTTTGACTGGGTGAGCAGGA	57
AE008826	fljB_1,x	Serotyping	Filament structural protein, detects 1,x antigens	CCAATAATGGTACTACACTGGATGTATCG-GGTCTTGATGATGCAGCTATTAAAGCGGCT	59
AJ292277	fljB_l,w-l,v	Serotyping	Filament structural protein, detects l,w and l,v antigens	ACAGTGGTATTAGTGCTGCTGATGCTGCAAAAG-GTCAATTAGTTACGATGTCTTATACGGA	61
AF118107	floR	Resistance	Putative efflux protein: FLO/CHL resistance	CTGATGGCTCCTTTCGACATCCTCGCTTCACT-GGCGATGGATATTTATCTCCCTGTC	57
AF246666	gipA	Pathogenicity	Gifsy-1 Peyer's patch-specific virulence factor	CTGATTAACGATAACCAAGTTGTATGCGTC-GAATCCCTGAAAGTGAGGAACATGATC	57
AE008720	glxK	Metabolism	Glycerate kinase II	GAAACGGGTTTCTGTGGACGTTAGCGGGCCGAT-GGGGGAAAAAGTAAACGGATTTTAT	58
AE008818	gogB	Pathogenicity	Gifsy-1, leucine-rich repeat protein	TGGGACAGGAAGAATAGAGCCGTGTTTAATAAA-GATGAGAAGATAGCAGAAAGATTGAATGA	62

(continued)

Table 1 (continued)

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
AF254762	gtgA	Pathogenicity	Gifsy-2 prophage protein	TTCCAGACCTTCCAGAACACCAAGATAATCCT-TCGCAATTACGCCTCCAACATGATG	57
AL513383	HCM1.71	Mobility	Putative periplasmic protein, on pHCM1 plasmid (R27)	CCATGTAATTTCAATATGTACGCCAAGTTAGGT-CAAAGACTGGTGGGATCTCGGGAA	57
AE008831	hilA	Pathogenicity	SPI 1- encoded transcription activator	TGATGATTTCATACTCAACATGGACGGCTCCCT-GCTACGCTCAGAAAAGAAAGTCAATA	59
AE008902	hilD	Pathogenicity	Putative AraC-type DNA binding domain containing protein	ACTTTTCGGCCCCATTAACAAAACCGAC-GACAAAACATCTGTTAGCGCCAATAGAAA	57
AE008826	hin	Serotyping	Regulator for fljA: DNA invertase hin	TACATGAACGTGGAGCTCACTTCCATTCTTTAAC-CGATAGTATTGATACCAGTAGCGCG	59
AY462995	hldD_ DT104	Pathogenicity	DT104- specific phage- encoded protein	AAAGGTCAATGACCATTGTTCTGTTCATCGCAT-AGGTTTCAGCAGACTCTATAAGCG	57
AE008913	hsdM	Metabolism	DNA methylase M, host modification	AAAACTACGTCAATGAACTCGCCTCGCTGCT-GTTTTTGAAAATGTGCAAAGAGACCG	57
AY144490	htrE	Pathogenicity	Probable porin/fimbrial assembly protein	TTGTGCGTGGTATTAACAATGCTGGTGAACT-CATCGTTCGTTGGTATGAAGAAGGTC	57
L16014	hydH(stn)	Pathogenicity	Enterotoxin sensory kinase	ATTCAGGGAGTGAGTAATAATATCATTGAGGT-TAACCGTCTGGAGCGTCAGATGCGC	57
AE008831	iagB	Pathogenicity	SPI 1- encoded invasion associated protein IAGB precursor	CATAACCGAGATGGTTCAACCGATCTTGGCCT-GATGCAAATTAACAGCTTCCATATG	57
AF261825	int_SG1	Resistance	Integrase from Tn*4555*, present in *S.* Typhimurium DT104 SGI 1	CTATCTCTACGAGAACCCAAAGACACAAGCA-GAGCGTCAGCACAATAAAGAAATGTTGC	59
X12870	int1	Resistance	DNA Integrase 1: Iintegron associated	GATCTGCTCGGCCATTCCGACGTCTCTACGAC-GATGATTTACACGCATGTGCTGAAAG	58

L10818	int2	Resistance	DNA Integrase 2: Integron associated	GCAAGAACTCTTAGGGCATAACGATGTTAAGAC-CACGCAAATCTATACGCATGTGTTGG	59
M90846	invA	Pathogenicity	SPI 1- encoded invasion protein	TGTTCGTCATTCCATTACCTACCTATCTGGTT-GATTTCCTGATCGCACTGAATATCGTACTGG	63
AE008832	invH	Pathogenicity	SPI 1- encoded invasion protein	AACCCGGAAGTAAGAATTTAAGCATATATCA-GACGTTACTTGCTGCCCATGAAAGACTGCAA	62
AE008832	invI	Pathogenicity	SPI 1- encoded secreted protein	CAGACAGCTCAGTCGTGAGGAAATTTATACGT-TATTACGTAAGCAGTCTATTGTTCGCCGG	61
AE008826	iroB	Pathogenicity	Putative glycosyl transferase	GTCGTTGGACCACTGATTGCCGCTAAGTATGA-CATTCCGGTAGTGATGCAAACCGTC	57
AY328029	irsA	Pathogenicity	Putative transcriptional regulator, internal stress response element, prophage encoded	GGTCTATAAGGCTGTGCTGAACGTCCTGT-GGAACTGGATTCTGTTTCGTAAATTTTC	57
X07037	IS150	Mobility	Insertion element, unnamed protein ORF B	TTGTCTGGATAATGCTGTGGTGGAGTGTT-TCTTTGGAACCTTAAAGTCGGAGTGTTT	57
AJ746361	ISCR1	Mobility	Insertion sequence common region ISCR1	GAAGATCCGAAGGTCATTGAGCAGATTCTCAAG-CATCTGAAACAGAAAACAGCCAAG	58
AF231986	ISCR2	Mobility	Insertion sequence common region ISCR2	GATCGGCGCTCAATCTGAATGTTCACTTCCA-CATGCTGTTTCTCGACGGTGTGTATG	57
AF261825	ISCR3	Mobility	Insertion sequence common region ISCR3	CGACGACAGCATGGATGGGCTGCGGATGAGT-TCGATCACCTACC	57
AY341249	ISCR4	Mobility	Insertion sequence common region ISCR4	CTGCCATGCGGAACGATTGGTGGCGTTCTCGT-GCAAGAAG	44
AY114142	ISCR6	Mobility	Insertion sequence common region ISCR6	GAACTTCCTATACCCTTCTCCTGTACCT-GCGAATTGTGCCATGCTGATACGTCGAAA	40
AJ250371	ISCR7	Mobility	Insertion sequence common region ISCR7	TGAACGAGCATGTGCATTTCCATTGCTGTGT-CATCGACGG	47

(continued)

Table 1 (continued)

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
AF028594	ISCR8	Mobility	Insertion sequence common region ISCR8	GCAAAGAGGGAAGCAAACTGGTGTACCGCTGT-GCCAAACA	40
AF106956	rep_iterons_ FIA	Mobility	F Plasmid pmk115, mini F plasmid replication origin, IncFIA replicon	CATGACGGTATCTGCGAGATCCATGTT-GCTAAATATGCTGAAATATTCGGATTGACC	40
AE008868	rep_iterons_ HI2	Mobility	Plasmid R478, incompatibility group HI2 (IncHI2 subgroup)	GACTTAATAGGCTCACTACCGTTGTCATCCTG-TAAGTTAAGAGGTTGATCTGCTCAA	57
AF106566	rep_ iterons_P IncP	Mobility	Plasmid RK2, transposon insertion site (Tn*1723*) plasmid incompatibility group	CACAGATGATGTGGACAAGCCTGGGGATAAGT-GCCCTGCGGTATTGACACTTGAGGG	57
K03089	leuO	Pathogenicity	Transcription regulator, component of ilvIH-leuO-leuABCP promoter relay region	GCTGGGCGTGATAAAGGGCATCAATGGATGGAA-GATTTATTAGTCTCTGTTTGTAAGCGATA	57
AF106566	lpfD	Fimbrial	Long polar fimbrial operon protein	GGTGGAACCTATGCGATGTCCTGTGAATGCCCT-GATGATACCTCTCTTATAAATGAC	62
AE008875	marT	Pathogenicity	SPI 3- encoded putative transcriptional regulator	AAAGTGTGGGAAGAAGACGGCATGGTGGT-GTCGGCAAATACGCTTTATCAGAATATC	57
AF161317	merA	Resistance	Hg(II) reductase: Mercury resistance	ATGAGCACTCTCAAAATCACCGGCATGACTT-GCGACTCGTGCGCAGTGCATGTCAAG	57
AE008725	mgtC	Pathogenicity	SPI 3- encoded putative transcriptional regulator	CAGTGGTTACTGAATATCGTAAAAGAG-GCCGCGATCTGTTTACAAGGGTTAGGTTCG	57
AE008799	misL	Pathogenicity	SPI 3- encoded protein	AACCGATTATTCCTGATCCAGTAGACCCTGT-TATCCCTGACCCTGTCGTTCCCGATC	57

NC_003197	mntH	Metabolism	NRAMP manganese transport protein	GATAATTGTCGTGCTGGTCGTCGCCCT-GAATATCTGGTTGCTGGTTGGAACGGTGAT	57
AE008800	mrdA	Metabolism	Cell elongation- specific transpeptidase of PBP 2	GATCGCCTGTCAGAATGGATGGGTAAGTTCG-GCTATGGTCACTACACCGGAATTGAT	57
X60666	msgA	Pathogenicity	SsrB-regulated factor	GATATCATTCTGGGAGAATTGACTAAGAGGGT-TCACCGGATCTTCCCCGATGCTGAT	57
X60665	nanH	Resistance	Fels-1- encoded neuraminidases	TGGTCATTGCCATCAGGATACTGTGAGGGTTTT-GGTTCAGAAAATAACATCATAGAA	57
AF378725	oafA	Metabolism	Acetylation of the O:5- antigen	GCACATCTTATGCCAGGGCTGAAATCGGTATTT-GGAAATTCACTTAACATTACGCAGAGAAC	57
AE008831	orf 17.4	Serotyping	β- O-Polysaccharide polymerase: Sserogroups E_1 and E_4	TCACTTTATCACTAAATGGTCCGTTCCTGTCTA-CATTGCATTAGGTTTGCTACTGCCTCT	62
AE016845	orf 9.6	Serotyping	α- O-Polysaccharide polymerase: Sserogroup E_1	AGGTGGGCAGTTTATATTCACAGAGGCTTT-TCATTCACTTGGTTATGTCGGAGTATTCCTG	60
AE008792	orf84	Phages	Putative prophage Cro protein	CCGCCTCATGTGAACGATAGCAGAACCATATT-AGCGAAGGTGAACAACCATGATCAC	61
AF013776	orgA	Pathogenicity	SPI 1- encoded oxygen-regulated invasion protein	TTCACCTGAACAATTTGTTGAAACGGCAGTAG-GCGTCATTAAGCATCATCTTGATGAACTTC	57
AE008783	oxyR	Pathogenicity	Hydrogen peroxide-inducible regulon activator	GCTGCATATTGGTTTAATCCCAACAATTGGTC-CCTATTTGCTGCCGCTTATCATTCC	62
AF250878	pagA(udg)	Metabolism	UDG-glucose/GDP-mannose dehydrogenase	GGTGGCGTTAGACATTGTTCCTTCCCGTGTT-GAACTGTTAAATGATCGGATATCTCC	57
AB041905	pagJ	Pathogenicity	Gifsy-3 encoded, PhoP-activated gene	GGCTTTAATATTACCATCCTCACTATATCTT-GCTCTTACAATAGCGGCAGACTCCCAA	57
AE008892	pagK	Pathogenicity	PhoPQ-activated protein	TTTGGCCTGCTGGCATACCCTTCCCTGAAGATT-GGTTTAAAATGTGTAGAGGTTATTG	58

(continued)

Table 1
(continued)

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
AE008753	parA-parB IncHI1	Mobility	Putative partition protein on antibiotic resistance plasmid	GTTCCCGATATGCTGCTCATCACTCATACATTAC-CCCCTTGCTTCACTGAAAAACTC	59
AE008753	pefA	Fimbrial	Virulence plasmid- encoded fimbrial protein	AACCAGGTTGTTCAGTTAGGTACTGTTCAG-GCAGGTCAGGAAGGTACGGCTGTTGATT	57
NC_006431	pflD	Metabolism	Putative pyruvate formate lyase II	ATGACCCATCGTATTCAACGCCT-CAAAGCCGCTCTGTTTCAGAATCACCGT-GAAATT	58
NC_004631	phoP	Pathogenicity	Response regulator in two-component regulator system with phoQ	GAATACACCATTATGGAAACGCTTATCCG-TAACAACGGTAAAGTGGTCAGCAAAGATTCGC	57
AE008747	phoQ	Pathogenicity	Sensory kinase protein in two-compo-nent regulatory system with PhoP	TGATGGGCAACGTACTGGACAACGCTTG-TAAATATTGTCTGGAGTTTGTCGAGATTTC	61
AY532917	pilR	Pathogenicity	SPI 7- encoded nucleotide-binding protein, putative sigma 54 interac-tion protein	AATGTGGTTATGCCTTCCCCTCAAAA-GAAGCCGTGAACTATCTCTCTATGGTCAGTG	58
NC_003197	pilV	Pathogenicity	SPI 7- encoded prepilin peptidase	CAAACCAATCGTTTCACTCAGGCAGTCTCATC-CTATGTTGGAAAGTTTTATCCGACG	57
NC_003197	pipA	Pathogenicity	SPI 5- encoded protein	AGAAGGCAGGAAAGTTATTTGTCTCAATCT-GGACGATTCTGATGATTCATATACCGAACA	57
U66901	pipB2	Pathogenicity	T3SS translocated protein	GCTGGACAAGTTATTGTACGAGTCAGTAAAG-GCGACCATTCTGAGACAAGAGAAATTCCG	60
AF261825	pipD	Pathogenicity	SPI 5- encoded protein	CAAACAGCTAAGCAGCAGTATAAGATGGAGCA-GAGCTATCTGAGATTATATGCGTCG	57
AY906856	prgH	Pathogenicity	SPI 1, needle complex inner mem-brane protein	AAAGAGAAGACGATAACAAGCCCAGGGCCATA-CATAGTTCGATTACTTAACAGCTCA	57

AM234698	Prot6E	Fimbrial	*Salmonella* Enteritidis fimbrial biosynthesis protein	TGTGGGTCGTAACGCACAAGTGAAATTTACCCTGAGGGAGGCTTATGGTAATAATAATTTGG	57
AM234722	qacEΔ1	Resistance	Qac multidrug exporter, encoding Et-Br and quaternary ammonium resistance	GCAATAGTTGGCGAAGTAATCGCAACATCCGCATTAAAATCTAGCGAGGGCTTTACTAA	62
NC_003197	qnrA	Resistance	Confers quinolone resistance, plasmid located	ATGGATATTATTGATAAAGTTTTTCAGCAAGAGGATTTCTCACGCCAGGATTTGAGC	59
AE006471	qnrB2_B1	Resistance	Confers quinolone resistance, recognisizes both *qnrB1* and *qnrB2*, plasmid located	CAGCAAACTTCACACATTGCGATCTGACCAATTCGGAGTTGGGTGACTTAGATATTC	57
AE008837	qnrS	Resistance	Confers quinolone resistance, plasmid located	ATGGAGAGGGTTTGTTTAGAAAAATGTGAGTTGTTTGAAAATCGCTGGATAGGAACG	57
J01724	ratB	Pathogenicity	Putative outer membrane protein	CTATTTATCTAAATCAACCTCAAGCGGCGGCATGTATTGCGGCGTAGATGAGAATAC	57
BX664015	rck	Pathogenicity	Resistance to complement killing protein, encoded on pSLT plasmid	GTACAGTTTAATCCGGTGGAAAATGTGGTCATCGATCTGGGCTATGAGGGAAGTAAAGT	59
M20134	recC	Mobility	DNA replication protein	CTCATGGGGAAAACTGGGGCGGGATTATATCTACCTGCTATCTGAACTGGAAAACAG	61
Y00768	rep_ori_γ IncX	Mobility	IncX replicon - plasmid incompatibility group	GTTAGCCATGAGGGTTTAGTTCGTTAAACATGAGAGCTTAGTACGTTAAACATGAGAGC	57
M20413	rep_SG1	Pathogenicity	Replication protein encoded by SGI 1	TTCAAGAGTAAATGGATCTGAGTTTGAGAGGCGTTGTGGTGCTGCTGGTCTATACAT	59
M28718	rep_W	Mobility	Plasmid R388, class 1 integron In3, plasmid incompatibility group IncW	GTCTTGATGATCTCGTTGATACGATGGCCGGGGGCTTTGTTGTTCTTAGGCATGTTG	57
M93063	repA_A/C	Mobility	Replicon A, plasmid incompatibility group	GGACCACCAGCTAGAAAGTATTAACGGAACAATCATGAGTAAGAGAACCAAAGACAAA	57

(continued)

Table 1 (continued)

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
AF261825	repA_B_C IncL/M	Mobility	Plasmid pMU407.1, plasmid incompatibility group, Inc L/M	TATACTCAGGTGGTTATAGTCGTTTGTAGTCGGAGGGCTTGTGAGCAGAGTAGTTGAG	58
U12441	repA_FIB	Mobility	IncF plasmid RepFIB replicon, plasmid incompatibility group	GAAGTAAGTTAATGACATAAACTATGGTCAGTATGCCAGACTCAGTTGTTAAATACAGGCTGC	58
X73674	repA_FIIs	Mobility	pSLT, plasmid incompatibility group	CATTATGATCCACTGGCCAACCGTGTACAGAGATCCATCACCAATCTGGCTATAGAG	63
U27345	repA_N	Mobility	IncN plasmid R64, plasmid incompatibility group	TATCTGGGAAATCGAATTTAACCATAAACTCCTGCGGTACATTTACGGCCTGACGAA	57
M26308	repA_repB IncF	Mobility	Plasmid pRK100, plasmid incompatibility group	GTACTGCGAGAGAGAGGGGATAACACAGGCTCAGTTCGTTGAGAAAATCATCAAAGAT	57
AE006471	repA_T	Mobility	Plasmid Rts1, plasmid incompatibility group IncT	TAATCAAAGTAGTATAACTCCCATAATCGCTCGTCTGCTTCCAGTTCCACAACGTCTGTATCG	58
NC_003292	repA_Y	Mobility	IncY plasmid P1, plasmid incompatibility group	TCTTTACGCAGACATTGAAAGTAAGGCAAAAGAACTAACAGTTAATTCAAACAACACT	63
AY234375	repA2_FIC	Mobility	Plasmid F, putative replication protein genes	ATCTTCACATTGATTCCAGCAAGTATCCTCACCCGTTTTGCAGCCTTCTCCAGAAAA	58
K00053	repC_DT193	Mobility	DNA replication protein	AAGCACATACAGGACTGCATCGAGCGCCTTTGGAAGGTATCCATCATCGCCCAGAAT	57
K02380	repC_R64	Mobility	DNA replication protein of plasmid R64	GGAAAATACTTCGGTCAACGCCCTAGCCGAACGCTTCCTCGATGACGGCCTGAAAAC	57
M16168	rfbD	Serotyping	TDP-dehydrorhamnose synthetase: Sserogroups A, B, C_2-C_3, D_1, D_2	TGACCTTATTCTGCCTCAATGGGAATTAGGAGTTAAGCGTATGCTGACTGAAATGTT	57

AY524415	rfbE	Serotyping	CDP-tyvelose-2-epimerase: Serogroups A and D	ACAGTGGTGTTCAGGCATTCATCAATGTATGGT-GGGAGACAGTTTGCTACTTATGATC	60
AP005147	rhaA	Metabolism	L-rhamnose isomerase	GTCTGATCCCAGGCCCAAAACGGCTGAACCTA-CACGCCATTTACCTTGAGTCGGATA	57
AE008792	rhuM	Pathogenicity	SPI 3- encoded putative cytoplasmic protein	GAAGCCGAAGGTGAGAAGGATATCGCCGGTTT-GCTACAATGGGAAACAGAACCTAAA	58
AL627273	rep_RNAI IncI1	Mobility	Plasmid incl-1 mini-replicon, plasmid incompatibility group	CATAAGCGACAGCTTGTGGCAGGTCTGAA-GAATACTCCATATAACGCAGTACACTGG	57
AE008889	rep_RNAI_ BO	Mobility	Plasmid pMU720, IncB mini-replicon encoding RNA I, RNA II, and pro-motor regions	TCACATAAGGATGTATCTGTGGCAAGAGCGAA-GATAAGCAGTTGAATAGATCGTTATATT	57
AE008874	rep_RNAI_ K/B	Mobility	Plasmid R387, replication-associated protein, plasmid incompatibility group	CAGCTTGTGGCAGGTCTGAAGAATACTTCAT-ATAACGCAGTACACTGGAGTCAGTTAGC	57
AE008833	rpoS	Metabolism	Major sigma factor during stationary phase	GTCTGCTGGGATATGAAGCTGCGACACTGGAA-GATGTAGGCCGTGAAATCGGTCTTA	60
AE008708	safC	Fimbrial	Putative fimbriae usher protein	GTAAGTGCTAGTTGGCAGATGACTTCACCAT-CACACGGTGGTCAGACGCAACAAGTG	59
M63169	sat (Tn7)	Resistance	Streptothricin acetyltransferase of Tn*7*	AAGAGCTTGTCGGGAAGATTGAACTCAACTCAA-CATGGAACGATCTAGCCTCTATCG	57
AB161461	sat1 (int2)	Resistance	Streptothricin acetyltransferase linked to integrase 2	CAAGCTATGAGCCAGGTCAAGCTCCATATTC-CGTTGAAGAATTAGCAGATGATGTGG	57
NC_004313	SB10	Phages	Encoded by ST64B phage	GTTATGTGCTCTCCCGCGAATTGCGAAAAT-CACTCCGTCCTCAGCGAAGGAAGAAAA	57
NC_004313	SB54	Phages	Encoded by ST64B phage	TTGAACTACTGGCAATCAGATATGCAGCATGGAT-TAAGCCGGAATTTGAAATCGAAG	57
L11008	sefA	Fimbrial	Fimbrial protein encoded by *S.* Enteritidis, *S.* Dublin and *S.* Gallinarum	TTTCCGTGGGCGTATTCAGGGAGCCAATAT-TAATGACCAAGCAAATACTGGAATTGA	57

(continued)

Table 1 (continued)

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
AF239978	sefR	Fimbrial	Sef14 fimbrial regulator	AGCCGTTGTGGAAATTGAATATATGGATGACATT-GAATCATTTGACATTATTACTTTGCCAGA	57
Xxxxxx[1]	SEN4287	Metabolism	Putative restriction endonuclease gene *Salmonella* Enteritidis unique PT4	GTCGGTAGTGATTAGCGTACCGCGAATGGCTTT-GTGGTAGGGTAGTGCACCGCGTAG	57
AE008719	sfbA	Pathogenicity	Putative periplasmic iron-binding lipoprotein	CGACGCCAACCTCTTTCAACATACCCTCTATTTT-GACAAATTCACCGCTGACAAAGG	63
NC_003197	sgbE	Metabolism	Ribulose-5-phosphate 4-epimerase	GAAATTAACGGCGAGTACGAGTATCAGACCG-GCGAGGTGATTATTAAAACCTTTGAA	57
NC_003197	shdA	Pathogenicity	Fecal shedding factor	CAATATGGTTTCTACAACAACAGCGTAGA-GAGCGGTGATGCGGGATCTGAATCTTAT	57
U51867	sifA	Pathogenicity	Lysosomal glycoprotein (lgp)-containing structures	CAAGAAAAGGCAACCTACCTGGCAGCGAAAAT-TCAGTCTGGGATTGAAAAGACAACG	57
AE008831	sipA	Pathogenicity	SPI 1- encoded *Salmonella* (cell) invasion protein	GCAGTAACCATAGCGTGGATAACAGTAAGCATAT-TAACAATAGCCGAAGCCATGTCG	57
AF026035	sirA	Pathogenicity	Invasol SirA: Regulator of invasion proteins	GTAAAGGTGGGGTACGTAATGATGACACATATC-CGAATGCCAGTAACAATGCCGAAG	57
AF128999	sitA	Pathogenicity	Invasion SirA: Regulator of invasion proteins	GATATTAAACGAGCGCAGGGGGCACAGCTTATC-CTCGCGAATGGTCTGAACCTGGAG	57
AF127079	slrP	Pathogenicity	Leucine-rich repeat protein	TATGATAACAGCATAAGGACACTGCCAGCA-CATCTTCCGTCAGAGATTACCCATTTGAATG	57
AE008762	slyA	Pathogenicity	MarR family transcriptional regulator for hemolysin	GAGCTTCTGATTAAACTTATCGCCAAACTT-GAACACAATATTATGGAATTGCACTCTCACGA	57
AF007380	sodC1	Pathogenicity	Gifsy-2- encoded, copper/zinc superoxide dismutase	TATCCGTTACTGGCACCACGCCTTAAATCACT-GTCAGAACTGAAAGGTCACTCATTG	61

AF254764	sodCIII	Pathogenicity	Fels-1- encoded putative Cu/Zn superoxide dismutase precursor	TATTACAATTACCGAAACAGAATATGGCTTGT-TATTCACCCCACATTTGTCCTCACTTCC	62
AF121227	sopA	Pathogenicity	Secreted outer protein	CAGATAATTTCCTGCTGCTTTCCTCCCAAGAT-TCAGACACGGCGATGATGCTCTCCA	57
AE008747	sopB	Pathogenicity	SPI 5 invasion gene D protein	CGGCAAAGATCGTACAGGGATGATGGATTCA-GAAATCAAGCGAGAGATCATTTCCTTACATC	60
AE008834	sopD	Pathogenicity	Secreted outer protein	TGCCCGGCTCATCAAGATCTGTTTACTATCAA-GATGGACGCTTCTCAGACACAATTT	57
AL627268	sopD2	Pathogenicity	Secreted effector protein, sopD homologue (pseudogene)	TGGAATGTGTTGAATGGAATGGTACGCTTACT-GAAGAGGAGATGAATAAACTACGCTGTCT	63
L78932	sopE1	Pathogenicity	Translocated effector protein, encoded by P2-like cryptic bacteriophage	ACGTTTTATTTCGCATAAGAACACTGAATCTTCT-GCAACACACTTTCACCGAGGAAGC	57
AF200952	sopE2	Pathogenicity	Secreted outer protein	GTGACTAACATAACACTATCCACCCAGCACTACA-GAATCCATAGAAGTGACGTTGAACCAGT	61
AE008832	spaS	Pathogenicity	SPI 1- encoded surface presentation antigen	ATATTGTAGGTATTGCCGTCATTTGGCGT-GAACTTCTCCTCGCATTGGTATTAACTTGC	58
AF060869	siiD (spi4_D)	Pathogenicity	SPI 4 HlyD family secretion protein, predicted cation efflux pump	ATCTCTTTCTAAAGGAGGGACGATACAAGATATT-TATGTAGCCGAGGGTGATACTGT	62
AF060869	siiE (spi4_F)	Pathogenicity	SPI 4- encoded protein	TAATGGTATTGCTGTCGGTCAGGCTGTAACGGA-TAGTTTGGGTAACTTCACCTTTAC	59
AF060869	siiF (spi4_R)	Pathogenicity	SPI 4- encoded putative ABC-type bacteriocin/lantibiotic exporter	GGGCGTGAGTTATCAGTATGATGCTCAATCTC-CGATGATTATTAACCGACTGTCTAT	57
NC_003197	sprB	Pathogenicity	SPI 1- encoded transcriptional regulator for Type III Secretion System (TTSS)	TGTGTGCTGCAATATTTTGGCGTTATGGATTAT-GTTTTAAAAGACGAACTATCCTGCG	57
AE008831	sptP	Pathogenicity	SPI 1- encoded tyrosine phosphatase	ACCTTATTAAGAGCAAGGATAATGTTGGTGT-CAGGAATGCCGCTTTAGTCATAAAAGGC	57

(continued)

Table 1
(continued)

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
AE006471	spvC_a	Pathogenicity	*Salmonella* plasmid virulence: hydrophilic protein	GGTTACGATGTTTTCATCCATGCTCGTCGA-GAATCACCTCAGTCTCAGGGCAAATTT	58
AE006471	spvC_b	Pathogenicity	*Salmonella* plasmid virulence: hydrophilic protein	CGGTGACAAGTTCCACATCAGTGTGCTCAGGGA-TATGGTGCCACAAGCATTTCAAGC	59
AE006471	spvR	Pathogenicity	*Salmonella* plasmid virulence: regulation of spv operon, lysR family	ACAAAGACTCTTTATACGGAAGAATGGCACTCT-TATCCCAACCGAATTTGCACAAACT	57
AE008907	srfJ	Pathogenicity	Putative virulence factor, activated by transcription factor SsrB	TGACATGATTGGTAATTTCAAATCGGGTTG-TAGCGGGTTTATCGACTGGAATCTGCTG	57
AE008761	ssaQ	Pathogenicity	SPI 2- encoded secretion system apparatus protein	ATACCACAACAGGTGCTCTTTGAGGTCGGACGT-GCGAGTCTGGAAATTGGACAATTA	58
NC_003197	sseC	Pathogenicity	SPI 2- encoded translocation machinery component, required for systemic infection	AAATAGAGCAATTAATAACTCAGCAACGGTTTCT-GGATTTCATAATGCAACAAACAGA	58
AE008761	sseF	Pathogenicity	SPI 2- encoded secretion system effector	ATTCATATTCCGTCAGCGGCAAGTAATATAGTC-GATGGTAATAGTCCTCCTTCCGATATA	57
AE008743	sseI	Pathogenicity	Gifsy-2 prophage putative type III secreted protein	GCAACAGAACCGGGAGTGGAACGCACAGA-TATAACTTACAACCTAACCAGTGATATTGAT	58
AF294582	sseJ	Pathogenicity	Translocated effector protein regulated by SPI 2	CACATCATATCTTACCCTCCTATGGT-CAATACTTTGGCGGAAGGTTTACTAATGGATT-TACC	60
AE008894	sseK1	Pathogenicity	T3SS *Salmonella* secreted effector K1 secreted by SPI 2	GGGATAATAGCTGTTGATCGCAATAACCACCCG-GCTTTACTTGCTGGATTAGAAATAATGC	60

AE008795	sseK2	Pathogenicity	*Salmonella* secreted effector K1	TTTCATGTCAAAGTAATACTCAAACCATCGCAC-CTACGCTCAGTCCACCTTCATCAG	62
AF013776	sspH1	Pathogenicity	Gifsy-3- encoded leucine-rich repeat protein, *Salmonella*-secreted protein H1	CTTACCTTCCCCGCGTGGGAGGAGAATAT-TCAGTGTAACAGGGATGGTATAAATCAG	61
AE008800	sspH2	Pathogenicity	Leucine reach repeat protein, *Salmonella*-secreted protein H2	GATGTCTTCCCGCCACCATCAGTAATCGCCG-CATTTATCGTATTGCCTGGTCTGATA	57
AE008761	ssrB	Pathogenicity	SPI 2- encoded protein: secretion system regulator	CCTGTTGTGCATACGAGCCTGACATACTTATC-CTTGATCTTAGTCTACCTGGCATCA	57
AL627265	staA	Fimbrial	Fimbrial protein encoded by *S.* Typhi CT18	CGGCTGATGTAACTGATGCCACTAAGGCT-TCTCTGGTAATGGATTCGTCATTCTCTA	57
AE008710	stbD	Fimbrial	Fimbrial usher protein	AATATCGGTTTGCCAACGGTGATTAGCGTCAG-TAATAGTGAAACATTTCAACCCTCCG	57
AE008795	stcC	Fimbrial	Paral putative outer membrane protein	TGTAGTAGATCATCATGGTCATAATGTGGGCATT-GTTGGACAAGGTAGTCAGCTATTTATTCG	57
AE008839	stdB	Fimbrial	Putative outer membrane usher protein	AATTACTGGAACGCACAGTCCAACAATAACTA-CATGCTCAGCCTCAACAAGGTGTTC	58
AL627276	steB	Fimbrial	Outer membrane fimbrial usher protein encoded by *S.* Typhi CT18	CAGCCCGGATCAGAGTAACTATAACCT-GTCTCTTTCCTGGTACTTCGACTTAGGGTC	63
AE008703	stfE	Fimbrial	Putative minor fimbrial subunit	GGCGGTGAGGTGGAATTTGGCAATGTGTTGAC-GACGAAAGTGGATGGGGTGAATTAC	57
AL627280	stgA	Fimbrial	Fimbrial protein encoded by *S.* Typhi CT18	GATTCTGCGTATAGCACGATTGATACCACAGCG-GGTACGGCTTCTATGGAGTTTATC	57
AE008702	stiC	Fimbrial	Putative fimbrial usher protein	TGAACTACAGCTTCAGCGGCTATAAAGAGTACG-GTTCCAGTGAGGATTCCGACGATG	57
AE008915	stjB	Fimbrial	Putative fimbrial usher protein	GGTTATTACACTTATCAGGCTACGGATAAT-GACAACGACTCTCGCAGTATAATGGCTTCCT	57

(continued)

Table 1 (continued)

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
NC_006511	stkC	Pathogenicity	Outer membrane usher protein	CGGAACCTATCGTGTGAAAGTGAATCTTAACAATGCGTTGAAATCTACCTCTGAAATTACC	57
AE0098709	STM0305	Serotyping	Discrimination between *Salmonella* subspecies I and non- subspecies I	CACCTGTTCAAAGATTTTCTGAAGGCGCAGGAGTATTCATTACTGATATCCTCCATT	61
AE008710	STM0330	Metabolism	Putative 3-isopropylmalate isomerase, (dehydratase), subunit with LeuC	TGCGAAATTACGACGGCAGAGGAAACGATCTCCTTTGTGATCAGTGAACTCAAACGG	61
AE008737	STM0900	Phages	Fels-1 prophage- encoded protein	GTAAGCAACTACATCAGACACAAACTTATCAGATATGCAAAAGGAAAGGGGAAGGCGGC	57
AE008784	STM1896	Metabolism	Putative cytoplasmic protein	TTTCAGTAGATGTTTCCGACAATGGTATTTCTGGCGTGGCAAAAGAGTGGCTTAAAG	57
AE008819	STM2616	Pathogenicity	Gifsy-1 prophage- encoded protein	ACCACTCAAATCTCTGTCGAAACTCTTTCCCCGATTACCCATAACCAAATTCCCGTTA	59
AE008823	STM2701	Pathogenicity	Fels-2 prophage- encoded protein	GAATGCTGATCTGGCCTGACTTCATCAACTTTGACACCGTGCTGAAAGCAGACGCGA	57
AE008824	STM2740	Pathogenicity	Fels-2 prophage- encoded protein	CTACAGAACGCTTCTATCCGCAAGGAAACCCGCTGCCTTATCGAATGGGAGCTACTG	58
AE008842	STM3098	Serotyping	Discrimination between *Salmonella* and non-*Salmonella*	GCTATGGGAAGACCAGATTATCTATATTTATCACTCTACGCCGGGTTCGCAGGGAAG	57
AE008876	STM3782	Metabolism	Putative PTS system galactitol-specific enzyme IIC component	AGGTAACCCAGCCATTATATCTACAGCACTGATTCTGACACCTATCTCTGTCTTTATTGC	57
AE008889-1	STM4057	Serotyping	Discrimination between *Salmonella* subspecies I and non- subspecies I	TGATCATTACGTTGTGATTTATTCCAACGGTACGCTGTATGGGGAATGGCCC	57

AE008896	STM4200	Phages	Putative phage tail fiber protein H	TGGAGGTGGAGGGCATACGAGTAATACAGAT-GGTCTTCTCTATTGTTCAGGTGGTAA	60
AE008896	STM4210	Phages	Putative methyl-accepting chemotaxis prophage- encoded protein	GATGAACTGCTGAGCGTCGTTGAAGAGGGTAT-GCGTGAAGCCAAAGAGATGATGGAT	52
AE008911	STM4497	Metabolism	Putative cytoplasmic protein	AAAAACAACGGCTCCGGTAATGAGATTGGGT-TCTGGATTTTTGATTATCCTGCTCAG	57
AE008916	STM4595	Fimbrial	Putative fimbrial chaperone protein	GATTACGTTCAATGGCAAAATTTACGATCAG-GCGTGTACGGTTCAGGTGAATGGCTC	57
M28829	strA	Resistance	Aminoglycoside-6 ″-phosphotransferase, encoding STR resistance	GAACAGCAGATCGCTATGCCGATTTGGCACT-CATGATTGCTAACGCCGAAGAGAACT	57
M28829	strB	Resistance	Aminoglycoside-3 ″-phosphotransferase, encoding STR resistance	GGACTCCTGCAATCGTCAAGGGATTGAAAC-CTATAGAAGACATTGCTGATGAACTGC	57
AL627279	STY3672	Phages	Hypothetical phage protein encoded by phage cs73 of *S.* Typhi CT18	ACAGAAGATTCCATTACAGATGTGTTTAAC-CAAGTGCCTTCATCGTGTATATCCGACAGTGC	57
AL627279	STY3676	Phages	Phage protein, putative capsid scaffolding protein	CTGATTAACCTTGAGCACATCAAGTCTTATCT-GCCGGACAGCACCTTTAACCGCTAC	57
AL627281	STY4221_1	Metabolism	Putative amino-transferase	ATTAATGGATTTCCAGTAATACCTATGTCTGTT-GCAGATACCGATTTAGTTTCTCCCGTGG	62
AL627283	STY4625	Phages	Phage protein, major capsid in phage P2, homologue with Fels-2 protein	CGATCCGACGCTGATGGAAGACGTGGAATA-CAAATGCGAGCAGACCAACTTTGATAC	57
AL627283	STY4631	Phages	Hypothetical phage protein encoded by phage cs73 of *S.* Typhi CT18	ACATAAGCCTGAAGGTAAGAAGCAGAAG-CACACGCATCCTGTAGATGTTGTATTTAGT	61
AF106566	sugR	Pathogenicity	SPI 3- encoded putative ATP- binding protein	CGCATTTCCACTAATCCAGTTTATTGTCACTAC-CCATAGCCCGCAGGTTATCAGCAC	57

(continued)

Table 1 (continued)

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
X12869	sul1	Resistance	Dihydropteroate synthase encoding SUL resistance	CTCTTAGACGCCCTGTCCGATCAGATGCACCGT-GTTTCAATCGACAGCTTCCAACCG	58
M36657	sul2	Resistance	Dihydropteroate synthase encoding SUL resistance	TTCTATCCGCAATTGGCGAAATCATCT-GCCAAACTCGTCGTTATGCATTCGGTGCAA	57
AJ459418	sul3	Resistance	Dihydropteroate synthase encoding SUL resistance	AAATAACTGGAACCGATGTGAAATCTCGTTTAG-CACCAACTCTTGCAGCAGAAATGTATGC	57
AL627266	tcfA	Fimbrial	Typhi coloniszation factor, putative fimbrial protein	GTATGCCACAGGAGAAGGAGGTACCAGCAG-GGAATGATATAGAGACAGGACTTGTTG	57
X61367	tet(A)	Resistance	Efflux pump, encoding TET resistance	ATCGTCGGACCCCTCCTCTTCACGGCGATCTAT-GCGGCTTCTATAACAACGTGGAAC	61
V00611	tet(B)	Resistance	Efflux pump, encoding TET resistance	TTGGATGGAATAGCATGATGGTTGGCTTTTCATT-AGCGGGTCTTGGTCTTTTACACT	57
J01749	tet(C)	Resistance	Efflux pump, encoding TET resistance	CATGACTATCGTCGCCGCACTTATGACTGTCT-TCTTTATCATGCAACTCGTAGGACA	57
L06798	tet(D)	Resistance	Efflux pump, encoding TET resistance	CGGAGCAGAAACAAGAAAGCGCAGGTAT-CAGCTTTATCACACTGCTTAAACCTCTGG	57
L06940	tet(E)	Resistance	Efflux pump, encoding TET resistance	CGGCGTTATTACGGGAGTTTGTTGGAAAG-GCTAATGTTGCAGAGAACTACGGTGTTT	57
S52437	tet(G)	Resistance	Efflux pump, encoding TET resistance	GCCTCACCAATCTAAGCTCTATCGCAGGAC-CGCTTGGCTTCACAGCACTCTATTCTG	57
NC_006816	tnp_Cf	Mobility	Transposase insertion sequence IS	CATCGACCAGTGCTTGGATCTCAGTGATC-TACGTGCCTACCTGGCAGATTTCTATAG	57
NC_006816	tnp_IS1	Mobility	Transposase insertion sequence IS*1*	GGTGGAGCTGCATGACAAAGTCATCGGGCAT-TATCTGAACATAAAACACTATCAATA	57

NC_002056	tnp_IS102	Mobility	Transposase insertion sequence IS*102*	GTGAATATGCAGACCGTAACCGTGCAGT-GGCTAATCAGCGAATGACCGGGAGTAATG	57
NC_003384	tnp_IS1202	Mobility	Transposase insertion sequence IS*1202*	CTATTTTGAAGCGACCCGTGGCTATATCGA-GAAATACGGTAAGCCCATGATCCTTTA	57
NC_006816	tnp_IS1294	Mobility	Transposase insertion sequence IS*1294*	TTTTAAGATGGTGAGGTACTTCGGGTTCCTT-GCCAACCGTGTGTGTGGAGAGAAGCT	57
NC_003198	tnp_IS1351-like	Mobility	Transposase insertion sequence IS*1351*like	GAATCACAAAAGGCTCCACCGTATTTACTGTCT-GCTCAAGCTGAATTTTCGCCGTAA	57
NC_002305	tnp_IS30	Mobility	Transposase insertion sequence IS*30*	GCTAAACAACAGACCGAGAAAGACACTGAAGT-TCAAAACACCGAAAGAGATAATTGA	57
AJ310778	tnp_Orf341E	Mobility	Transposase Orf341E	CTCAATGTCCACTACCACATGCTGTTTCTCGAT-GGTGTCTATGCCGAAGATGACTAT	57
AJ634602	tnp_pFPTB1	Mobility	Transposase insertion sequence on plasmid pFPTB1	TACTTTGGTAATAACAGAGGGATCACCTGGTA-CAACTTTGTGTCCGATCAGTATTCC	57
NC_006511	tnp_SPA2465	Mobility	Transposase SPA2465	GCTGAATGAGGTGCGGGAAATTACGGATAAAG-GGTTATCAGAATATAACTGCGAACG	57
NC_003198	tnp_STY343	Mobility	Transposase STY343	CAGATCATCGCTGTGATTAGATCAGTTGAATC-CGGACGGACTGTTAAAGATGTCTAC	57
NC_003384	tnp_Tn2680	Mobility	Transposase of Tn*2680*	CTTTGAATGGGTTCATGTGCAGCTCCATCAG-CAAAAGGGGATGATAAGTTTATCACC	57
NC_003384	tnpA_IS1-like	Mobility	Transposase A of insertion sequence IS1 like	TGCAGTTCACTTACACCGCTTCTCAACCCGG-TACGCACCAGAAAATCATTGATATGG	57
AJ746361	tnpA_IS1696	Mobility	Transposase A of insertion sequence IS*1696*	TCATATCAACCCGAAGTATGGAAGCAGTCCAG-GACGGACTTTCTACACCCATATCTC	57
NC_006816	tnpA_IS186	Mobility	Transposase A of insertion sequence IS*186*	CGCTGAATGGCGACTACATATGGGATATGATC-CTCATACCTGTCAGTTCACTGATTT	57

(continued)

Table 1
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Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
AY341107	tnpA_IS200	Mobility	Transposase A of insertion sequence IS*200*	AAATACCGAAGACAAGCGTTCTATGGAGA-GAAGCGTAGGGCAGTAGGCAGCATATTAA	57
NC_006816	tnpA_IS26	Mobility	Transposase A of insertion sequence IS*26*	TTGAACACCGACAGATTAAGTACCG-GAACAACGTGATTGAATGCGATCATGGCAAAC	57
AY509004	tnpA_IS3/IS911-like	Mobility	Transposase A of insertion sequence IS3/IS*911* like	GAGGAAGAGAACACCAGACTCAAGAAGCTGCTT-GCCGAAGCCATGCTGGATAAAGAG	57
AY509004	tnpA_IS4	Mobility	Transposase A of insertion sequence IS*4*	AAGTTTGATTCCTTGGACTCTTCAGAATACA-GACAGCAAATAAAGACCTTTCGTTTGA	57
AY509004	tnpA_IS406	Mobility	Transposase A of insertion sequence IS*406*	AAATGTATGTCAAAGGAGTAAGTACCCGCAG-GGTCTCGGATATCGTCGAAATTCTTT	57
AF261825	tnpA_IS6100	Mobility	Transposase A of IS*6100*, SGI 1	CCGATCACGGAAAGCTCAAGATACTGAT-CAAGCCGGTGCGCGGTTTCAAATCGATCC	57
AF071413	tnpA_Tn21	Mobility	Transposase A of Tn*21*	GACTCCAAGGACGACCTGATCCGACATTACA-CATTCAACGATACCGACCTCTCGATC	58
AY509004	tnpA_Tn3	Mobility	Transposase A of Tn*3*	GTGTTCTTCAACCGCCTTGGGGAAATCAG-GGATCGGAGCTTCGAG	57
AP005147	tnpR_IS10	Mobility	Resolvase of insertion sequence IS*10*	CATGGTATAAATCCGTTGAGAAGCTGGGTTGG-TACTGGTTAAGTCGAGTAAGAGGAA	45
AF261825	tnpR_SG1	Mobility	SGI 1 resolvase	AGGGAGATTAGGGCATTACTCAAAGATGGTTC-TATTCCTGTATCTGATGTTGCTAGGCGA	57
AY144490	tpase1	Mobility	SPI 3, transposase 1 similar to transposase A	CGATTGTTAGGTTAAGGACACACCCTAATAC-CCCATTTGTTTCTGCTATCCTCAAAC	57

AE00647	traT	Mobility	pSLT plasmid- encoded conjugative transfer surface exclusion	GCGATGAGCACAGCAATCAAAAAGCGTAATCTT-GAAGTGAAAACCCAGATGAGTCAG	57
AF261825	trhH	Pathogenicity	SGI 1- encoded putative pilus assembly protein	ATAACAGCCTGCTTGAAGCCATGATGTC-TATAACTGGTGCCGTTGTCATTGGTGATT	57
M84642	wbaA_C1	Serotyping	O-antigen- polymerase: Serogroup C_1	GTGCTTGGTGCCATTCTATCATTGCCTTTGTCA-CATTATTTATCAGATATAATTTCTTCCGTT	63
X60665	wbaO_E1	Serotyping	Manosyl transferase (β 1-4 linkage): Sserogroups E_1 and D_2	ATCTTTGGATTTTAGGCTTGGCTCCCAGACCTT-TACTGAAGTTGATCGGCAAGTGTATATC	61
X56793	wbaU_B	Serotyping	Manosyl transferase (α 1-4 linkage): Serogroups B and D_1	TGCCTGATGCAATTTCCCGATTTAACAACTAT-GTCGCACGGTATGACTTTGATAATATGAAGC	63
X56793	wbaV_B	Serotyping	Abequosyl transferase: Serogroup B	CGGGTGTGATTTAGTTGAGATTAGAAACCCT-CATCGTTCTTGGCTCAGAACAGATGATGAAC	62
M65054	wbaV_D1	Serotyping	Tyvelosyl transferase: Serogroup D	TCGGCGATGGTTAAATGGTGGCAGTAGAT-TATTTTCTTTTAGCAATGAAGCTGATTTGA-TAGA	63
D14156	wcdA	Pathogenicity	UDP-glucose/GDP mannose dehy-drogenase	GATTATTGGGCTGGGATATGTTGGGCTTCCTCT-GGCAGTTGAATTTGGCAAATCTCG	57
D14156	wcdE	Pathogenicity	Required for translocation of the Vi polysaccharide to the cell surface	TACTCAAACAAGAGGATTGGGAGGGGCTAT-GCCTCTATTTCAGTCAGCATCCCGAAAA	58
D14156	wzf	Pathogenicity	Vi polysaccharide export inner-membrane protein	GATTCTGTCCGTAGAGCGTCATTAGTTATTAAA-GAGCGACGTGTTCAGCAAGCCAAG	59
AY334017	wzx_O6,14	Serotyping	O antigen flippase: O6,14 serovar factor	AAAGCGACCTTGAGTATTGGGCTCACTGCTG-TAGTAGTTATAATTTATAGTAGAGTGGA	57
AF017148	wzy(O27)	Serotyping	α- 1-6 polymerase: O27 serovar factor	TGAGTCTTTATTTAATCAAATATCTTTTATGCG-GATGCTGGATTGGCTACATCAAGGGGCAGT	63
AE008758	wzy_B	Serotyping	α- 1-2 polymerase: Serogroup B	TGGCGAATTACTCGGATTATACCCGTAATGCT-GTTCTTGTTGCTTCCTCAAACTTTG	57

(continued)

Table 1 (continued)

Accession No.	Probe name	Marker group	Gene function	Probe sequence	Length
U04165	wzy_D2/E1	Serotyping	Putative O antigen polymerase: Ssero-groups D2 and Serogroup E1	AGGCGGTCAGTTTATATTCACAGAGGCTTT-TCATTCACTTGGTTATGTCGGAGTATTCCTG	61
AE008706	yafD	Metabolism	Putative cytoplasmic protein	TGGTAGTAAATGTTCATGCGGTAAATTTTAGTCT-GGGCGTGGACGTATACAGTAAGC	57
			Control oligonucleotides		
AF282268	ttrC	positive. Cco-ntrol	Tetrathionate reductase subunit C	ATGACGCATTCACTCATCATTGAAGAAGTGCT-GGCTCACCCGCAGGACATTAGCTGG	57
X58149	PRKase	Negative Ccontrol	PRKase of *Arabidopsis thaliana*, nega-tive control probe	TAACCTCTTCTTCTTCTTCCAAACAAGTCTTC-CTCTACCGTCGTCAACCACAAACCA	57
M86720	rca	Negative Ccontrol	RCA of *Arabidopsis thaliana*, negative control probe	GATGATGAAGTGAGGAAGTTCGTTGAGAGCCTT-GGAGTTGAGAAGATCGGAAAGAGG	57
NM_121758	rcp1	Negative Ccontrol	RCP1 of *Arabidopsis thaliana*, negative control probe	AGGTGTTAGGTTTGTAGGGTCTTTATCTGGAT-GGACAGCAACTCTTATGTTCATGTGGATG	57

Note: The table indicates the accession numbers, the probe names, corresponding marker group, a short description of the gene function, the probe sequence, and the oligonucleotide length of each probe. Sequence of SEN4287 is available from www.sanger.ac.uk/projects

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Chapter 20

Methods for Data Analysis

William Paul Hanage and David Michael Aanensen

Abstract

The molecular epidemiology of infectious diseases uses a variety of techniques to assay the relatedness of disease-causing organisms to identify strains responsible for outbreaks or associated with particular phenotypes of interest (such as antibiotic resistance) and, it is hoped, provide insights into where and how these strains have emerged. The correct analysis of such data requires that we understand how the assayed variation accumulates. We discuss this with specific reference to three classes of methods: those based on gel electrophoresis of fragments generated by restriction enzymes or polymerase chain reaction (PCR), those based on microsatellites and other repeat elements, and raw sequence data from protein-coding genes. We also provide a simple example of how the likely origin of an apparently novel antibiotic-resistant strain may be identified and conclude with a discussion of some popular analysis packages and the more interesting prospects for the future in this rapidly developing field.

Key words: Analysis software, clustering, eBURST, homoplasy, molecular epidemiology, molecular typing, phylogenetics, population biology.

1. Introduction

The precise way in which molecular data are analyzed will obviously depend on the technique used. There are, however, some general features and considerations that we briefly discuss, along with some potential pitfalls.

Here are some typical questions in molecular epidemiology:

Is a case of disease due to an outbreak strain or an unrelated infection?

Is a case of tuberculosis, in a patient previously thought to be cured of the disease, due to reinfection with a new strain or due to a failure to clear the original infection?

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How is an antibiotic-resistant strain of a pathogen related to those previously known? Is it a result of *de novo* acquisition of resistance?

Can we identify lineages associated with traits such as virulence?

2. The Importance of Clustering

The key to all of these questions is the relatedness of the isolates in the data set under study. Having used some methods (amply covered in the previous chapters) to assay genetic variation in the population of a pathogen, the next step is to cluster the organisms by relatedness. Analysis of molecular epidemiology data is mainly concerned with this. Depending on the type of variation assayed and the nature of the pathogen under study, there are several options available. One should be particularly aware of evolutionary processes that may make two isolates appear more related than they really are, such as recombination or convergent evolution. These processes will both lead to homoplasy—character states that are similar for reasons other than descent from a common ancestor.

To begin, consider two extreme outcomes of an investigation. At one end, all isolates are indistinguishable by the method used, while at the other end all are different and, moreover, equally different from each other. If either of these results is obtained, it is likely that the technique applied is not appropriate, offering in one case insufficient discrimination and in the other too much. While this may seem like a mere thought experiment, examples very like these two hypothetical data sets are to be found in the literature ***(1,2)***.

In a more successful study, some isolates will be indistinguishable, some will be very similar to them, and others will be very different. The processes by which we cluster these isolates require that we understand the way in which the assayed variation is generated so we can translate it into a genetic distance. Three types of variation are discussed next: (i) banding patterns produced by methods such as restriction fragment length polymorphisms (RFLPs) and amplified fragment length polymorphisms (AFLPs); (ii) microsatellites and other repeat elements; and (ii) raw DNA/RNA sequences.

2.1. Banding Patterns

Techniques such as pulsed-field gel electrophoresis (PFGE) and AFLP assay the presence of unique recognition sequences (sites where restriction enzymes cut or where polymerase chain reaction [PCR] primers bind) in the genome of a microorganism. The distances between these recognition sequences vary among strains, leading to DNA fragments of varying size produced by restriction digests or PCR with appropriate primers. These DNA fragments may be readily compared by electrophoresis, and the resulting banding patterns are the final data. Inherent to all these techniques is the ability to "bar code" the genomes of isolates of interest and the subsequent comparison of the bar codes provides the distance measure utilized for clustering.

The variation in this case is in the length of the DNA separating the recognition sequences. These sequences may be lost or gained by mutation, or indeed by recombination with related strains, and by other events, such as insertions, deletions, or large-scale genomic rearrangements that can alter the distances between recognition sequences. All of these mechanisms will alter the banding pattern.

As a result, these techniques offer very little information about the deep relatedness between strains with no recent common ancestor; unless two banding patterns are very similar, it is impossible to know how many events separate them or what those events might be. Moreover, the genes in which the assayed sequences lie are usually unknown; consequently some of the observed changes may be more likely to occur than others as a result of selection. For purposes such as outbreak analysis, this is not generally a problem as one is only attempting to identify the outbreak strain and any arising from it over the period of the outbreak. Therefore, one needs to assay variation that accumulates rapidly enough so that these can be distinguished from other unrelated infections, and consequently, one is looking for identical or very similar banding patterns. Interpretive criteria for how similar banding patterns should be for strains to be considered part of the same cluster vary between methods, but generally the banding patterns are required to show very high similarity. For an example, see the work of Tenover et al. ***(3)***.

By comparing the number of bands in common between profiles (inherently a somewhat subjective process that is in part automated in programs such as BioNumerics), it is possible to produce a distance matrix and from this a dendrogram (*see*, for example, ***ref. 4***). This should be avoided because it suggests more confidence about deep relationships than is appropriate given the considerations discussed.

2.2. Repeat Sequences

For organisms with limited sequence variation, including many eukaryotes, the existence of repeat elements in the genome offers an attractive source of variation for a typing method. In particular, the rates with which changes in these repeats occur are typically several orders of magnitude greater than mutations in protein-coding genes. As a result, organisms that are otherwise difficult to type because they are almost identical may be readily resolved. An example in bacteria is spoligotyping of *Mycobacterium tuberculosis* ***(5)*** and in several fungal species multilocus microsatellite typing (MLMT) ***(6)***. When analyzing data from such methods, it is important as ever to consider the mechanisms by which variation arises. For example, variation at a single microsatellite is constrained to a minimum and maximum number of repeats. Given the high rate of mutation, it is not at all unlikely that at a single locus, an identical allele (i.e., same number of repeats) could arise

by chance. This will produce homoplasy (*see* **Subheading 2.**) and has an effect similar to recombination in that it can make two distantly related isolates appear more closely related than they actually are. Adding more loci may improve the resolution, and this is the rationale underlying the inclusion of multiple microsatellites in MLMT. The limits of the size of the microsatellite array also mean that genetic distances at microsatellites become saturated more quickly than variation in coding sequences. For example, estimates of the divergence time between the two fungal species *Coccidioides immitis* and *Coccidioides posadasii* obtained from microsatellites, on one hand, and their flanking regions, on the other hand, differed by an order of magnitude (7.6×10^5 and 12.8×10^6 yr, respectively). As expected, this discrepancy was not found when considering more closely related populations *(7)*.

In the case of other target sequences, such as the variable direct repeats assayed by spoligotyping, it is thought that variation arises through deletion. Therefore, theoretically, one should be able to compare closely related sequences and not only cluster them but also infer the direction of change (because those with fewer direct repeats must be derived from those with more). However, in this and similar cases, phylogenies cannot be created because the repeats violate the assumption of independence among the variable sites: Adjoining repeats may be lost together *(8)*. A final general comment about repeat regions of this kind and others is that their function is rarely understood. Hence, the selective consequences of variation at these loci are an open question.

2.3. Sequence Data

With the increasing availability and decreasing expense of DNA sequencing, the direct determination of sequence data is becoming more and more prominent as a method for epidemiological typing. It might be thought that this makes data analysis easier than the examples given, but this is not necessarily so.

It is now known that many bacteria undergo frequent horizontal transfer of homologous genes *(9)*. As a result, if we examine a single locus and find two isolates that are identical at that locus, this may be no reflection of the overall relatedness of these isolates because the relevant genetic material could have been imported relatively recently. This (and other reasons) has led to the development of multilocus sequence typing (MLST) *(10)*, by which sequences are determined for multiple loci. This buffers against the distorting effect of horizontal gene transfer: Even if one locus changes through recombination, then the others do not and remain to give a better account of the relationship between the isolate in question and the rest of the population.

If the recombination rate is high enough, it is again very difficult to draw conclusions about deep branches in the tree. For many species, a dendrogram produced from the pairwise differences between MLST profiles contains minimal phylogenetic information beyond relatively close linkage distances. While it is possible

to produce a tree from concatenates of the sequences at the MLST loci, this has the problem that a single recombinational import can introduce many nucleotide changes. As a result, in addition to problems with the branching order or topology of the tree *(11)*, branch lengths can be artificially inflated as many polymorphisms are introduced by a single event. Two approaches to this problem are the programs eBURST *(12)* and CLONALFRAME *(13)*. The former focuses on changes at the very tips of the tree, and the latter attempts to identify and account for recombinational imports.

eBURST is discussed in detail elsewhere *(14–16)* and in the example given in **Subheading 3.3.** It handles recombination by focusing on single-allele changes and weighting them equally whether they introduce a single base change or many (the latter is likely to be recombination). In contrast, CLONALFRAME attempts to identify recombination events and account for them by simply excluding them from the analysis. The sequence which remains is the 'clonal frame'. In simulations it performs well, but it makes the assumption that recombination imports arise from *outside* the sample. Its performance in a situation when most recombinant alleles are present in the sample is not clear. Both programs become less reliable with very high recombination rates *(13,16)*.

No investigator should overlook recombination when interpreting data and deciding on a method of analysis. However, in some species, such as *Staphylococcus aureus*, it appears to be sufficiently rare that conventional phylogenies can be constructed with few qualms (although, as usual, care should be taken to select and deploy an appropriate model of nucleotide substitution). Such organisms may also show considerable stability in terms of clonal structure. Thus, the same clones can be recognized over a long period of time, and band-based methods produce similar results to MLST *(17)*. In very rapidly mutating and highly recombinogenic organisms, such as *Helicobacter pylori*, this is not possible, and every epidemiologically unconnected strain is different.

Similar considerations apply to viruses for which recombination can also be frequent and confuse matters. RNA viruses require particular attention when reconstructing phylogenies. In contrast to many of the problems faced by phylogeneticists, in molecular epidemiology we usually consider closely related members of the same species. This means that for bacteria, for example, one can be more relaxed about problems that arise when considering very distantly related taxa (such as long-branch attraction; *18,19)*. RNA viruses, however, are the most rapidly evolving organisms known: Substantial viral diversity arises in a single human immunodeficiency virus (HIV) 1 patient. Properly accounting for different rates of substitution and selection is of great importance in this field and is far beyond the scope of this chapter. A useful start point for interested readers is **ref.** *20,* and investing in a phylogenetics textbook (e.g., **ref.** *19*) is recommended if such techniques form a large part of future plans.

3. Example: Origin of Resistant Clones of *Streptococcus pneumoniae*

3.1. Background

Streptococcus pneumoniae (the pneumococcus) is a major childhood pathogen. A vaccine has recently been devised that is highly effective at preventing disease due to 7 of its more than 90 serotypes (4, 6B, 9V, 14, 18C, 19F, 23F). Vaccination has almost totally removed the vaccine serotypes from the population, including many important antibiotic-resistant clones, which expressed vaccine serotypes *(21)*.

Pneumococcal clones can change their serotypes by recombination events that insert the relevant serotype loci from other clones, a process known as *serotype switching (22)*. As a result, simply recording serotype is insufficient to tell us how a given isolate is related to others. While vaccination initially has a marked and beneficial effect on the prevalence of antibiotic resistance (because vaccine serotypes were more likely to be associated with resistance) *(23)*, investigators are beginning to record increased resistance to antimicrobials among serotypes not included in the vaccine.

In 2007, Pichichero et al. reported a pneumococcal isolate retrieved from a case of ear infection that was resistant to all classes of antibiotics licensed for pediatric use in the United States. The serotype of the isolate was 19A, a nonvaccine serotype *(24)*. This finding raised the following question: Has resistance been acquired *de novo,* or does this clone arise from serotype switching allowing a vaccine-serotype-resistant clone to acquire a new, nonvaccine serotype (in this case 19A)?

3.2. Choice of Method and Results

Techniques such as PFGE are unlikely to enable us to say what this strain has derived from because, in the pneumococcus, variation assayed by this method accumulates very quickly. Instead, MLST, which assays sequence variation at multiple sites around the genome and assigns an allelic profile (*see* **Chapter 11**), is the method of choice. Variation at these sites accumulates relatively slowly, so it should be informative regarding the relationship over the longer term. The MLST database also contains a large number of strains with which the results can be compared.

Subjecting the isolate in question to MLST, it was found to have the allelic profile 7, 11, 10, 15, 6, 8, 1, corresponding to sequence type (ST) 2722. To find whether there are any other records of this ST, we can go to the MLST database at http://spneumoniae.mlst.net/ and enter this ST via the advanced query page (http://spneumoniae.mlst.net/advanced/). Doing so reveals no additional records from strains with that ST isolated prior to the time of writing (January 15, 2008). This means that this is the first time an ST 2722 strain has been reported. If there was another strain in the database with a vaccine serotype and a similar resistance profile, it could have suggested that ST 2722 had been derived from it by serotype switching.

However, to conclude with certainty that serotype switching has not been involved, we must consider how ST 2722 is related to the rest of the pneumococcal population and consider whether there are any other likely parent strains.

3.3. Clustering: eBURST

eBURST groups genotypes defined by MLST or similar methods into "clonal complexes" through a simple parsimony-based method. Briefly, clonal complexes are defined on the basis of genetic similarity: All members of a clonal complex must share genetic information to a specified degree of similarity (which may be specified by the user) with at least one other member. Within clonal complexes, the genotype with the largest number of minor variants is assigned as the most likely ancestor, and the patterns of descent from this ancestor are defined according to specified rules.

To find whether eBURST can predict a putative ancestor for ST 2722, simply visit http://spneumoniae.mlst.net/eburst/ and select the option to run eBURST on the whole *S. pneumoniae* MLST database. Once the program has opened, select the analysis window and click "compute" using the default parameters. The group or clonal complex into which ST 2722 falls is then simply located using the "Find ST" feature illustrated in **Fig. 1**. At the time of writing, ST 2722 falls within group 1 (*see* **Subheading 3.5.**).

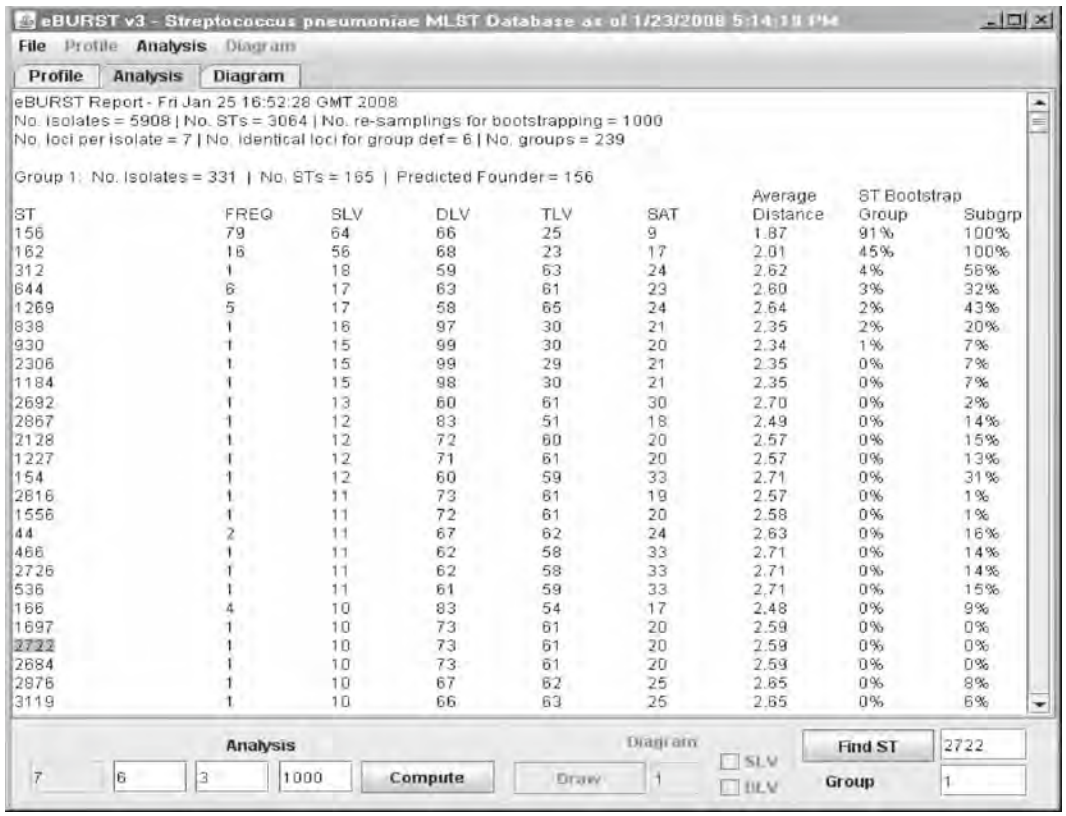

Fig. 1. Textual output from eBURST analysis of the entire pneumococcal MLST database. The "Find ST" function is at bottom right of the window.

3.4. Graphical Representation

To produce a graphical representation of the group containing ST 2722, select the Diagram window, enter the group number in the dialogue box at the bottom of the page, and click draw. The results should resemble **Fig. 2**. Again, ST 2722 may be located using the Find ST feature as shown.

The blue circle in the center represents ST 156, and those arranged around it are the STs differing from ST 156 at one of the seven MLST loci. The nature of the difference (single-base change or many) is not taken into consideration. Hence, this method is refractory to distortion by recombination.

eBURST implies that the most likely ancestor of this clonal complex, and therefore of ST 2722, is ST 156. This is interesting because this is the ST of one of the major multiresistant clones circulating prior to introduction of the vaccine *(25)*. Specifically, it is the Spain 9V-3 clone. As implied in its name, the serotype of the majority of the strains with this ST is 9V, one of the targeted vaccine serotypes.

By clicking on ST 156 and selecting "database" from the diagram pull-down menu as shown, we may link to the MLST

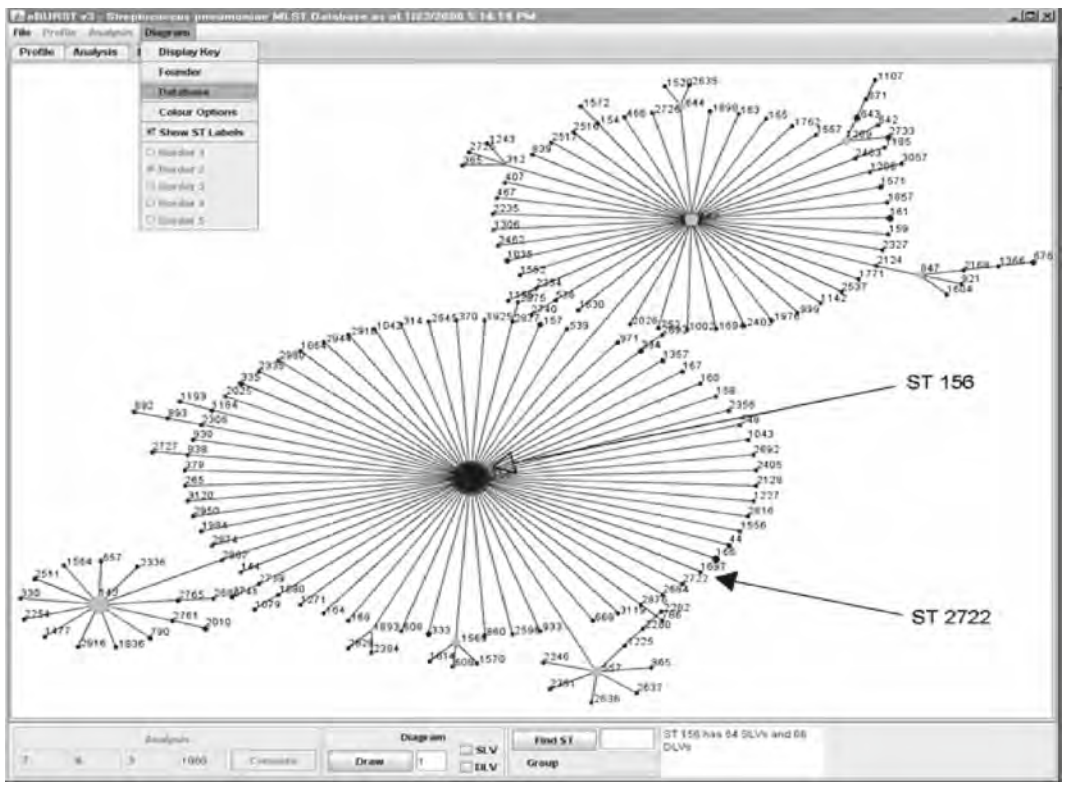

Fig. 2. Screenshot showing Group 1, the largest clonal complex in the pneumococcal MLST database at the time of writing. The predicted overall ancestor of the clonal complex ST 156 and its single-locus variant ST 2722 are both indicated. The pull-down menu at top left shows how the MLST database may be accessed within eBURST.

database. The results retrieved are those that have been submitted to the MLST database by sources worldwide. As can be seen, to date these do not include 19A strains. We can therefore suggest that the multiresistant phenotype of ST 2722 was inherited from its ancestor, ST 156, but that it has acquired a new capsule suited to the postvaccine era.

3.5. Caveats

While it is almost certainly true that ST 2722 is derived from an ST 156 strain, there are several caveats that should be mentioned. At present no isolates of ST 2722 prior to that reported by Pichichero et al. *(24)* have been recorded, but they may be in the future and then provide a more accurate picture of how, where, and when this lineage acquired a new capsule. At the time of writing, ST 2722 fell within the largest clonal complex in the MLST database. As more records are entered, the composition of this group will change, and in time it may surrender its position as the largest clonal complex. It should also be appreciated that considerable variation could be present within ST 156 (all we know for sure is that all ST 156 strains have identical sequences at seven gene fragments).

Finally, the clonal complex in question illustrates how sampling can bias analyses. Because ST 156 is highly resistant, it has been frequently reported to the MLST database, along with numerous minor variants, like ST 2722, which are also resistant. This inflates the number of resistant strains in the database and the number of minor variants of ST 156. As a result, eBURST identifies ST 156 as the ancestor of this clonal complex. However, the true overall ancestor, which gave rise to ST 156, is ST 162. This ST is not, however, a multiresistant clone, so it and its susceptible variants are underrepresented in the database. For a more thorough discussion of this issue, *see* **ref. *15.***

4. Looking Forward

A survey of this kind is inevitably destined to be obsolete in a few years as new analysis methods and opportunities arise and become widespread. Here, we summarize some of the methods that seem particularly interesting.

4.1. Population Analysis

The genetic data that are collected for molecular epidemiology are suitable, in fact ideal, for approaches that aim to identify discrete populations within a species. From the point of view of epidemiology, and when combined with data on the infected hosts, this can conceivably define groups within which contact is more likely. The proof of principle for this is a study of *H. pylori*

in which a close concordance was found between the strains colonizing human hosts and the ethnic origin of those human hosts *(26)*. The reason for this relation is that *H. pylori* is normally acquired from only very close contacts, often vertically, and hence can be used to recapitulate human population movements. Popular programs for this sort of analysis include STRUCTURE *(27)* and BAPS *(28,29)*. An excellent review of these and others, together with a discussion of what a population identified by such methods actually is, may be found in **ref. 30**.

4.2. Spatial Analysis

For many infections, one may identify certain strains or lineages of the pathogen that are endemic to a particular region. In some cases, this may be the result of clear phylogeographic structure, while in other cases it may be due to seeding of an epidemic from elsewhere. In both circumstances, it is helpful to be able to visualize the spatial location of the cases and the associated genotypes of the disease-causing organisms. This is becoming possible through several initiatives that link epidemiological data with mapping and other data (e.g., GENELAND; *31*). One possible limitation of such analyses is concern over linking disease data to a specific location (e.g., methicillin-resistant *S. aureus* within a particular hospital), which may limit their applications; compromises will have to be negotiated.

4.3. Inferring Evolutionary History from Trees

Trees can tell us more than how closely taxa are related. They can also be used to estimate the time at which two lineages diverged, given assumptions about the rate with which substitutions accumulate. Moreover, the distribution of mutations on the tree (which relate to the lengths of the branches in question) will vary depending on the selective and demographic history of the population. What can we learn from examining the shape of the genealogy?

As a first step, some statistical description of the shape of the genealogy is needed. This is offered by the coalescent. This approach, developed by Kingman *(32)*, describes the distribution of "coalescent events" going back in time from the present. Coalescent events are where two lineages coalesce, for instance, internal nodes in a genealogy. These represent the point at which two lineages shared a common ancestor. The way the rate of coalescence relates to variation in the population size is illustrated in **Fig. 3**. In the case of sequences from a population that experienced a severe bottleneck (**Fig. 3a**), the part of the genealogy that corresponds to this time will have a higher number of coalescent events because the chance of two lineages sharing a common ancestor becomes higher in the smaller population during the bottleneck. In the case of a rapidly expanding population, the tree will resemble **Fig. 3b**, with long terminal branches and short internal ones.

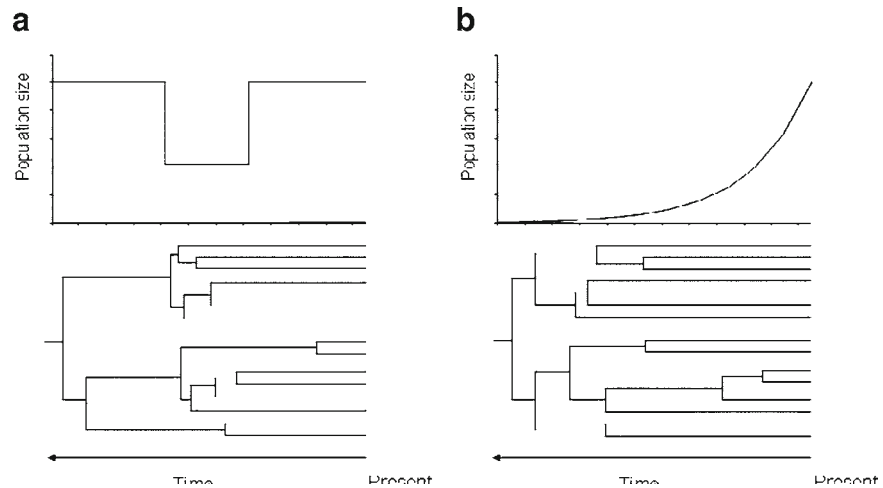

Fig. 3. Two examples of how changing population size can affect the shape of the genealogy of a set of sequences. Time is depicted on the *x* axis. (**a**) The consequences of a population bottleneck. The majority of the coalescent events take place in the part of the genealogy corresponding to the period of the bottleneck, shown here directly below it. (**b**) A population growing in exponential fashion. Again, the smaller population is associated with an increased rate of coalescence, leading to a genealogy in which the majority of terminal branches are quite long.

A fertile field in modern epidemiology is using these insights to explore the history of a set of sequences and to produce "sky-line plots" ***(33)***, which track the population size over the history of the tree (for some nice examples of this approach, *see* **refs. *34*** and ***35***). In a particularly exciting development, it has become possible to incorporate information about the time at which a sample was collected and to study what are sometimes called "measurably evolving populations," such as HIV-1 infecting an individual host ***(36)***. A comprehensive discussion of coalescent theory is far beyond the scope of this chapter, but interested readers are referred to one of the several books (e.g., ***37,38***) becoming available on this exciting topic.

4.4. Population Genomics and Single-Nucleotide Polymorphisms

It is becoming ever easier to sequence a genome, especially if at least one genome of the species in question has already been sequenced. While whole-genome comparisons, as a matter of routine for epidemiology, are still some way off, these studies do furnish us with a comprehensive catalogue of the sites at which the reference genomes differ. Single-nucleotide polymorphisms (SNPs) so identified may be targeted and used as the basis of a typing scheme. When considering SNP data one should, as ever, consider the evolutionary forces generating the assayed differences (is there evidence of homoplasy or selection, for instance? Are the assayed SNPs at synonymous sites?). A further, very important consideration in SNP analysis is phylogenetic discovery

bias. Under the approach outlined, our typing scheme will seek out only those SNPs that differ between the sequenced strains, that is, those that occurred on the evolutionary path separating the reference strains. Just as a microarray based on one or a few genomes cannot, by definition, be used to detect or study genes that are not present in those genomes, this approach cannot identify diversity that is present within branches leading to unsequenced strains. This problem is less acute for organisms in which recombination is common because here the genomic variation present in the rest of the species will have been sampled along the evolutionary path separating the reference strains. Of course, recombination leads to other problems, as discussed in **Subheading 2.3.** Discovery bias has been demonstrated in both real and theoretical situations ***(39, 40)*** and may be combated by a polymorphism discovery approach in which many genes are sampled in diverse isolates (for an example, *see* **ref.** *41*).

4.5. Work Flows and Web Services

Programmatic interfaces for many common bioinformatics procedures, such as BLAST and CLUSTAL, are located on Web servers across the globe (e.g., the National Center for Biotechnology Information [NCBI] has Web services for accessing database searches under the EFetch facility). This allows software developers to build tools that utilize these procedures without the need to program such functions themselves. Data are simply sent to the service provider and the results returned in an appropriate format. This method of reusable functionality is similar in concept to the approach utilized at the repository of modules for the freely distributed R statistics software found at CRAN (http://cran.r-project.org/). However, rather than downloading and using modules on a single computer, Web services allow the development of truly distributed software. A Web service can be described at its most basic as a set of three parts: (i) a definition of inputs (e.g., a FASTA file containing a set of sequences); (ii) the algorithms utilized on the inputs (e.g., a CLUSTAL alignment); and (iii) the outputs produced from step ii (e.g., an alignment file and tree definition file).

To allow less programmatically inclined users to access such facilities, programs have been developed that provide graphical interfaces to the wealth of Web services available. One of the more promising examples is Taverna ***(42)***, developed by the European Bioinformatics Institute (EBI). Taverna allows multiple Web services to be chained together into a work flow, using a graphical interface to define a series of steps that are undertaken on an initial input set of data. A work flow is built up as a flowchart with the inputs for each step defined and the outputs passed into the next step of the work flow. The resulting work flow is viewed as a flowchart, and the work flow "definition" can be saved as a text file, allowing reuse.

More usefully, the work flow can be uploaded to a Web site that allows others to take advantage, test, and amend the original user's experimental steps (see http://www.myexperiment.org). Many standard procedures involved in molecular epidemiological studies could be developed in such a fashion, with the advantage of user amendments, review, and testing. An example of such a work flow can be seen at http://www.myexperiment.org/workflows/124. This allows a single novel sequence to be entered along with a list of EMBL accession numbers for sequences on which a homology search is to be performed. Sequences are retrieved from GenBANK, translated into protein sequences, followed by an all-versus-all BLAST to identify homology. Subsequently, a CLUSTAL alignment is undertaken on a nonredundant set of BLAST matches and, finally, a neighbor-joining (NJ) tree or unweighted pair group method with arithmetic means (UPGMA) tree is produced along with the alignment files as final output. Should anyone wish to run this work flow, they can simply paste a URL into Taverna, and the work flow is imported. Access to the hundreds of Web services available means work flows are only limited by the users' questions. Combined with peer review of submitted work flows, this offers a very powerful complement to current software development methodologies.

5. Packages for Analysis of Molecular Epidemiological Data

A multitude of options exists when it comes to programs for data analysis. Some are summarized in **Table 1**, which presents a list that is by no means exhaustive; it focuses on those mentioned in this chapter. Most of the commonly utilized software packages have overlapping functionality, and very similar analyses can be undertaken using more than one package. As mentioned, interpretation, particularly when inferring relationships between strains and population-level analysis, is dependent on understanding the algorithms used. Some of the criticisms directed at published results are more likely due to inappropriate use of methodology rather than at the programs themselves.

One distinctive feature of many molecular epidemiology laboratories is high throughput of samples, with a requirement that experiments be conducted and results recorded in a standardized fashion. Some packages contain additional features to help in project management. These are generally the proprietorial packages, which may be quite expensive. However, many free alternatives are available for the majority of analyses. The advantages of the former, in addition to the inclusion of laboratory information management software (LIMS), is clear

Table 1
Programs Mentioned in the Text

Name	Web site	Functions	Cost	Comments
BioNumerics	http://www.applied-maths.com/bionumerics/bionumerics.htm	LIMS and numerous bioinformatics modules including MST and 1 and 2D gel analysis	$$$	Includes scripting language
CLCBio	http://www.clcbio.com/	LIMS-MLST module available for additional fee	$$$	
Geneious	http://www.clcbio.com/	LIMS	$$	Includes scripting language
Phineus	http://www.phineus.org/	LIMS and MLST analysis		In development
eBURST	http://eburst.mlst.net/	Patterns of recent descent		
CLONALFRAME	http://www2.warwick.ac.uk/fac/sci/statistics/staff/research/didelot/clonalframe/	Accounting for recombination in genealogies		
BAPS	http://web.abo.fi/fak/mnf//mate/jc/software/baps.html	Identifying populations and admixture between them using genetic data		
STRUCTURE	http://pritch.bsd.uchicago.edu/structure.html	Identifying populations and admixture between them using genetic data		
MEGA	http://www.megasoftware.net/	Phylogenetics		
PAUP*	http://paup.csit.fsu.edu/	Phylogenetics	$	
PAML	http://abacus.gene.ucl.ac.uk/software/paml.html	Phylogenetics		
MrBayes	http://mrbayes.csit.fsu.edu/	Phylogenetics		
PHYLIP	http://evolution.genetics.washington.edu/phylip.html	Phylogenetics		
BEAST	http://beast.bio.ed.ac.uk/			
SPLITSTREE	http://www.splitstree.org	Production of reticulate phylogenies that display conflict in the data		Includes Neighbor-net

Note: This is not an exhaustive list, and new options are continually becoming available.

documentation and user support, which in the case of free packages is inevitably limited by how much time the developer has available to devote to it.

In terms of proprietorial packages, the industry leader is BioNumerics. A tool for bioinformatics applications in general, BioNumerics has several modules specifically tailored to epidemiological investigations (including MLST and *spa* typing). SQL databases are used to store results from a remarkable variety of tests, including sequences, 1D and 2D gels, metabolic tests, microarray data, and more. Furthermore, BioNumerics includes a scripting language that allows users to manipulate core features of the software programmatically. While undoubtedly powerful, most laboratories will probably struggle to make use of many of its features. Alternative packages include CLCBio, which is again focused on general bioinformatics applications. However, plug-ins for epidemiological applications are becoming available (e.g., MLST).

Of stand-alone phylogenetics programs, the best known is probably MEGA *(43)*, with PAML *(44)*, MrBayes *(45,46)*, PAUP* *(47)*, and PHYLIP *(48)* also popular for certain specific purposes and within some user communities. The most recent version of MEGA *(43)*, still free to download, contains an integrated trace file editor and alignment tools. It uses a relatively intuitive interface with dialogue boxes and pull-down menus. In contrast, most other packages are operated via the command line, which can nevertheless be useful (e.g., if preparing batch analysis procedures, obviating the need for repeated clicking). A common feature of all these is that while they can take sequences or distance matrices as input, results from gel-based methods must be converted to a distance matrix beforehand. The BEAST *(49)* package (with related programs) is a well-supported, easy-to-use program for the sorts of analyses briefly discussed in **Subheading 4.3.** A recent and excellent review has surveyed the population genetics packages available together with their strengths and weaknesses *(50)*. While not all of these will be useful to researchers in this field, those who are interested in population genetic questions will find them invaluable.

In representing sequences where recombination is known or suspected, methods such as CLONALFRAME *(13)* or eBURST *(12)* should be used, but the drawbacks of each of these should be appreciated. CLONALFRAME, in detecting anomalous DNA, must assume that it arises from outside the sample, which may produce problems when both donor and recipient are present in the sample. eBURST, in contrast, tells you nothing about the relatively deep branches that separate the clonal complexes identified. Both programs perform poorly under

conditions of very high recombination. BioNumerics allows the construction of minimum-spanning trees (MSTs), which build on eBURST results to link clonal complexes via hypothetical unsampled intermediates. How secure this assumption is and whether it introduces an additional source of error into such analyses remain to be tested. Finally, programs such as Splitstree or Neighbor-net *(51,52)* find a reticulate phylogeny for the data and so represent recombinant genealogies better than a bifurcating tree. However, it should be noted that the signal that these programs detect may be produced by processes other than recombination, and that they are on their own not an acceptable test for recombination.

6. Concluding Remarks

The analysis of data is as important as their collection, and it is important that the correct tools are used and their underlying assumptions made plain. The discussion in this chapter focuses on clustering because this is the most important aspect in the majority of epidemiological questions. The degree to which microorganisms can be clustered and the appropriate tools to do it depend on the type of variation assayed, the rate of recombination, and so on. It is increasingly common for data collected as part of epidemiological investigations to be used for population genetic purposes, that is, to investigate hypotheses regarding the evolution of pathogens. In the opposite direction, insights derived from population genetics may be used to study pathogen spread.

Whichever technique is applied and however the data are analyzed, one should remember that a program of laboratory work should not be undertaken without a thorough understanding of the procedures that are to be used. This applies just as much to the tools that are used to interpret the data.

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Chapter 21

Internet-Based Sequence-Typing Databases for Bacterial Molecular Epidemiology

Keith A. Jolley

Abstract

As the use of nucleotide sequence-based typing has become more widespread in the investigation of microbial epidemiology, there has been a natural requirement for curated Internet-based databases that can act as central authorities for nomenclature and type definitions. These facilitate the sharing and comparison of data between laboratories without the need for reference samples. Here, the use of the most common multilocus sequence typing (MLST) and antigen sequence databases are described. In particular, for MLST, the steps required for allele sequence and profile identification are explained along with a detailed overview of searching and matching isolate records. BLAST searching of antigen sequence databases is also described.

Key words: Bacterial typing, databases, Internet, MLST, nucleotide sequencing.

1. Introduction

The genotyping of strains is a central requisite of global molecular epidemiology and is required for the detailed study of transmission dynamics. In an outbreak situation, strain identification of the etiological agent is useful for determining treatment regimens and prophylactic measures, but sometimes the overriding requirement is to determine whether two isolates are either identical or clonally related to each other. For this, many highly discriminatory comparative techniques with continuous values may be used, such as pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism, or random amplification of polymorphic DNA. Continuous values, such as the measurement of

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electrophoretic mobility of proteins or DNA in a gel, suffer from potential ambiguity resulting from the level of precision that the measurement can be made. Measurements can be affected by extraneous factors, so their use requires standard markers to calibrate the experiment if comparisons are to be made with existing data. This is problematic for long-term epidemiology and portability of data, particularly the use of online databases.

In contrast, epidemiological surveillance requires that the same biological markers are used routinely so that results are reproducible over time and between laboratories. Such library typing techniques preferably make use of markers with categorized values, for example, DNA sequences in multi- or single-gene sequence typing or integers representing the number of repeats in variable-number tandem repeat analysis. Databases that use continuous values of data do exist for molecular epidemiology; PulseNet *(1)*, utilizing PFGE data for enteric bacteria, is perhaps the best-known example, although its use is computationally intensive, and the uploading of large image files for processing is required to make comparisons.

Increasingly, the use of nucleotide sequence technology is becoming predominant in surveillance as it offers significant advantages over competing methodologies, namely, in reproducibility, portability of data, and resolution. Nucleotide sequences are highly amenable to electronic transmission and storage in databases, while a range of freely available software is available to facilitate its comparison. With near-ubiquitous access to the Internet, the use of online sequence databases for rapid identification and comparison of microorganisms has increased rapidly.

A distinction should be made between archival and actively curated databases. Archival databases, such as GenBank, often accept direct submissions without oversight of data quality and store data for publication and general identification purposes. These are invaluable for identifying genes and species but have little to offer for epidemiological purposes. Actively curated databases that have been set up specifically for strain typing, however, such as those for multilocus sequence typing (MLST) or for specific antigen genes, are essential for accurate microbial identification required for surveillance.

2. Methods

2.1. Multilocus Sequence Typing

MLST indexes the neutral variation in sequences of housekeeping gene fragments *(2,3*; *see also* **Chapter 11** for practical details). The use of multiple loci provides a robust method of typing organisms that undergo frequent recombination that

would otherwise invalidate phylogenetic methods using a single locus. As with any method that relies on nucleotide sequence data, results are unambiguous and portable, making them amenable to electronic storage. MLST databases are now available for at least 40 organisms, mainly bacteria, ensuring a uniform nomenclature. More than half of these are hosted at the University of Oxford in the United Kingdom (http://pubmlst.org) ***(4)***, with other schemes hosted at the United Kingdom's Imperial College (http://www.mlst.net) ***(5)***; the Environmental Research Institute, Cork, Ireland (http://mlst.ucc.ie); and the Pasteur Institute, Paris (http://www.pasteur.fr/mlst/).

There are three main types of query that MLST databases address: (i) allele sequence identification and comparison; (ii) allelic profile or sequence type (ST) identification and comparison; and (iii) matching of isolates. All MLST Web databases offer these functions, but the exact steps required and additional functions available vary. Here, the steps used on the PubMLST and Pasteur sites that use the mlstdbNet software ***(4)*** and the mlst.net site are specifically described.

2.1.1. Allele Sequence Identification

After trace files have been assembled, they are generally trimmed so that the sequence starts and finishes at the endpoints of the defined MLST locus under consideration. This can be done manually (*see* **Note 1**) or by using automated tools such as STARS (http://sara.molbiol.ox.ac.uk/userweb/mchan/stars/) or Phineus (http://www.phineus.org). Identification of the allele can then be determined as described next.

2.1.1.1. PubMLST Site

1. Select "Single locus query" within the profiles database.
2. Choose the appropriate locus, paste the sequence into the Web form, and click the "Submit Query" button.
3. If the sequence has been defined previously, the identity of the matching allele will be displayed along with a "Find similar" link to discover similar alleles and the nucleotides at which they vary. If the sequence has not been defined, the Web site will display the name of the allele to which it is most similar, along with a list of the nucleotides that vary so they can be confirmed (*see also* **Note 2**).

2.1.1.2. mlst.net Site

1. Select "Single Locus" from the "Locus Query" drop-down list box on the database front page.
2. Choose the appropriate locus, paste the sequence into the Web form, and click the "Submit" button.
3. If the sequence has been defined previously, the identity of the matching allele will be displayed. If the sequence is new, a message will inform you of the most similar allele along with its percentage identity.

4. To determine the nucleotide differences between the query sequence and the nearest match, click the "Sequence analysis" button. This opens a Java applet window with the query sequence aligned with known alleles. Visual inspection of this alignment will identify where the sequences vary (*see* **Note 3**).
5. Confirmed new sequences should be submitted to the database curator for inclusion.

2.1.2. Allelic Profile Identification and Comparison

Once alleles have been identified for each of the loci, the ST can be determined.

2.1.2.1. PubMLST Site

The batch profile function of the profiles database provides the easiest method for profile determination (even if you are only determining the ST for one profile).

1. Select "Batch profile query" within the profile database.
2. Copy and paste the sample identifier and allelic profile directly from a spreadsheet into the Web form. You can copy as many samples as you wish together; each sample should be on a separate row with columns separated by any amount of white space.
3. Click "Submit." A table displaying the sample identifier, allelic profile, ST, and clonal complex, if appropriate, will be returned (*see* **Note 4**).

2.1.2.2. mlst.net Site

1. Select "Allelic" from the "Profile Query" drop-down list box on the database front page.
2. Enter the allele numbers for each locus of your profile in the appropriate boxes of the form.
3. Ensure that the query type is set for "Exact or nearest match."
4. Click "Query list of distinct STs." A table will be displayed showing either an exact match, if available, or the nearest matching profiles otherwise.

2.1.3. Searching the Isolate Databases

The MLST isolate databases offers various search capabilities to find isolates that match any criteria of interest.

2.1.3.1. PubMLST Site

1. Click "Search database" from the isolate database front page.
2. The search form allows values to be selected from a number of fields that can be combined so that either all or any (and/or) are matched. The values can be specified to match exactly, match partially, to be greater or less than, to be not, or to not contain the selected value (*see* **Note 5**). Results can be ordered by any field, and the number of records per page can be set. To search for an empty field, the value "<blank>" can be specified. Click "Submit" once search criteria have been entered (*see* **Note 6**).
3. A page of results is returned. To navigate to the next set of results, click the ">" button on the page bar at the bottom of

the page. Further information about any particular isolate can be found by clicking its hyperlinked ID number.

4. The results can be broken down by individual fields by clicking the "Breakdown dataset" button. This generates charts for each field showing the frequency for each field value. More detailed analyses can be performed by clicking the "Advanced breakdown" button. This provides options to break the data set down against any two fields, so, for example, the frequency of strain type fields can be determined by country. The frequency of field combinations can also be shown where any selection of fields can be chosen. This can be particularly useful, for instance, in determining the surface antigen repertoire of a collection of isolates.

2.1.3.2. mlst.net Site

1. Select "Database query" from the "Profile Query" drop-down list box on the database front page.
2. The search form allows values to be selected from a number of fields that can be combined so that either all or any are matched. The values can be specified to match exactly (not case sensitive), to be greater or less than, or to be not. Click "Submit" (*see* **Note 7**).
3. The query results will be displayed in a table (*see* **Note 8**). More detailed information about each isolate can be found by clicking the hyperlinked ID number.

2.2. Antigen Sequence Typing

Sequencing of antigen genes is being used increasingly in place of serological characterization of isolates in bacterial typing schemes. Nucleotide sequencing has the advantage that every variant can be identified, whereas many isolates can be nontypable using monoclonal antibody panels. Curated Web databases for specific antigens, such as the *Neisseria meningitidis* serotyping (PorB) ***(6)***, serosubtyping (PorA) ***(7)*** and FetA ***(8)*** proteins, *Campylobacter jejuni* FlaA ***(9)*** and MOMP, *Streptococcus zooepidemicus* seM ***(10)***, and *Wolbachia* Wsp ***(11)*** proteins are available. These databases make use of the agdbNet software ***(12)***, which offers BLAST ***(13)*** querying of nucleotide or peptide variants and linking to isolate data.

The sequence query page of these databases allows either a nucleotide or a peptide sequence to be entered and compared to all known alleles or variants using the BLAST algorithm. This works whether the variants are defined by their nucleotide or peptide sequence. In some databases, multiple loci may be defined, and these can all be queried at the same time, or the user can specifically select the locus to search against. Depending on the database, isolates with a matching antigen variant can also be retrieved.

1. Select "Single sequence query" (or in some databases "Identify variable region").

2. Select the locus of interest if the database contains multiple loci. Alternatively, a value of "all" will search all loci together.
3. Copy and paste either a nucleotide or a peptide sequence into the Web form and click "Submit Query."
4. If identical matches are found, these will be listed (*see* **Note 9**) along with a link to the BLAST results output. If no exact matches are found, a list of partial matches will be displayed along with their percentage identity, the number of mismatches, the number of gaps, and the length of the alignment.
5. Results are hyperlinked, so clicking these will navigate to further information about the particular sequence or variant. This may include GenBank accession numbers, publications in which the sequence is described, or links to matching isolates.

3. Notes

1. The "locus explorer—polymorphic site analysis" function of a PubMLST profiles database shows a schematic of a particular MLST locus, clearly identifying start and end points and showing all known mutations within the gene fragment, colored by their relative abundance within defined alleles. This information can be very useful when trimming allele sequences manually.
2. On the PubMLST site, MLST sequences can also be queried against all known alleles using BLAST, available within the profiles database. This has the advantage that sequence trimming is not required, and it is not necessary to specify the locus. This is important if the start point cannot be identified and confirmation is needed that the sequence obtained from the sequencer is the correct locus and not something else due to a mix-up of samples. The disadvantage is that one does not get a simple list of nucleotide differences to the nearest known allele.
3. In three of the databases on www.mlst.net (*Enterococcus faecalis*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*), the single-locus query offers choices of "Simple results" or "View DNA mismatches." The first simply states the nearest allele and its percentage identity in the case of an unknown allele sequence. The latter displays a list of the defined alleles along with the positions of nucleotides that vary. If available, the latter display makes confirming nucleotide differences easier than using the "Sequence analysis" applet.
4. Using the batch profile function of a PubMLST database to identify STs from profiles has a major advantage over the standard "allelic profile query" in that allele numbers do not need to be

manually transcribed into the Web form. The standard allelic profile query does, however, allow partial matching of profiles to be performed to identify related STs.

5. In a PubMLST isolate database, if combinations of four fields are not sufficient, the interface can be customized by going to the options page and changing the number of fields to include (up to 20 fields can be used). If a field is of particular interest, the interface can be customized further to include a drop-down box of that field's values to be selected. These options are remembered between sessions, so the interface can be customized to a user's preferences.
6. Isolate data sets can also be retrieved from a PubMLST database by using a list query. Here a long list of attributes, usually ID numbers or isolate names, can be pasted into a Web form. This makes it particularly easy to retrieve specific records, especially if a list has already been prepared.
7. In a database on mlst.net there is no documented way to search for an empty field.
8. On larger databases on mlst.net, exercise restraint in making queries that are likely to return a large proportion of the database. The system does not offer a way to break the results into multiple pages, and you may find your Web browser locks up when attempting to display hundreds or thousands of rows of data.
9. BLAST searches of antigen databases can sometimes return multiple exact matches. This occurs if variants have been defined that are identical to preexisting sequences with the exception of missing end motifs, sometimes seen in variants defined by a variable-loop sequence. The software uses the BLAST algorithm to simply identify the presence of an exact sequence and does not have a definition of the start or end points of the variant or allele. In cases such as these, the longest matched sequence is usually the correct one, but the user should check by trimming the sequence back to the defined end points of the allele and then query again. The correct variant will be an exact match and the same length as the query sequence.

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