Are genome-wide association studies of infection any value?

Host genetic effects on susceptibility to infectious diseases has long been recognised. Genes encoding sickle-cell traits or β-thalassaemia protect against malaria, and polymorphism of the T-cell receptor CCR5 affects susceptibility to plague, smallpox, and HIV. Knowledge of genetic associations has helped to inform understanding of disease pathogenesis and inspired new approaches to treatment. The past year or so has seen an increased focus on the finer aspects of genetic influences on disease susceptibility with a growing number of genome-wide association studies. But will this approach have any clinical implications for infectious disease?

Until the sequencing of the human genome in 2003, studies of disease susceptibility focused on genes or regions of DNA identified by linkage studies. Such genes would by default always be those with a substantial easily detected association with a disease (eg, the sickle cell trait and malaria). In genome-wide association studies, researchers compare the genomes of patients with particular diseases with those of healthy controls to identify small genetic changes, single-nucleotide polymorphisms (SNPs), that differ in prevalence between the two groups. The studies allow researchers to detect more subtle influences of differences in genetics than determined major susceptibility loci. The approach has been applied widely in the investigation of non-communicable diseases including diabetes, cardiovascular disease, and rheumatoid arthritis, but infectious diseases have received relatively little attention from people doing such studies.

Two recent genome-wide association studies, published in early August, investigated susceptibility to infectious disease. The first identified polymorphisms in regions encoding complement proteins that influence susceptibility to invasive disease caused by *Neisseria meningitidis* in European populations, and the second identified a locus on chromosome 18 at which a particular variant, rs4331426, was associated with increased susceptibility to tuberculosis in Africans but not in other populations. These two studies follow on the heals of other studies investigating susceptibility to Kawasaki disease, malaria, and leprosy published in the past year.

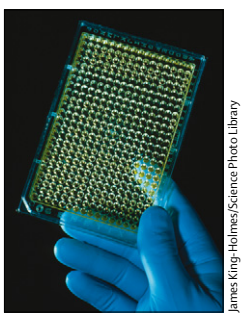
Infectious diseases actually offer some advantages over other disease for research with genome-wide association studies. For non-communicable diseases, confirmation of whether a person has a disease, will never develop a disease, or will later develop disease can be difficult, but confirmation of active infectious disease (symptoms in the presence of an infectious organism), non-invasive colonisation (presence of organism without symptoms), or past infection (by serology) allows researchers an additional degree of confidence in their findings.

Nonetheless, genetic variants that determine susceptibility in one human population might not be present in another, as shown in the recent study of tuberculosis. For infectious diseases the picture is further complicated by the presence of a second genome, that of the microorganism. Due to the constant evolution of pathogens in response to the population they effect, a variant that confers resistance to people in one region might not have any effect elsewhere, because of difference in the prevalent strains of a microorganism.

Finally, as with all uses of genetic data, there are important ethical caveats. If human genetic sequence data gathered during such research are to be made open access as outlined in the Fort Lauderdale agreement, can the techniques be adequately explained to the populations being investigated to obtain consent for such dissemination of data?

Genome-wide association study is a powerful method for generating data. But turning lists of DNA variants into clinically useful information may not be so straightforward. The identification of an association between variations in a complement factor with susceptibility to *N meningitidis* might easily lead to hypotheses about bacterial invasion, but identification of an association between rs4331426 in a gene-poor region only generates more questions leading to more research, and only a long way down the line might a clinical impact be realised; for non-communicable diseases, clinical results of these studies have been slow in emerging.

Given the fierce competition for research funds for infectious diseases and the disparity in access to treatment the world's rich and poor, investigators must clearly make the case for the value of genetic association studies relative to research with more immediate clinical impacts. ■ *The Lancet Infectious Diseases*



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For the **genome-wide association study of susceptibility to *Neisseria meningitidis*** see *Nat Genet* 2010; DOI:10.1038/ng.640

For the **genome-wide association study of susceptibility to tuberculosis** see *Nat Genet* 2010; DOI:10.1038/ng.639

For the **Fort Lauderdale Agreement** see http://www.genome.gov/Pages/Research/WellcomeReport0303.pdf

The latest threat in the war on antimicrobial resistance

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Enterobacteriaceae, most notably *Escherichia coli* and *Klebsiella pneumoniae*, are among the most important causes of serious nosocomial and community-associated bacterial infections in people, and resistance of these bacteria to antimicrobial drugs is a serious concern.[1] Of particular concern is development of resistance to the carbapenems (ie, imipenem, meropenem, ertapenem, and doripenem), because these drugs are often the last line of effective treatment available for infections with multiresistant Enterobacteriaceae.[2] Resistance is due to carbapenemases, of which there are three types: *K pneumoniae* carbapenemases, metallo-β-lactamases, and oxacillinases.[2]

The production of metallo-β-lactamases has mostly been associated with *Pseudomonas aeruginosa* and *Acinetobacter* spp and is rare in Enterobacteriaceae, except in isolates from Mediterranean Europe.[3] Metallo-β-lactamases hydrolyse many β-lactams, such as penicillins, cephalosporins, and carbapenems, but not the monobactams (ie, aztreonam). The two most common types of metallo-β-lactamases are Verona integron-encoded metallo-β-lactamase and IMP.[3]

New Delhi metallo-β-lactamase (NDM) 1 was identified in *K pneumoniae* and *E coli* recovered from a Swedish patient who was admitted to hospital in New Delhi, India.[4] In *The Lancet Infectious Diseases* today Kumarasamy and colleagues[5] provide evidence that NDM-producing Enterobacteriaceae (mostly *K pneumoniae* and *E coli*) are widespread in India and Pakistan. They also identify patients in the UK infected with NDM-producing bacteria who had recently travelled to India for several types of medical procedures. The patients presented with various nosocomial and community-associated infections, mostly urinary tract infections. The NDM-producing bacteria were multiresistant to many groups of antibiotics, including fluoroquinolones, aminoglycosides, and β-lactams (especially carbapenems), but were sensitive to colistin and tigecycline. The NDM gene was present on plasmids, which were easily transferred between bacteria.

NDM-producing Enterobacteriaceae have recently been isolated from patients in the USA,[6] the Netherlands,[7] Australia,[5] and Canada (unpublished) in patients who had received medical care in India. Many people choose to undergo surgical procedures in countries such as India, China, and South Korea to avoid long waiting times and to save money.[8] This so-called medical tourism to India might grow by 30% each year over the next 5 years.[9] Medical tourism has some risks, including ethical and legal issues as well as the quality of postoperative care.

The worldwide spread of multi-resistant NDM-producing Enterobacteriaceae will have serious implications for the empirical treatment of hospital-associated and community-associated infections. To make matters worse, there are very few antibiotics in development with activity against Gram-negative bacteria.[10] Of particular concern is that NDM enzymes are present in *E coli*; the most common cause of community-associated urinary tract infections.

The spread of these multiresistant bacteria merits very close monitoring and worldwide, internationally funded, multicentre surveillance studies, especially in countries that actively promote medical tourism. Patients who have had medical procedures in India should be actively screened for multiresistant bacteria before they receive medical care in their home country. If this emerging public health threat is ignored, sooner or later the medical community could be confronted with carbapenem-resistant Enterobacteriaceae that cause common infections, resulting in treatment failures with substantial increases in health-care costs. The consequences will be serious if family doctors have to treat infections caused by these multiresistant bacteria on a daily basis.

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I declare that I have no conflicts of interest.

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ACTs for schistosomiasis: do they act?

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There is evidence that crude extracts of the medicinal herbs *Artemisia annua* and *Artemisia apiacea* were used in China more than 2000 years ago.[1] Indeed, herbalists prescribed tea that included dried leaves of the *Artemisia* plants to treat fever and other ailments.[1] However, the active component of *A annua* was only identified more recently and named Qinghaosu, now known as artemisinin. This breakthrough discovery made in the early 1970s stemmed from screening more than 2000 Chinese herb preparations by the phytochemist You-You Tu.[2]

The true importance of these discoveries was only recognised in the mid-1980s, facilitated by a review in the journal *Science*[3] that included confirmatory data mostly generated by the Walter Reed Army Institute of Research in the USA. Key features of the artemisinins are their rapid onset of action, high parasite reduction ratio, broad parasite stage specificity, and effectiveness.[4] Hence the conclusion that the artemisinins are the most powerful antimalarials discovered so far.[5] Nonetheless, the artemisinins have liabilities, such as suboptimum biopharmaceutical properties due to their short half-lives. Frequent treatments are thus needed, which compromise compliance and bear the risk of resistance development.[4] In view of these concerns, and to avert a malaria disaster, leading malariologists emphasised, more than a decade ago that artemisinin-based combination therapies (ACTs) are the way forward.[6]

Artemisinins are also active against blood flukes, the cause of schistosomiasis. This discovery dates back 30 years and was also not recognised by conventional medicine for many years.[7,8] In-depth laboratory investigations showed that juvenile stages of the parasite (ie, schistosomula) were most severely affected by the artemisinins, by contrast with praziquantel, which mainly kills adult schistosomes. A series of clinical trials in China showed that repeated oral artemether or artesunate prevented patent *Schistosoma japonicum* infections.[7,8] At the turn of the new millennium, the activity of the artemisinins was

	Number of patients	Age	Infection intensity	ACT	Cure rate	Egg reduction rate
S haematobium						
Niakhar site, Senegal, 2007[12]	12	15–74 months	0·233 eggs per 10 mL of urine	Artesunate (4 mg/kg for 3 days) plus sulfadoxine (25 mg/kg) and pyrimethamine (1·25 mg/kg)	100%	100%
Niakhar site, Senegal, 2007[12]	15	15–74 months	0·403 eggs per 10 mL of urine	Artesunate (4 mg/kg for 3 days) plus amodiaquine (10 mg/kg for 2 days then 5 mg/kg on the third day)	87%	92%
Djalakorodji, Mali, 2009[13]	392	6–15 years	42·2 eggs per 10 mL of urine	Artesunate (100 mg) plus sulfalene (250 mg) and pyrimethamine (12·5 mg) daily for 3 days	44%	93%
Guéssiguié, Côte d'Ivoire, 2010[14]	18	7–13 years	42 eggs per 10 mL of urine	Artesunate (100 mg) plus mefloquine (250 mg) once daily for 3 days	61%	96%
S mansoni						
New half, Sudan, 2008[11]	6	6–40 years	45 eggs per g of stool for all three treatment groups	Three doses of artesunate (200 mg) one every 12 h plus sulfadoxine (500 mg) and pyrimethamine (25 mg/kg) every 12 h	100%	100%
New half, Sudan, 2008[11]	5	6–40 years	45 eggs per g of stool for all three treatment groups	Three doses of artesunate (200 mg) one every 24 h for 3 days plus sulfadoxine (500 mg) and pyrimethamine (25 mg/kg) for 3 days	100%	100%
New half, Sudan, 2008[11]	3	6–40 years	45 eggs per g of stool for all three treatment groups	Artemether (80 mg twice daily for 3 days) plus lumefantrine (480 mg twice daily for 3 days)	100%	100%
New half, Sudan, 2009[15]	46	8–17 years	100 eggs per g of stool	Artesunate (4 mg/kg for 3 days) plus sulfadoxine (25 mg/kg) and pyrimethamine (1·25 mg/kg) on day 0	59%	79%
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Table: **Results obtained thus far with different artemisinin-based combination therapies (ACTs) against *Schistosoma mansoni* and *Schistosoma haematobium***

6 CDC. Detection of Enterobacteriaceae isolates carrying metallo-beta-lactamase—United States, 2010. *MMWR Morb Mortal Wkly Rep* 2010; **59:** 750.
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Table: **Results obtained thus far with different artemisinin-based combination therapies (ACTs) against *Schistosoma mansoni* and *Schistosoma haematobium***

confirmed against *Schistosoma mansoni* and *Schistosoma haematobium* in the first randomised, placebo-controlled trials in Africa.[9,10] More recently, two exploratory trials[11,12] showed that patients co-infected with *Plasmodium falciparum* and either *S haematobium* or *S mansoni* were cleared of *P falciparum* after being given ACT, and high cure and egg reduction rates were seen against schistosome infections (table). The first large-scale trial with an ACT (artesunate plus sulfalene and pyrimethamine)[13] specifically designed to treat schistosomiasis reported a moderate cure rate and a high egg reduction rate against *S haematobium* in school-aged children in Mali. A single dose of praziquantel—the current drug of choice against schistosomiasis—showed a significantly higher cure rate.[13] The trial by Obonyo and colleagues,[16] published today in *The Lancet Infectious Diseases*, in Kenyan schoolchildren infected with *S mansoni*, compared the same ACT with praziquantel as in the aforementioned Mali trial. The recorded cure rate and egg reduction rate are disappointing. Two issues warrant special mention: the ACT used is neither the first-line nor second-line ACT in Kenya and has not yet met WHO prequalification standards; and underlying assumptions for sample size calculation can be challenged, particularly the expected cure rate of 95% in the ACT group.

Taken together, the evidence suggests that the efficacy of ACTs against the two major schistosome species is only moderate. Unfortunately, larger clinical trials, including school-aged children who are the target group for schistosomiasis control interventions, could not confirm the promising results obtained in small exploratory trials. Despite the inferiority of the ACTs to praziquantel, we believe that the ACTs can have some role against schistosomiasis. First and foremost, the ACTs must be reserved for the prevention and control of malaria, and efforts are underway for universal coverage in malaria-endemic settings (ie, access to quality ACTs within 24 h of a clinical malaria episode), facilitated by national malaria control programmes with support from the Global Fund to Fight AIDS, Tuberculosis and Malaria. Second, in regions where both malaria and schistosomiasis coexist (eg, over large parts of sub-Saharan Africa) and where the efficacy and safety of new ACTs are being investigated or are already used at large scale against malaria, investigators should specifically look for potential ancillary benefits against schistosomiasis, as shown when the first respective findings were published and we saw the beginning of large-scale use of ACTs in Africa.[7,17] Third, the health and demographic surveillance systems of the INDEPTH network, particularly its integrated phase 4 trial platform, offers a unique platform to monitor malaria incidence and changing patterns of schistosomiasis as a function of first-line and second-line ACTs and attained coverage in specific settings. Finally, in a recent randomised, exploratory open-label trial,[14] an artesunate–mefloquine combination resulted in high cure and egg reduction rates against *S haematobium*. Importantly, mefloquine, unlike the other drugs accompanying the artemisinin compound, has shown activity against *S manoni* in vivo[18] and targets both schistosomula and adult worms; hence additional research is warranted with this ACT.

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Research into tuberculosis diagnosis in children

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Tuberculosis is an important cause of morbidity and mortality in children worldwide, but estimates of disease burden are inaccurate because most cases are not confirmed.[1] The most common form of childhood tuberculosis is pulmonary disease, and in tuberculosis-endemic communities, most cases present in young children. Collection of respiratory specimens for laboratory diagnosis is difficult in this age group. Most cases are paucibacillary, and therefore mycobacterial culture of specimens is required to optimise diagnostic yield. Studies of tuberculosis diagnosis in children are hampered by the lack of a gold standard for tuberculosis disease and infection, with the limitations of mycobacterial culture and tuberculin skin test well recognised. During the past decade, there has been a surge in development of new diagnostics aiming to provide more accurate and rapid diagnosis. Despite the challenges, new diagnostic techniques need to be studied in children.[2]

In a study by Richard Oberhelman and colleagues[3] in *The Lancet Infectious Diseases* today, multiple specimens were obtained from Peruvian children (median age 3 years) with clinically diagnosed pulmonary tuberculosis for comparison of diagnostic methods. This research group has previous experience with new diagnostic techniques, including nasopharyngeal aspiration for sputum collection and the use of broth culture by microscopic-observation drug-susceptibility (MODS).[4,5] MODS provided a significantly higher and faster yield than did Lowenstein-Jensen culture for confirmation of pulmonary tuberculosis diagnosis. Nasopharyngeal aspiration after cough induction provided a lower yield of culture-positive specimens than did gastric aspiration. The induced sputum technique might have improved the yield because it also includes the use of chest physiotherapy and hypertonic nebulised saline before cough induction. This method was not used in this study but has shown promise compared with gastric aspiration in South African children.[6,7] The lowest diagnostic yield was from stool specimens, including with the use of PCR.

Duplicate PCR was done on all specimens and the results show that the role of PCR in diagnosis of tuberculosis in children is unknown. The potential advantage of PCR over culture would be more rapid diagnosis with possibly greater sensitivity. In this study, the proportion of samples that were positive by PCR was higher than that for culture in all specimens, especially in those from children with a lower clinical score. This finding might suggest improved sensitivity; however, PCR was negative in 38% of culture-positive children with strong clinical evidence of pulmonary tuberculosis. Furthermore, PCR was positive in a small proportion of controls, raising doubts about specificity of PCR for disease compared with infection.

The low overall yield of culture-confirmed cases in this study of a large number of children, many with strong clinical evidence of pulmonary tuberculosis, emphasises the ongoing diagnostic challenges for research. Patients with a higher diagnostic score were more likely to have tuberculosis confirmed on culture, but this finding does not necessarily mean that those with a higher score were more likely to have tuberculosis. These patients might have pulmonary tuberculosis disease at time of presentation that is more likely to be culture positive than infected patients with a lower score. Without a gold standard, the yield from culture is usually reported in relation to clinical diagnosis, which is affected by selection of patients and definitions used for clinical categorisation.

There are many clinical definitions or scoring systems used for diagnosis of tuberculosis in children, including for diagnostic research purposes that have not been validated and are not comparable.[8,9] Although there is inherent consistency within studies, comparison of a particular diagnostic technique is impossible between studies in different settings that use different clinical definitions for comparison. A distinction needs to be made between a clinical definition for reporting of research findings and an

15 Mohamed AA, Mahgoub HM, Magzoub M, et al. Artesunate plus sulfadoxine/pyrimethamine versus praziquantel in the treatment of *Schistosoma mansoni* in eastern Sudan. *Trans R Soc Trop Med Hyg* 2009; **103:** 1062–64.

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Research into tuberculosis diagnosis in children

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See Articles page 612

Tuberculosis is an important cause of morbidity and mortality in children worldwide, but estimates of disease burden are inaccurate because most cases are not confirmed.[1] The most common form of childhood tuberculosis is pulmonary disease, and in tuberculosis-endemic communities, most cases present in young children. Collection of respiratory specimens for laboratory diagnosis is difficult in this age group. Most cases are paucibacillary, and therefore mycobacterial culture of specimens is required to optimise diagnostic yield. Studies of tuberculosis diagnosis in children are hampered by the lack of a gold standard for tuberculosis disease and infection, with the limitations of mycobacterial culture and tuberculin skin test well recognised. During the past decade, there has been a surge in development of new diagnostics aiming to provide more accurate and rapid diagnosis. Despite the challenges, new diagnostic techniques need to be studied in children.[2]

In a study by Richard Oberhelman and colleagues[3] in *The Lancet Infectious Diseases* today, multiple specimens were obtained from Peruvian children (median age 3 years) with clinically diagnosed pulmonary tuberculosis for comparison of diagnostic methods. This research group has previous experience with new diagnostic techniques, including nasopharyngeal aspiration for sputum collection and the use of broth culture by microscopic-observation drug-susceptibility (MODS).[4,5] MODS provided a significantly higher and faster yield than did Lowenstein-Jensen culture for confirmation of pulmonary tuberculosis diagnosis. Nasopharyngeal aspiration after cough induction provided a lower yield of culture-positive specimens than did gastric aspiration. The induced sputum technique might have improved the yield because it also includes the use of chest physiotherapy and hypertonic nebulised saline before cough induction. This method was not used in this study but has shown promise compared with gastric aspiration in South African children.[6,7] The lowest diagnostic yield was from stool specimens, including with the use of PCR.

Duplicate PCR was done on all specimens and the results show that the role of PCR in diagnosis of tuberculosis in children is unknown. The potential advantage of PCR over culture would be more rapid diagnosis with possibly greater sensitivity. In this study, the proportion of samples that were positive by PCR was higher than that for culture in all specimens, especially in those from children with a lower clinical score. This finding might suggest improved sensitivity; however, PCR was negative in 38% of culture-positive children with strong clinical evidence of pulmonary tuberculosis. Furthermore, PCR was positive in a small proportion of controls, raising doubts about specificity of PCR for disease compared with infection.

The low overall yield of culture-confirmed cases in this study of a large number of children, many with strong clinical evidence of pulmonary tuberculosis, emphasises the ongoing diagnostic challenges for research. Patients with a higher diagnostic score were more likely to have tuberculosis confirmed on culture, but this finding does not necessarily mean that those with a higher score were more likely to have tuberculosis. These patients might have pulmonary tuberculosis disease at time of presentation that is more likely to be culture positive than infected patients with a lower score. Without a gold standard, the yield from culture is usually reported in relation to clinical diagnosis, which is affected by selection of patients and definitions used for clinical categorisation.

There are many clinical definitions or scoring systems used for diagnosis of tuberculosis in children, including for diagnostic research purposes that have not been validated and are not comparable.[8,9] Although there is inherent consistency within studies, comparison of a particular diagnostic technique is impossible between studies in different settings that use different clinical definitions for comparison. A distinction needs to be made between a clinical definition for reporting of research findings and an

approach to clinical diagnosis that is more individualised, flexible, and usually needs to be made before culture results are available. The recognised difficulties with confirmation of diagnosis have contributed to a common, and perhaps erroneous, perception that the diagnosis of childhood tuberculosis is always difficult. Clinical diagnosis is fairly straightforward in many patients and this unnecessarily negative perception can be a barrier for improving clinical management, supporting child tuberculosis research, and reporting of disease burden.

A response to the global emergence of drug-resistant tuberculosis is the increasing availability of culture facilities in high tuberculosis-endemic settings. This development provides an important opportunity for child tuberculosis research. In the absence of a gold standard, other analytical approaches also need to be considered and investigated.[10,11] A more consistent approach to diagnostic methods would be very helpful for the purposes of reporting and more meaningful comparison across diagnostic research studies.

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2 Zar HJ, Connell TG, Nicol M. Diagnosis of pulmonary tuberculosis in children: new advances. *Expert Rev Anti Infect Ther* 2010; **8:** 277–88.
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Biosafety and tuberculosis laboratories in Africa

As a major concern of laboratory biosafety, *Mycobacterium tuberculosis* has been classified as a category 3 pathogen, meaning that culture-based work can only be done in a biosafety level 3 (BSL3) laboratory (smear microscopy can be done in BSL2 laboratories because the risk of significant aerosolisation is low).[1,2] However, most tuberculosis burden is in low-income countries, such as those in sub-Saharan Africa, that do not have the financial resources to build or maintain expensive and complex BSL3 laboratories (table).[1–4] Some people have even suggested that extensively drug-resistant (XDR) strains of tuberculosis should be classified as category 4 organisms;[4] however, because a strain cannot be identified as an XDR before culture and sensitivity tests, all culture-based work with *M tuberculosis* would have to be done in a BSL4 laboratory.

BSL3 laboratories differ from BSL2 laboratories in three areas: safe laboratory practices, safety equipment, and the facilities themselves.[1–4] Although the first two categories are intended to protect laboratory workers inside the laboratory, the facility itself includes several containment measures (table). Most of the required laboratory measures are inexpensive and easily built and maintained. The most expensive measure is the unidirectional ventilation, which requires negative pressure inside the laboratory. In western European and North American BSL3 laboratories, this ventilation is achieved by installing a heating, ventilation, and air-conditioning control (HVAC) system,[2,3] which creates negative air pressure that is independent from the biological safety cabinet. The HVAC system usually raises the cost of a BSL3 into the region of €150 000–€250 000, a price that does not include complex maintenance and

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repair, which require high levels of infrastructure and expertise that might be difficult to obtain in remote and resource-poor settings. Many people equate a BSL3 laboratory with the high-level BSL3 laboratories in affluent countries; however, biosafety guidelines do not require such complex and advanced infrastructure.[2,3] Some confusion remains regarding what elements are really necessary to comply with BSL3 category requirements.

Some studies have reported the successful conversion of existing infrastructures into diagnostic tuberculosis laboratories,[5] but others have called for more investment, strategic planning, and strengthened collaborations.[6,7] Little practical information or guidance is available to explain exactly what the infrastructure of BSL3 laboratories should look like. The European Union (EU) Directive on biosafety[1] does not include the same recommendations as do WHO or Centers for Disease Control and Prevention guidelines.[2,3] This directive states that ventilation-related measures, such as negative pressure and HVAC, are only recommended, not compulsory. In many affluent EU countries, however, most BLS3 laboratory facilities exceed the EU directive recommendations. But why were those recommendations not made compulsory for all EU states?

We find it peculiar that the building of tuberculosis laboratories in Africa often appears to require higher standards than recommended by the EU; this clearly illustrates some of the confusion that surrounds infrastructure and the measures needed to obtain BSL3 category status. Are there any alternative BSL3 solutions? Alternatives to the expensive HVAC system that retain the other features of a BSL3 laboratory have been suggested, with the biological safety cabinet to create the HVAC unidirectional airflow system. The cabinet can be ducted to the outside, ideally using a thimble and secondary ventilator in the exhaust ducting, as part of the solution. Such an infrastructure could possibly be built for less than €20 000–30 000 and importantly is much simpler to run and maintain—the expertise required for repair is substantially less than would be required in a HVAC-equipped laboratory. The Stop TB Partnership have endorsed this solution in their summary report guidance on biosafety related to tuberculosis laboratory diagnostic procedures;[8] however, this theoretical guidance should be supplemented with practical suggestions. Basic guidelines are needed on the size of the biological safety cabinet, adequate thimble duct extraction, solutions for air entry (including basic calculations of air exchanges), ventilator power, types and size of air entry and exhaust filters, and eventually, cost-related guidance. Although this information would not be a blueprint for construction, it could be very valuable when discussing and negotiating an alternative BSL3 construction.

	EU Directive 2000/54/EC[1]	WHO biosafety manual[3]	Centers for Disease Control biosafety manual[2]
Workplace should be separate from other activities in the same building	Recommended	Yes	Yes
Double door entry	Not mentioned	Yes	Yes
Anteroom	Not mentioned	Yes	Yes
Autoclave on site	Not mentioned*	Yes	Yes
Sealable for decontamination	Recommended	Yes	Yes
Ventilation (inward ventilation or negative pressure)	Recommended	Yes	Yes

* The European Union (EU) Directive recommends that a BLS3 laboratory should contain its own equipment.

Table: **Example of infrastructure requirements for a BSL3 laboratory**

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We have no conflicts of interest.

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It'll be fine with a bit of ice and lemon

The presumptive coliform count, the classic foundation of water testing, has long been supplanted by more sophisticated membrane filtration and most-probable-number tests. Yet these modern versions of the traditional technique are still based on the idea of distinguishing between water containing coliforms, which should not be consumed, and water lacking them, which is probably safe. Although *Escherichia coli* dies out in a watery environment outside the body, it does not die more quickly than bacterial pathogens.

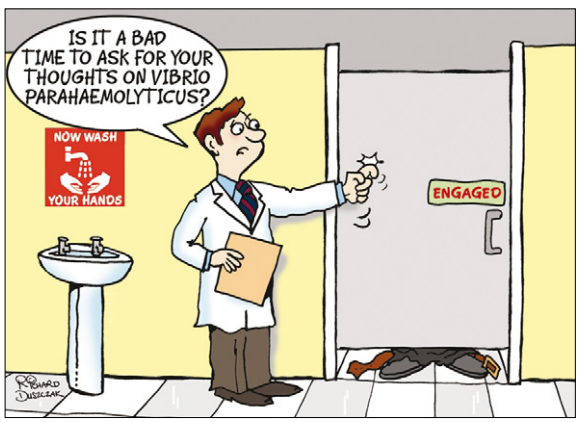
What many textbooks still fail to emphasise, however, is that although the persistence of so-called indicator bacteria in water systems is comparable to that of disease-causing bacteria, it correlates rather poorly with that of potential viral pathogens. Yet enteric viruses are responsible for a substantial proportion of the 50 000 deaths caused each day throughout the world by water-borne diseases.

Martella Du Preez of the Council for Scientific and Industrial Research in Pretoria, South Africa, and Lawrence Goodridge of Colorado State University, USA, have also been concerned that indicator bacteria, whose presence is taken to signal faecal pollution, can occur in unpolluted soil and watery environments as well. So these investigators have turned to F-specific RNA (FRNA) bacteriophages, whose survival characteristics and morphology closely resemble those of enteric viruses affecting people. Although phages are the most ubiquitous microorganisms on the planet, this specific group might be harnessed in the interests of routine water assessment.

Previous investigators have shown that the detection in water of genotypes belonging to groups 1 and 4 suggests contamination by animal faeces, whereas genotypes of groups 2 and 3 indicate human faecal contamination. Hoping to develop an integrated concentration and detection method on the basis of this distinction, Du Preez and Goodridge did a study earlier this year based on rivers in Pretoria and Phola Township (adjacent to a sewage works). The results, presented to a recent Society for Applied Microbiology (SfAM) meeting in Brighton, UK, suggest that the test they have developed is both sensitive and rapid. It could prove especially useful for microbiologists investigating water supplies in the aftermath of adverse weather events such as monsoons and hurricanes.

The duo used anionic exchange resin beads to concentrate FRNA phages in river water samples and detected bound virions by isolating viral RNA. They then did reverse transcriptase real-time PCR (RT-RT-PCR) with primers designed to pinpoint the four phage genogroups.

"With an enrichment step, the assay detected FRNA phages belonging to genogroups 2 and 3 from the Phola



Township samples, clearly indicating the presence of human faecal pollution", Du Preez and Goodridge reported. "When we performed RT-RT-PCR directly on the resin beads, without enrichment, FRNA phages belonging to genogroups 2, 3, and 4 (Phola Township) and 2 and 4 (Pretoria) indicated the presence of animal faecal pollution in addition to human pollution. The entire concentration and detection assay (without enrichment) was completed within eight hours."

SfAM's Brighton audience also learned of new findings that might lead to a much-needed test to distinguish virulent and avirulent isolates of *Vibrio parahaemolyticus*. Although people ingesting this organism in shellfish can develop severe diarrhoea and vomiting a few hours later, most isolates from the sea are harmless. Now Colin Munn and colleagues at the University of Plymouth, UK, have taken modest steps in showing that there are indeed differences that might facilitate the development of a screening method.

For example, sodium dodecyl sulphate polyacrylamide gel electrophoresis showed additional whole-cell protein bands in clinical isolates to those found in environmental isolates. Further work highlighted one particular feature: an extra band at 36 kDa noted in 47% of clinical isolates but only in 11·5% of environmental isolates. But, although haemolytic and enzymatic activities were clearly important in cytotoxicity, no single virulence factor distinguished virulent from avirulent strains.

Clearly this (together with the development of molecular typing) is still work-in-progress. Everyone who has ever been assailed by *V parahaemolyticus* will wish the Plymouth group well.

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Books

Health law, human rights, and public health

As shown in this two-volume set of previously published articles, public health is a vast field, encompassing most areas of human activity. Public health covers poverty and warfare to genetics and climate change through governmental actions taken to prevent disease and improve people's quality of life. When discussion turns to public health ethics, bioethicists and health lawyers are often called upon to give comment—the editor, Michael Freeman, Professor of English Law at University College London, UK, has done just that to compile this book.

Both bioethics and public health ethics are products of World War 2: bioethics grew out of the 1945–46 Nazi Doctors' Trial and public health ethics grew out of the 1948 Universal Declaration of Human Rights. Jonathan Mann, the former head of the WHO's global AIDS programme, remarked that the language of bioethics was well suited to the practice of medicine, whereas the language of human rights was needed for public health. As Rebecca Cook and Bernard Dickens note in their powerful essay The Injustice of Unsafe Motherhood, Jonathan Mann's legacy is "his focus on how social inequality, economic powerlessness, social exclusion, and denial of human dignity condition preventable disease, disability, and premature death". This is the modern view of public health and human rights. A more traditional (one might even say old-fashioned) view is that public health is about government coercion, usually in response to epidemic disease.

Public health is, of course, based on the actions of governments—it is empowered (and constrained) by human rights laws globally, and constrained by domestic laws nationally (eg, the Constitution of the USA). People often incorrectly refer to public health ethics when they are talking about either public health law (often constitutional law) or government policy.

Sofia Gruskin's article describes the use of a human rights framework to assess public health policy issues. Such practice puts emphasis on several foundational human right precepts: equality, which prohibits any form of discrimination, and the special status of women and children, which requires governments to prioritise their needs and provide not only basic medical care, but also food, clean water, housing, and education. The universal scope of public health is matched only by that of human rights.

Freeman has gathered 72 previously published articles, including 17 on infectious diseases. Unfortunately, they are all presented as photocopies of the originally published essays, which makes some of them almost unreadable when shrunk from journal-sized to book-sized pages. The definition of the virtually self-defining field of public health remains elusive to most of the authors, and the application of ethics to public health even more so. Knowing about public health is not a requirement for writing about public health, but it should be. As in all applied ethics fields, good ethics begin with good facts; the lack of public health facts means most of these essays are not terribly useful for public health practitioners. It is, nonetheless, an invaluable collection for anyone beginning an exploration into the meaning of ethics in public health.

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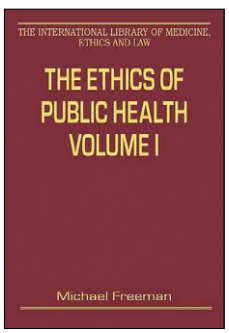


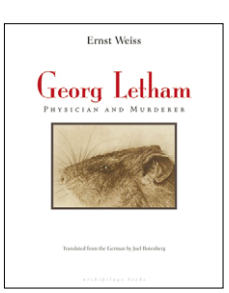
The Ethics of Public Health, Volumes I and II (The International Library of Medicine, Ethics and Law)
Edited by Michael Freeman.
Ashgate, 2010. Pp 1166. £315.
ISBN 978-0-7546-2605-3

Georg Letham: physician and murderer

His life story itself is the stuff of novels. Born in 1882 to a well-to-do Jewish family in what was then the Austro-Hungarian Empire—now the Czech Republic—Ernst Weiss spent his youth in some of central Europe's most agreeable cities: Prague, Brno, Litomerice, and Berlin. He studied medicine in Vienna and later became a surgeon. 1912 saw Weiss take up a berth on a ship bound for India and Japan. When he returned to Europe, the storm clouds were gathering. He served with distinction as a military physician in the Great War: they awarded him the Golden Cross for bravery. Afterwards, he settled in Prague, but he didn't want to be a doctor anymore.

Before the war, Weiss had struck up a friendship with Franz Kafka, who said of him "what an extraordinary writer he is". Not everyone agreed: 23 publishers turned down Weiss' first novel *The Galley* (1913). He moved to Berlin—where he wrote *Georg Letham: Physician and Murderer*—but by 1934 things were looking dangerous for central Europe's Jews, and Weiss fled to Paris. There he lived an impoverished existence, eased by handouts from literary supporters such as Thomas Mann. In 1938, Weiss wrote *The Eyewitness*, his last novel, which contained a thinly veiled portrait of Hitler; a final act of defiance perhaps, for as the Nazis invaded Paris, Weiss drank poison. He died the following evening.

Astonishingly, it has taken until now for *Georg Letham: Physician and Murderer* to be translated into English. Joel Rotenberg has done a fine job of rendering Weiss' snappily sardonic prose. It is presented in a handsome binding by Archipelago books. The eponymous antihero



Georg Letham: Physician and Murderer by Ernst Weiss.
Archipelago Books, 2010.
Pp 560. £12.99.
ISBN 978-0-9800330-3-8

is a bacteriologist who murders his wife. He does so partly for money, partly because she repulses him, but mainly, you can't help but feel, because he wants to spill blood. Letham is condemned to spend the rest of his life on a far distant penal colony, known only as C, where yellow fever is rampant.

It's a distinctive and vivid work. Weiss has a remarkable facility for conjuring up chilling scenes of desolation and decay. There's an eerie account of a doomed expedition to the North Pole that brings to mind Coleridge's *Ancient Mariner*. Here, the sailors are forced to give over their vessel to the insatiable horde of rats that have overrun the ship. "The ship does not understand the rodents in its belly. They merrily go on living. They are not looking for any pole. They are not interested in meteorology, not in dialects, not in Eskimo folktales, not in Christianity. Food to be taken is all that exists for them. If a weaker, good-tasting creature is alive and they can catch it, then they kill it".

The descriptions of the yellow fever patients are a uniquely piquant mixture of cold medical terminology and visceral human suffering: "the conjunctivae were yellow, shot through with distended scarlet venules. He gave off the foul carrion-like stench that is characteristic of the disease. The tongue and oral mucosa were unspeakably raw, as though the top dermal layers had been removed with a grater, taken down to the bare meat."

The author questions whether scientific detachment be brought to bear outside the laboratory. "I will hold up a mirror to myself. With a steady hand. With the exacting eye of a scientist" Letham explains in the book's foreword. In reality, of course, this is a man in thrall to his passions, though he despises himself for it. This novel, it should be noted, was first published in 1931 in a Germany not yet immersed in the terrible collective mania of the Nazi era, against which reason was no match.

There's more than a hint of Dostoevsky to the book: murderous, itchily neurotic characters, scenes of animal maltreatment and human degradation; indeed, the passages concerning the prisoners' voyage to C are more brutal and hopeless than anything in *Memoirs from the House of the Dead*. And like *Crime and Punishment*, *Georg Letham* reads in places like a thriller. But there's none of the Russian's religiosity: Letham looks to science for his salvation.

Freud's influence also looms large: there are dream sequences and lengthy passages concerning formative incidents from the protagonist's childhood. It adds up to a heady journey into the recesses of a tortured soul. But it's the imagery that stays with you—a remarkable, haunting work. An extraordinary writer indeed.

Talha Burki
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Trial results finally show potential for microbicidal HIV gel

Salim and Quarraisha Abdool Karim, husband and wife, and co-principle researchers on the Centre for AIDS Programme of Research in South Africa (CAPRISA) trial, received a standing ovation at the recent International AIDS Society Conference in Vienna when they announced their results, which showed—for the first time—that the use of an antiretroviral microbicidal gel can protect against HIV transmission. Mathematical modelling suggests that, in South Africa alone, this gel can prevent up to 1·3 million new infections and 8000 HIV-related deaths during the next 20 years.

The randomised, double blind, placebo-controlled trial followed 889 women without HIV infection in KwaZulu-Natal, South Africa, for 30 months. Women who used the 1% tenofovir vaginal gel, applied no more than 12 h before vaginal sex and as soon as possible, but no later than 12 h after, had a 39% lower rate of HIV infection than did those who used an unmedicated placebo gel. In those participants who used the tenofovir gel more consistently, protection rose to 54%. Additionally, the trial showed that use of the microbicide reduced the risk of herpes simplex virus 2 infection—an infection that doubles an individual's chance of contracting HIV—by 51%.

WHO estimates that 60% of the African HIV burden is borne by women. Accordingly, there has been much effort to offer HIV prevention to women who cannot always negotiate mutual monogamy or condom use—in the past 20 years there have been 11 unsuccessful trials with six candidate microbicide gels. The CAPRISA researchers caution that this is just a proof-of-concept trial, and urge the need for further trials to confirm these findings and to explore ways of improving effectiveness without compromising safety—the only side-effect in the CAPRISA trial was mild diarrhoea.

"It is a game changer—a huge step forward and a tremendous scientific achievement", said Mitchell Warren, executive director of AVAC, a non-governmental organisation involved in global advocacy for HIV prevention. "We now have proof-of-concept for microbicides and that is a fundamental building block for the future." He stresses the need to begin attempts, even at this early stage, to ensure that this successful clinical trial can translate into successful public health interventions: "no biomedical strategy, however effective, will have a lasting impact unless we also address stigma by using an evidence-based, human-rights focused approach. The proof-of-concept is actually the beginning and not the end of the road".

"The important issue is to make sure that vaginal microbicides do not acquire the negative connotations, such as promiscuity, disease prevention, and unfaithfulness, that make condoms so unacceptable in relationships defined as regular", Robert Pool (Barcelona Centre for International Health Research and the Microbicides Development Programme, Barcelona, Spain) told *TLID*. "Men argue against condom use by saying that it implies that their partner does not trust them."

Pool cites evidence from other trials that the lubrication offered by vaginal gels can lead to increased sexual enjoyment for both partners, and that "there needs to be an effort to get the partners of vulnerable women to want their women to use the microbicide". He argues that promoting the positive connotations of vaginal gels, such as increased pleasure and intimacy, and not just their ability to protect women against demonised promiscuous partners, will lead to more consistent use, and that the resulting improved communication about sex may also empower women to negotiate condom use.

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Salim Abdool Karim at the International AIDS Society Conference

"Of course there are also many women who may want to use a microbicide covertly", notes Pool, "there needs to be a whole range of products available for different tastes and scenarios." Another trial, the Vaginal and Oral Interventions to Control the Epidemic (VOICE) study, results for which are due in 2013, is assessing the use of a daily applied tenofovir-based gel and pre-exposure prophylaxis tablets. In addition to providing additional supportive data on the effectiveness of vaginal microbicides, results from this trial are eagerly anticipated because the alternative dosing regimens may permit greater privacy and convenience of use than does the tenovofir gel formulation used in the CAPRISA trial.

Nonetheless, this landmark CAPRISA trial is certainly cause for excitement. "Tenofovir gel potentially adds a new approach to HIV prevention as the first that can be used and controlled by women. It can help empower women to take control of their own risk of HIV infection", Salim Abdool Karim announced. "Once confirmed and implemented, tenofivir gel has the potential to alter the course of the HIV epidemic."

Dara Mohammadi

For the **CAPRISA tenofovir study** see *Science* 2010; DOI:10.1126/science.1193748

For more on **AVAC** see http://www.avac.org/

For more on the **Microbicides Development Programme** see http://www.mdp.mrc.ac.uk/

Smallpox: should we destroy the last viral stocks?

For the **WHO smallpox factsheet** see http://www.who.int/mediacentre/factsheets/smallpox/en/

For the **Institute of Medicine 2009 report *Live Variola Virus: Considerations for Continuing Research*** see http://iom.edu/Reports/2009/LiveVariolaVirusContinuingResearch.aspx

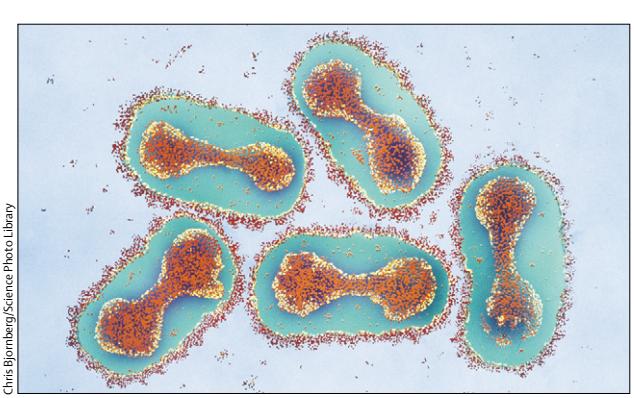
An International Symposium held in Rio de Janeiro, Brazil, on August 24–27, has focused on the lessons learned from the smallpox experience and featured debates on one very thorny issue still surrounding smallpox—whether we should destroy all stocks of the viral strains held in the world. WHO declared smallpox officially eradicated 30 years ago and it is still the only infectious disease that vaccination has succeeded in completely removing from the human population worldwide. Smallpox was highly virulent and killed 20–60% of those it infected, and caused long-term disability, such as deafness and blindness, in many of the rest. "Originally, stocks of the smallpox virus were held at dozens of locations but this was reduced to only two WHO Collaborating Center repositories—the State Research Centre of Virology and Biotechnology (VECTOR) in Russia and one at the Centers for Disease Control (CDC) in the USA", notes Gilberto Hochman (Oswaldo Cruz Foundation, Rio de Janeiro, Brazil), one of the conference organisers. "The others were destroyed after the accidental infection and death of a lab worker in Birmingham in the UK in 1978."

The decision to destroy all smallpox virus stocks has been made, but since 1999 several World Health Assembly (WHA) resolutions, such as WHA Res 60.1, have put off this final action. "All authorise further temporary retention of the two official WHA-sanctioned stocks of live variola to facilitate outcome-oriented, essential public health research; they also require review and approval of the research by a technical committee", explains Inger Damon, Chief of the Poxvirus and Rabies Branch at CDC (Atlanta, GA, USA). Research towards the development of diagnostics, antivirals, less reactogenic vaccines, and animals in which to best study these countermeasures, continues only under WHO auspices. "The continued retention of the official stocks will again be addressed in 2011, when WHO will provide a major review of research completed, products developed, and considerations for further essential research for global public health, through the WHO Executive Board, to the WHA", Damon told *TLID*.

The benefit of destroying all remaining stocks would be to further reduce the already unlikely possibility that a lethal epidemic might result from the theft or accidental release of the virus from one of the remaining laboratory stocks. "However, the governments of both the USA and Russia have thus far resisted any recommendations for destruction of their stockpiles because of the very serious drawbacks that such action would have", comments Raymond Weinstein (George Mason University, VA, USA).

One major disadvantage is that we would lose the smallpox virus as a research tool. Smallpox is one of the oldest diseases known and the human immune system evolved under the influence of smallpox. "To see the importance of smallpox in human immune evolution one need only look at the success of the mutation in the CCR5 chemokine receptor, which first appeared in a single northern European individual about 1000 years ago and is now present in around 10% of all northern Europeans", notes Weinstein. People who have it lack the cellular transmembrane immunological receptor CCR5 and show substantial resistance to smallpox—and nearly complete immunity to HIV. "We are only just beginning to unravel the complex pathophysiology and virulence mechanisms of smallpox but we do know that the variola virus produces important short-term and long-term alterations in the human immune system. Gaining a better understanding of these changes could lead to new therapeutic options for a host of diseases, but if all smallpox samples are forever destroyed the medical and scientific communities would lose a potentially valuable research tool", adds Weinstein. "It is certainly true that smallpox research has had important spin-offs for other viral infections; the research is not only applicable to a disease that no-one can catch anymore", stresses David Evans (University of Alberta, Edmonton, AB, Canada).

Complete destruction of the smallpox virus would also be difficult because no-one knows exactly what viable stocks still remain. "At least 461 isolates of variola have been deposited with the CDC; the DNA of nearly 50 of these virus isolates have been sequenced and are in the public domain, but less is known about viral sequences of the stocks in VECTOR", says Robert Drillian (IGBMC, Illkirch, France). Undisclosed stocks of smallpox virus may also exist. "It has been virtually impossible to verify whether or not the Russians fully destroyed their weapons-grade variola stocks that were created by Biopreparat in the 1980s", says Grant McFadden (University of Florida, Gainsville, FL, USA). Theft or movement of stocks during the breakup of the former USSR could have resulted in terrorist acquisition of smallpox viral stocks. It is state-sponsored terrorism that we have to worry about here, rather than



Chris Bjornberg/Science Photo Library

TEM of the smallpox virus (Poxviridae)

splinter groups in hiding, believes Evans. "Unless it is stored carefully at very low temperatures, the smallpox does not survive well and needs to be cultured every few years to remain viable. Stocks that have been stored improperly for the last 10 years are likely to be dead by now. To maintain a terrorist threat, groups would need to have access to the highest level containment facilities and the expertise to passage and re-store the virus", he explains.

The argument for destroying remaining smallpox virus stockpiles may also be rendered moot by two possibilities. "Creating smallpox, of course, would be unethical and probably illegal but certainly could be done from a technical point of view. To create live poxviruses from scratch, all you need is time (maybe 6–12 months), money (maybe US$100 000 at today's prices), and the technical know-how (pretty much available to anyone with basic knowledge in molecular biology and synthetic gene constructions)", explains McFadden. A further sobering thought is that nature might do the job for us. "Studies suggest that both variola and vaccinia probably emerged from either camelpox or taterapox, an enzootic poxvirus of a small African rodent; it is not impossible that nature might once again create variola from a near relative poxvirus or even create a distinct, smallpox-like virus from a more distant yet clinically similar poxvirus such as monkeypox", adds Weinstein.

The need to protect the global population from the smallpox threat, whatever its source, has led to some consensus. "Broad international agreement has enabled the development of up-to-date methods of virus detection, safer vaccines, and effective therapeutics, the latter being in existence before eradication", comments Drillian. Stockpiles of second-generation smallpox vaccine ACAM2000 have been amassed by many countries in the world, and by WHO. Despite that, we still have much to fear; immunity to the smallpox vaccine 40 years after population-wide vaccinations ceased in most countries would be low in all age groups. Even if vaccination could be mobilised quickly enough, people with HIV/AIDS, transplant recipients, or anyone with immunosuppression could not be protected. "The only validated vaccine we have is a live vaccine and this would certainly be fatal to the immunocompromised", concludes Evans.

Kathryn Senior

Infectious disease surveillance update

Pan(dem)ic over

On August 10, the WHO Director-General's statement after the ninth meeting of the Emergency Committee declared the 2009–10 influenza A H1N1 pandemic over. WHO estimates that 18 449 people have died as a result of the virus in 214 countries and overseas territories or communities.

Polio in Angola

As of July 19, the Angolan Ministry of Health had reported 15 cases of wild poliovirus type 1 (WPV1) since February 2010. All cases were detected in the capital Luanda or in provinces previously free of polio (Bie, Bengo, Huambo, Lunda Norte, and Lunda Sul). A region of DR Congo bordering Angola (Kasai Occindentale) reported a genetically related case.

Since 2007 there has been a persistent outbreak of WPV1 in Angola. And with the recent expansion of the outbreak, WHO judges it of high risk of further international spread into neighbouring countries. Angola was free of polio between 2001 and 2005, genetic markers suggest importation of the virus from India.

In 2010, Angola targeted high-risk provinces from May 7–9 with a subnational round of immunisation with monovalent oral polio vaccine type 1 (mOPV1). From June 11–13 there was a national round of immunisation with a combination of trivalent oral polio vaccine and mOPV1. Mop-up immunisation campaigns with mOPV1 were done in response to cases of WPV1.

Measles in Greece

As of July 25 there have been 126 cases of measles reported to the Greek Centre for Disease Control and Prevention in 2010 related to a recent outbreak in Bulgaria. Infected individuals were mostly unvaccinated. 77 of the reported cases were laboratory confirmed, with remaining cases classified as probable (31 cases) or possible (18) on the basis of the European Union case definition of 2008. All 19 cases genotyped by the Greek National Measles Reference Laboratory were genotype D4.

78 of the reported cases were in children age 0–14 years, 34 of which were in children in the 1–4 year group, and ten were in children younger than 1 year. 106 people with the infection had known vaccination status: 93 were unvaccinated and 13 had received at least one dose of the combined measles, mumps, and rubella vaccine.

Information on foreign travel was available for 114 of the people with measles: 104 people had no recent history of foreign travel, eight people had recently travelled to Bulgaria, one to France, and one to an unspecified destination.

In response to the outbreak vaccination campaigns have targeted groups with low vaccine coverage, prioritising high-risk communities in affected areas.

Onisillos Sekkides

For **the WHO Director-General's statement** see http://www.who.int/csr/disease/swineflu/9th_meeting_ihr/en/index.html

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Research brief

Basophils: tick protectors

Ticks transmit several pathogens to people and animals. Many animals develop immunity to ticks after repeat infestations but the nature of this acquired immunity is unclear. Researchers now report that antibodies and IgFc receptor expression on basophils is required for acquired resistance to *Haemaphysalis longicornis* ticks in mice. Furthermore, mice that lack basophils fail to acquire resistance to this vector for the human pathogens that cause Q fever, babesiosis, and Russian encephalitis. The discovery that basophils are essential for antibody-mediated immunity against ticks might suggest new strategies for the control of tick-borne diseases.

For more on **anti-tick immunity and basophils** see *J Clin Invest* 2010; **120:** 2867–75; DOI:10.1172/JCI42680

For more on **cross-species transmission of viruses** see *Science* 2010; **329:** 676–79; DOI:10.1126/science.1188836

For more on **immune responses to HIV-1 Env trimers** see *J Exp Med* 2010; DOI:10.1084/jem.20100025

For more on **ancient viral sequences in vertebrate genomes** see *PLoS Pathog* 2010; **6:** e1001030; DOI:10.1371/journal.ppat.1001030

For more on **genetic susceptibility to meningococcal disease** see *Nat Genet* 2010; DOI:10.1038/ng.640 and see **Leading Edge** page 577

For more on **type IIA topoisomerase inhibitors** see *Nature* 2010; DOI: 10.1038/nature09197

For more on **the development of the human gut microbiome** see *Proc Natl Acad Sci USA* 2010; DOI:10.1073/pnas.1000081107

Species hopping by viruses

Cross-species transmission of RNA viruses underlies the recent emergence of several new human diseases such as SARS. It is generally thought that rapid evolution helps RNA viruses to leap between species but a new study suggests that the genetic similarities between donor and recipient hosts might be a more important determinant of cross-species viral transmission. An analysis of the nucleoprotein gene sequences from 372 rabies viruses collected from 23 North American bat species shows that the likelihood of initial infection and ongoing transmission decreases as the phylogenetic distance between bat species increases.

Commonsource

Relatedness predicts cross-species transmission of rabies in bats

HIV-1 vaccine strategies

So far, efforts to elicit broadly neutralising antibodies against HIV-1 by immunisation have been disappointing. To gain insights into how to design vaccines that can stimulate protective HIV-1 immune responses in people, researchers have been investigating the immunogenic properties of soluble HIV-1 envelope glycoprotein trimers in rhesus macaques. Trimer inoculation in adjuvant, they report, stimulates robust memory B-cell responses and high titres of circulating antibodies that are improved by booster inoculations. However, protection against rectal simian-HIV challenge is modest, a result that highlights the challenges of eliciting adequate protective immune responses at mucosal sites.

Fossil viruses

A new study unexpectedly reveals that vertebrate genomes contain multiple insertions of ancient single-stranded RNA virus sequences. Researchers matched 5666 viral genes from all known non-retroviral families with single-stranded RNA genomes with the germline sequences of 48 vertebrate species. They found almost 80 examples of RNA virus sequences in 19 of the vertebrates. Most of these integrations, which occurred about 40 million years ago, were related to Bornavirus, Ebolavirus, and Marburgvirus sequences. Integration of these sequences might give the vertebrates harbouring them a selective advantage.

Meningitis susceptibility

Meningitis and septicaemia caused by *Neisseria meningitidis* cause substantial death and disability worldwide. Genetic factors contribute to the susceptibility to and the outcome of meningococcal disease but the genes responsible for disease development are largely unknown. Now, a genome-wide association study in 475 UK cases and two replication studies in western European and south European cohorts identify a cluster of single nucleotide polymorphisms in the complement factor H region of the human genome that is associated with susceptibility to meningococcal disease.

Antibiotics in action

Bacterial type IIA topoisomerases, which regulate DNA topology during replication and transcription, are major antibacterial drug targets. Researchers now report the crystal structure of a potent new bacterial topoisomerase inhibitor (GSK299423) in complex with *Staphylococcus aureus* DNA gyrase and DNA. The inhibitor, which "bridges" the DNA and a transient non-catalytic pocket at the gyrase dimer interface, lies close to the enzyme's active site and the binding site of fluoroquinolone antibiotics. These results provide important insights into how topoisomerases work and a platform for the design of a new class of antibiotics.

Baby bugs

The gut microbiome is implicated in human health. For example, it can contribute to excess adiposity. But how does it develop during early childhood? In a recent study, 60 faecal samples were collected from a single healthy infant during the first 30 months of life and the bacterial composition of the samples profiled using 16S rRNA gene and metagenome analysis. These analyses show that the phylogenetic diversity of the microbiome increased gradually and smoothly over time but that abrupt shifts in the distribution of major taxonomic groups occurred in response to dietary and health changes.

Jane Bradbury

Books

Health law, human rights, and public health

As shown in this two-volume set of previously published articles, public health is a vast field, encompassing most areas of human activity. Public health covers poverty and warfare to genetics and climate change through governmental actions taken to prevent disease and improve people's quality of life. When discussion turns to public health ethics, bioethicists and health lawyers are often called upon to give comment—the editor, Michael Freeman, Professor of English Law at University College London, UK, has done just that to compile this book.

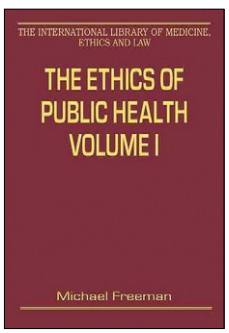
Both bioethics and public health ethics are products of World War 2: bioethics grew out of the 1945–46 Nazi Doctors' Trial and public health ethics grew out of the 1948 Universal Declaration of Human Rights. Jonathan Mann, the former head of the WHO's global AIDS programme, remarked that the language of bioethics was well suited to the practice of medicine, whereas the language of human rights was needed for public health. As Rebecca Cook and Bernard Dickens note in their powerful essay The Injustice of Unsafe Motherhood, Jonathan Mann's legacy is "his focus on how social inequality, economic powerlessness, social exclusion, and denial of human dignity condition preventable disease, disability, and premature death". This is the modern view of public health and human rights. A more traditional (one might even say old-fashioned) view is that public health is about government coercion, usually in response to epidemic disease.

Public health is, of course, based on the actions of governments—it is empowered (and constrained) by human rights laws globally, and constrained by domestic laws nationally (eg, the Constitution of the USA). People often incorrectly refer to public health ethics when they are talking about either public health law (often constitutional law) or government policy.

Sofia Gruskin's article describes the use of a human rights framework to assess public health policy issues. Such practice puts emphasis on several foundational human right precepts: equality, which prohibits any form of discrimination, and the special status of women and children, which requires governments to prioritise their needs and provide not only basic medical care, but also food, clean water, housing, and education. The universal scope of public health is matched only by that of human rights.

Freeman has gathered 72 previously published articles, including 17 on infectious diseases. Unfortunately, they are all presented as photocopies of the originally published essays, which makes some of them almost unreadable when shrunk from journal-sized to book-sized pages. The definition of the virtually self-defining field of public health remains elusive to most of the authors, and the application of ethics to public health even more so. Knowing about public health is not a requirement for writing about public health, but it should be. As in all applied ethics fields, good ethics begin with good facts; the lack of public health facts means most of these essays are not terribly useful for public health practitioners. It is, nonetheless, an invaluable collection for anyone beginning an exploration into the meaning of ethics in public health.

George J Annas
annasgj@bu.edu



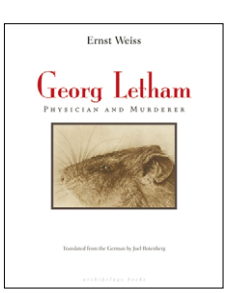
The Ethics of Public Health, Volumes I and II (The International Library of Medicine, Ethics and Law)
Edited by Michael Freeman.
Ashgate, 2010. Pp 1166. £315.
ISBN 978-0-7546-2605-3

Georg Letham: physician and murderer

His life story itself is the stuff of novels. Born in 1882 to a well-to-do Jewish family in what was then the Austro-Hungarian Empire—now the Czech Republic—Ernst Weiss spent his youth in some of central Europe's most agreeable cities: Prague, Brno, Litomerice, and Berlin. He studied medicine in Vienna and later became a surgeon. 1912 saw Weiss take up a berth on a ship bound for India and Japan. When he returned to Europe, the storm clouds were gathering. He served with distinction as a military physician in the Great War: they awarded him the Golden Cross for bravery. Afterwards, he settled in Prague, but he didn't want to be a doctor anymore.

Before the war, Weiss had struck up a friendship with Franz Kafka, who said of him "what an extraordinary writer he is". Not everyone agreed: 23 publishers turned down Weiss' first novel *The Galley* (1913). He moved to Berlin—where he wrote *Georg Letham: Physician and Murderer*—but by 1934 things were looking dangerous for central Europe's Jews, and Weiss fled to Paris. There he lived an impoverished existence, eased by handouts from literary supporters such as Thomas Mann. In 1938, Weiss wrote *The Eyewitness*, his last novel, which contained a thinly veiled portrait of Hitler; a final act of defiance perhaps, for as the Nazis invaded Paris, Weiss drank poison. He died the following evening.

Astonishingly, it has taken until now for *Georg Letham: Physician and Murderer* to be translated into English. Joel Rotenberg has done a fine job of rendering Weiss' snappily sardonic prose. It is presented in a handsome binding by Archipelago books. The eponymous antihero



Georg Letham: Physician and Murderer by Ernst Weiss.
Archipelago Books, 2010.
Pp 560. £12.99.
ISBN 978-0-9800330-3-8

The real cost of counterfeit medicines

In April 2010, the Cambodian Ministry of Health announced that the previous 5 months had seen the enforced closure of nearly 65% of the illegal pharmacies operating in the country. The US Agency for International Development (USAID)-backed Promoting the Quality of Medicines Programme—which is active in 30 nations—had gathered evidence that these pharmacies were a significant source of counterfeit and substandard medicines in Cambodia.

4 years earlier, *Tropical Medicine and International Health* published a study examining the authenticity of antimalarial drugs in Burma, Laos, Vietnam, Cambodia, and Thailand. The researchers purchased 188 packets labelled artesunate from vendors in the five countries, and found that 53% of the samples did not contain artesunate at all. The authors noted that the fake blister packets were "often hard to distinguish from their genuine counterparts", adding that the previous few years had seen a sharp increase in the problem. "An alarmingly high proportion of antimalarial drugs bought in pharmacies and shops in mainland South East Asia are counterfeit", they concluded. In 2006, a study restricted to Cambodia collected 488 sample antimalarial drugs, 27% of which failed thin layer chromatography and disintegration tests.

"Counterfeiting is still a major problem in Cambodia", says Sylvia Meek (Malaria Consortium, London, UK), "although the government is trying hard to deal with it." The Thai authorities responded to similar concerns by debarring private shops from selling antimalarials—a measure that Meek believes is proving effective but isn't practical for neighbouring Laos or Cambodia, where the need is greater and the public health-care system is unable to ensure comprehensive access to the required drugs. Indeed, it is those who are most at-risk from malaria—the rural poor and migrant population—who are most likely to encounter counterfeit drugs. The Malaria Consortium estimates that some 60–70% of Cambodians obtain treatment from the private sector, where fake medications abound.

Whether or not patients suspect that their antimalarials are fake (sometimes counterfeit medications are sold alongside genuine ones, but priced more affordably), the consequences of taking such products can be severe. It could mean death, of course. Then there is the issue of resistance. The relationship has not been proven, but it seems likely. Counterfeiters may include, say, 5% of active ingredient to mislead those tests that measure presence rather than quantity. This amounts to incomplete treatment. Counterfeits also weaken faith in genuine drug regimens, prompting changes in treatment-seeking behaviour that can exacerbate already tricky situations.

"Cambodia is doing a lot of work in improving their capacity for testing drugs and taking action against counterfeiters", Meek explains. This requires a cadre of trained inspectors, enough to have a deterrent effect, and concomitant strengthening of regulatory bodies. No small task, of course, but in the short term a scheme in which village-based volunteers are trained to dispense authenticated antimalarial medications seems to be paying dividends.

The scheme addresses key reasons people turn to counterfeit drugs: access and availability. Take a sub-Saharan nation in which the pharmacies are concentrated in the cities. A merchant can travel from village to village selling fake medications. For the villagers, the system is cheap, convenient and confidential, and they can buy a few tablets at a time. For the merchant, it is an easy profit with little risk. After all, not all countries have legislated against this trade, and in those that have, it is often regarded as a trademark infringement, for which the sanction is a small fine.

Yet this is a criminal enterprise—traces of MDMA (ecstasy) in fake medications attest to the fact that the same machines used to press narcotics are hauled into service for counterfeit drugs—and it is a serious threat to public health. The Cambodian studies are rare exceptions: elsewhere the extent of the counterfeit trade is unknown. The consequences, on the other hand, can be for all to see. During its 1995 meningitis epidemic, Niger unknowingly inoculated some 50 000 citizens with a fake vaccine, resulting in 2500 needless deaths. The same year, dozens of children in Haiti died after consuming a paracetamol cough syrup that contained a poisonous chemical used in antifreeze.

Antiretrovirals are another example of drugs that have drawn the attention of counterfeiters. "If you look for counterfeits, you'll find them", points out Hans Hogerzeil (WHO, Geneva, Switzerland). "The trend seems to be that we're finding more and more", he told *TLID*, but he noted that this could be down to improved detection methods.

"It is extremely difficult to quantify the prevalence of counterfeiting around the world, or even in the USA", states Ilisa Bernstein (Food and Drug

For **WHO's report on counterfeit drugs** see http://www.oecd.org/dataoecd/43/17/35650095.pdf

An official examines medicine in a pharmacy on the Thai–Cambodian border

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Shoppers buying smuggled, counterfeit medicine in Abidjan, Cote d'Ivoire

Administration, Silver Spring, MD, USA). "Counterfeiters have access to sophisticated technologies and are able to make very, very good copies of the real drug and packaging." Even if a patient falls sick after taking a counterfeit drug, doctors may not always suspect that the drug is to blame. Besides, to get an accurate idea of the variety and scale of counterfeiting requires a study of an enormous size and detail; and given the adaptability of those involved in the trade, it could only provide an insight into the situation there and then. The pharmaceutical industry does its own research, but they do not tend to place their findings in the public domain lest it erode trust in authentic medications.

So what estimates do exist? A 2005 Organisation for Economic Co-operation and Development report suggested the trade was worth at least US$32 billion per year, adding that by 2010 this figure might reach $75 billion, but the authors conceded that their figures were "guesstimates—[there's] no hard data on incidence, prevalence, or economic burden". In 2008, WHO affirmed that "many countries in Africa and parts of Asia and Latin America have areas where more than 30% of the medicines on sale can be counterfeit". Some experts place the value of the entire counterfeiting industry at $600 billion per year, up to 7% of international trade. But whereas the trade in fake pharmaceuticals accounts for part of this figure, there are vital differences between this trade and that in, for example, counterfeit jeans

It comes down to an issue of semantics, but it is one which colours the debate and often leads to producers of fake medicines facing punishments much too lenient for the severity of their crime. For counterfeit jeans, the trademark violation represents the entire infraction. But this is not how WHO defines counterfeit. "Counterfeit medicines are medicines that are deliberately and fraudulently mislabelled with respect to identity and/or source", states WHO. Certainly, this entails a trademark violation but the important point is that the producers have hidden their identity: regulators have no way of monitoring drug quality. This, stresses Hogerzeil, is wholly different to those medications that violate patents. "There is a real fear in developing countries that the counterfeit discussion and legislation in rich countries is being used to frustrate generics", he explains. The pharmaceutical industry often conflates the two issues, claiming that producing unapproved generics is tantamount to counterfeiting. "WHO is not bothered at all about any trademark infringement—that's for other organisations to address—our concern is the public health", Hogerzeil told *TLID*.

Last year, authorities in the Netherlands and Germany used counterfeiting and intellectual property legislation to seize shipments of pharmaceuticals lawfully produced by countries that don't recognise drug patent rights, mainly India. "We at WHO deplore these interceptions", Hogerzeil said categorically. "We don't want counterfeit legislation to be used to stop a generic product which is in itself perfectly legitimate in its trade."

Enacting enforceable and appropriate legislation is a crucial element of any national response. Countries should establish central reporting systems, says Hogerzeil, so that they can collect data. Regulators need to disseminate information on counterfeiting to concerned parties, including neighbouring countries; and pharmacists have to be trained to recognise fake products. "It all fits within a general pattern of promoting regulation capacity", affirms Hogerzeil. There's an overspill into law enforcement, and at the beginning of this year Interpol—who have been active in tracing counterfeit medicines in southeast Asia back to their source factory—established a unit for Medical Products Counterfeiting and Pharmaceutical Crime.

There are a lot of sectors that need to be coordinated here: medical regulatory authorities, pharmaceutical companies, law enforcement agencies, non-governmental organisations, and politicians. Counterfeit medications can also cross several borders on the way to their final destination. The seemingly impossible task of stemming the trade in narcotics hints at the magnitude of the task at hand.

Then again, the fact that these drugs can be acquired legally offers an important tool to those fighting counterfeiters. "Price is extremely important to the people buying the drugs, especially in remote areas", explains Meek. She points out that the Affordable Medicines Facility for Malaria could reduce the cost of treatment to around $0.1. "It could take away a lot of the incentive for counterfeiting." Meek emphasises the need for international cooperation to tackle counterfeiting. Moreover, the emergence of artemisinin-resistant malaria in Cambodia adds urgency to the fight. "It's really important that we do everything we can to reduce resistance", she told *TLID*. "The association between fake drugs and resistance is hard to understand, but there's a clear risk and if resistance spreads to Africa we will be set back by years."

Talha Burki

Invasive infections and sickle-cell disease

The burden of disease caused by bacterial infections in patients with sickle-cell disease has long been neglected. Meenakshi Ramakrishnan and colleagues[1] emphasise the scarcity of data for the association between sickle-cell disease and invasive bacterial disease in Africa. However, four of the seven studies included in this meta-analysis were from Kinshasa, Democratic Republic of the Congo (formerly Zaire).[2–5] Although the reviewers present these as independent studies, the same data are shared in these reports. The first study by Eeckles and colleagues,[2] published in 1967, included data for blood and cerebrospinal fluid (CSF) infections, recorded from 1959 to 1966. The second study, by Lontie and colleagues[3] published in 1973, included data for CSF samples collected from 1959 to 1972. The third study, by Omanga[4] published in 1981, from the same clinics as the first and second studies, included data for all types of invasive infections; the investigators do not mention dates of sample collection. In a fourth study by Omanga[5] published in 1989, data were collected from 1964 to 1985. This study reported 69 patients with sickle-cell disease and bacteraemia. At least 14 of these 69 patients also had positive CSF cultures. A fifth study, published in 1977 (not included in Ramakrishnan and colleagues review), also by Omanga and colleagues,[6] examined meningitis in 47 patients with sickle-cell disease, nine of whom also had positive blood cultures. These CSF samples were collected from 1964 to 1974. Because Eeckles and colleagues' 1967 study,[2] reported 45 patients with sickle-cell disease, few new cases are likely to have been added in the last study published in 1989[5] (conflict in the Democratic Republic of the Congo meant microbiology laboratories might not have been functional).

The combined odds ratios for bacterial infections calculated in Ramakrishnan and colleagues' study therefore included duplicate, triplicate, and perhaps quadruplicate data. This fact clearly shows the need for a more careful analysis of this published work, but more importantly, the need for new studies to accurately establish the relative burden of disease in patients with sickle-cell disease in Africa.

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The revised pooled odds ratio (OR) for all types of invasive bacterial infections was 19·9 (95% CI 13·9–28·5), which is similar to the previous finding of 18·7 (14·7–23·9). The revised pooled OR for invasive pneumococcal disease was 26·1 (12·6–53·9), which is lower than the previous result of 35·7 (20·0–63·6). The revised pooled OR for invasive *Haemophilus influenzae* type b was 17·4 (4·6–66·2), higher than the previous result of 12·8 (5·6–29·1).

Despite differences in point estimates, these results are consistent with our original findings and reinforce our conclusion that invasive bacterial disease is strongly associated with sickle-cell disease in African people.

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Cervical cancer incidence can increase despite HPV vaccination

We read with interest the conclusion that Chris Bauch and colleagues[1] presented, indicating that the incidence of cervical cancer would be very unlikely to increase as a result of human papillomavirus (HPV) vaccination. The investigators conclude that screening coverage in a population is unlikely to decline to levels that would be necessary to lead to an increase cervical cancer incidence—this being an 80% decrease in screening with 30% vaccine coverage. We refute their conclusions and present data from the UK and Finland that show that screening less than 20% of young women resulted in a substantial increase in cervical cancer incidence in an unvaccinated female population. With increasing background exposure to high-risk HPV, the incidence of cervical cancer did not decrease in the UK until at least 70% of the population were appropriately screened.[2,3] Likewise, when less than 70% of the population is screened, only individual benefits result and the population incidence of cervical cancer is not reduced.

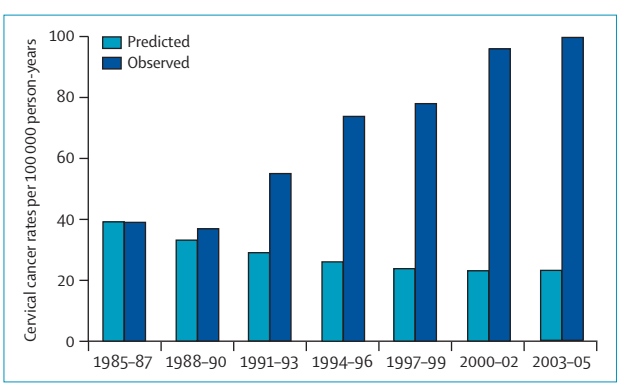

Figure 1: **Sentinel warning**
By 2005, the incidence of cervical cancer had increased more than four times over the rate predicted by historical screening patterns for 30–39 year old Finnish women per 1 million person-years.[7]

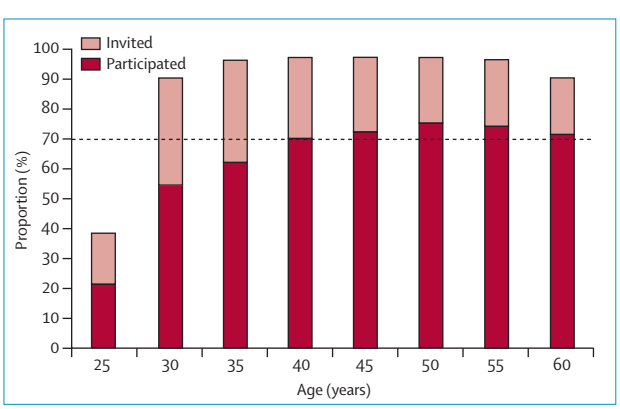

Figure 2: **Coverage of the 2003 Finnish cervical cancer screening programme in 2003**
The dashed line represents the 70% minimum coverage rate that is needed to gain a population benefit from organised cervical cancer screening programmes.[4,5]

Finland adopted organised cervical cancer screening in the early 1960s. For the first decade of organised screening, incidence was 14–16 per 100 000 woman-years,[4] effectively finding the prevalent cancer cases in the previously unscreened population. By 1970, organised screening coverage had reached at least 70% of the target population, and the incidence of invasive cervical cancer dropped sharply by 75% over the next 20 years. Incidence rose again to an overall level of 4 per 100 000 woman-years over the next 10 years.[5] Why did this happen?

In 1985, when cervical cancer incidence was still quickly declining, the actual incidence corresponded to predictions that were based on previous decreases;[6] however, for every subsequent year, the rate of cervical cancer was higher than predicted for women aged 30–39 years[4,5]—the peak age group for cervical cancer. In Finland, every year the rate continued to increase by 3 per 100 000 woman-years until it was more than four times higher than the expected rate per 1 million person-years for 30–39 year old women (figure 1); a real step backwards for cervical cancer prevention in the population. In contrast, over the same period, cancer rates for women over 50 years old continued to decrease as expected because over 70% of these women still actively attended screening. Reasons for increases in cervical cancer in younger women were a willful lack of screening and rapid increases in HPV16 incidence and prevalence.[7] The Finnish Mass Screening Registry[5] reported that less than 60% of women less than 40 years of age accepted the invitation to be screened in 2003, and only 20% of the women 25–30 years of age participated in the screening programme (figure 2). Assuming low vaccine coverage (30%), which gains no herd benefit,[8] these data fulfil the thresholds that Bauch and colleagues[1] defined for cervical cancers to increase after HPV vaccination. Willful lack of screening participation is already occurring in our youngest women, who have the lowest awareness of the magnitude of the morbidity and mortality of cervical cancer. An increase in cervical cancer can be seen as soon as 5 years after a willful decrease of participation in screening programmes and an ongoing high-risk HPV epidemic.

Organised HPV vaccination combined with screening could potentially prevent most cervical cancer. Vaccinations alone will not prevent cervical cancer unless their efficacy is longer than 15 years; if the duration of efficacy is shorter and efficient boostering is not organised, the onset of the cancer in women is merely postponed.[8] Previous studies have already shown that a willful cessation of screening in a generation of women who had little contact with friends who were developing or dying from invasive cervical cancer has resulted in increases in cervical cancer rates measurable at the population level. Misunderstanding of the

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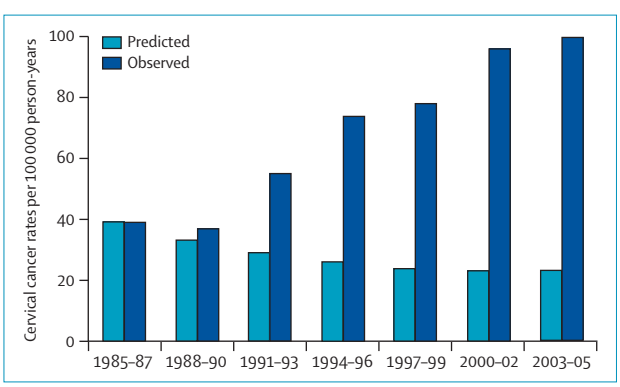

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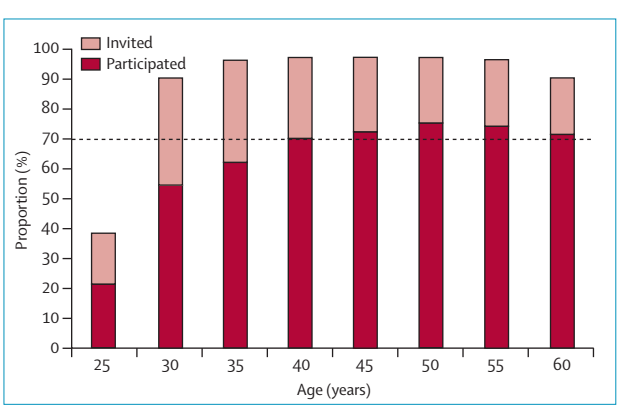

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benefits of individual HPV vaccination is real. If even more young vaccinated adolescents mature into women who willfully refuse cervical cancer screening, the population rates of cervical cancer will increase.

DH received a speaker's bureau, travel and accomodation expenses, and consultancy fees for an advisory board and clinical trial support from Merck and Co and GlaxoSmithKline. ML received grant support and travel and acommodation expenses from Merck and Co and GlaxoSmithKline Biologicals. PN received fees for consultancy and travel and accomodation support from GlaxoSmithKline (member of the external end point committee of the GlaxoSmithKline HPV vaccine study). JP declared no conlicts of interest.

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Author's reply

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Harper and colleagues use data showing increasing cervical cancer incidence in Finland and the UK, either directly related to declining screening coverage or despite rising screening coverage. They conclude that incidence of cervical cancer can increase in vaccinated populations and our model must therefore be wrong; however, this reasoning is also questionable. The data used are from the pre-vaccine era when there was no other public health interventions to counterbalance surges in cervical cancer cases caused by reduced screening adherence or changes in sexual behaviour. Of course cervical cancer incidence will increase in such conditions. Our model was devised to understand when immunisation would be sufficient to outweigh the effects of a decline in screening adherence in vaccinated women.

Every model makes assumptions and ours is no exception. It assumes that cervical cancer incidence is constant or already declining before the HPV vaccine is introduced. This is true for many countries,[3,4] but the data presented by Harper and colleagues show that this assumption does not apply to certain age groups in Finland. To address this example adequately, our model would have to include a scenario of a pre-existing rise in incidence before vaccine introduction. Even without this inclusion, the organised screening scenario of our model suggests that Finland might be more vulnerable than other countries to increases in cervical cancer incidence in vaccinated women who reduce their cervical screening adherence. This insight—together with the pre-vaccine data presented by Harper and colleagues—suggests that HPV immunisation programmes implemented in Finland should be monitored closely to ensure that this increase in incidence does not occur.

CTB has received travel and accomodation expenses, consultancy fees, and a research grant from GlaxoSmithKline.

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benefits of individual HPV vaccination is real. If even more young vaccinated adolescents mature into women who willfully refuse cervical cancer screening, the population rates of cervical cancer will increase.

DH received a speaker's bureau, travel and accomodation expenses, and consultancy fees for an advisory board and clinical trial support from Merck and Co and GlaxoSmithKline. ML received grant support and travel and acommodation expenses from Merck and Co and GlaxoSmithKline Biologicals. PN received fees for consultancy and travel and accomodation support from GlaxoSmithKline (member of the external end point committee of the GlaxoSmithKline HPV vaccine study). JP declared no conlicts of interest.

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Acute bacterial meningitis

I read with great interest Kwang Sik Kim's excellent Review[1] of acute bacterial meningitis in infants and children in *The Lancet Infectious Diseases*. I am grateful for Kim's contribution to the knowledge of bacterial meningitis in children; however, he states that concomitant "dexamethasone and vancomycin can reduce penetration of vancomycin into the CSF [cerebrospinal fluid] by virtue of the anti-inflammatory activity of dexamethasone". Findings from one multicentre study[2] have shown that the crossing of the blood–brain barrier by vancomycin is unaffected by steroid use. Furthermore, in agreement with previous data,[3,4] after administration of the conjugate antipneumococcal vaccine, some pneumococcal serotypes are replaced via the emergence of serotypes that are not included in the vaccine. Hence, in severe cases of pneumococcal meningitis, vancomycin should be added despite concerns of a decrease in blood–brain barrier penetration with the concomitant use of steroids, because it can reduce the incidence of neurological sequelae.[5] This finding should be kept in mind, especially after the increase of pneumococcal serotype 19A that has led to increased antimicrobial resistance to β lactams.[3]

I declare that I have no conflicts of interest.

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Tuberculosis and diabetes mellitus: is vitamin D the missing link?

We read with interest Kelly Dooley and Richard Chaisson's review[1] of the association between diabetes mellitus and tuberculosis. Although the investigators discussed several mechanisms that might underlie this association, we feel that that one potentially important consideration was omitted—vitamin D.

Vitamin D is a secosteroid that is either synthesised in the skin by the action of sunlight or ingested in the diet; dietary intake accounts for a much smaller proportion of total vitamin D, because it is scarce in foods.[2] Vitamin D is generated by the action of ultraviolet B radiation on the precursor, 7-dehydrocholesterol, in the skin, and subsequent hydroxylation in other organs. The vitamin has pleiotropic effects on many organs, including the adaptive immune system and the innate immune system.

An estimated 1 billion people worldwide have vitamin D deficiency or insufficiency due to reduced sun exposure or inadequate intake; and only now are we becoming aware of the effects of this deficiency. Vitamin D deficiency increases the risk of type 1 and type 2 diabetes mellitus, and supplementation is protective against both types.[2–4] Vitamin D is thought to affect pancreatic β cell function (insulin synthesis and secretion) and immune response;[5,6] low concentrations of vitamin D are associated with insulin resistance and glucose intolerance.[5] In chronic disease, vitamin D is used to restore health in organ systems, the risk of deficiency is therefore increased. Because vitamin D deficiency is associated with active tuberculosis (odds ratio 2·9, 95% CI 1·3–6·5),[7] diabetes-related deficiency might lead to susceptibility to tuberculosis infection and vice versa. In vitro, the actions of monocytes and macrophages on *Mycobacterium tuberculosis* are heavily dependent on vitamin D concentrations.[2] If these concentrations become too low, phagocytosis cannot occur.

The strong association of vitamin D concentrations with tuberculosis and diabetes mellitus means that vitamin D deficiency might explain some of the association between these two diseases, thus, population-wide supplementation measures to prevent both diseases should be considered.

We have no conflicts of interest.

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Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study

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Summary

Background **Gram-negative Enterobacteriaceae with resistance to carbapenem conferred by New Delhi metallo-β-lactamase 1 (NDM-1) are potentially a major global health problem. We investigated the prevalence of NDM-1, in multidrug-resistant Enterobacteriaceae in India, Pakistan, and the UK.**

Methods **Enterobacteriaceae isolates were studied from two major centres in India—Chennai (south India), Haryana (north India)—and those referred to the UK's national reference laboratory. Antibiotic susceptibilities were assessed, and the presence of the carbapenem resistance gene bla_{NDM-1} was established by PCR. Isolates were typed by pulsed-field gel electrophoresis of XbaI-restricted genomic DNA. Plasmids were analysed by S1 nuclease digestion and PCR typing. Case data for UK patients were reviewed for evidence of travel and recent admission to hospitals in India or Pakistan.**

Findings **We identified 44 isolates with NDM-1 in Chennai, 26 in Haryana, 37 in the UK, and 73 in other sites in India and Pakistan. NDM-1 was mostly found among *Escherichia coli* (36) and *Klebsiella pneumoniae* (111), which were highly resistant to all antibiotics except to tigecycline and colistin. *K pneumoniae* isolates from Haryana were clonal but NDM-1 producers from the UK and Chennai were clonally diverse. Most isolates carried the NDM-1 gene on plasmids: those from UK and Chennai were readily transferable whereas those from Haryana were not conjugative. Many of the UK NDM-1 positive patients had travelled to India or Pakistan within the past year, or had links with these countries.**

Interpretation **The potential of NDM-1 to be a worldwide public health problem is great, and co-ordinated international surveillance is needed.**

Funding **European Union, Wellcome Trust, and Wyeth.**

Introduction

Bacteria from clinical and non-clinical settings are becoming increasingly resistant to conventional antibiotics. 10 years ago, concern centred on Gram-positive bacteria, particularly meticillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* spp. Now, however, clinical microbiologists increasingly agree that multidrug-resistant Gram-negative bacteria pose the greatest risk to public health. Not only is the increase in resistance of Gram-negative bacteria faster than in Gram-positive bacteria,[1,2] but also there are fewer new and developmental antibiotics active against Gram-negative bacteria[3–6] and drug development programmes seem insufficient to provide therapeutic cover in 10–20 years.[7–9]

The increase in resistance of Gram-negative bacteria is mainly due to mobile genes on plasmids that can readily spread through bacterial populations. Standardised plasmid typing methods are enhancing our understanding of the host ranges of these elements and their worldwide distribution.[10,11] Moreover, unprecedented human air travel and migration allow bacterial plasmids and clones to be transported rapidly between countries and continents.[12,13] Much of this dissemination is undetected, with resistant clones carried in the normal human flora and only becoming evident when they are the source of endogenous infections. The CTX-M-15 extended-spectrum β-lactamase (ESBL) encoded by $bla_{CTX-M-15}$ was first reported in India in the mid-1990s.[14,15] The gene jumped from the chromosome of its natural hosts, *Kluyvera* spp, to plasmids that have subsequently spread widely,[10,16] establishing CTX-M-15 as the globally-dominant ESBL and the primary cause of acquired resistance to third-generation cephalosporins in Enterobacteriaceae.[17,18]

Recent surveys have identified ESBLs in 70–90% of Enterobacteriaceae in India and; although these collections might be a biased sample, they do suggest a serious problem, making the widespread use of reserved antibiotics such as carbapenems necessary.[15,19] Rates of cephalosporin resistance are lower in other countries but the growing prevalence of ESBL producers is sufficient to drive a greater reliance on carbapenems. Consequently, there is selection pressure for carbapenem resistance in Enterobacteriaceae, and its emergence is a worldwide public health concern since there are few antibiotics in

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reserve beyond carbapenems.[20] Already *Klebsiella pneumoniae* clones with KPC carbapenemase are a major problem in the USA, Greece, and Israel, and plasmids encoding the VIM metallo-carbapenemase have disseminated among *K pneumoniae* in Greece.[21]

We recently reported a new type of carbapenem resistance gene, designated bla_{NDM-1}.[22] A patient, repatriated to Sweden after admission to hospital in New Delhi, India, was colonised by *K pneumoniae* and *Escherichia coli* with bla_{NDM-1} on plasmids of varying size, which readily transferred between bacterial strains in vitro. We sought molecular, biological, and epidemiological data on New Delhi metallo-β-lactamase 1 (NDM-1) positive Enterobacteriaceae in India and Pakistan and investigated importation of the resistance gene into the UK by patients returning from the Indian subcontinent.

Methods

Bacterial isolates

Isolates of bacteria were identified from Chennai and Haryana in India. UK isolates were identified from referrals to the Antibiotic Resistance Monitoring and Reference Laboratory by UK microbiology laboratories between 2003 and 2009. We also identified isolates from other sites around Bangladesh, India, and Pakistan.

Procedures

Bacteria were identified via the Phoenix automated phenotypic identification criteria (Becton Dickinson, Oxford, UK) or with API 20E strips (bioMerieux, Basingstoke, UK). Minimum inhibitory concentrations (MICs) and carbapenem resistance were established by microbroth dilution (Phoenix), British Society for Antimicrobial Chemotherapy (BSAC) agar dilution, or disc diffusion.

Modified Hodge (cloverleaf) test involving distorted carbapenem inhibition zones and imipenem-EDTA synergy tests by disc, or the MBL Etest (AB bioMerieux, Solna, Sweden) were used to screen for metallo-β-lactamase production.[23] The presence of bla_{NDM-1} was established by PCR with specific primers targeting the gene.[22] PCR and sequencing were used to identify other resistant genes (bla_{CMY-4} and $bla_{CTX-M-15}$) carried by the bacterial isolates.

Conjugational transfer of antibiotic resistance to the laboratory strain *E coli* J53 was done on blood agar without selection. After 18 h, the mixed cultures were washed from the plates, suspended in saline, and plated onto MacConkey agar containing sodium azide (100 mg/L) and meropenem (2 mg/L). Transconjugants were confirmed to have bla_{NDM-1} by PCR analysis. Plasmids were subsequently isolated and typed on the basis of their origins of replication, as described by Carattoli and colleagues.[11]

Genomic DNA was prepared in agarose blocks and digested with the restriction enzyme XbaI (Roche Diagnostics, Mannheim, Germany). DNA fragments were separated by pulsed-field gel electrophoresis (PFGE) on a CHEF-DR III apparatus (Bio-Rad, Hercules, CA, USA) for 20 h at 6 V/cm at 14°C with an initial pulse time of 0·5 s and a final pulse time of 30 s. Dendrograms of strain relatedness were created with BioNumerics software.

Genomic DNA in agarose blocks was digested with the restriction enzyme S1 (Invitrogen, Abingdon, UK). DNA fragments were separated by PFGE as above. In-gel hybridisation was done with a bla_{NDM-1} probe labelled with ^{32}P (Stratgene, Amsterdam, Netherlands) with a random-primer method.[22] Plasmid DNA bands that hybridised with bla_{NDM-1} were cut from the gel, purified, and typed as described by Carattoli and colleauges.[11]

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

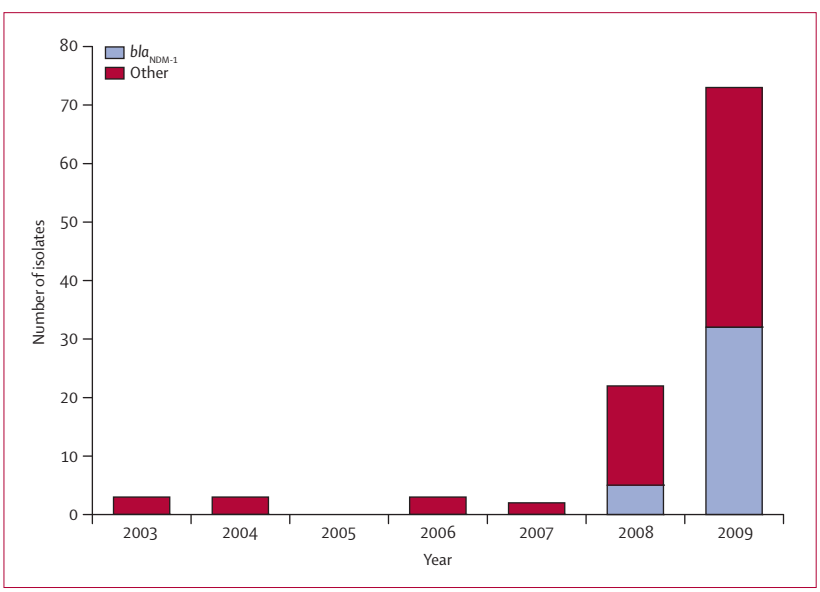

***Figure 1*: Numbers of carbapenemase-producing Enterobacteriaceae referred from UK laboratories to the UK Health Protection Agency's national reference laboratory from 2003 to 2009**
The predominant gene is bla_{NDM-1}, which was first identified in 2008. The other group includes diverse producers of KPC, OXA-48, IMP, and VIM enzymes.

Results

From Chennai, 75 *E coli*, 60 *Klebsiella* spp, and six other Enterobacteriaceae resistant to carbapenems were isolated from 3521 (4%) Enterobacteriaceae analysed throughout 2009. Of these 141 carbapenem-resistant Enterobacteriaceae, 44 (19 *E coli*, 14 *K pneumoniae*, seven *Enterobacter cloacae*, two *Proteus* spp, one *Citrobacter freundii*, and one *Klebsiella oxytoca*) were NDM-1 positive (about 1% of all resistant isolates). During that same period, 47 carbapenem-resistant isolates (24%) of 198 from Haryana were identified; from these, 26 (13%) were positive for NDM-1, and all were *K pneumoniae*. The Indian isolates from Chennai and Haryana were primarily from

community acquired urinary tract infections, pneumonia, and blood-stream infections. The age range was 4–66 years with a mean of 36 years (SD 20) and a female to male ratio of about two to one.

In the UK resistant isolates increased in both 2008 and 2009 (figure 1). Isolates with the NDM-1 enzyme, which was first detected in the UK in 2008, became the predominant carbapenemase-producing Enterobacteriaceae in 2009, accounting for 32 (44%) of 73 carbapenemase producers. During 2008–09, 37 Enterobacteriaceae isolates with the NDM-1 enzyme were referred from 25 laboratories across England with single representatives also from Scotland and Northern Ireland. These were identified as *K pneumoniae* (21 isolates), *E coli* (seven), *Enterobacter* spp (five), *Citrobacter freundii* (two), *Morganella morganii* (one), and *Providencia* spp (one). They were from 29 patients and had been isolated from urine (15 patients), blood (three), burn or wound swab (four), sputum (two), central line tip (one), throat swab (one), or unknown specimens (three). The mean age of the patients was 60 years (SD 24; range 1–87), with 17 male patients and 12 female patients. At least 17 patients had a history of travelling to India or Pakistan within 1 year, and 14 of them had been admitted to a hospital in these countries. Reasons for these admissions included renal or bone marrow transplantation, dialysis, cerebral infarction, chronic obstructive pulmonary disease, pregnancy, burns, road traffic accidents, and cosmetic surgery.

Isolates, NDM-1-positive bacteria from Mumbai (32 isolates), Varanasi (13), and Guwahati (three) in India, and 25 isolates from eight cities in Pakistan (Charsadda, Faisalabad, Gujrat, Hafizabad, Karachi, Lahore, Rahim Yar Khan, and Sheikhupura) were also analysed in exactly the same manner but in laboratories in India and Pakistan. These isolates were from a range of infections including bacteraemia, ventilator-associated pneumonia, and community-acquired urinary tract infections.

All the isolates producing the NDM-1 enzyme were resistant to several antibiotic classes (table). The 37 UK isolates were all resistant to imipenem and ertapenem, although a single *M morganii* isolate remained susceptible, at least in vitro, to meropenem (MIC 2 mg/L). Only four UK isolates remained susceptible to the monobactam aztreonam (MICs ≤1 mg/L), which is unaffected by metallo-carbapenemases including NDM-1; the other UK isolates were all resistant to all β-lactams, including aztreonam, suggesting the concurrent presence of additional β-lactamases including ESBLs and AmpC enzymes—identified by sequencing as mainly $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CMY\text{-}4}$. All 37 isolates were resistant to amikacin and tobramycin, although one isolate was susceptible to gentamicin and three to ciprofloxacin. MICs of minocycline were consistently 2 mg/L or greater, interpreted as non-susceptible with the BSAC and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for doxycycline, but most (33 of 37) were susceptible to colistin (MICs ≤4 mg/L) and 26 were suseptible to tigecycline (MICs ≤1 mg/L; figure 2).

The 44 isolates from Chennai were similarly resistant to all β-lactam antibiotics, fluoroquinolones, and aminoglycosides, apart from two that were sensitive to gentamicin. 39 were resistant to minocycline with MICs >2 mg/L, 19 to tigecycline, and three to colistin (table and figure 2). Two of the three isolates resistant to colistin were *Proteus* spp, which are intrinsically resistant, and the third was a *K pneumoniae* strain (colistin MIC >32 mg/L;

	UK (n=37)		Chennai (n=44)		Haryana (n=26)	
	MIC_{50}; MIC_{90} (mg/L)	Proportion susceptible*	MIC_{50}; MIC_{90} (mg/L)	Proportion susceptible*	MIC_{50}; MIC_{90} (mg/L)	Proportion susceptible*
Imipenem	32; 128	0%	64; 128	0%	32; 128	0%
Meropenem	32; 32	3%	32; >32	3%	>32; >32	3%
Piperacillin-tazobactam	>64; >64	0%	>64; >64	0%	>64; >64	0%
Cefotaxime	>256; >256	0%	>256; >256	0%	>256; >256	0%
Ceftazidime	>256; >256	0%	>256; >256	0%	>256; >256	0%
Cefpirome	>64; >64	0%	>64; >64	0%	>64; >64	0%
Aztreonam	>64; >64	11%	>64; >64	0%	>64; >64	8%
Ciprofloxacin	>8; >8	8%	>8; >8	8%	>8; >8	8%
Gentamicin	>32; >32	3%	>32; >32	3%	>32; >32	3%
Tobramycin	>32; >32	0%	>32; >32	0%	>32; >32	0%
Amikacin	>64; >64	0%	>64; >64	0%	>64; >64	0%
Minocycline	16; >32	0%	32; >32	0%	8; 16	0%
Tigecycline	1; 4	64%	4; 8	56%	1; 2	67%
Colistin	0·5; 8	89%†	1; 32	94%†	1; 2	100%†

MIC=minimum inhibitory concentration. *Susceptibility defined by British Society for Antimicrobial Chemotherapy and European Committee on Antimicrobial Susceptibility Testing breakpoints; doxycycline breakpoints were used for minocycline. †Colistin-resistant UK isolates were one isolate of *Morganella morganii* and one *Providencia* sp (both intrinsically-resistant species), also one *Klebsiella pneumoniae* and one *Enterobacter* sp.

Table: **Antibiotic susceptibilities for NDM-1-positive Enterobacteriaceae isolated in the UK and north (Chennai) and south India (Haryana)**

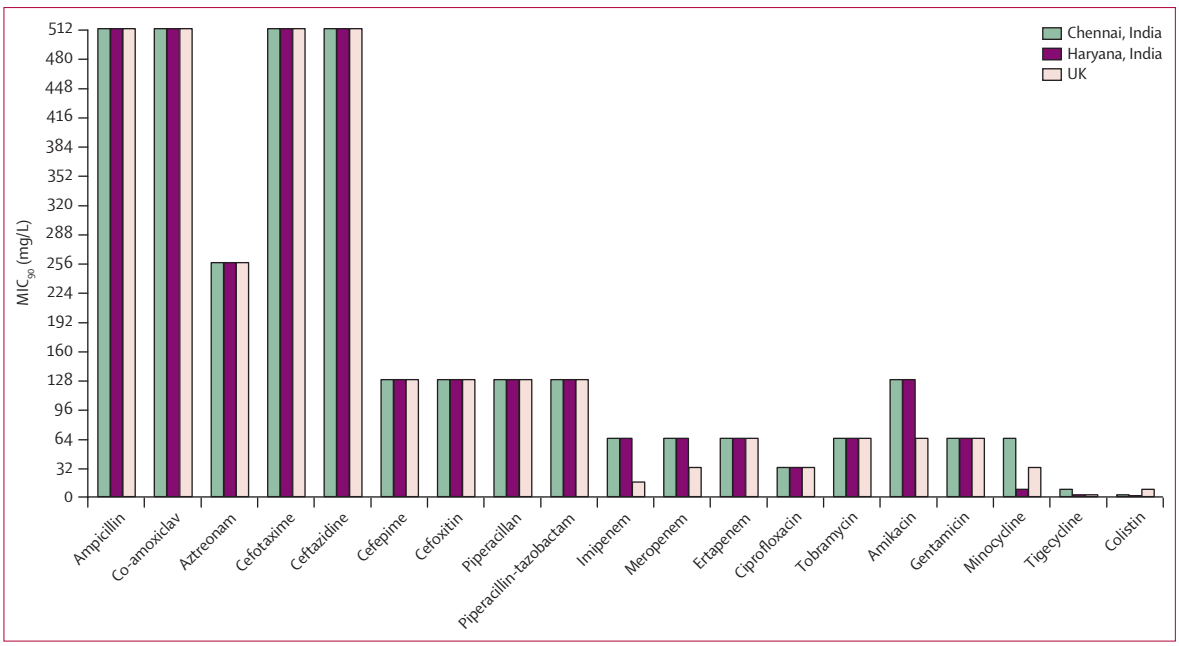

***Figure 2*: 90% minimum inhibitory concentration (MIC_{90}) for Enterobacteriaceae from Chennai and Haryana, India, and the UK**

tigecycline MIC 8 mg/L). Although several reports from Greece have noted *K pneumoniae* isolates as colistin resistant, we believe our isolate is truly pan-resistant.[24,25] Most of the 26 Haryana isolates were resistant to all β-lactam and non-β-lactam antibiotics, although three were susceptible to aztreonam and one to ciprofloxacin and amikacin. Minocycline MICs for the Haryana isolates were 8–16 mg/L and ten isolates had intermediate resistance (2 mg/L) to tigecycline by EUCAST criteria. None were resistant to colistin (table and figure 2).

The 21 *Klebsiella* isolates from the UK had different PFGE profiles and were typed to 19 distinct groups with only two related pairs, both of which included isolates from epidemiologically linked patients, probably representing cases of cross-infection. All the UK *E coli* isolates were different. The Chennai isolates were also very different, with none similar to each other. By contrast, the 26 NDM-positive *K pneumoniae* isolates from Haryana belonged to a single PFGE profile suggesting clonal spread. We could not prove statistically significant strain relatedness between the Indian and UK isolates.

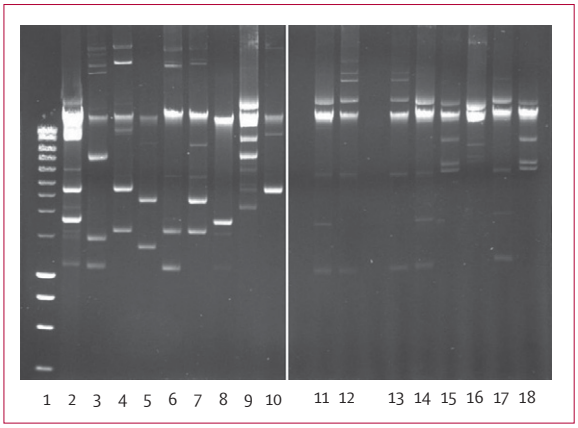

***Figure 3*: The difference in plasmid numbers from a selection of Indian isolates**
Tracks 1–10 show the number of plasmids in isolates from Chennai (south India) and tracks 11–18 show the number of plasmids in isolates from Haryana (north India). Most isolates contained up to seven plasmids, and in Chennai there was greater variation than in isolates from Haryana showing the bacterial clonality of NDM-1 carriage in Haryana.

Isolates from Chennai, Haryana, and the UK's Antibiotic Resistance Monitoring and Reference Laboratory were analysed for the location of the bla_{NDM-1} gene by S1 digestion of DNA, and then PFGE and direct probing of the gels with a radiolabelled bla_{NDM-1} gene. Each of the three groups of isolates typically carried several plasmids, with some isolates having up to eight plasmids (figure 3).

Indian isolates had bla_{NDM-1} exclusively on plasmids. Plasmids from the non-clonal Chennai isolates ranged from 50 kb to 350 kb, whereas those from the clonal *K pneumoniae* from Haryana were predominately either 118 kb (54%) or 50 kb (36%). The UK isolates had a more diverse range of plasmid sizes, 80 kb to greater than 500 kb. Three UK isolates also carried bla_{NDM-1} on their chromosome, suggesting in-situ movement of bla_{NDM-1}. There were many plasmids of identical size in isolates collected from India and the UK (data not shown), suggesting plasmid movement between bacterial isolates. In some isolates, bla_{NDM-1} was carried on more than one plasmid (figure 4).

47 isolates from Chennai (33) and Haryana (14) were randomly chosen for further investigation with PCR and DNA probing to verify the origin of replication (incompatibility type) for plasmids carrying bla_{NDM-1}.[11,22] Plasmids carrying bla_{NDM-1} from the 14 isolates from Haryana could not be typed. 13 of the 33 isolates from

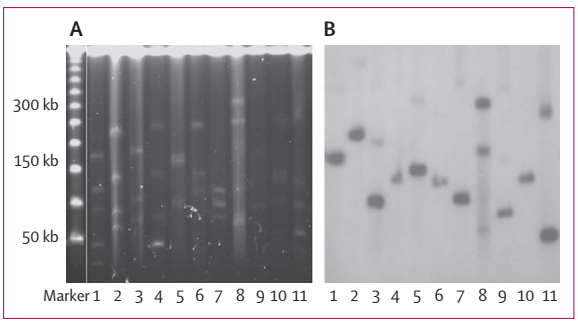

***Figure 4*: Hybridisation results of UK isolates with bla_{NDM-1}**
Pulsed-field gel of S1-treated plasmid DNA of UK isolates M15–M27 stained with ethidium bromide (A). Molecular weight marker is Lambda concatamer 50–1000 kb. The chromosome of each isolate is the bright band at the top of each lane and bright bands below are plasmids of various sizes. Autoradiogram of gel A probed with a bla_{NDM-1} showing individual or multiple plasmids in each strain carrying bla_{NDM-1} (B).

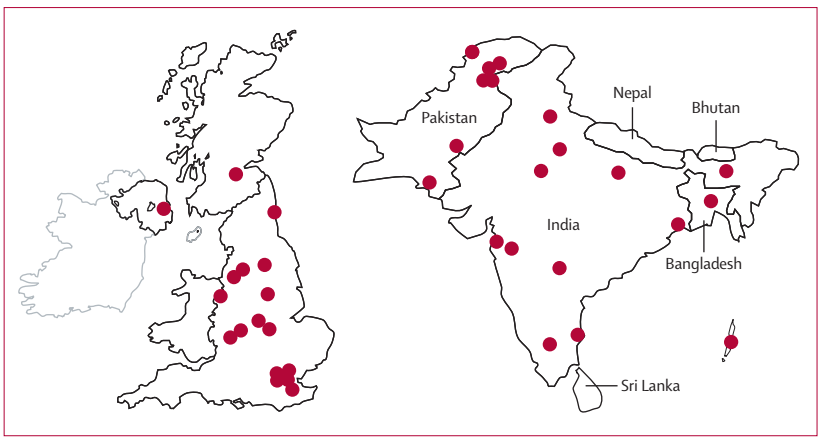

***Figure 5*: Distribution of NDM-1-producing Enterobacteriaceae strains in Bangladesh, Indian, Pakistan, and the UK**

Chennai carried bla_{NDM-1} on A/C-type plasmids and one bla_{NDM-1} positive plasmid was incompatibility type FI/FII. Similarly, when the 32 randomly selected UK isolates were assessed with the same methods, 22 carried A/C type plasmids. The other bla_{NDM-1} positive plasmids from India and the UK that were A/C and FI/FII negative could not be typed.

Transconjugants were created in *E coli* J53 from the 33 Chennai and 32 UK isolates; however, the isolates from Haryana did not produce transconjugates.[22] All transconjugants were shown by PCR to contain bla_{NDM-1}. We compared the sizes of the plasmids in the clinical strains with those of the transconjugants and, in about 10% of cases, the plasmid had altered in size during transfer. In most cases the plasmid had lost DNA but two of 102 had gained DNA during transfer.

In addition to the collections of isolates from Chennai and Haryana detailed above, we have confirmed by PCR alone the presence of genes encoding NDM-1 in carbapenem-resistant Enterobacteriaceae isolated from Guwahati, Mumbai, Varanasi, Bangalore, Pune, Kolkata, Hyderabad, Port Blair, and Delhi in India, eight cities (Charsadda, Faisalabad, Gujrat, Hafizabad, Karachi, Lahore, Rahim Yar Khan, and Sheikhupura) in Pakistan, and Dhaka in Bangladesh (figure 5) suggesting widespread dissemination.

Discussion

Enterobacteriaceae with NDM-1 carbapenemases are highly resistant to many antibiotic classes and potentially herald the end of treatment with β-lactams, fluoroquinolones, and aminoglycosides—the main antibiotic classes for the treatment of Gram-negative infections. Only a few isolates remained sensitive to individual aminoglycosides and aztreonam, perhaps owing to the loss of resistance genes (eg, those encoding aminoglycoside modifying enzymes, 16S rRNA methylases, or bla_{CMY-4}).[12,22] Most isolates remained susceptible to colistin and tigecycline.

Typing did not identify common strain types of *E coli* or *K pneumoniae* between the Indian subcontinent and the UK or between north and south India. Nevertheless, the NDM-1-positive *K pneumoniae* isolates from Haryana were clonal, suggesting that some strains could potentially cause outbreaks. Most bla_{NDM-1} positive plasmids were readily transferable and prone to rearrangement, losing or (more rarely) gaining DNA on transfer. This transmissibility and plasticity implies an alarming potential to spread and diversify among bacterial populations. Curiously, many of the plasmids were incompatibility A/C types—a group not commonly associated with multidrug-resistant phenotypes.

Although antibiotic resistance in China has been highlighted as a concern,[4] the rapid emergence of bla_{NDM-1} deserves equal attention. A recent editorial by Abdul Ghafur[26] highlights the widespread non-prescription use of antibiotics in India, leading to huge selection pressure, and predicts that the NDM-1 problem is likely to get substantially worse in the foreseeable future. This scenario is of great concern because there are few new anti-Gram-negative antibiotics in the pharmaceutical pipeline and none that are active against NDM-1 producers.[20] Even more disturbing is that most of the Indian isolates from Chennai and Haryana were from community-acquired infections, suggesting that bla_{NDM-1} is widespread in the environment.[27]

The introduction of NDM-1 into the UK is also very worrying and has prompted the release of a National Resistance Alert 3 notice by the Department of Health on the advice of the Health Protection Agency.[28] Given the historical links between India and the UK, that the UK is the first western country to register the widespread presence of NDM-1-positive bacteria is unsurprising. However, it is not the only country affected. In addition to the first isolate from Sweden, a NDM-1-positive *K pneumoniae* isolate was recovered from a patient who was an Australian resident of Indian origin and had visited Punjab in late 2009. The isolate was highly

resistant and carried bla_{NDM-1} on an incompatibility A/C type plasmid similar to those in India and the UK.

Several of the UK source patients had undergone elective, including cosmetic, surgery while visiting India or Pakistan. India also provides cosmetic surgery for other Europeans and Americans, and bla_{NDM-1} will likely spread worldwide. It is disturbing, in context, to read calls in the popular press for UK patients to opt for corrective surgery in India with the aim of saving the NHS money.[29] As our data show, such a proposal might ultimately cost the NHS substantially more than the short-term saving and we would strongly advise against such proposals. The potential for wider international spread of producers and for NDM-1-encoding plasmids to become endemic worldwide, are clear and frightening.

Contributors

TW, MT, and KK did MIC determinations, all genetic analysis, and plasmid profiling. Authors from HPA Centre for Infections undertook strain typing, MIC determinations, and follow-up for UK cases. DP proofread the manuscript and provided data from Australia. PK and MT (Chennai) and MS and UC (Haryana) characterised strains and provided clinical details. UR and AK provided prevalence data and demographics. All authors were involved in the compiling of the report and approved the final version.

Conflicts of interest

KK has received a travel grant from Wyeth. DML has received conference support from numerous pharmaceutical companies, and also holds shares in AstraZeneca, Merck, Pfizer, Dechra, and GlaxoSmithKline, and, as Enduring Attorney, manages further holdings in GlaxoSmithKline and Eco Animal Health. All other authors declare that they have no conflicts of interest.

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Efficacy of artesunate with sulfalene plus pyrimethamine versus praziquantel for treatment of *Schistosoma mansoni* in Kenyan children: an open-label randomised controlled trial

Charles O Obonyo, Erick M O Muok, Pauline N M Mwinzi

Summary

Background **Schistosomiasis is an important parasitic disease in Kenya. Decreasing susceptibility of schistosomes to praziquantel, the major drug used to reduce disease morbidity, has made assessment of new antischistosomal drugs a priority. We aimed to assess the safety and efficacy of an artesunate-based combination drug in the treatment of schistosomiasis.**

Methods **In this open-label randomised trial in Rarieda district of western Kenya, we enrolled school children (aged 6–15 years) who had *Schistosoma mansoni* infection according to duplicate Kato-Katz thick smears from a stool sample. Computer-generated block randomisation was used to assign children (1:1) to receive artesunate (100 mg) with sulfalene (also known as sulfamethoxypyrazine; 250 mg) plus pyrimethamine (12·5 mg) as one dose every 24 h for 3 days or one dose of praziquantel (40 mg/kg per day). The primary efficacy endpoint was the number of participants cured 28 days after treatment. Analysis was by intention to treat. This trial is registered with ClinicalTrials.gov, number NCT01054651.**

Results **Between October and December, 2009, 212 children were enrolled and assigned to receive artesunate with sulfalene plus pyrimethamine (n=106) or praziquantel (n=106). 69 patients (65%) were cured in the praziquantel treatment group compared with 15 (14%) in the artesunate with sulfalene plus pyrimethamine treatment group (p<0·0001). Adverse events were less common in patients taking artesunate with sulfalene plus pyrimethamine than in those taking praziquantel (22% [n=23] *vs* 49% [n=52], p<0·0001), and no drug-related serious adverse events occurred.**

Interpretation **The standard treatment with praziquantel is more effective than artesunate with sulfalene plus pyrimethamine in the treatment of children with *S mansoni* infection in western Kenya. Whether artemisinin-based combination therapy has a role in the treatment of schistosomiasis is unclear.**

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Introduction

Human schistosomiasis is a chronic debilitating disease caused by trematode worms of the genus *Schistosoma*. There are five species of schistosomes that can infect man, but *Schistosoma mansoni, Schistosoma haematobium*, and *Schistosoma japonicum* are the most important. Schistosomiasis is also one of the most prevalent parasitic infections in sub-Saharan Africa, where it is exceeded in prevalence only by malaria. An estimated 779 million people are at risk worldwide, and 200 million people are infected, of whom 120 million are symptomatic and 20 million have severe disease.[1–4] About 85% of people infected with schistosomiasis live in sub-Saharan Africa, where *S mansoni* and *S haematobium* are endemic.[4,5] In western Kenya more than 90% of infections are due to *S mansoni*, which is the focus of this report.[6]

In the absence of a vaccine, the global control strategy for schistosomiasis is to reduce morbidity by use of chemotherapy. Praziquantel is the drug of choice for treatment of all five species of human schistosomiasis, it can be given as a single dose and is affordable, safe, and effective. Praziquantel is effective against invasive stages and adult worms, but is ineffective against the young developing stages of the parasite (schistosomules).[7,8] Evidence from laboratory studies and field trials shows reduced susceptibility of schistosomes to praziquantel, usually manifesting as low rates of cure and egg reduction.[9–11] Such findings have raised concerns over the possible emergence of drug resistance or increased parasite tolerance to praziquantel.[12–14] Together with the danger of reliance on one drug for treatment and control, these results have prompted the scientific community to focus on the development of new drugs to replace praziquantel. Potential alternatives to praziquantel are scarce.

The artemisinin derivatives artesunate and artemether are promising candidates for treatment and chemoprophylaxis of schistosomiasis.[15,16] This class of drugs is the most potent for treatment of malaria, for which they are used in combination with another drug with a different mechanism of action to delay or prevent the development of drug resistance. Artemisinins have antischistosomal activity in vitro and in vivo.[17] Unlike praziquantel, they are specifically effective against young developing forms of the parasite.[7,8] Few trials have

investigated the effect of artemisinin derivatives (used either alone or in combination) in the treatment of schistosomiasis in Africa. In clinical trials of treatments for *S mansoni*[18] or *S haematobium* infection,[19] higher cure rates were recorded with a combination of artesunate and praziquantel than when either of these drugs was used alone. In one trial in Gabon, artesunate provided no benefit over praziquantel alone for treatment of *S haematobium* infection.[20]

The standard of care for malaria treatment is chemotherapy with a combination of antimalarial drugs that includes an artemisinin derivative. In areas where malaria and schistosomiasis coexist, whether widespread use of artemisinin-based combination therapies (ACTs) for malaria control will confer some benefit to schistosomiasis control is unclear. Data on the safety and efficacy of ACTs in the treatment of schistosomiasis are scarce. Evidence for the benefit of ACTs for treatment of schistosomiasis is from two antimalarial drug studies in which a few patients with malaria were also infected with either *S mansoni* (n=14 patients)[21] or *S haematobium* (n=27).[22] 1 month after treatment for malaria with artesunate with sulfadoxine plus pyrimethamine, artemether plus lumefantrine, or artesunate plus amodiaquine, more than 93% of participants were also cured of schistosomiasis. In two trials assessing ACTs compared with praziquantel for schistosomiasis, ACT was associated with significantly lower cure rates than was praziquantel: 58·6% (artesunate with sulfadoxine plus pyrimethamine) versus 100% in 92 Sudanese children infected with *S mansoni*;[23] and 43·9% (artesunate with sulfalene [also known as sulfamethoxypyrazine] plus pyrimethamine) versus 53·0% in 800 Malian children infected with *S haematobium*.[24] Artesunate with sulfalene plus pyrimethamine is an effective coformulated ACT with a simple dosing regimen (one tablet daily for 3 days) that is being introduced in several African countries for the treatment of uncomplicated malaria.[25]

Before consideration of the role of ACTs in schistosomiasis control, research is needed to establish the safety and efficacy of ACTs on different schistosome species in settings with various burdens of malaria and schistosomiasis. We present the results of an open-label randomised trial to assess the safety and efficacy of artesunate with sulfalene plus pyrimethamine versus praziquantel for the treatment of *S mansoni* infection in school children in Rarieda district of western Kenya. In this region, more than 96% of people are from the Luo ethnic group, many of whom are fishermen and small-scale subsistence farmers, and 87–95% of fishermen are infected with *S mansoni* (PNMM, unpublished). In neighbouring Asembo Bay, *S mansoni* was the most common schistosome species in school children, with a mean school prevalence of 16% (range 0–80%).[6] Groups at high risk of *S mansoni* infection in western Kenya include both adults and children.

Methods

Participants

Children (aged 6–15 years) were enrolled from five primary schools located in the Lwanda Kotieno region in Rarieda district, western Kenya, and treated between October and December, 2009. The schools were selected because of their accessibility and proximity (within 2 km) to Lake Victoria. The study area has year-round transmission of malaria, but our study was done during the season in which malaria transmission is low, with prevalence of *Plasmodium falciparum* malaria in screened children of 18%.

Eligible children were healthy at enrolment (as assessed by the study clinician), could provide sufficient volume of stool sample at enrolment and follow-up, had *S mansoni* infection (eggs excreted in stool), and could take oral treatment. Children were excluded if they weighed more than 50 kg, were pregnant or lactating at the time of the study, had co-infection with *P falciparum,* had a severe illness (such as cerebral cysticercosis), or had signs of severe malnutrition (defined as children with weight-to-height ratio below three SDs or 70% of the median of WHO's standardised reference values, or with bilateral pedal oedema). Other exclusion criteria included hypersensitivity to artesunate, use of sulfonamides or praziquantel, use of another antimalarial or anti-schistosomal drug during the study, or previous participation in the same study.

After a series of meetings held at schools with the parents or guardians of potential participants to explain the objectives, procedures, and potential risks of the study, children were approached through the school administration and parent–teacher associations. All parents and guardians who permitted participation of their children in the study received further detailed explanation of the study and provided written informed consent. Oral informed assent for screening and enrolment was obtained from all children. This study received approval from the ethical review committee of the Kenya Medical Research Institute (KEMRI scientific steering committee number 1582).

Randomisation and masking

Children were randomly assigned (1:1) to receive artesunate with sulfalene plus pyrimethamine or praziquantel. The randomisation sequence was computer generated in blocks of four by the study sponsor. The sequence was generated from a prespecified list of consecutive numbers assigned to the study treatments. Once eligibility of a child was confirmed, the study clinician assigned the child the next lowest study number to which a treatment group had been allocated. The study nurse gave the assigned study drug after confirming the treatment allocation from the randomisation sequence. The study nurse, who gave the study drug, and study participants were unmasked to treatment assignment, but the study

clinician and laboratory technicians assessing study outcomes were masked to treatment assignment throughout the study.

Procedures

At screening, every child provided a fresh stool sample (about 5 g), which was used to detect the presence of *S mansoni*, hookworms, *Ascaris lumbricoides*, and *Trichuris trichiura*. The study clinician took a medical history, did a physical examination, and checked the eligibility of every child. A capillary blood sample was taken by fingerprick for estimation of the haemoglobin concentration by use of a portable haemoglobinometer (HemoCue, Angelholm, Sweden). Thick blood films were Giemsa stained and examined for malaria parasites; to calculate malaria density, parasites and leucocytes were counted in the same field until 300 cells were counted. Children whose stools tested positive for *S mansoni* eggs and who met all eligibility criteria were invited to participate in the study.

At enrolment, every child provided a fresh stool sample to confirm *S mansoni* infection, and the study clinician took a standardised medical history, obtained a fingerprick capillary blood sample, and did a clinical examination, including weight (with a digital weighing scale) and temperature measurements and assessment of liver and spleen size. Participants assigned praziquantel (Biltricide, Bayer Healthcare, Leverkusen, Germany) received one dose of 40 mg/kg per day to the nearest half tablet (tablets of 600 mg). Participants assigned artesunate with sulfalene plus pyrimethamine (Co-Arinate Junior FDC [fixed-dose combination], Dafra Pharma, Turnhout, Belgium) received one tablet every 24 h for 3 days. Artesunate with sulfalene plus pyrimethamine is a fixed-dose combination of 100 mg artesunate with 250 mg sulfalene plus 12·5 mg pyrimethamine, packaged as three tablets per packet. Children received slices of bread and a glass of orange juice to improve bioavailability before drug ingestion.[26] All study drugs were given orally in the presence of the study nurse, who also recorded the exact time of drug ingestion. Children were observed for 1 h after taking the drug to ensure retention and check for any immediate adverse events. If vomiting occurred within 1 h of drug ingestion, a second full dose was given. Children with repeated vomiting were withdrawn from the study. Children with geohelminthic infections were treated with 400 mg albendazole. At the end of the study, all children who were still excreting *S mansoni* eggs (ie, not cured) were treated with praziquantel.

Children were followed up for 28 days. They were visited at school or at home during the first 2 days after enrolment to complete the adverse events questionnaire or to receive the second and third doses of artesunate with sulfalene plus pyrimethamine. At the follow-up visit at 28 days (range 26–30) after enrolment, children provided an early morning stool sample, and the study clinician took a medical history, did a clinical examination, and obtained a fingerprick blood sample. Participants who did not return for scheduled follow-up visits were visited at home.

The appearance (colour, consistency, and presence of worms, blood, mucus, or pus) of all stool samples was recorded. Duplicate slides were prepared from stool samples provided at enrolment and day 28, and were examined under a microscope independently by two experienced laboratory technicians. *S mansoni* and soil-transmitted helminth eggs were quantified by use of the Kato-Katz faecal thick-smear technique,[27] with a template containing about 41·7 mg of faeces when filled. The mean number of *S mansoni* eggs counted per slide was expressed as eggs per gram (EPG) of faeces. Intensity of infection was categorised on the basis of WHO classification as light (1–99 EPG), moderate (100–399 EPG), or heavy (≥400 EPG).[28] As a quality control measure for inter-observer variability, a third technician reread a random selection of 10% of slides, and all slides for which the readings varied by more than 20% between the two technicians.

Blood samples obtained at enrolment and on day 28 were used for haematological and biochemical assessments: haemoglobin concentration, white blood cell count, red blood cell count, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration, mean corpuscular haemoglobin, lymphocyte count, platelet count, alkaline phosphatase concentration, and creatinine concentration. Haematological measurements were done with Coulter Act Diff 2 Hematology Analyzer (Beckman Coulter, Brea, CA, USA), and biochemical tests were done with Reflotron Plus Chemistry Analyzer (Roche Diagnostics, Basel, Switzerland).

The primary outcome was the proportion of patients cured (cure rate) between enrolment and day 28. Cure was defined as the proportion of children infected with *S mansoni* at enrolment who were not excreting eggs at

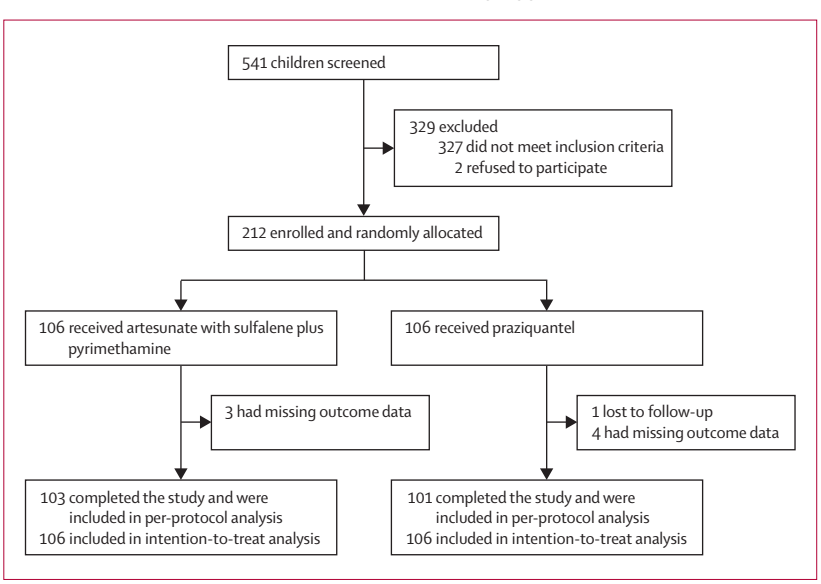

Figure: **Trial profile**

28 days after treatment. Secondary outcomes 28 days after treatment were the proportion of participants excreting *S mansoni* eggs, the egg reduction rate, the egg load (infection intensity), and the frequency of adverse events (mild, moderate, severe, or life threatening) in each treatment group. Egg reduction rate is the proportional reduction in number of *S mansoni* eggs in stool samples:

$$\left(1-\left[\frac{\text{geometric mean egg count after treatment}}{\text{geometric mean egg count at enrolment}}\right]\right)\times 100$$

An adverse event was defined as a sign, symptom, intercurrent illness, or abnormal laboratory finding that was not present at enrolment but occurred during follow-up. The relation of adverse events to study treatment was assessed by the study clinician (unmasked to assigned treatment at the end of the study), and designated as definite, probable, possible, unlikely, not related, or unknown. A serious adverse event was defined as an adverse event that was lethal, life threatening, disabling, or required hospital admission.

Statistical analysis

With 80% power and a two-sided α error of 5%, we calculated that 88 children would be needed in each treatment group to detect a significant difference in cure rates, assuming a cure rate of 80% with praziquantel and 95% with artesunate with sulfalene plus pyrimethamine. An additional 18 children (20%) per treatment group were included to allow for loss to follow-up and non-compliance.

Data collected from participants were recorded on paper-based case report forms, entered into computers by use of Epi Info (version 3.2.2), and analysed with SPSS for Windows (version 12.0). Primary and secondary outcomes, baseline measurements, and adverse events were analysed for all patients who received at least one dose of study drug whether they completed the study or not. The effect of infection intensity on cure rate was analysed per protocol (ie, all participants who completed the study).

For each treatment group, the cure rate was calculated as the proportion of participants randomly assigned to treatment who were cured at 28 days after treatment. Cure rates, stratified by sex, bodyweight (≤25 kg *vs* >25 kg), and infection intensity before treatment, were compared between treatment groups by use of the Pearson χ^2 test for contingency tables and were summarised as relative risks (RR) with 95% CIs. The relative risk was calculated as the proportion of participants cured on artesunate with sulfalene plus pyrimethamine divided by the proportion cured on praziquantel. Changes in continuous variables between day 0 and day 28 were examined with ANOVA. For all participants excreting *S mansoni* eggs at baseline and follow-up, the geometric mean egg count was calculated. Reduction in the egg count was expressed as a percentage. Two-sided p values of less than 0·05 were regarded as significant.

This trial is registered with the ClinicalTrials.gov, number NCT01054651.

	Praziquantel (n=106)	Artesunate with sulfalene plus pyrimethamine (n=106)
Male sex	55 (52%)	51 (48%)
Age (years)		
Mean (SD)	11·7 (1·6)	11·6 (1·7)
Median (range)	12 (8–15)	12 (7–15)
Primary schools		
Lenrose	10 (9%)	9 (8%)
Mirau	43 (41%)	42 (40%)
Naya	16 (15%)	18 (17%)
Nyaondo	15 (14%)	16 (15%)
Ranyala	22 (21%)	21 (20%)
Enlarged spleen	5 (5%)	12 (11%)
Bodyweight (kg)		
Mean (SD)	35·9 (7·9)	35·3 (7·1)
Median (range)	35 (22–50)	35 (19–50)
Haemoglobin concentration (g/L)	123 (18)	121 (23)
S mansoni infection intensity		
Light (1–99 EPG)	29 (27%)	25 (24%)
Moderate (100–399 EPG)	31 (29%)	31 (29%)
Heavy (≥400 EPG)	46 (43%)	50 (47%)
Geometric mean egg count on day 0 (EPG; 95% CI, range)	257·9 (198·2–335·8, 12–3270)	300·9 (231·6–390·9, 12–4800)

Data are number (%) or mean (SD), unless otherwise indicated. EPG=eggs per gram.

Table 1: **Baseline characteristics**

Role of the funding source

This study was funded by Dafra Pharma. The sponsors had no role in the study design, data collection, data analysis, data interpretation, writing of the report, or the decision to submit the paper for publication. The corresponding author had full access to all the data and responsibility for the decision to submit for publication.

Results

541 children were screened for eligibility, of whom 464 (86%) were infected with *S mansoni*. 212 children (39%) were enrolled and randomly assigned treatment, of whom only one child in the praziquantel group did not complete the study (figure). Stool samples from four children in the praziquantel group and three in the artesunate with sulfalene plus pyrimethamine group were not available on day 28. At baseline, age, sex, bodyweight, haemoglobin concentration, and geometric mean egg count were well balanced between treatment groups (table 1). 43 children (20%) had heavy schistosome infections of 1000 EPG or more: 20 (19%) treated with praziquantel, and 23 (22%) treated with artesunate with sulfalene plus pyrimethamine.

The cure rate in the artesunate with sulfalene plus pyrimethamine group was significantly lower than in the praziquantel group (RR 0·217, 95% CI 0·133–0·355; table 2). 128 children (60%) were not cured: 37 (35%) treated with praziquantel, and 91 (86%) given artesunate with sulfalene plus pyrimethamine. In children who were not cured, the geometric mean egg count was significantly higher and the egg reduction rate was significantly lower in the artesunate with sulfalene plus pyrimethamine group than in the praziquantel group (table 2).

The cure rate did not differ when stratified by sex for praziquantel (p=0·462) or artesunate with sulfalene plus pyrimethamine (p=0·216). For children weighing more than 25 kg, a significantly higher proportion were cured on praziquantel than on artesunate with sulfalene plus pyrimethamine, but for those weighing 25 kg or less few patients were cured in both groups (table 2).

In the per-protocol analysis, excluding children with missing outcome data, 22 (76%) of 29 patients given praziquantel were cured versus nine (36%) of 25 who took artesunate with sulfalene plus pyrimethamine (p=0·003) for light infections, 87% (26/30) versus 10% (3/30; p<0·0001) for moderate infections, and 50% (21/42) versus 6% (3/48; p<0·0001) for heavy infections. For children who were not cured, reduction in infection intensity was higher for recipients of praziquantel than for recipients of artesunate with sulfalene plus pyrimethamine recipients (table 3).

No severe or serious adverse events occurred within 1 h of treatment and no child needed immediate medical attention during the study. Both treatments were well tolerated. 75 children (35%) had adverse events: 52 (49%) on praziquantel, and 23 (22%) on artesunate with sulfalene plus pyrimethamine (p<0·0001). 95 events occurred, of which 59 (62%) were related to the study drug (table 4). The proportion of drug-related adverse events was higher for praziquantel recipients (46/95 [48%]) than for recipients of artesunate with sulfalene plus pyrimethamine (13/95 [14%]). In children on praziquantel, most adverse events were moderate (41/65 [63%]), and the proportion of moderate events was significantly higher than in children on artesunate with sulfalene plus pyrimethamine (11/30 [37%], p<0·0001). The most common adverse events were abdominal pain, diarrhoea, and headache in praziquantel recipients and abdominal pain, diarrhoea, and cough in recipients of artesunate with sulfalene plus pyrimethamine. Abdominal pain was significantly more common in children taking praziquantel than in those taking artesunate with sulfalene plus pyrimethamine (30/106 [28%] *vs* 6/106 [6%]; p<0·0001).

Haematological and biochemical measurements were similar between treatment groups at enrolment; but at day 28, white blood cell and lymphocyte counts were significantly different between treatment groups (table 5). Furthermore, in each treatment group, white blood cell count increased and lymphocyte count decreased significantly between enrolment and day 28.

	Praziquantel	Artesunate with sulfalene plus pyrimethamine	χ^2	p value
Cured	69/106 (65%)	15/106 (14%)	57·5	<0·0001
By bodyweight (kg)				
≤25	7/10 (70%)	2/6 (33%)	2·05	0·152
>25	62/96 (65%)	13/100 (13%)	55·2	<0·0001
By *S mansoni* infection intensity before treatment				
Light	22/29 (76%)	9/25 (36%)	8·73	0·003
Moderate	26/31 (84%)	3/31 (10%)	34·3	<0·0001
Heavy	21/46 (46%)	3/50 (6%)	20·1	<0·0001
Geometric mean egg count on day 28 (EPG; 95% CI)	41·1 (23·6–71·3)	195·5 (154·3–247·8)	30·2	<0·0001
Egg reduction rate by day 28	84·1%	35·0%	49·8	<0·0001

Data are n/N (%), unless otherwise indicated. EPG=eggs per gram.

Table 2: **Primary efficacy outcomes (intention-to-treat population)**

	Non-cure rate	Infection intensity after treatment		
		Light infection	Moderate infection	Heavy infection
Praziquantel				
Light	7/29 (24%)	5	1	1
Moderate	4/30 (13%)	4	0	0
Heavy	21/42 (50%)	17	2	2
All infections	32/101 (32%)	26	3	3
Artesunate with sulfalene plus pyrimethamine				
Light	16/25 (64%)	4	10	2
Moderate	27/30 (90%)	9	13	5
Heavy	45/48 (94%)	9	19	17
All infections	88/103 (85%)	22	42	24

Data are n/N (%) or number.

Table 3: **Intensity of *S mansoni* infection after treatment in children who were not cured, by infection intensity before treatment (per-protocol population)**

Discussion

In this randomised trial assessing treatment for *S mansoni* infection in western Kenya, we recorded a significantly lower cure rate with artesunate with sulfalene plus pyrimethamine than with praziquantel. In both treatment groups, cure was affected by bodyweight and infection intensity before treatment. With the treatment doses used, children weighing more than 25 kg were less likely to be cured with artesunate with sulfalene plus pyrimethamine than with praziquantel, and children in both treatment groups were more likely to be cured if they had light intensity infections than if they had heavy intensity infections before treatment. Both treatments were well tolerated, but the occurrence of adverse events was substantially lower in children who took artesunate with sulfalene plus pyrimethamine than in those who took praziquantel.

In our setting of high *S mansoni* prevalence (86%), praziquantel was significantly more efficacious than was artesunate with sulfalene plus pyrimethamine for treatment of *S mansoni* infection. The cure rate with

	Praziquantel		Artesunate with sulfalene plus pyrimethamine	
	Not related to drug	Related to drug	Not related to drug	Related to drug
Overall	19	46	17	13
Abdominal pain				
Mild	3	3	1	2
Moderate	2	22	1	2
Diarrhoea				
Mild	0	2	1	2
Moderate	0	4	0	3
Cough				
Mild	2	1	3	1
Moderate	1	0	1	1
Dizziness				
Mild	0	0	1	0
Headache				
Mild	2	4	3	1
Moderate	1	5	0	0
Itchy skin				
Mild	0	1	0	0
Moderate	0	1	0	0
Body weakness				
Mild	1	0	0	0
Moderate	1	0	1	0
Skin rash				
Mild	3	0	3	0
Moderate	2	1	1	0
Nausea				
Mild	0	0	0	1
Vomiting				
Mild	0	1	0	0
Moderate	0	1	1	0
Anorexia				
Mild	1	0	0	0

Table 4: **Adverse events**

praziquantel in our study (65%) is consistent with the range of 60–90% in other studies of praziquantel for *S mansoni* infection.[12,29,30] Similarly, the rate of 66% (range 36–82) was recorded in a longitudinal study of adult participants in western Kenya who were infected with *S mansoni* and received one praziquantel dose.[14] The egg reduction rate that we recorded with praziquantel (84·1%) is also consistent with previous studies.[12,14,31,32]

Although artesunate with sulfalene plus pyrimethamine is an effective artemisinin-based antimalarial drug combination,[21,25] it had a low cure rate (14%) for *S mansoni* infection. We also noted that the proportion of children with moderate infections before treatment had substantially increased after treatment with artesunate with sulfalene plus pyrimethamine (table 3). With a 3 day course of this treatment, we recorded substantially lower cure and egg reduction rates than those recorded in school children in Sudan (58·6% and 79%, respectively) in an area with low *S mansoni* prevalence (16%).[23] In a study of 800 children with *S haematobium* infection in Mali, the cure rate was 43·9% with artesunate with sulfalene plus pyrimethamine treatment given as a single dose of three tablets versus 53% with praziquantel treatment.[24] Both trials support our finding that ACTs have lower cure rates than does praziquantel.

In our study, artesunate with sulfalene plus pyrimethamine was associated with fewer adverse events, drug-related adverse events, and moderate adverse events, suggesting that this treatment was safer than was praziquantel. Our ability to confidently compare treatment safety in this trial was limited by the sample size and the duration of follow-up. However, our finding is consistent with results of a study in Mali in which children treated with artesunate with sulfalene plus pyrimethamine had fewer adverse events than did those treated with praziquantel.[24] In our study, a high proportion of the adverse events were classified as mild or moderate and resolved within 1–2 days of treatment. The most common side-effect of abdominal pain with praziquantel is consistent with the findings of other treatment studies.[30–35] Results of these studies also showed a positive correlation between worm burden and the intensity of adverse events, but we did not assess this correlation in our study.

The findings of our study raise important issues about the efficacy of artesunate with sulfalene plus pyrimethamine in western Kenya, and we propose possible explanations for the low cure rates recorded with this treatment. First, in almost all previous trials,[20,23,24] treatment with an artemisinin derivative was associated with significantly lower cure rates than was praziquantel, probably confirming that artemisinin derivatives have weaker antischistosomal activity against adult worms.[7] Although artemisinin derivatives have some effect on adult worms, they are most effective on juvenile stages of the worm, whereas praziquantel is most effective against adult worms, suggesting that the two drugs could have a complementary effect in combination.[36,37] Second, the prevalence and intensity of infection in our study population was much higher than in studies in Sudan or Mali.[23,24] At enrolment, 45% of children in our study had heavy schistosome infections, and 20% were excreting more than 1000 EPG. Cure rate after one treatment course is negatively correlated with intensity of infection for all schistosome species, with high cure rate usually associated with light infection.[13,30,32] In our study, 94% of children with heavy infection before treatment were not cured by artesunate with sulfalene plus pyrimethamine. Third, for malaria treatment with artesunate with sulfalene plus pyrimethamine, the fixed dose of artesunate (junior formulation) is 100 mg/kg per day for 3 days; because we recorded a significant difference in cure rates between the treatment groups in children weighing more than 25 kg, this dose might not be sufficient to have an effect on *S mansoni* in heavier children. Last, praziquantel was

taken as one dose whereas artesunate with sulfalene plus pyrimethamine required three doses, which might have affected compliance, but our study team supervised ingestion of all study drugs.

Our study addressed an important public health problem in a rigorous and ethical manner, with follow-up of all but one participant. However, our study was limited by the open-label design, timing of the efficacy assessment, and use of only one stool sample per child to diagnose *S mansoni* infection and assess efficacy. Masking of study participants to treatment assignment is essential to reduce bias from subjective reporting of outcomes, or enthusiasm for participation or follow-up. For example, the knowledge that a child was receiving a new medicine could have contributed to the reporting of adverse events. The optimum time for assessment of treatment efficacy in schistosomiasis trials has not been established.[38,39] We measured efficacy at 28 days after treatment in the context of continued disease transmission, although some researchers have suggested that 3 weeks might be best.[39] However, without validated molecular markers, differentiation between reinfection and recrudescence is difficult in settings that are highly endemic for schistosomiasis. Partly for logistical reasons, we requested one stool sample per child at enrolment and follow-up, with duplicate Kato-Katz smears, which might have reduced the sensitivity for diagnosis of infected children or classification of infection intensities.[40] However, the best number of stool samples for measurement of the effect of treatment is unknown, and most studies use one stool sample per child.[32,38] Standardisation of assessment protocols is needed to help interpret cure rates and aid comparison of findings between different research settings. For sample size calculation, we assumed a conservative cure rate of 95% with artesunate with sulfalene plus pyrimethamine on the basis of the study in Sudan in which a cure rate of 100% was recorded in 14 participants with both malaria and *S mansoni* infection.[21] Our study was therefore overpowered to detect the 51% difference in cure rates with the number of participants that we enrolled.

Our study has raised several questions. Most notably, do ACTs have any role in control of schistosomiasis? At present, ACTs should be restricted to malaria control because they are probably the last effective chemotherapy for malaria. A global effort to scale up implementation of ACTs to eliminate malaria in endemic areas will mean that more people will receive ACTs in future. The regions at risk of malaria and schistosomiasis closely overlap, so the effect of widespread ACT implementation on the burden of schistosomiasis needs to be monitored.[41] Patients with both schistosomiasis and malaria might benefit from one drug combination for both diseases. If ACTs were effective for schistosomiasis, they could prove useful in various situations, including in areas where schistosomiasis is endemic but malaria is not, in patients with both malaria and schistosomiasis, as chemoprophylaxis for travellers to areas endemic for schistosomiasis, and as second-line treatment in cases of praziquantel failure or unavailability. In some studies, combination of artesunate with praziquantel was associated with significantly higher cure rates than was praziquantel alone,[19,20] but this combination should not be recommended in areas endemic for malaria

	Praziquantel		Artesunate with sulfalene plus pyrimethamine		p value
	Number tested	Mean (SD)	Number tested	Mean (SD)	
White blood cell count (10⁹/L)					
Day 0	103	9·16 (4·48)	105	8·42 (2·73)	0·149
Day 28	95	10·6 (3·76)	89	12·9 (5·77)	0·001
p value	..	0·016	..	<0·0001	..
Lymphocyte count (10⁹/L)					
Day 0	102	50·9 (9·41)	105	49·3 (9·28)	0·221
Day 28	95	47·7 (10·6)	89	39·3 (12·3)	0·0001
p value	..	0·026	..	<0·0001	..
Red blood cell count (10⁹/L)					
Day 0	103	5·03 (0·71)	103	4·94 (0·63)	0·340
Day 28	95	5·08 (0·53)	89	4·97 (0·42)	0·146
p value	..	0·606	..	0·697	..
Haemoglobin concentration (g/L)					
Day 0	106	123 (18)	106	121 (23)	0·344
Day 28	95	124 (16)	89	123 (13)	0·867
p value	..	0·925	..	0·355	..
Haematocrit					
Day 0	106	0·392 (0·0587)	106	0·389 (0·0803)	0·691
Day 28	95	0·398 (0·0398)	89	0·395 (0·0798)	0·595
p value	..	0·418	..	0·462	..
Mean corpuscular volume (fL)					
Day 0	105	78·9 (11·3)	105	78·2 (10·1)	0·610
Day 28	95	78·8 (8·3)	89	79·8 (3·02)	0·366
p value	..	0·915	..	0·202	..
Mean corpuscular haemoglobin (pg)					
Day 0	103	24·9 (4·28)	104	24·8 (3·67)	0·789
Day 28	95	24·4 (2·44)	89	24·9 (3·02)	0·284
p value	..	0·317	..	0·845	..
Mean corpuscular haemoglobin concentration (g/L)					
Day 0	102	317 (49·1)	104	315 (18·5)	0·658
Day 28	95	309 (9·9)	89	311 (10·5)	0·239
p value	..	0·162	..	0·158	..
Platelet count (10⁹/L)					
Day 0	104	446·2 (121·2)	104	447·7 (135·6)	0·933
Day 28	95	453·8 (100·1)	88	449·8 (84·0)	0·768
p value	..	0·632	..	0·903	..
Creatinine concentration (μmol/L)					
Day 0	90	55·6 (6·94)	95	56·0 (6·84)	0·677
Day 28	78	55·6 (8·31)	77	56·1 (8·12)	0·698
p value	..	0·984	..	0·868	..
Alkaline phosphatase concentration (U/L)					
Day 0	70	230·9 (90·6)	70	223·9 (90·4)	0·648
Day 28	87	234·5 (69·3)	87	235·3 (77·8)	0·942
p value	..	0·778	..	0·393	..

Table 5: **Haematological and biochemical assessments**

because such monotherapy is unsuitable for people at risk of malaria. Furthermore, fear of accelerated development of antimalarial drug resistance means that mass community-based distribution of ACTs is unlikely.

The best dosing schedule of ACTs for treatment of schistosomiasis is unclear, which could have implications for the possible use of ACTs for treatment of schistosomiasis in areas where malaria is endemic. The efficacy of different ACTs on various species of *Schistosoma* could also be investigated. The benefit of ACTs combined with praziquantel (sequentially or simultaneously) for schistosomiasis is unknown, but this combination could target different developmental stages of the worm. This approach needs efficacy, safety, and economic assessment in future studies.

Contributors

COO developed the study protocol, oversaw data collection, analysed data, and drafted the report, which was commented on by all coauthors. EMOM contributed to development of the protocol, supervised data collection, contributed to data interpretation, and revised the report. PNMM contributed to development of the protocol, supervised laboratory staff, contributed to data interpretation, and revised the report.

Conflicts of interest

We declare that we have no conflicts of interest.

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Diagnostic approaches for paediatric tuberculosis by use of different specimen types, culture methods, and PCR: a prospective case-control study

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Summary

Background The diagnosis of pulmonary tuberculosis presents challenges in children because symptoms are non-specific, specimens are difficult to obtain, and cultures and smears of *Mycobacterium tuberculosis* are often negative. We assessed new diagnostic approaches for tuberculosis in children in a resource-poor country.

Methods Children with symptoms suggestive of pulmonary tuberculosis (cases) were enrolled from August, 2002, to January, 2007, at two hospitals in Lima, Peru. Age-matched and sex-matched healthy controls were enrolled from a low-income shanty town community in south Lima. Cases were grouped into moderate-risk and high-risk categories by Stegen-Toledo score. Two specimens of each type (gastric-aspirate, nasopharyngeal-aspirate, and stool specimens) taken from each case were examined for *M tuberculosis* by auramine smear microscopy, broth culture by microscopic-observation drug-susceptibility (MODS) technique, standard culture on Lowenstein-Jensen medium, and heminested IS6110 PCR. Specimens from controls consisted of one nasopharyngeal-aspirate and two stool samples, examined with the same techniques. This study is registered with ClinicalTrials.gov, number NCT00054769.

Findings 218 cases and 238 controls were enrolled. 22 (10%) cases had at least one positive *M tuberculosis* culture (from gastric aspirate in 22 cases, nasopharyngeal aspirate in 12 cases, and stool in four cases). Laboratory confirmation of tuberculosis was more frequent in cases at high risk for tuberculosis (21 [14·1%] of 149 cases with complete specimen collection were culture positive) than in cases at moderate risk for tuberculosis (one [1·6%] of 61). MODS was more sensitive than Lowenstein-Jensen culture, diagnosing 20 (90·9%) of 22 patients compared with 13 (59·1%) of 22 patients (p=0·015), and *M tuberculosis* isolation by MODS was faster than by Lowenstein-Jensen culture (mean 10 days, IQR 8–11, *vs* 25 days, 20–30; p=0·0001). All 22 culture-confirmed cases had at least one culture-positive gastric-aspirate specimen. *M tuberculosis* was isolated from the first gastric-aspirate specimen obtained in 16 (72·7%) of 22 cases, whereas in six (27·3%), only the second gastric-aspirate specimen was culture positive (37% greater yield by adding a second specimen). In cases at high risk for tuberculosis, positive results from one or both gastric-aspirate PCRs identified a subgroup with a 50% chance of having a positive culture (13 of 26 cases).

Interpretation Collection of duplicate gastric-aspirate specimens from high-risk children for MODS culture was the best available diagnostic test for pulmonary tuberculosis. PCR was insufficiently sensitive or specific for routine diagnostic use, but in high-risk children, duplicate gastric-aspirate PCR provided same-day identification of half of all culture-positive cases.

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Introduction

Although incidence of tuberculosis in adults has decreased in many high-burden countries, assessment of tuberculosis incidence in children remains difficult because of the absence of a dependable gold-standard test. Children account for an estimated 20% of the total tuberculosis caseload in high-incidence communities,[1–3] but most surveillance programmes only count acid-fast bacillus smear-positive cases, excluding more than 95% of presumptive tuberculosis cases in children younger than 12 years.[4] Adequate diagnosis of paediatric tuberculosis is difficult because of the lack of sputum production and paucity or absence of organisms in respiratory secretions, since tuberculosis bacilli typically remain confined to perihilar nodes that do not rupture into the bronchus.[5]

Technical and economic factors compound these diagnostic problems. The best available diagnostic tests are costly, whereas traditional methods are slow and insensitive. Even under optimum circumstances, *Mycobacterium tuberculosis* is isolated in fewer than 50% of children thought to have tuberculosis clinically.[6–10] Consequently, physicians often rely on poorly validated scoring systems.[11–13] Even in 2010, we still mainly depend on tools available since the 1950s to presumptively diagnose paediatric tuberculosis: purified protein derivative (PPD) skin test, chest radiography, history, and physical examination, usually without bacteriological confirmation.

The primary goal of this study was to assess new methods for diagnosis of pulmonary tuberculosis in

children, including a heminested PCR assay (for potential rapid results), improved culture methods, and alternative clinical specimens. We analysed three outcomes in Peruvian children with suspected pulmonary tuberculosis. First, we examined *M tuberculosis* recovery from non-invasive specimens, such as nasopharyngeal aspirates and stool samples, compared with gastric aspirates, from single and duplicate specimens. Our previous data suggest that nasopharyngeal aspirates are a useful and less invasive alternative to gastric aspirates for diagnosis of tuberculosis in children,[14] and stool is potentially useful for tuberculosis PCR. Second, we analysed the speed and sensitivity of *M tuberculosis* isolation by microscopic-observation drug-susceptibility (MODS) culture compared with conventional Lowenstein-Jensen culture, and determined the added benefit of repeated testing. This study allowed us to expand our previous experience of the MODS technique in children[15] to establish the extent to which the enhanced microbiological sensitivity of this technique translates into clinical usefulness for diagnosis of tuberculosis in children. Third, we assessed the sensitivity, specificity, and predictive values of a heminested PCR assay compared with *M tuberculosis* culture. PCR technology might be available in reference laboratories in resource-poor countries, providing results within 6 h that can be used in initial clinical decision making. An age-matched and sex-matched control group was included to determine test specificity.

Methods

Cases and controls

The study design is summarised in figure 1. This study included cases with clinical evidence suggestive of pulmonary tuberculosis and healthy controls matched for age and sex. Children with evidence of HIV infection or AIDS were excluded. Cases were enrolled between April, 2002, and January, 2007, at the Instituto de Salud del Niño and the Hospital Nacional Cayetano Heredia in Lima, Peru. Paediatricians assessing children with respiratory illness in clinics at the two participating hospitals referred patients that they suspected of having pulmonary tuberculosis to a physician co-investigator or study nurse, who then assessed their eligibility for participation. Cases were classified according to the clinical criteria of Stegen and colleagues[16] (ie, Jones score in Africa) for diagnosis of paediatric tuberculosis as revised by Toledo and colleagues,[17] with modifications. This Stegen-Toledo scoring system is widely used throughout Latin America, and criteria used to determine Stegen-Toledo score are shown in table 1. Inclusion criteria for cases were age 12 years or younger, Stegen-Toledo score of 5 points or more, and absence of antituberculosis therapy. Although positive *M tuberculosis* culture is one of the Stegen-Toledo criteria, culture results were a primary outcome of this study and were not available at enrolment; therefore, this criterion was not included in our modified scoring system for participant eligibility. Cases were classified as either at moderate risk (Stegen-Toledo score 5–6 points) or high risk (score ≥7 points) for tuberculosis.

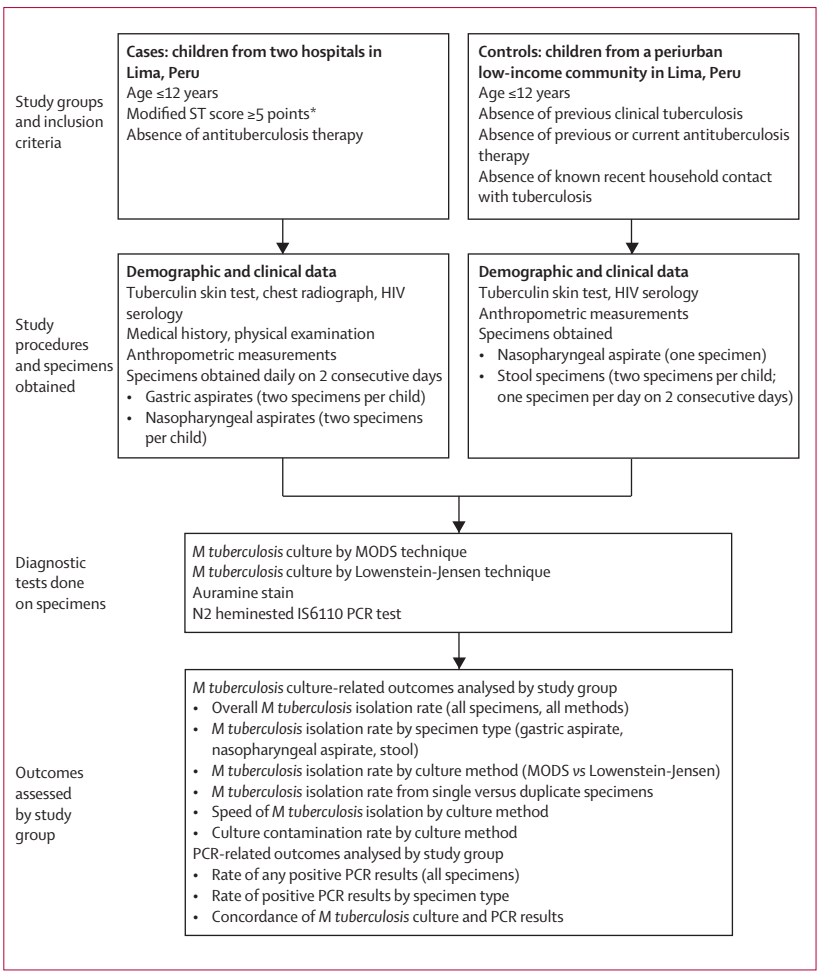

Figure 1: **Study design**
*Patients were classified according to the Stegen-Toledo (ST) score as moderate risk (score 5–6 points) and high risk (score ≥7 points). MODS=by microscopic-observation drug-susceptibility.

Empiric treatment for tuberculosis was given according to standard guidelines of WHO and the Peruvian Ministry of Health. Therapeutic decisions including inpatient versus outpatient management and treatment protocols used were established by local hospital physicians. The study procedures did not necessitate admission to hospital.

Age-matched and sex-matched controls were enrolled on a continuing basis from the Pampas de San Juan, a low-income shanty town community in south Lima that is within the area served by one of the hospitals from which cases were recruited. Inclusion criteria were an absence of chronic cough, fever, or evidence of pulmonary disease; absence of previous clinical tuberculosis; no previous or current treatment for tuberculosis; and absence of recent (past 2 months) household contacts with known or suspected pulmonary tuberculosis.

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	High-risk cases (ST score ≥7; n=153)	Moderate-risk cases (ST score 5–6; n=65)	p value*	Controls (n=238)	p value†
Modified ST criteria‡					
Primary lesion by radiography (4 points)§	34/153 (22·2%)	0/65	<0·0001	NA	NA
Tuberculin skin test positive (>10 mm; 3 points)	144/152 (94·7%)	53/64 (82·8%)	0·0047	13/235 (5·5%)	<0·0001
Contact with patient with tuberculosis in past 2 years (2 points)	141/153 (92·2%)	42/65 (64·6%)	<0·0001	0/235	<0·0001
Suggestive radiograph (2 points)§	118/153 (77·1%)	26/65 (40·0%)	<0·0001	NA	NA
Cough >2 weeks (2 points)	80/153 (52·3%)	21/65 (32·3%)	0·0068	NA	NA
Admitted to hospital	13/153 (8·5%)	7/65 (10·8%)	0·5949	NA	NA
Income¶					
<400 Peruvian soles	68/141 (48·2%)	28/59 (47·5%)	..	190/238 (79·8%)	..
≥400 Peruvian soles	73/141 (51·8%)	31/59 (52·5%)	0·9209	48/238 (20·2%)	<0·0001
Mother's education					
Primary or less	34/153 (22·2%)	10/64 (15·6%)	..	82/238 (34·5%)	..
Secondary or more	119/153 (77·8%)	54/64 (84·4%)	0·2704	156/238 (65·5%)	0·0098
BCG vaccination					
Yes	124/150 (82·7%)	55/64 (85·9%)	..	216/235 (91·9%)	..
No	26/150 (17·3%)	9/64 (14·1%)	0·5537	19/235 (8·1%)	0·0059
ST score	7 (7–14)	5 (5–6)	<0·0001	NA	NA
Tuberculin skin test size (mm)	15 (12–20)	13 (10–18)	0·0301	0 (0–2)	<0·0001
Age (years)	3 (0–12)	4 (0–11)	0·7593	4 (0–12)	0·7134
Sex (male)	78/153 (51·0%)	34/65 (52·3%)	0·8577	128/238 (53·8%)	0·5882
Percentage weight-for-age	97% (88–106)	94% (85–104)	0·2244	94% (87–104)	0·0635
Percentage height-for-age	98% (95–101)	98% (95–103)	0·5555	96% (93–99)	<0·0001

Data are number (%) or median (range). For case groups, the number of cases positive for each of the factors used to determine the modified Stegen-Toledo (ST) criteria are reported. NA=not applicable. *Comparison of high-risk and moderate-risk groups. †Comparison of high-risk group and control group. ‡Standard interpretation for ST score is high risk for tuberculosis ≥7 points; moderate risk for tuberculosis 5–6 points; low risk for tuberculosis 3–4 points; unlikely tuberculosis 0–2 points. §In each case points were assigned for only one of these radiographic criteria. ¶400 Peruvian soles=US$142 or £93 (date of conversion July 14, 2010).

Table 1: **Demographic features and modified Stegen-Toledo criteria[18,19]**

Cases and controls were screened for HIV infection by duplicate commercial assays. HIV DNA PCR tests were done in children younger than 18 months old. Screening for HIV infection was requested, but not required if parents refused the test. Cases and controls who declined HIV testing were included and classified as HIV negative if they had no history of HIV exposure and no clinical evidence of HIV/AIDS.

Written informed consent was obtained from all cases and controls or their parents or guardians. This research adhered to human experimentation guidelines of the US Department of Health and Human Services. The protocol and consent forms were approved by the Institutional Review Boards of Tulane Medical Centre, Johns Hopkins Bloomberg School of Public Health, Asociación Benéfica PRISMA, the US Naval Medical Research Center Detachment (Lima, Peru), Hospital Nacional Cayetano Heredia, and the Instituto Nacional de Salud del Niño (Lima, Peru).

Data collection

Demographic and clinical data, including tuberculin skin test results, were obtained from cases and controls. Data collected from cases consisted of medical history, physical examination findings, and a chest radiograph read by a paediatric radiologist. Specimens obtained from cases for tuberculosis cultures and PCR were gastric aspirates, nasopharyngeal aspirates, and stool samples.

Gastric aspirates were obtained on two successive early mornings (0600–0700 h) by brief (<10 min) nasogastric intubation after an overnight fast. The volume of gastric aspirates was augmented as needed by injecting 5 mL sterile water and aspirating back. Nasopharyngeal aspirates were obtained daily for 2 days by inserting a soft flexible nasopharyngeal tube into the nasopharynx, lavaging with 5 mL saline solution, and aspirating with an electrical suction device or hand-held aspirator. The nasopharyngeal aspirate procedure induces a cough and sputum production, which is then aspirated from the nasopharynx. Stool specimens were collected daily for 2 days.

All specimens from cases were obtained in the hospital within 5 days of enrolment, before starting antituberculosis treatment. Blood samples were taken from cases for measurement of serum albumin, as an indicator of nutritional status.

Specimens taken from controls for tuberculosis cultures and PCR assays were one nasopharyngeal-aspirate and two stool samples. No gastric-aspirate specimens were obtained from controls because of the invasive nature of the test, and only one nasopharyngeal aspirate was taken

	High-risk cases (ST score ≥7)	Moderate-risk cases (ST score 5–6)	Adjusted odds ratio (95% CI)*	p value*	Controls	Adjusted odds ratio (95% CI)*	p value*
Culture							
Any specimen							
Positive	21/149 (14·1%)	1/61 (1·6%)	12·1 (1·5–97·1)	0·0189	0/238	..	<0·0001
Negative	128/149 (85·9%)	60/61 (98·4%)	Reference	..	238/238 (100·0%)	..	..
Nasopharyngeal aspirate							
Positive	11/152 (7·2%)	1/63 (1·6%)	6·2 (0·7–53·6)	0·0974	0/208	..	<0·0001
Negative	141/152 (92·8%)	62/63 (98·4%)	Reference	..	208/208 (100·0%)	..	..
Gastric aspirate							
Positive	21/152 (13·8%)	1/64 (1·6%)	13·1 (1·6–105·6)	0·0156	..	..	..
Negative	131/152 (86·2%)	63/64 (98·4%)	Reference	..	..	..	..
Stool sample							
Positive	3/148 (2·0%)	1/62 (1·6%)	1·3 (0·1–12·8)	0·8293	0/230	..	<0·0001
Negative	145/148 (98·0%)	61/62 (98·4%)	Reference	..	230/230 (100·0%)	..	..
PCR							
Any specimen							
Positive	40/152 (26·3%)	15/64 (23·4%)	1·2 (0·6–2·4)	0·6161	15/227 (6·6%)	5·0 (2·6–9·9)	<0·0001
Negative	112/152 (73·7%)	49/64 (76·6%)	Reference	..	212/227 (93·4%)	Reference	..
Nasopharyngeal aspirate							
Positive	22/153 (14·4%)	4/65 (6·2%)	2·8 (0·9–8·6	0·0763	5/228 (2·2%)	7·2 (2·5–20·2)	0·0002
Negative	131/153 (85·6%)	61/65 (93·8%)	Reference	..	223/228 (97·8%)	Reference	..
Gastric aspirate							
Positive	27/152 (17·8%)	8/65 (12·3%)	1·6 (0·7–3·8)	0·2622	..	..	..
Negative	125/152 (82·2%)	57/65 (87·7%)	Reference	..	..	..	..
Stool sample							
Positive	13/150 (8·7%)	4/64 (6·2%)	1·5 (0·5–5·1)	0·5067	10/237 (4·2%)	2·4 (1·0–5·9)	0·0578
Negative	137/150 (91·3%)	60/64 (93·8%)	Reference	..	227/237 (95·8%)	Reference	..

Data are number of children (%) with at least one positive *Mycobacterium tuberculosis* culture or PCR result in a clinical specimen, grouped by type of specimen. Analyses by specimen type limited to cases with complete data for specimens of the indicated type by the method used (culture or PCR). Any specimen indicates all specimen types combined (for cases, limited to those with two gastric-aspirate, nasopharyngeal-aspirate, and stool specimens; for controls, limited to those with one nasopharyngeal-aspirate and two stool specimens). *For the comparison between the high-risk group and the moderate-risk group, the odds ratios and p values were adjusted for confounding differences in patient status (inpatient *vs* outpatient). For the comparison between the high-risk group and the control group, the odds ratios and p values were adjusted for confounding differences in income and mother's education.

Table 2: **PCR and culture results in high-risk and moderate-risk cases and controls**

from each control because this test is unpleasant for some children, and repeat nasopharyngeal-aspirate testing of controls was not acceptable to many parents.

Tuberculosis diagnostic techniques

Specimens were decontaminated with 0·5% N-acetyl-L-cysteine, 2% sodium hydroxide, and 1·45% sodium citrate, and the centrifuged pellet was resuspended in 2 mL 0·2% bovine serum albumin.[18] Before decontamination, stool samples were prepared by suspension of 0·1 g in 6 mL phosphate buffered saline, homogenisation, and settling for 10 min to separate, after which the supernatant was processed. This stool processing technique was shown in pilot work to provide the best possible compromise between detection sensitivity versus culture contamination. Specimens were tested by four methods. To reduce the risk of bias, samples were obtained from cases and randomly selected matched controls, and technicians were not aware of clinical characteristics, identity of participant groups, or the results of other tests.

In the first method, microscopic-observation drug-susceptibility (MODS),[19,20] 500 µL of each decontaminated specimen was inoculated into modified Middlebrook 7H9 media and separated into four 1·2 mL samples that were cultured in a sterile 24-well plate. Plates were placed in a plastic resealable bag, incubated at 37°C, and examined every other day without opening for up to 30 days by inverted light microscopy. Presumptive *M tuberculosis* isolates with cording morphology were reported as positive and all were subsequently confirmed by heminested IS6110 *M tuberculosis* PCR. Direct concurrent drug susceptibility testing was not done.

The second method was Lowenstein-Jensen agar culture. 250 µL of each decontaminated specimen was inoculated onto a Lowenstein-Jensen slant, incubated at 37°C, and examined two times a week from the first to eighth week after inoculation.

In the third test, auramine stain (smear test), two drops of each decontaminated specimen were dried on a microscope slide, stained with 0·1% auramine O (Sigma

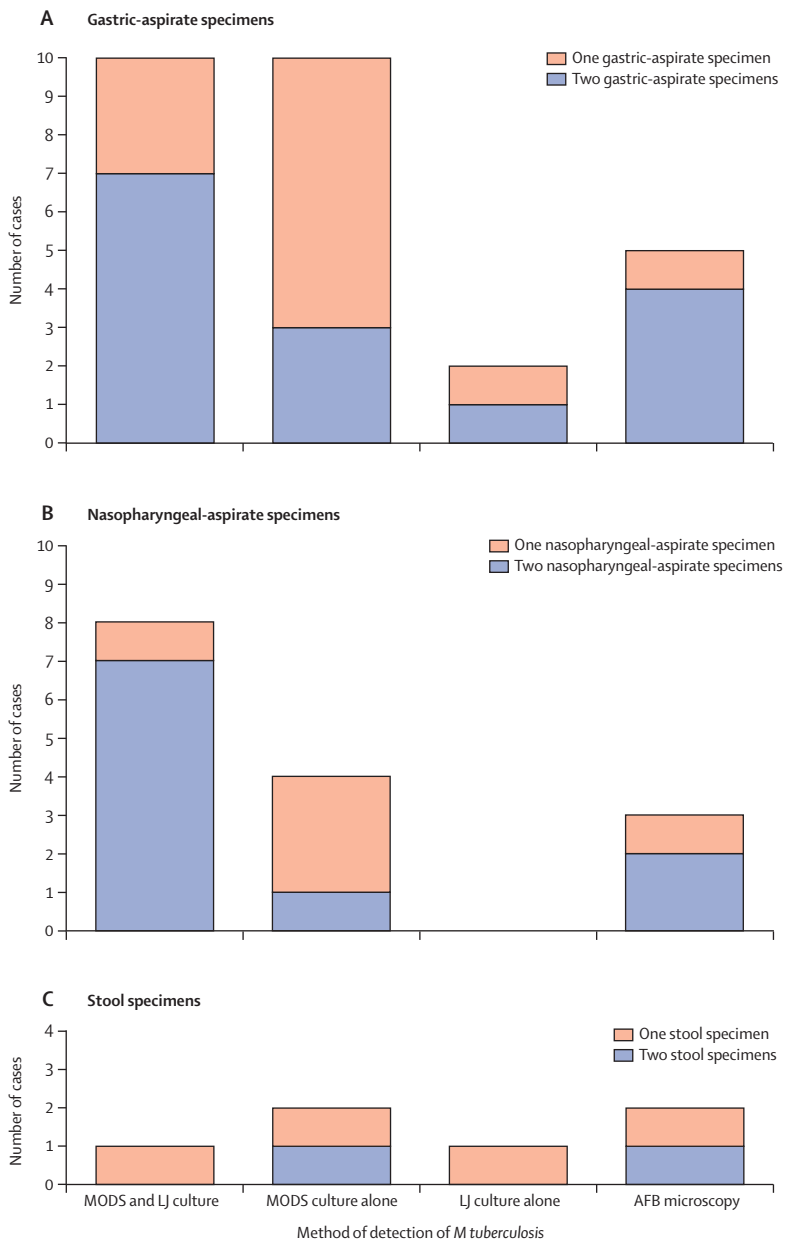

Figure 2: **Number of cases of tuberculosis detected by culture and microscopy, by specimen**
MODS=microscopic-observation drug-susceptibility. LJ= Lowenstein-Jensen agar. AFB=acid-fast bacilli.

Aldrich, St Louis, MO, USA), and examined at 100-times magnification. Positive tests had five or more bacilli counted in 300 fields.

N2 IS6110 PCR procedure (referred to as PCR testing) was the fourth method.[21] DNA was extracted from 500 µL of each decontaminated specimen, and two consecutive nested IS6110 PCRs were done with outer and inner primers. To reduce the risk of false-positive PCR reactions, separate rooms were used for DNA extraction, PCR mix preparation, amplification, and electrophoresis with protective clothing. Every heminested PCR assay included positive controls of genomic *M tuberculosis* DNA and negative controls of water added to the PCR reagents in place of the sample DNA.

Statistical analysis

We calculated a sample size to detect a 15% difference in sensitivity of detection of *M tuberculosis* between two tests done in the same individuals (eg, gastric aspirates *vs* nasopharyngeal aspirates) or between detection methods (eg, MODS *vs* Lowenstein-Jensen culture; culture *vs* PCR) or between cases and controls. On the basis of 80% power and an α error of p=0·05, 189 cases and 189 controls were required. To compensate for non-evaluable individuals (eg, second sample not provided), we increased the study sample by 10% to a target sample of 210–220 cases and an equal number of controls.

Demographic and clinical data, and culture and PCR results were compared in cases and controls by Stegen-Toledo risk group (moderate risk *vs* high risk). Culture and PCR results were compared by sample and by person. A negative culture result was deemed valid (ie, readable and contamination-free) if at least one valid Lowenstein-Jensen and one valid MODS result was available. A case with negative cultures was valid if all three types of specimen (gastric aspirate, nasopharyngeal aspirate, and stool sample) had valid results for duplicate specimens. For controls, nasopharyngeal-aspirate and stool cultures required valid results for inclusion in the analysis. χ^2 and McNemar's tests were used for categorical variables; two-tailed *t* test or Wilcoxon rank sum test were used for continuous variables. Multiple logistic regression was used to adjust for potentially confounding variables. Recovery rates for *M tuberculosis* were compared by culture method for cases and specimens, grouped by specimen type (gastric aspirate, nasopharyngeal aspirate, stool sample) and by auramine stain result. Cases with at least one clinical specimen that was culture positive for *M tuberculosis* by any method were included in the culture-positive group. Data analysis was done with STATA version 11 and EpiInfo version 6.

This study is registered with ClinicalTrials.gov, number NCT00054769.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study, had final responsibility for the decision to submit for publication, and takes responsibility for the data and the accuracy of the data analysis.

Results

The study population consisted of 456 children with complete demographic information and, for most participants, complete laboratory data, with 218 presumptive tuberculosis cases (20 [9%] inpatients;

198 [91%] outpatients) and 238 controls. 81 potential cases were not enrolled because participation was declined by their parents, and three controls without valid Lowenstein-Jensen results were excluded. These children were similar in age and sex distribution to enrolled participants.

Demographic characteristics of cases and controls and distribution of the characteristics used to determine the modified Stegen-Toledo criteria are shown in table 1. 153 (70%) cases had a Stegen-Toledo score of 7 points or more (high risk for pulmonary tuberculosis), and 65 (30%) cases had scores of 5–6 points (moderate risk). Most cases at high risk for tuberculosis had abnormal results on chest radiograph, and the most common findings were interstitial markings (83 [54%] cases) and hilar adenopathy (67 [44%]). A primary Ghon complex was present in 34 (22%) high-risk cases. Among moderate-risk cases, 26 (40%) had suggestive findings on chest radiograph (all interstitial markings or hilar adenopathy), and none had a primary Ghon complex.

Rate of BCG vaccination was lower in cases than in controls (p=0·006). Compared with high-risk cases, controls had lower levels of income and maternal education. HIV test results were negative in 179 (82%) cases and the test was declined in 39 (18%) cases. No cases or controls were HIV positive on the basis of screening tests.

22 (10%) cases had at least one positive *M tuberculosis* culture, on the basis of MODS and Lowenstein-Jensen results combined. *M tuberculosis* was isolated from gastric-aspirate specimens in all 22 cases, nasopharyngeal-aspirate specimens in 12 cases, and stool specimens in four cases. In all, 58 culture-positive specimens were obtained from cases, consisting of 33 gastric aspirates, 20 nasopharyngeal aspirates, and five stool specimens. 17 (29%) of these 58 specimens were positive on auramine microscopy.

There was a significant association between high-risk group and positive tuberculosis culture results (table 2). One (1·6%) of 61 moderate-risk cases with complete specimen collection was culture positive compared with 21 (14·1%) of 149 high-risk cases. The modified Stegen-Toledo scores in cases with a positive culture for *M tuberculosis* (median 9 points, range 6–13) were significantly greater than in culture-negative cases (7 points, 5–14; p<0·0001). 13 (8·5%) of 153 high-risk cases and seven (10·8%) of 65 moderate-risk cases were admitted to hospital, and hospital admission was associated with higher rates of positive *M tuberculosis* cultures (seven [36·8%] of 19 inpatient cases *vs* 15 [7·9%] of 191 outpatient cases; odds ratio 6·8, 95% CI 2·3–19·9, p<0·0001). None of the samples from the 238 controls were *M tuberculosis* culture positive.

Culture-positive cases were similar to culture-negative cases in terms of sex distribution and age. Median serum albumin concentrations (42 g/L [IQR 37–43] *vs* 45 g/L [43–47]) and prevalence of serum albumin lower than 45 g/L (17 [81%] of 21 *vs* 74 [40%] of 184) were both significantly different between culture-positive and culture-negative cases (p=0·0001 and p=0·0015, respectively).

	Culture positive (n=21)	Culture negative (n=128)	Sensitivity	Specificity	Positive predictive value	Negative predictive value	κ
Any specimen							
PCR positive	13 (62%)	26 (20%)*	61·9%	79·7%	33·3	92·7	0·306
PCR negative	8 (38%)	102 (80%)	..	..	..	..	..
Nasopharyngeal aspirate							
PCR positive	9 (43%)	13 (10%)*	42·9%	89·8%	40·9	90·6	0·321
PCR negative	12 (57%)	115 (90%)	..	..	..	..	..
Gastric aspirate							
PCR positive	13 (62%)	13 (10%)*	61·9%	89·8%	50·0	93·5	0·471
PCR negative	8 (38%)	115 (90%)	..	..	..	..	..
Stool sample							
PCR positive	4 (20%)‡	9 (7%)†	20·0%	93·0%	30·8	88·1	0·152
PCR negative	16 (80%)‡	119 (93%)	..	..	..	..	..

*p≤0·0001 for comparison of culture-positive and culture-negative groups. †p=0·0567 for comparison of culture-positive and culture-negative groups. ‡Stool samples were unavailable from one culture-positive case.

Table 3: **Agreement of PCR and culture results, and predictive values of PCR for detection of children with at least one positive culture among high-risk cases**

Mean time from sample processing to isolation of *M tuberculosis* was significantly shorter for MODS cultures (mean 10 days, IQR 8–11) than for Lowenstein-Jensen cultures (25 days, 20–30; p=0·0001). MODS cultures were not interpretable because of bacterial or fungal contamination less often than were Lowenstein-Jensen cultures (14 [1·1%] of 1292 *vs* 102 [7·9%] of 1292, p<0·0001) and there were no significant differences in contamination rates between stool, nasopharyngeal-aspirate and gastric-aspirate specimens. MODS was more sensitive than was Lowenstein-Jensen culture, diagnosing 20 (90·9%) of 22 patients compared with 13 (59·1%) of 22 patients (p=0·015). Nine (41%) of the 22 culture-positive cases were detected by MODS culture only, two (9%) cases by Lowenstein-Jensen culture only, and in 11 (50%) cases at least one specimen was culture-positive by both methods (nine of 13, or 70% more cases detected by MODS that were missed by Lowenstein-Jensen culture).

As in our preliminary analysis,[15] the MODS technique was more sensitive than were the other tests. Here we extend previous results to establish how this test performance affected patient diagnosis. Figure 2 shows recovery of *M tuberculosis* from the 22 culture-positive cases by culture method and specimen type. Although all 22 cases were culture positive in at least one gastric-aspirate specimen, 11 of these 22 cases only had positive cultures from one of two gastric-aspirate specimens (figure 2). When results were analysed on the basis of the order of collection for duplicate specimens, *M tuberculosis* was isolated from the first gastric-aspirate specimen

obtained in 16 (72·7%) of 22 cases, whereas in six (27·3%), only the second gastric-aspirate specimen was culture positive (37% greater yield by adding a second specimen). Addition of a second nasopharyngeal-aspirate specimen increased yield by 50%.

Table 2 shows PCR results and culture results for high-risk and moderate-risk cases and controls. The proportions of patients with at least one positive PCR result were similar between high-risk and moderate-risk cases, and both groups had significantly higher proportions of positive PCR compared with the control group (p=0·0001).

15 of 238 controls had at least one positive PCR result (five from nasopharyngeal-aspirate specimens; ten from stool samples). No controls had more than one positive PCR result. 14 controls had a positive PPD skin test, but only one of these 14 children also had a positive PCR result (nasopharyngeal-aspirate specimen). All controls with a positive PPD were assessed by a physician, examined by chest radiography, assessed not to have tuberculous disease, and given preventive therapy. None of these controls with positive PPDs or PCRs developed further evidence of clinical tuberculosis during the year after enrolment. Controls had twice as many stool samples as nasopharyngeal aspirate samples per person, and since ten (4·2%) controls had a PCR-positive stool and five (2·1%) had a PCR-positive nasopharyngeal aspirate, this suggests that the rate of false-positive PCR results in any individual specimen (from these highly selected children with low risk of tuberculosis) was about 2%.

Table 3 compares PCR results in the 21 culture-positive cases compared with the 128 culture-negative cases with complete culture and PCR data for all specimens. Culture-positive cases were three times more likely to have at least one PCR-positive specimen than were culture-negative cases. Similar results were noted in analyses limited to each specimen type. To assess the potential for use of PCR to identify high-risk paediatric cases likely to be culture positive, PCR sensitivity, specificity, and predictive values were analysed with culture as the reference standard (table 3). In this high-risk group, a positive PCR from a gastric aspirate was associated with a positive predictive value (for a positive culture result) of 50%. Sensitivity of PCR in this subgroup was 62% for gastric aspirates and for any specimen, but false-positive PCR results (with culture as the reference standard) equalled or exceeded true positives for all specimen types. However, both specificity and positive predictive values must be reduced by the limited sensitivity of culture as a reference standard.

Discussion

This study presents a direct comparison of culture methods and PCR, with different specimen types for both approaches, for diagnosis of paediatric tuberculosis in a resource-poor area. In these Peruvian children, MODS roughly doubled the diagnostic sensitivity of culture and halved the time required compared with traditional Lowenstein-Jensen culture. 70% more cases were diagnosed as having culture-positive tuberculosis by MODS compared with Lowenstein-Jensen culture, and all specimens from these additional cases were auramine smear negative. The speed and sensitivity of the MODS technique results from the use of culture broth with microscopic detection of positive cultures before they are large enough to be visible to the naked eye and the capacity of this technique to culture a large volume of each clinical specimen.[20] This increased diagnostic yield in children with culture-proven tuberculosis by addition of MODS greatly exceeds the 10–20% additional yield seen in high-risk Peruvian adults,[20] as would be expected for diagnostic samples from children that usually contain few mycobacteria. Our results lend support to WHO recommendations that suggest that liquid tuberculosis culture techniques are superior to agar-based techniques.[18] MODS performed better than did Lowenstein-Jensen culture for auramine-negative specimens, which include most culture-positive paediatric specimens.

M tuberculosis recovery by culture from gastric aspirates (22 of 22 cases) was clearly superior to recovery from stool samples (four of 22 cases) and from nasopharyngeal aspirates (12 of 22 cases). The 37% additional yield of culture-positive patients identified by a second gastric aspirate greatly exceeded the 5–10% additional yield from a second sputum culture in Peruvian adults.[20] One explanation of this major incremental benefit from a second specimen in children is that adults tend to be already smear positive and with high bacillary loads at detection, whereas children with paucibacillary disease benefit much more from a sensitive method like MODS. In almost half the patients studied, gastric aspirates were the only specimens that were culture positive, and in five of these cases only one specimen was culture positive, showing the importance of testing at least duplicate specimens.

We noted that high-risk clinical scores were associated with higher likelihood of a positive *M tuberculosis* culture. Larger numbers of moderate-risk cases would be useful to substantiate this finding, since only one moderate-risk culture-positive case was identified in this study. Although this result suggests that the Stegen-Toledo score might correlate with rates of isolation of *M tuberculosis* in children, it also emphasises the fact that, even with intensive culture methods, 85% of high-risk cases were still culture negative. Determining how many of these children have culture-negative pulmonary tuberculosis is difficult, but data from cases with positive cultures (eg, the predominance of smear-negative specimens, the high proportion of cases confirmed by a single MODS culture-positive gastric aspirate) suggest that many of these cases are tuberculosis. Other new

techniques, such as induced sputum with increased recovery of *M tuberculosis* by culture in African children,[22,23] might help to confirm these suspicions.

The N2 IS6110 PCR assay was positive more often in subgroups with greater clinical suspicion of tuberculosis or positive cultures than in those at low risk or with negative cultures. Our findings corroborate those of a previous retrospective study of IS6110 stool PCR,[24] and this larger prospective study also shows that sensitivity of PCR to detect culture-positive children was greater for both nasopharyngeal aspirates and gastric aspirates than with stool specimens. However, several healthy controls had positive PCR results despite having no evidence of tuberculosis exposure or disease, implying that these were false positives, occurring in about 2% of specimens despite extensive precautions to prevent PCR contamination. These results raise the possibility that the PCR assay is detecting the acquisition or presence of latent tuberculosis or early, asymptomatic disease. Our 1-year follow-up of controls for signs of clinical tuberculosis did not suggest this, but additional studies might be needed to better assess this possibility.

Despite these non-specific positive results, in some cases we noted that PCR might be useful for identification of children who are likely to have culture-positive pulmonary tuberculosis. Among high-risk children, a positive PCR result from a gastric-aspirate specimen was associated with a positive predictive value (for a positive culture result) of 50%—ie, it identified a subgroup of high-risk patients with a 50% rate of positive cultures with a test result that can be available within hours. By contrast with these encouraging results, eight (38%) of 21 children in the high-risk, culture-positive group were PCR negative in all specimens. Thus, our results do not support the routine use of PCR for diagnosis of paediatric tuberculosis, but suggest that the same-day gastric-aspirate PCR test might be useful as a screening test for high-risk children because it rapidly identifies those children in the high-risk group who are three times more likely to have culture-proven tuberculosis.

The results reported in this study are subject to several limitations. First, cases were enrolled on the basis of the Stegen-Toledo score, which although widely used is poorly validated. The association between Stegen-Toledo risk group and proportion of culture-confirmed cases was notable, somewhat diminishing this limitation. Second, potential participants who did not enrol might have been different from those who did enrol in the study. Most parents of the potential cases and controls who declined to participate were unwilling or unable to return to the clinic for subsequent sample collection, because participation required several days' attendance. This situation was usually the result of parental work demands or distance from home to the clinic. These children were not different from enrolled participants in terms of age or sex, but there could have been differences in other variables such as income and level of education. Third, we chose to include cases and controls who declined HIV testing and lacked evidence of HIV/AIDS. Although we cannot exclude occult HIV infection, there is substantial cultural stigma associated with HIV testing and a very low HIV seroprevalence in Lima (about 0·5% in adults and <0·1% in children younger than 12 years),[25] so including these children in the analysis seemed appropriate. Finally, controls were only assessed with a single nasopharyngeal-aspirate specimen and no gastric-aspirate specimens, whereas two nasopharyngeal aspirates and two gastric aspirates were obtained from cases. The extended sampling strategy for cases is justified from a standard of care perspective, since there is potential direct benefit to the patient from extended sampling. We did not believe that the discomfort of gastric aspiration was justified for a well child. Nasopharyngeal aspiration is unpleasant for some, and many parents were unwilling to agree to repeated sampling.

Although our sampling strategy therefore differed between cases and controls, the study provided the most extensive data so far on specificity of nasopharyngeal-aspirate culture for tuberculosis in children, providing samples from more than 200 children in a population in which *M tuberculosis* infection is common. Among cases with positive nasopharyngeal-aspirate cultures, around two-thirds were culture positive in both nasopharyngeal-aspirate specimens collected, suggesting that examination of a single nasopharyngeal-aspirate specimen was reasonable in view of the logistical and ethical considerations.

Thus, MODS culture increased sensitivity and speed of diagnosis of pulmonary tuberculosis compared with conventional Lowenstein-Jensen culture. Gastric-aspirate cultures improved case detection compared with nasopharyngeal-aspirate cultures. Although most children treated for presumed pulmonary tuberculosis were culture-negative in all specimens, MODS culture of duplicate gastric aspirates substantially improved the yield of laboratory confirmation. PCR was insufficiently sensitive and specific for routine diagnostic use, but in high-risk children duplicate gastric-aspirate PCR rapidly identified a subgroup with a 50% rate of positive cultures. Collection of duplicate gastric-aspirate specimens from high-risk children for MODS culture increased microbiological diagnosis of tuberculosis by more than a third.

Contributors

GS-C participated in enrolment of participants, collection of study data, and quality control. MEC, LK, ES-L, and EN contributed to participant referral and medical support. TDP contributed to interpretation of radiographs. SM, LC, VAL-T, DAJM, and RHG contributed to laboratory support and diagnostic testing. MS undertook data analysis. DAJM, CAE, and RHG participated in study design and data interpretation. The principal investigator (RAO) was involved in study design, quality control, data analysis, data interpretation, and reporting.

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Conflicts of interest

We declare that we have no conflicts of interest.

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Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis

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Multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis are generally thought to have high mortality rates. However, many cases can be treated with the right combination and rational use of available antituberculosis drugs. This Review describes the evidence available for each drug and discusses the basis for recommendations for the treatment of patients with MDR and XDR tuberculosis. The recommended regimen is the combination of at least four drugs to which the *Mycobacterium tuberculosis* isolate is likely to be susceptible. Drugs are chosen with a stepwise selection process through five groups on the basis of efficacy, safety, and cost. Among the first group (the oral first-line drugs) high-dose isoniazid, pyrazinamide, and ethambutol are thought of as an adjunct for the treatment of MDR and XDR tuberculosis. The second group is the fluoroquinolones, of which the first choice is high-dose levofloxacin. The third group are the injectable drugs, which should be used in the following order: capreomycin, kanamycin, then amikacin. The fourth group are called the second-line drugs and should be used in the following order: thioamides, cycloserine, then aminosalicylic acid. The fifth group includes drugs that are not very effective or for which there are sparse clinical data. Drugs in group five should be used in the following order: clofazimine, amoxicillin with clavulanate, linezolid, carbapenems, thioacetazone, then clarithromycin.

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Introduction

The period between 1950 and 1970 was a turning point in the battle against tuberculosis: most of the current antituberculosis drugs were discovered and new therapeutic regimens made tuberculosis a curable disease.[1] The initial optimism of the tuberculosis-control community began to wane when drug-resistant *Mycobacterium tuberculosis* strains emerged. Tuberculosis strains classified as multidrug-resistant (MDR) are those resistant to at least the two most potent first-line antituberculosis drugs—ie, isoniazid and rifampicin.[2–5] Extensively drug-resistant (XDR) tuberculosis strains are resistant to either isoniazid or rifampicin (like MDR tuberculosis), any fluoroquinolone, and at least one of three second-line antituberculosis injectable drugs—ie, capreomycin, kanamycin, and amikacin.[2–5] The treatment of tuberculosis becomes more complicated as the antibiotic resistance profile of *M tuberculosis* broadens, especially in the case of MDR and XDR tuberculosis. MDR and XDR tuberculosis are generally thought to have high mortality rates. With the exception of the fluoroquinolones, no new antituberculosis drug has been introduced in the past 45 years.[2,6] The notion of patients with incurable tuberculosis (ie, totally drug-resistant tuberculosis) is now regularly referred to.[7] The probability of successful treatment further decreases with the emergence of new drug-resistant strains. However, prudent use of combinations of available drugs could improve chances of cure,[8] even in patients with MDR[2,3,9–15] or XDR[4,5,16–18] tuberculosis, and even in areas with broad-spectrum resistance.[9,13,17] Clinicians treating patients with XDR tuberculosis are confronted by frustrations similar to those faced before antibiotics were available,[19] when host immunity, fresh air, rest, and good nutrition were relied on for treatment. However, data and clinical experience from the past few years now show drug-resistant tuberculosis can largely be cured with the right combination and rational use of available antituberculosis drugs.[2,20,21]

In this Review we discuss the evidence that rational use of antituberculosis drugs can treat MDR and XDR tuberculosis. Other clinically relevant topics (eg, the need to diagnose MDR tuberculosis as early as possible, the ability of laboratories serving MDR and XDR tuberculosis reference centres to do quality first-line and second-line drug susceptibility testing, and the clinical skills necessary to combine the drug susceptibility testing information about fluoroquinolones, injectables, and ethambutol with a patient's history of prescribed antituberculosis drugs), have been reviewed and discussed elsewhere[22–25] and are not included in this Review. When appropriate, evidence grades for the recommended treatment regimens are given in accordance with the grading system of the Scottish Intercollegiate Guidelines Network.[26]

Treatment overview

There are several basic rules for the management of patients with MDR or XDR tuberculosis. The recommended regimen is a combination of at least four drugs to which the *M tuberculosis* isolate is likely to be susceptible, although more than four might be necessary. Drugs are chosen with a stepwise selection process through five groups of antituberculosis drugs (table), on the basis of efficacy, safety, and cost. The duration of the intensive phase of treatment (when an injectable drug is given) should be at least 6 months (or 4 months after culture conversion). The continuation phase (without the injectable drug) should last until 18 months after culture conversion. Surgery could be considered under specific conditions (eg, when less than four second-line drugs are available, when the patient has localised lesions, and when the patient has enough respiratory reserve to tolerate surgery).[2,21] These principles are the same for the treatment

	Daily dose
Group one: first-line oral antituberculosis drugs (use all possible drugs)	
Isoniazid	5 mg/kg
Rifampicin	10 mg/kg
Ethambutol	15–25 mg/kg
Pyrazinamide	30 mg/kg
Group two: fluoroquinolones (use only one, because they share genetic targets)	
Ofloxacin	15 mg/kg
Levofloxacin	15 mg/kg
Moxifloxacin	7·5–10 mg/kg
Group three: injectable antituberculosis drugs (use only one, because they share very similar genetic targets)	
Streptomycin	15 mg/kg
Kanamycin	15 mg/kg
Amikacin	15 mg/kg
Capreomycin	15 mg/kg
Group four: less-effective second-line antituberculosis drugs (use all possible drugs if necessary)	
Ethionamide/Prothionamide	15 mg/kg
Cycloserine/Terizidone	15 mg/kg
P-aminosalicylic acid (acid salt)	150 mg/kg
Group five: less-effective drugs or drugs on which clinical data are sparse (use all necessary drugs if there are less than four from the other groups)	
Clofazimine	100 mg
Amoxicillin with clavulanate	875/125 mg every 12 h
Linezolid	600 mg
Imipenem	500–1000 mg every 6 h
Clarithromycin	500 mg/12 h
High-dose isoniazid	10–15 mg/kg
Thioacetazone	150 mg

Table: **Categories of antituberculosis drugs**

of both MDR and XDR tuberculosis, but the treatment of patients with XDR tuberculosis is much more difficult, because more patients need group five drugs and surgery. The panel summarises the role of the different drug groups in the treatment on MDR and XDR tuberculosis.

Group one: first-line antituberculosis drugs

The first group includes the four key drugs that make up the initial phase of the ideal short-course chemotherapy regimen: isoniazid, rifampicin, pyrazinamide, and ethambutol.[2,20,21] Most patients with MDR or XDR tuberculosis will have already been mismanaged with one or more cycles of combined drugs, including pyrazinamide and ethambutol.[3] On the basis of this assumption and the low reliability of the susceptibility tests to ethambutol and pyrazinamide,[21,27] resistance to these two drugs should be suspected and they should, therefore, never be included among the four drugs used to treat patients with MDR or XDR tuberculosis. In recent years evidence has emerged that these first-line drugs (including isoniazid) are of value in the treatment of patients with MDR or XDR tuberculosis.[10,28,29]

Isoniazid

Isoniazid is a prodrug and is not effective against *M tuberculosis* unless it is activated by catalase-peroxidase, which is regulated by the *katG* gene. Therefore, mutation of the *katG* gene results in a very high level of resistance to isoniazid (resistant to concentrations greater than 1 mg/L). Ser315Thr is the most widespread *katG* mutation in clinical isolates, but there are many mutations that result in inactivation of catalase-peroxidase. When the *katG* gene is not mutated, activated isoniazid acts on several *M tuberculosis* genes, of which those in the *inhA* promoter region are the most important.[30] Because the *inhA* gene is also the target of ethionamide and prothionamide (two antituberculosis drugs),[31,32] its mutation (or mutations in the *inhA* promoter region) results in low-level resistance to isoniazid (0·2 mg/L) and resistance to ethionamide. Therefore, strains that are highly resistant to isoniazid because of the *katG* mutation are probably susceptible to ethionamide and prothionamide. Conversely, strains that are resistant to low concentrations but susceptible to high concentrations of isoniazid in vitro are usually resistant to ethionamide, but susceptible to high doses of isoniazid (10–15 mg/kg) in vivo.[33] Resistance to low but not high doses of isoniazid with resistance to ethionamide is common in up to 15% of patients with isoniazid-resistant tuberculosis,[28,29] in whom high doses of isoniazid might, despite in vitro resistance,[28,30,31] be clinically useful to overcome the potential problem of the cross-resistance to ethionamide.

The use of high-dose isoniazid to overcome any potential cross-resistance with ethionamide has been studied in a randomised clinical trial.[28] Individuals receiving a high dose of isoniazid as part of a standard MDR tuberculosis regimen became sputum smear-negative 2·38 times more rapidly (95% CI 1·45–3·91; p=0·001) and were 2·37 times more likely (1·46–3·84, p=0·001) to achieve sputum smear conversion at 6 months than were those who did not receive isoniazid. These patients showed radiological improvement without an increased risk of isoniazid toxicity. Although findings were encouraging, the study's sample size was unfortunately too small to control for other predictors of outcome such as the participants' age and drug susceptibility status. Moreover, isoniazid is a very cheap and well-tolerated drug and susceptible testing to it is very reliable.[2] A high dose of isoniazid should be considered in patients with difficult to treat MDR or XDR tuberculosis (grade B evidence), and is effectively used for this purpose in some countries, such as Bangledesh and countries in Africa and South America.[34,35] Ideally, high-dose isoniazid should be included in the treatment regimen for patients in whom susceptibility to this dose is proven. This recommendation is not included in the WHO MDR tuberculosis treatment guidelines.[21]

Systematic prescription of high-dose isoniazid to the MDR or XDR tuberculosis treatment regimen could be a reasonable recommendation, but further study is needed. The use of high-dose isoniazid and ethionamide increases the chance of successful treatment. Strict monitoring of potential hepatotoxic and neurotoxic effects of the drugs is necessary, especially in at-risk populations.

Rifampicin and others rifamycins

Three different rifamycins are commercially available: rifampicin, rifabutin, and rifapentine. *M tuberculosis* develops resistance to all three through a mutation in the 81 bp region of the RNA polymerase b-subunit (*rpoB*) gene.[36] However, even if most of the rifampicin-resistant *M tuberculosis* isolates are also resistant to rifapentine, about 15–20% of them are likely to be susceptible to rifabutin.[37] This finding has been substantiated in clinical studies.[38–40] Unfortunately, rifabutin is expensive and its drug susceptible testing procedure and cutoff value have never been clinically validated. Therefore, the clinical response to rifabutin is uncertain in patients whose mycobacterial isolates are susceptible to rifabutin but resistant to other rifamycins. Unfortunately, use of rifabutin to treat patients with MDR or XDR tuberculosis is limited by its high cost and the restricted availability of both the drug and procedures for susceptibility testing in many developing countries. For these reasons, the use of rifampicin or other rifamycins is not recommended in the treatment of patients with MDR or XDR tuberculosis (grade D evidence).

Pyrazinamide

Pyrazinamide was widely used between 1950 and 1970 to treat patients with *M tuberculosis* resistant to isoniazid and streptomycin. This period was before the advent of rifampicin and fluoroquinolones; clinicians treating such patients would, therefore, face a situation similar to that of those treating patients with XDR tuberculosis nowadays.[2,8] At the time, three studies[8,41–43] reported excellent cure and bacteriological conversion rates by combining ethionamide, cycloserine, and pyrazinamide. Presumably, pyrazinamide had a major role in this regimen as it would have remained active for the entire treatment duration.[8] Many *M tuberculosis* strains are susceptible to pyrazinamide in patients with MDR tuberculosis. Many initial treatment failures in patients with MDR tuberculosis are because, even though the patients were resistant only to isoniazid at the beginning of treatment, they developed resistance to rifampicin during the continuation phase (when isoniazid and rifampicin are given together).[44] This could explain the results of two studies in patients with MDR[10] and XDR[29] tuberculosis, which showed that the addition of pyrazinamide, ethambutol, or streptomycin to the treatment regimen of patients remaining susceptible to these drugs improves prognosis.

The susceptibility testing method for pyrazinamide has not been established, but because of its low cost and low toxicity, it is recommended to be included in MDR tuberculosis treatment regimens during the entire duration of treatment and not just during the intensive phase (grade C). This recommendation is included in the WHO MDR tuberculosis treatment guidelines.[21] However, because a reliable drug susceptibility test is not yet widely available, pyrazinamide should not be one of the four main drugs used in the treatment of drug-resistant tuberculosis. Its effectiveness should be assessed individually in patients at high risk of hepatotoxicity (especially elderly people and people who misuse alcohol). The practice of prescribing pyrazinamide to patients with MDR tuberculosis (irrespective of drug susceptibility test results) needs to be assessed to establish if a real clinical benefit exists and if this benefit justifies the possible increased toxicity.[17]

Ethambutol

Ethambutol has an excellent tolerability profile and resistance in treatment-naive patients with tuberculosis is very rare in most countries.[45] Furthermore, patients with *M tuberculosis* resistant only to isoniazid who have initial treatment failure and become MDR are likely to remain susceptible to ethambutol.[44] In view of the uncertain clinical reliability of the susceptibility test for ethambutol[21,27] and the low cost and toxicity of the drug, the inclusion of ethambutol (at a dose of 15–25 mg/kg)[21,46] in the treatment regimen for patients with MDR tuberculosis is justifiable (grade C). However, ethambutol is not recommended if susceptibility tests show resistance to ethambutol and if patients have previously taken the drug. These recommendations are included in the WHO MDR tuberculosis treatment guidelines.[21] Ethambutol should not be included as one of the four main drugs that form the basis of drug-resistant tuberculosis regimens, and if patients are already taking many pills adherence to the regimen might be negatively affected by the inclusion of ethambutol.

Group two: fluoroquinolones

Resistance to fluoroquinolones increases the risk of treatment failure and death in patients with MDR or XDR tuberculosis.[47] Fluoroquinolones are the mainstay of treatment for patients with MDR or XDR tuberculosis and deliver better clinical outcomes than do the drugs in the other groups.[2,3,8,12] Because all fluoroquinolones share a genetic target—the *gyrA* gene[48]—the use of only one of them is justified.[2,20,21]

Comparative effectiveness

The fluoroquinolones include ciprofloxacin, ofloxacin, levofloxacin, and moxifloxacin. There is little information on whether all fluoroquinolones are equally effective in the treatment of MDR and XDR tuberculosis. Ciprofloxacin is somewhat less effective[6,48] than ofloxacin,

levofloxacin, and moxifloxacin and is therefore not recommended.[21] Only one clinical study has compared two fluoroquinolones—ofloxacin and levofloxacin. Levofloxacin is more effective than is ofloxacin for tuberculosis with confirmed susceptibility to ofloxacin and ofloxacin-resistant strains.[49] The finding that levofloxacin is effective against ofloxacin-resistant strains suggests that cross-resistance between fluoroquinolones is not always complete. The first pharmacodynamic data showed that levofloxacin was less effective than moxifloxacin and gatifloxacin.[48] In these early studies levofloxacin was prescribed at a dose of 500 mg/day.[48] More recent studies show that, at the dose of 1000 mg/day, levofloxacin has the best early bactericidal activity in the group.[50,51] Other studies[44] have shown that moxifloxacin and gatifloxacin are better than ofloxacin, but do not include comparison with levofloxacin. Levofloxacin at high doses, but not low doses, seems to have better pharmacodynamic qualities than do either moxifloxacin or gatifloxacin.[50,51] Some animal data indicate that moxifloxacin has the best sterilising activity among the fluoroquinolones.[52]

Cross-resistance

Initially, cross-resistance among fluorquinolones was thought to be likely because they all target *gyrA*.[48] However, analysis of the different mutations of this gene (resistance mutations in other genes, such as *gyrB*, have been described) has shown that about half of the isolates resistant to ofloxacin could be susceptible to moxifloxacin[53,54] and to high doses of levofloxacin.[54] These findings might account for the effectiveness of levofloxacin in patients with *M tuberculosis* strains resistant to ofloxacin.

Selection of the best fluoroquinolone

Taking into account the cost of each drug, levofloxacin would be the ideal fluoroquinolone at doses of 1000 mg/day (not at the standard dose of 750 mg, unless the patient has a very low bodyweight; grade C evidence). This recommendation is included in the WHO MDR tuberculosis treatment guidelines.[21] The slightly better profile of high-dose levofloxacin than moxifloxacin and gatifloxacin is probably not clinically relevant, although a more in-depth clinical validation would be needed to verify this. Therefore, moxifloxacin (which probably has the best sterilising activity) and gatifloxacin could be deemed good options as well, even though the US Food and Drug Administration and other international drug agencies denied authorisation of gatifloxacin because of the risk of dysglycaemia in elderly patients.[55] Gatifloxacin is less expensive than moxifloxacin. In view of the low toxicity and price of these new generation fluoroquinolones (especially levofloxacin), always including one of them in treatment regimens for patients with MDR and XDR tuberculosis is justified. Although a fluoroquinolone is one of the four active drugs in any regimen designed to treat MDR tuberculosis, this is not the case for XDR tuberculosis, because of the risk of cross-resistance to ofloxacin. The issue of which fluoroquinolone to use (and at what dose) needs further clinical study, especially to assess the possible long-term adverse events, and the role of adding a new generation fluoroquinolone when levofloxacin resistance is noted and the addition of levofloxacin or moxifloxacin is not justifiable.

Group three: injectable antituberculosis drugs

This group of drugs is another mainstay in the treatment of MDR and XDR tuberculosis and includes the aminoglycosides (streptomycin, kanamycin, and amikacin) and the polypeptides (capreomycin and viomycin; unfortunatley viomycin is unavailable in most countries). The injectables are bactericidal with high extracellular activity, but some intracellular activity has also been shown,[56] which can be explained by their method of action. The aminoglycosides inhibit protein synthesis through irreversible binding to the 30S ribosomal subunit. The polypeptides seem to inhibit translocation of peptidyl transfer RNA and block the initiation of protein synthesis. Capreomycin has a different chemical structure and mechanism of action from the aminoglycosides, but its mechanism of antibacterial and metabolic activity is similar.[57] As with the fluoroquinolones, there is no reason to use more than one injectable in the treatment of MDR or XDR tuberculosis.[2,20,21] All second-line injectables display similar efficacy and adverse event profiles,[2,20,21,58] although capreomycin might cause electrolyte disorders more often (especially hypokalaemia).[59]

Cross-resistance

40 years ago, Tsukamura[60] reported that isolates resistant to low concentrations of kanamycin were susceptible to capreomycin and viomycin, many isolates resistant to high concentrations of kanamycin were resistant to capreomycin, and isolates resistant to capreomycin were susceptible to kanamycin but resistant to viomycin. In subsequent papers,[61,62] Tsukamura showed that unidirectional cross-resistance between the injectables is highly likely and emphasised the importance of choosing the correct first-choice injectables. More recent studies[63–65] of the mínimum inhibitory concentration of each injectable drug and the genetic mutations determining their resistance made several conclusions: isolates acquiring resistance to streptomycin are usually susceptible to kanamycin, amikacin, and capreomycin (although rare strains have single-step mutations conferring resistance to both streptomycin and kanamycin); isolates acquiring resistance to capreomycin are usually susceptible to kanamycin and amikacin (a few might also be resistant to kanamycin and even fewer might be resistant to amikacin); most isolates that acquire resistance to amikacin are resistant to both kanamycin and capreomycin; and isolates acquiring resistance to kanamycin show different levels of cross-resistance to amikacin and capreomycin.

Best use of injectable drugs

The treatment of MDR and XDR tuberculosis should always include an injectable drug, the choice of which depends on a patient's previous use of each drug and the likelihood of resistance, especially in patients with XDR tuberculosis. In most cases, an injectable drug should be one of the four key drugs that make up the treatment regimen, even for patients with XDR tuberculosis.[2,21] The little evidence available (laboratory studies only) suggests that, to avoid cross-resistance, which would prevent the activity of other injectables in subsequent treatment regimens, the best sequence in which to give these drugs for the treatment of MDR and XDR tuberculosis is streptomycin, capreomycin, kanamycin, and, finally, amikacin (grade D). This recommendation is not included in the WHO MDR tuberculosis treatment guidelines.[21] However, streptomycin should never be used to treat MDR or XDR tuberculosis, even if drug susceptibility tests indicate a susceptible isolate, because the clinical reliability of this test is not very high.[27] Primary resistance to streptomycin is very common, especially in patients with isoniazid-resistant strains of *M tuberculosis*, including MDR and XDR strains.[45] Other injectable drugs are available to assure the efficacy of this mainstay group of drugs. Hypothetically, the injectable drug of choice would be capreomycin, although it has several disadvantages: it is not available on a large scale worldwide; it has a short shelf-life (24 months); measurement of potassium concentrations is not always feasible or available; and it is more expensive than kanamycin. In many countries, kanamycin is the first-choice injectable, because it is cheaper and much more readily available. Amikacin is the most widely available injectable drug in all settings because it has excellent activity against a broad range of bacteria and not just *M tuberculosis*. Amikacin should always be avoided as a first choice because of the possible cross-resistance mentioned above and an increased risk of serious adverse events compared with other injectable drugs.

Group four: second-line antituberculosis drugs

This group includes compounds from three classes of drugs: the thioamides (ethionamide and protionamide), cycloserine or its derivative terizidone (a double molecule of cycloserine, which has nearly the same action but is cheaper), and aminosalicylic acid. Several clinical studies have assessed the efficacy and effectiveness of this group of second-line antituberculosis drugs. Because they belong to different drug classes with diverse genetic targets, the use of more than one of these drugs, if necessary, would be reasonable.[2,20,21] In an ideal sequence for the introduction of these drugs to a treatment regimen, the thioamides should be given first because compared with cycloserine and aminosalicylic acid they are more bactericidal, are less expensive, and have a more favourable ratio of toxic to therapeutic dose (grade C). After the thioamides, cycloserine should be introduced to the regimen, then aminosalicylic acid. They are introduced in this order on the basis of their effectiveness, adverse events, and cost (grade C). This recommendation is consistent with the current WHO MDR tuberculosis treatment guidelines.[21] Nevertheless, the second-line drugs are substantially less effective than first-line drugs, injectable drugs, and the flouroquinolones in treating tuberculosis.[57] Thioamide drugs seem to inhibit mycolic acid biosynthesis, but their precise mechanism of action has not been fully elucidated. The molecular target of ethionamide, like isoniazid, is *inhA*,[32] which possibly explains the cross-resistance with ethionamide and isoniazid in some isoniazid-resistant isolates. Another ethionamide resistance mechanism is a mutation in the *ethA* gene,[66] encoding the enzyme that activates the drug. Resistance develops rapidly if thioamides are used alone because there is complete cross-resistance between ethionamide and protionamide. They are generally well tolerated drugs, apart from gastric adverse events[20,21,67] and, as mentioned before, the risk of cross-resistance to isoniazid.[31,33,68–70] Thioamides are often used as basic antituberculosis drugs for patients with MDR or XDR tuberculosis. In fact, they are included in most standard regimens to treat MDR tuberculosis.[2,20,21]

Cycloserine is bacteriostatic and competitively blocks the enzymes that incorporate alanine into an alanyl-alanine dipeptide, an essential component of the mycobacterial cell wall.[57] Among the advantages of cycloserine are its high gastric tolerance (compared with the other drugs in this group) and the absence of cross-resistance to other compounds. The two main drawbacks of cycloserine are psychiatric adverse events (mainly psychotic reactions with suicidal tendencies),[20,21,67] which mean that a psychiatric interview is necessary before the drug is given, and its short shelf-life (24 months). Cycloserine is one of the cornerstones of treatment for MDR and XDR tuberculosis.[2,20,21,67] Terizidone (a combination of two molecules of cycloserine) might be less toxic,[20,21] although studies of this drug are scarce.

The first aminosalicylic acid compound to be investigated and used in different studies was its acid salt. However, in the 1950s and 1960s the use of the *P*-amino salicylate sodium, which requires doses 30% higher than the aminosalicylic acid, became progressively widespread. From the 1970s until 2000, aminosalicylic acid sodium was the compound used in most countries despite a well known gastric intolerance. However, in the past 10 years, after the substantial global demand for this drug to treat MDR and XDR tuberculosis, aminosalicylic acid was reintroduced, especially in the form of enteric-coated aminosalicylic acid granules.[71] Aminosalicylic acid has gradually replaced aminosalicylic sodium. Nonetheless, many countries use the sodium formulation, because its effectiveness has been proved around the world. The substantial demand for this compound has led to the use of both formulations (aminosalicylic acid and aminosalicylic sodium). The main advantage of

Panel: **Role of different drug groups in the treatment of multidrug-resistant and extensively drug-resistant tuberculosis**

Group one: first-line oral antituberculosis drugs
- The addition of high doses of isoniazid, ethambutol, and pyrazinamide to all therapeutic regimens is recommended
- Not to be counted as one of the four basic drugs of the regimen

Group two: fluoroquinolones
- A fluoroquinolone should always be included in the treatment of MDR or XDR tuberculosis
- Preferably use levofloxacin in doses of 750–1000 mg/day; moxifloxacin with or without gatifloxacin could also be a good choice
- A fluoroquinolone should be counted as one of the four basic drugs of the regimen in patients with MDR tuberculosis but not those with XDR tuberculosis

Group three: injectable antituberculosis drugs
- There should always be an injectable drug in the treatment of MDR and XDR tuberculosis
- An injectable drug should be one of the four basic drugs in the treatment of most patients
- Never use streptomycin
- Ideally, give preference to the following sequence: capreomycin first, kanamycin second, and amikacin third

Group four: second-line drugs
- Use all the necessary drugs to make up at least four active basic drugs; start with ethionamide or protionamide, then use cycloserine; aminosalicylic acid should be used last

Group five: less-effective drugs or drugs on which clinical data are sparse
- To be counted as half of one of the four drugs that make up a treatment regimen; when required, the use of at least two of these drugs is therefore necessary
- Introduce according to availability in the following order: clofazimine, amoxicillin-clavulanate, linezolid, imipenem or meropenem, clarithromycin, then thioacetazone

MDR=multidrug-resistant tuberculosis. XDR=extensively drug-resistant tuberculosis.

aminosalicylic acid seems to be a better gastric tolerance and lower dose requirement than aminosalicylic sodium, although it needs to be kept refrigerated (at 4°C), therefore requiring a cold chain, which is not always available in developing countries. By contrast, the major advantage of aminosalicylic sodium is its simple storage requirements with no need for a cold chain. Irrespective of these factors, aminosalicylic acid has very low effectiveness, is poorly tolerated, and is expensive, which mean it is the last-choice drug among the second-line group. However, despite its drawbacks, aminosalicylic acid is an important drug in the treatment of many patients with MDR tuberculosis and most patients with XDR tuberculosis.[2,20,21]

Group five drugs

Group five drugs are a very heterogeneous group of drugs, which are poorly studied in vivo in human beings or have low effectiveness or high toxicity. Therefore, the drugs in group five are thought of as minor or adjuvant drugs;[2,20,21] each one should be counted as only half of one of the four basic drugs needed to treat MDR and XDR tuberculosis. Therefore, when it is really necessary to use a drug from this group, at least two should be chosen. On the basis of effectiveness criteria, potential adverse events, and costs, group five drugs should be introduced into a treatment regimen in the following order of preference: clofazimine, amoxicillin with clavulanate (co-amoxiclav), linezolid, carbapenem, thioacetazone, then clarithromycin (grade C and D). This rational introduction of these drugs is not included in the WHO MDR tuberculosis treatment guidelines.[21]

Clofazimine

Despite restricted clinical experience with clofazimine to treat tuberculosis, its role is attracting interest because of its potential intracellular and extracellular activity.[72–74] Moreover, adequate dose management would help the control of adverse events, especially photosensitivity and gastric intolerance.[20,21,67] Clofazimine's low cost is an additional advantage, although the drug is not widely available because it has been almost exclusively restricted to leprosy treatment. In several of the countries in which the drug is available, clofazimine is included in the standard treatment regimen.[34] In view of its qualities, a large initiative aimed at ensuring its universal availability would be advisable.

Co-amoxiclav

Beta-lactam antibiotics are not regarded as useful drugs for the treatment of tuberculosis because *M tuberculosis* is resistant to most of these antibiotics in vitro. Resistance is thought to be mediated by a class of beta-lactamase enzymes that hydrolyse penicillins and cephalosporins.[75,76] Resistance can be overcome by either inhibiting the beta-lactamase or by use of an antibiotic that is not a substrate for the enzyme. Beta-lactamase can be inhibited with a combination of a beta-lactam and a beta-lactamase inhibitor, such as co-amoxiclav, which is active in vitro.[77] Anecdotally, co-amoxiclav combined with other second-line drugs has been successfully used to treat some patients with MDR tuberculosis.[4,78] This approach has met much scepticism, and the role, if any, of co-amoxiclav remains unclear.[76] Nevertheless, the absence of effective drugs for the treatment of patients with MDR or XDR tuberculosis and the good tolerability, low price, and low toxicity profile of this drug have made co-amoxiclav the drug of choice within group five.[2,20,21]

Linezolid

More than 10 years ago, studies in mice showed linezolid and other oxazolidinones to be effective against *M tuberculosis*,[79] despite their low early bactericidal activity.[80] These findings have been substantiated by several studies[81–84] of patients with MDR or XDR tuberculosis, although most of these studies included very few patients. The possibility of using linezolid as a

drug of choice in the management of MDR and XDR tuberculosis (even outside the limits of group five, to which the drug is currently assigned) is severely hampered by its high cost and high toxicity profile in the long term; up to 25–45% of patients reported severe anaemia with or without thrombocytopenia or peripheral and optic neuropathy.[21,67,81–84] Costs and toxicity can be reduced, without loss of effectiveness, by reducing the dose by 50% to 600 mg/day[83] or even to 300 mg/day.[84] Data for use of linezolid at low doses are scarce.[82,84] A recent cohort study of 85 patients with MDR or XDR tuberculosis from four European countries concluded that use of linezolid should be restricted to the most severe cases—eg, those resistant to more than seven antituberculosis drugs.[83]

Nevertheless, countries that can afford the drug and can monitor the adverse events use linezolid (600 mg/day) as a basic drug against XDR tuberculosis and probably in many cases of MDR tuberculosis.[83] Care should be taken during treatment because linezolid can reduce the patient's white cell and platelet count, which should both be monitored on a regular basis. If the drug is used for extended periods, patients should be advised to immediately report any unusual gum bleeding to their physician or clinic. Studies are needed to assess the use of low-dose linezolid for its antimycobacterial effect in patients with MDR or XDR tuberculosis.

Carbapenems

The carbapenems offer another approach (in addition to use of co-amoxiclav) to overcome the beta-lactam resistance of *M tuberculosis*. They are poor substrates for class A and class C beta-lactamases and two carbapenems, meropenem and imipenem, are active in vitro against *M tuberculosis*.[85] Effectiveness has been shown in some reports of patients with MDR tuberculosis treated with imipenem[76] and meropenem with clavulanic acid.[86] Despite these encouraging reports, restricted clinical experience, unknown long-term toxicity, high costs, and the need to treat intravenously restrict carbapenems to use in severe cases only.

Thioacetazone

Thioacetazone is one of the oldest, cheapest, and most widely used drugs that have been used in the treatment of tuberculosis,[1] even though its action has always been thought to be very weak because it has only bacteriostatic activity. This drug has high toxicity, especially in patients with HIV (in whom there is a high mortality rate),[87] and it is no longer used to treat tuberculosis. Moreover, thioacetazone is only partly cross-resistant to ethionamide.[21] The use of thioacetazone in patients with HIV is banned because of the high frequency of Stevens–Johnson syndrome.[80] The use of this drug should be restricted to cases with a broad drug-resistance profile, ensuring close follow-up of adverse events. It must never be given to patients with HIV and is now not generally used in clinical practice.

Search strategy and selection criteria

Articles cited in this Review were obtained though searches of PubMed and Google Scholar covering the period from November 1, 2000, until July 15, 2010. The keywords included "TB", "drug-resistant TB", "MDR-TB", and "XDR-TB". Reference lists of these articles were then reviewed to identify additional studies. The US Centers for Disease Control and Prevention's *Morbidity and Mortality Weekly Report* was searched from January 1, 1989, until July 15, 2010 for reports describing MDR and XDR tuberculosis cases, series, reports on epidemiological studies. The 2009 WHO Reports on drug-resistant tuberculosis and information from presentations at the 40th Union World Conference on Lung Health were also used.

Clarithromycin

Because clarithromycin is not very effective against *M tuberculosis*, it has no clear role in the treatment of tuberculosis.[67,73] Information about the role of clarithromycin is scarce, consisting of only a few studies of a small number of patients who received several other drugs in combination.[4,10,11] The unique advantage of this drug is its good tolerance and toxicity profiles. Clarithromycin use should be restricted to severe cases in which no other active drug is available; its contribution to a regimen in such cases will not be substantial.

Conclusions

In many cases, both MDR and XDR tuberculosis can be cured with the right combination of available antituberculosis drugs. Every case of tuberculosis, including those caused by *M tuberculosis* strains with a broad drug-resistance profile, can probably be treated if the available antituberculosis drugs are used rationally.[8] Several factors should be considered when choosing the appropriate drug, including availability of the drug, the rationale to introduce a given drug, the patient's resistance profile and previous use of the drug, the cost of the drug, and the possibility of toxic adverse events. Further information from prospective, controlled, clinical trials will elucidate the true role of each drug, and newer formulations, in the treatment of MDR and XDR tuberculosis, in patients with and without HIV.

Knowledge and best practice in this field are constantly changing, and changes in practice, treatment, and drug therapy might become necessary. Clinicians are advised to check the most current information provided on drugs with the manufacturer of each product given to patients to verify the recommended dose, method, duration of treatment, and any possible contraindications.

Contributors

GBM, AZ, and GS had the idea for, wrote, and revised the article. JAC did the data search, collection, analysis, and interpretation, and assisted with writing the article.

Conflicts of interest

We declare that we have no conflicts of interest.

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Do children infected with HIV receiving HAART need to be revaccinated?

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No official recommendations have been made on whether children infected with HIV on highly active antiretroviral therapy (HAART) should be revaccinated. We reviewed published work to establish whether these children have protective immunity to vaccine-preventable diseases and to assess short-term and long-term immune responses to vaccination of children given HAART. In general, children on HAART had low levels of immunity to vaccines given before treatment. Most children on HAART, however, responded to revaccination, although immune reconstitution was not sufficient to ensure long-term immunity for some children. These results suggest that children on HAART would benefit from revaccination, but levels of protective immunity might need to be monitored and some children might need additional vaccine doses to maintain protective immunity. Vaccination policies and strategies for children infected with HIV on HAART should be developed in regions of high HIV prevalence to ensure adequate individual and population immunity.

Introduction

As part of the Expanded Programme on Immunization (EPI), WHO recommends giving routine childhood vaccines to children infected with HIV,[1] with the exceptions of BCG vaccine to infants with confirmed HIV infection and measles vaccine to severely immunosuppressed children.[2,3] However, because of the progressive effects of HIV infection on the ability of the immune system to mount an effective response, many infected children have poorer responses to vaccines than do uninfected children.[4] In general, fewer children infected with HIV achieve protective immunity, and those who do might experience greater and more rapid waning of immunity.[4,5]

Highly active antiretroviral therapy (HAART) is effective in reducing morbidity and mortality in children infected with HIV by suppressing viral replication and restoring immune function.[6–10] However, immune reconstitution in children is primarily through the generation of naive T cells rather than expansion of memory T cells, as in adults.[11–13] Consequently, HAART might not restore vaccine-induced immunity established before the start of therapy. No recommendations have been issued on whether children infected with HIV on HAART should be revaccinated.

In low-income and middle-income countries, particularly those in sub-Saharan Africa that bear the greatest burden of HIV infection in children,[14] antiretroviral treatment programmes have been scaled-up substantially, increasing access to life-prolonging treatment for children infected with HIV.[15] However, these children often access treatment at a later stage in disease progression and at older ages than in more developed countries,[16,17] and will have received routine immunisations before treatment. As a result, revaccination might be important to ensure protection. In countries heavily affected by the HIV epidemic, children receiving HAART who remain susceptible to infection could become sufficiently numerous to sustain transmission of vaccine-preventable diseases and jeopardise control efforts.[18]

We reviewed published work (table 1)[12,19–55] to establish whether children taking HAART have protective immunity to vaccine-preventable diseases and to explore short-term (≤3 months) and long-term (>3 months) immune responses to immunisation. The implications of these findings for revaccination of children infected with HIV on HAART are discussed.

Studies of vaccines and HAART

We identified 38 studies that addressed at least one of the questions of interest (table 1). For the question of whether children taking HAART have protective immunity to vaccine-preventable diseases, studies were included if children were vaccinated before being started on HAART and measures of immunity were reported after the start of HAART but before revaccination. Studies of influenza were not included for this question because vaccine-induced immune responses could not be distinguished from those due to infection. For questions about the short-term (≤3 months) and the long-term (>3 months) immune response to vaccination on HAART, studies were included if children were revaccinated or received new vaccines to which they had no prior exposure after being started on HAART and either short-term or long-term immune responses were measured.

Immunity to vaccine-preventable diseases

Non-replicating vaccines

For non-replicating vaccines, including diphtheria tetanus pertussis vaccine (DTP), hepatitis B vaccine (HBV), pneumococcal vaccines, and conjugate *Haemophilus influenzae* type b vaccines (Hib), the proportion of children with an immune response, as defined by each study, after being started on HAART was highly variable, with no clear trend by type of vaccine (table 2). The proportion of children with an immune response after being started on HAART ranged from 38% to 77% for tetanus, 40% to 65% for diphtheria, 1% to 100% for HBV, and 25% to 87% by serotype for pneumococcal vaccines. The duration of HAART at the time antibody concentration was measured varied, with the average duration ranging from 28 weeks to 5·3 years.

	Vaccine	Number taking HAART/total	Age at study entry (years)	CD4 status at study entry	Question addressed in study*
Non-replicating vaccines					
Blazevic, 2001[19]	TT	11/11	Median 11·1; range 7·4–18·4	Median 598 cells per μL; range 63–835	A
Hainaut, 2003[20]	TT	19/19	Median 5·6; range 0·6–17·2	89% in CDC immune categories 2 and 3	A
Vigano, 2000[12]	TT	25/25	Median 9·7–9·8 by CDC stage	Median 5–27% by CDC stage	A
Peruzzi, 2002[21]	TT	7/7	Median 10·4; range 7·1–14·1	Range 15–42%	A
Ghosh, 2009[22]	TT	9/9	Not specified (mean 9·36 [SD 4·15] among full cohort)	Mean ARP 68·9	A, B
Essajee, 1999[23]	DT	25/25	Median 8·95; range 1·87–17·53	Median 2·0%; range 0–6	A, B
Rosenblatt, 2005[24]	DTaP	37/37	Median 6·1; range 2·9–10·9	Median 34%; range 14–51	B, C
Abzug, 2007[25]	DTaP	92/92	Median 9·3	Median 33%	B, C
Ching, 2007[26]	TT	15/15	Median 12·6	CR: median 35%; ICR: median 26%	A, B, C
Luzuriaga, 2000[27]	TT	17/17	Mean 1·9; range 0·5–3·0	Mean 41%; range 14–57%	B
Rigaud, 2008[28]	DTP, HAV	46/46	Median 13; range 3–17	Median 7%; range 1–14	A, B, C
Tangsinmankong, 2004[29]	Pneumococcus	41/41	Range 2–15; 39% between 2 and 6	Mean 31·9%; SD 10·2%	B
Tarrago, 2005[30]	Pneumococcus	56/56	Median 11; range 3–19	Median 29%	A, B
Costa, 2008[31]	Pneumococcus	38/40	Range 2–9	95% with CD4% ≥25%	A, B
Abzug, 2006[32]	Pneumococcus	225/225	Median 9·6	Median 33%	A, B, C
Fernandes, 2008[33]	HBV	42/58	Median 7; range 1·5–12	Mean 831 cells per μL; SD 604	A
Siriaksorn, 2006[34]	HBV	75/75	Mean 9·6; SD 2·5	Mean 25%; SD 5	A
Lao-araya, 2007[35]	HBV	63/63	Mean 10·1; SD 2·4	Mean 27·2%; SD 6·7	B
Pippi, 2008[36]	HBV	47/84	Median 4·7; 95% CI 4·2–5·2	40·4% in CDC category 1	C
Abzug, 2009[37]	HBV	204/204	Median 9·1	Median 34%	A, B, C
Weinberg, 2006[38] Weinberg, 2009[39]	HAV	152/152	Median 9·2	Median 32%	B, C
Siberry, 2008[40]	HAV	83/84	37% ≥13	65% with CD4% ≥25%	C
Tanzi, 2006[41]	Influenza	29/29	Mean 10·3: SD 4·3	83% had CD4 >500 cells per μL	B
Montoya, 2007[42]	Influenza	16/16	Mean 4·6; SD 2·5	Mean 1202 cells per μL; STD 844	B
Vigano, 2008[43]	Influenza	24/24	Mean 12·6; SD 4·6	Mean 36·9%; SD 9·1	B, C
Replicating vaccines					
Aurpibul, 2006[44]	MMR	93/93	Mean 9·7; SD 2·6	Mean 24·7%; SD 4·8	A
Berkelhamer, 2001[45]	MMR	14/28	Range 2·2–11	Range 10–45%	B
Lima, 2004[46]	MMR	15/15	Median 15·4; range 12·4–17·6	Median 1781 cells per μL; range 690–5137	B
Aurpibul, 2007[47]	MMR	51/51	Mean 10·2; SD 2·5	Mean 27·2%; SD 5·7	B
Bekker, 2006[48]	MMR	59/59	Median 4·3; IQR 1·4–8·8	..	A, C
Levin, 2006[49]	VZV	17/17	Median 6·2; 95% CI 3·3–6·7	Median 38%; 95% CI 34–48	B, C
King, 2001[50]	Influenza	24/24	Mean 4·7; range 1–7·9	79% CDC class 1	B
Both replicating and non-replicating vaccines					
Zaccarelli-Filho, 2007[51]	DTP, MMR, HBV	41/41	Good VLR:† mean 10·9; SD 3·3 Partial VLR: mean 7·4; SD 3·1 Poor VLR: mean 9·4; SD 3·9	Good VLR: median 33·0% Partial VLR: median 26·4% Poor VLR: median 21·6%	A
Farquhar, 2009[52]	TT, MMR	90/90	Median 4·9; IQR 2·6–6·5	Median 6·3%; IQR 3·0–10·6	A, B
Pensieroso, 2009[53]	TT, MMR, pneumococcus	64/70	Early:‡ mean 6·8; SD 3·2 Late control: mean 13·7; SD 4·1 Late failure: mean 15·8; SD 4·1	Early: median 35% Late control: median 33% Late failure: median 22%	C
Levin, 2008[54]	Influenza	243/243	LAIV: mean 11·4; SD 3·3 TIV: mean 11·9; SD 3·0	LAIV: mean 33·2%; SD 8·4 TIV: mean 34·1%; SD 8·1	B, C
Melvin, 2003[55]	DTP, MMR, Hib	19/19	Median 7; range 3–14	Median 26%; range 1–41	B, C

TT=tetanus toxoid. ARP=age-related percentage of peripheral blood CD4 T cells compared with CD4 T cells of healthy children. DT=diphtheria and tetanus. DTaP=diphtheria, tetanus, and acellular pertussis vaccine. CR=complete responder (patients with undetectable plasma HIV-RNA [≤2·6 log copies per mL] for at least 2 years before TT booster). ICR=incomplete responder (no change or increase in HIV plasma viraemia despite HAART). DTP=diphtheria, tetanus, and pertussis vaccine. HAV=hepatitis A virus vaccine. HBV=hepatitis B virus vaccine. MMR=measles, mumps, and rubella vaccine. VZV=varicella zoster virus vaccine. Hib=*Haemophilus influenzae* type B vaccine. VLR=viral load responder. LAIV=live attenuated influenza vaccine. TIV=inactivated trivalent influenza vaccine. *A: Do children taking HAART have protective immunity to vaccine-preventable diseases? B: What is the short-term (≤3 months) immune response to vaccination on HAART? C: What is the long-term (>3 months) immune response to vaccination on HAART? †Good VLRs are patients with HIV-RNA below 400 copies per mL for at least 12 months before tests. Partial VLRs are patients who showed at least 1 $\log_{10}$ reduction in HIV-RNA copies per mL after HAART initiation. Poor VLRs are patients who showed a decrease of <1 $\log_{10}$ of HIV-RNA copies per mL after being started on HAART. ‡Early: children who started HAART within the first year of life. Late control: children who were started on HAART after 1 year of age who achieved viral suppression. Late failure: children who were started on HAART after 1 year of age who did not achieve viral suppression.

Table 1: **Description of studies evaluating immunity to vaccine-preventable diseases among children in receipt of HAART**

Antibody concentrations defining an immune response were not consistent for each vaccine (table 2), further complicating comparisons.

Two studies were designed specifically to investigate the effect of HAART on immunity to vaccine-preventable diseases and reported antibody concentrations for tetanus

	Time on HAART	Assay used	Pre-HAART antibody measure	Post-HAART antibody measure	Definition of immune response
Non-replicating vaccines					
Tetanus					
Zaccarelli-Filho, 2007[51]	Good VLR:* mean 4·6 years (SD 1·0) Partial VLR: 3·7 (1·1) Poor VLR: 4·4 (1·0)	Double antigen ELISA	..	Good VLR: 71% protected Partial VLR: 77% protected Poor VLR: 73% protected	>0·1 IU/mL
Rigaud, 2008[28]	28 weeks	IgG ELISA kit	..	55% responded	>0·1 IU/mL
Ching, 2007[26]	Median 5·3 years; range 1·4–6·2	IgG EIA	..	38% protected	≥0·15 IU/ml
Farquhar, 2009[52]	6 months	In-house ELISA	78% positive	59% positive overall 31% of positives seroreverted 23% of negatives seroconverted	>0·01 IU/mL
Ghosh, 2009[22]	36 months (SD 20·2)	ELISA	Mean 0·25 IE/mL (SD 0·3)	Mean 0·67 IE/mL (SD 0·9)	
Diphtheria					
Zaccarelli-Filho, 2007[51]	Good VLR: mean 4·6 (SD 1·0) Partial VLR: 3·7 (1·1) Poor VLR: 4·4 (1·0)	Double antigen ELISA	..	Good VLR: 65% protected Partial VLR: 61% protected Poor VLR: 40% protected	>0·1 IU/mL
Hepatitis B virus					
Fernandes, 2008[33]	Median: 53 months; range 4–118	ELISA	..	17% protected	≥10mIU/mL
Zaccarelli-Filho, 2007[51]	Good VLR: mean 4·6 (SD 1·0) Partial VLR: 3·7 (1·1) Poor VLR: 4·4 (1·0)	ELISA	..	Good VLR: 100% protected Partial VLR: 100% protected Poor VLR: 91% protected	>10mIU/mL
Siriaksorn, 2006[34]	Mean 24 months (SD 4·4)	ELISA	..	1% protected	≥10mIU/mL
Abzug, 2009[37]	≥6 months	ETI-AB-AUK PLUS immunoassay	..	24% seropositive	≥10mIU/mL
Pneumococcus (PPV)					
Abzug, 2006[32]†	≥6 months	ELISA	..	31% responded (serotype 1), 58% (6B), 35% (14), 87% (19F), 25% (23F)	≥0·5 µg/mL
Costa, 2008[31]†	..	ELISA	..	Mean 0·343 ug/mL (serotype 4), 0·751 (6B), 0·453 (9V), 0·935 (14), 0·509 (18C), 1·513 (19F), 0·517 (23F)	
Tarrago, 2005[30]	..	ELISA	..	Mean 0·4 ug/mL (SD 0·8; serotype 6B); 1·3 (2·4; 14); 1·2 (4·0; 23F)	
Replicating vaccines					
Measles					
Farquhar, 2009[52]	6 months	ELISA	33% positive	42% positive overall 53% of positives seroreverted 40% of negatives seroconverted	>1·1 antibody index
Zaccarelli-Filho, 2007[51]	Good VLR: Mean 4·6 (SD 1·0) Partial VLR: 3·7 (1·1) Poor VLR: 4·4 (1·0)	Indirect ELISA	..	Good VLR: 43% protected Partial VLR: 44% protected Poor VLR: 45% protected	>0·12 IU/mL
Aurpibul, 2006[44]	Mean 24·5 months (SD 4·1)	ELISA	..	42% protected	≥320 mIU/mL
Bekker, 2006[48]	Median 205 weeks; IQR 124–359	Enzyme immunoassay	63% positive	40% of positives lost protective antibodies	≥9·0 AU/mL
Mumps					
Bekker, 2006[48]	Median 205 weeks; IQR 124–359	Enzyme immunoassay	52% positive	38% of positives lost protective antibodies	≥9·0 AU/mL
Rubella					
Bekker, 2006[48]	Median 205 weeks; IQR 124–359	Enzyme immunoassay	80% positive	11% of positives lost protective antibodies	≥10·0 IU/mL
Zaccarelli-Filho, 2007[51]	Good VLR: mean 4·6 (SD 1·0) Partial VLR: 3·7 (1·1) Poor VLR: 4·4 (1·0)	Indirect ELISA	..	Good VLR: 43% protected Partial VLR: 66% protected Poor VLR: 27% protected	>10·0 IU/mL

VLR=viral load responder. IU=international units. IE=internationale einheit. PPV=pneumococcal polysaccharide vaccine. AU=antibody units. *Good VLRs are patients with HIV-RNA below 400 copies per mL for at least 12 months before tests. Partial VLRs are patients who showed at least 1 $\log_{10}$ reduction in HIV-RNA copies per mL after being started on HAART. Poor VLRs are patients who showed a decrease of <1 $\log_{10}$ of HIV-RNA copies per mL after being started on HAART. †The study by Abzug and colleagues[32] included 25% who had not previously received pneumococcal polysaccharide or conjugate vaccine. The study by Costa and colleagues[31] included 40% who had not previously received pneumococcal polysaccharide vaccine.

Table 2: **Studies reporting humoral immunity to vaccine-preventable diseases after the start of HAART**

toxoid before and after initiation of HAART.[22,52] In the study by Farquhar and colleagues from Kenya,[52] 78% of children were seropositive before taking HAART. After 6 months of treatment, only 59% of children were seropositive, with 23% of children who were seronegative before HAART becoming seropositive after. Unexpectedly, 31% of children who were seropositive before HAART reverted to being seronegative after. In the study by Ghosh and colleagues from Germany,[22] mean antibody concentration rose from 0·25 IE/mL (SD 0·3) before HAART to 0·67 IE/mL (SD 0·9) after a mean of 36 months on HAART.

Predictors of immune response after starting HAART were reported in several studies.[25,32,37,51,52] Most studies assessed demographic characteristics as well as immunological and virological variables, including nadir values and values since starting HAART, in relation to immune responses to vaccines. Virological and immunological measurements at other potentially important times, such as the time of first vaccination, were not available in any of the studies. For tetanus toxoid, older age when starting HAART and greater increase in the proportion of CD4 T cells between start and 6 months of treatment were positively associated with an immune response after HAART in one study,[52] although the proportion of CD4 T cells, HIV-1 viral load, and anthropometric measures at the start of HAART were not associated with immunity.[52] Additionally, young age was associated with loss of immunity after being started on HAART. In another study, HAART response measured by viral suppression was not associated with immunity to tetanus toxoid.[51] For diphtheria and HBV, one study[51] found that children with good or partial responses to HAART, defined by long-term suppression of HIV-1 viral load, were more likely to have immunity than were children with poor responses to HAART, although this result was not statistically significant. In another study of HBV,[37] better immune status, defined by both pre-HAART nadir and study entry (after HAART) proportions of CD4 T cells, and shorter time between previous HBV vaccination and study entry were positively associated with immune response to HBV. When the components of immune status were examined, nadir proportions of CD4 T cells were more predictive than were those of CD4 T cells at study entry. For pneumococcal vaccines, immune status, as previously defined for HBV, was not predictive of immunity. Age, race, sex, duration of current HAART regimen, pre-HAART nadir proportions of CD4 T cells, and proportions of CD4 T cells and HIV-1 viral load at study entry (after HAART) were predictive for at most one serotype.[32]

Lymphoproliferative responses before and after initiation of HAART also were investigated for tetanus toxoid.[12,19–23,26,28] Responses before HAART ranged from 0% to 28% of children who had a stimulation index (SI) of either ≥3 or >4. Responses after HAART ranged from 0% to 71%. Four studies reported an increase in the proportion of children responding,[12,21,22,28] one study noted no change in response,[19] and two studies reported a decrease in lymphoproliferative responses after HAART.[20,23]

Several studies compared the immune responses of children infected with HIV taking HAART with control groups (figure). In the study by Ching and colleagues from the USA,[26] antibody concentrations and lymphoproliferative responses to tetanus toxoid among children infected with HIV receiving HAART were compared with those of healthy adults. Healthy adults were more likely to have protective antibody concentrations (100% *vs* 38%) and lymphoproliferative responses (100% *vs* 7%). In studies by Blazevic and colleagues[19] and Peruzzi and colleagues[21] adults and children not infected with HIV had higher lymphoproliferative responses (100% *vs* 11%)[19] to tetanus toxoid. In a study by Fernandes and colleagues,[33] HBV antibody concentrations in children with HIV who were receiving HAART were compared with those of children not receiving HAART (most of whom were receiving only two antiretroviral drugs) and healthy, uninfected, age and sex matched children.[33] Compared with children receiving HAART, infected children not receiving HAART (44% *vs* 17%) and uninfected children (87% *vs* 17%) were more likely to be seropositive for HBV. Differences due to HAART in children infected with HIV were postulated to be due to greater decline in CD4 T cells and immune function among children on HAART.

Live viral vaccines

For measles mumps rubella vaccine (MMR), the proportion of children with an immune response, as defined by each study, after starting HAART ranged from 42% to 45% for measles virus and 27% to 66% for rubella virus (table 2). Two studies reported antibody concentrations before and after HAART. For measles, the proportion of Kenyan children who were seropositive increased from 33% before HAART to 42% after HAART.[52] However, 53% of children who were seropositive before HAART lost protective immunity, whereas 40% of children who were seronegative or had borderline antibody concentrations became seropositive after receiving HAART for 6 months. In the study by Bekker and colleagues,[48] 63% of children were seropositive for measles before HAART, but 40% became seronegative after a median of 205 weeks on HAART. Similarly, 52% of children were seropositive for mumps before HAART and 80% for rubella, but 38% and 11% became seronegative after starting HAART.[48]

Predictors of immune responses for children on HAART were assessed in four studies, with few consistent results. For measles, lower HIV-1 viral load before HAART was predictive of an immune response after HAART in one study[52] but not another,[44] and HAART response (defined by long-term suppression of viral load) or viral load after HAART were not predictive of response in any study.[44,51,52] Higher proportions of CD4 T cells after HAART were marginally associated with an immune response in one study[52] but not another,[44] and the proportion of CD4 T

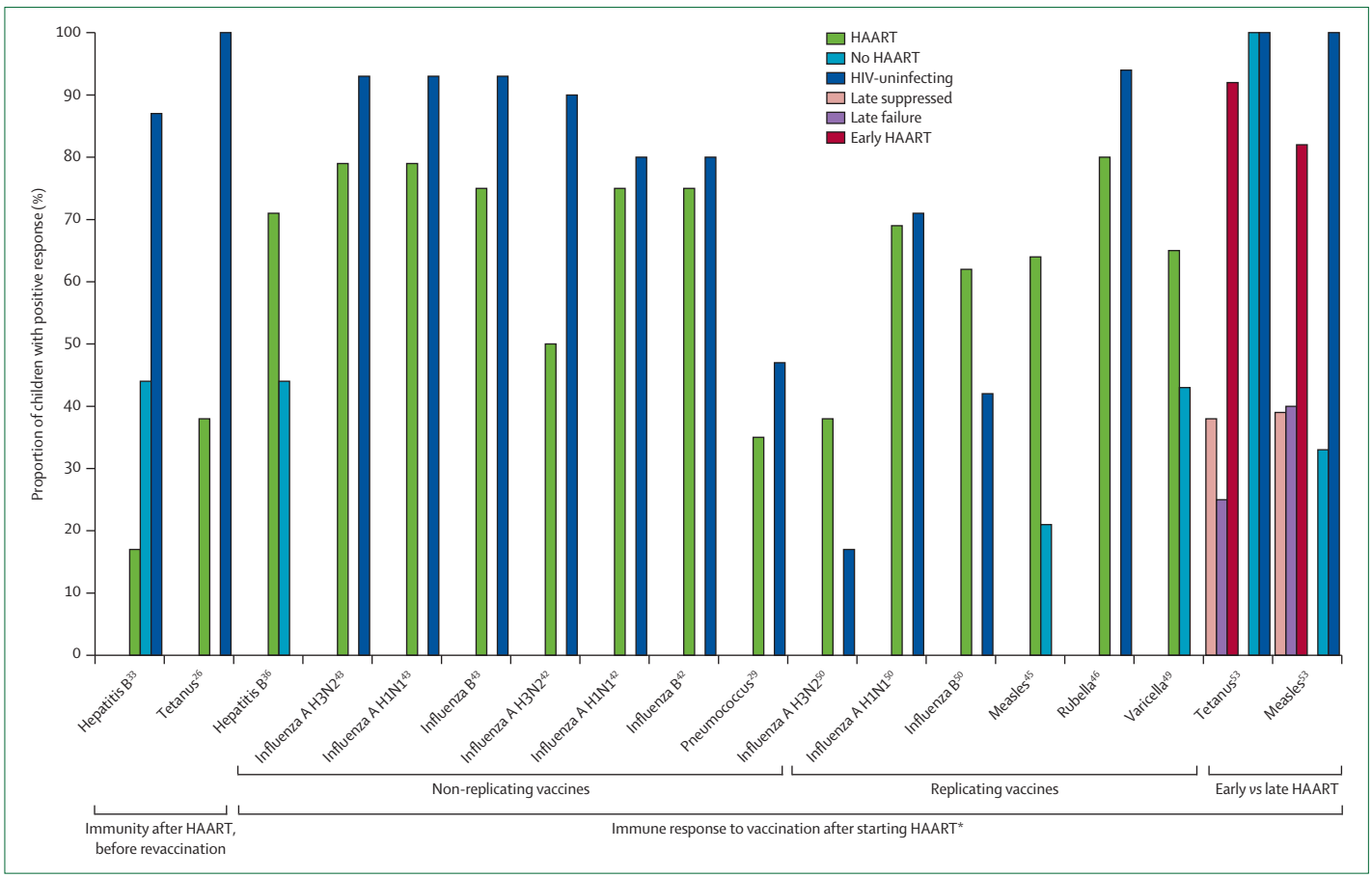

Figure: **Comparison of immune responses to vaccination between children infected with HIV on HAART and control groups**
Late suppressed=children who were started on HAART after age 1 year in whom viral suppression was achieved. Late failure=children who were started on HAART after age 1 year in whom viral suppression was not achieved. Early HAART=children who were started on HAART within the first year of life. HAART=all children on HAART. No HAART=children infected with HIV who either had no history of HAART or were not in receipt of HAART. HIV-uninfected=children in the HIV-uninfected control group. *Children may have received new vaccines or may have been revaccinated with prior vaccines.

cells before HAART was not predictive of response in either study.[44,52] Clinical status, either by Centers for Disease Control and Prevention (CDC) clinical category or anthropometric measures, was not predictive of immune response to measles vaccine.[44,52] Age at study enrolment was not predictive of immune response in several studies,[44,52] but younger age was associated with loss of immunity to measles virus in another.[48] For rubella, children with a good or partial response to HAART, defined by long-term viral suppression, were more likely to have protective immunity, although this difference was not statistically significant.[51] For measles, mumps, and rubella, low pre-HAART antibody concentrations were predictive of loss of immunity, although this finding was statistically significant only for measles and rubella.[48,52]

Response to vaccination after HAART

Non-replicating vaccines

Studies of DTP, conjugate Hib, HBV, pneumococcal, and inactivated influenza vaccines involved revaccination of children on HAART who had previously received the same vaccines before starting HAART (table 3). Within the first 3 months after revaccination, the proportion of children responding to vaccination, as defined by each study, was 53–100% for tetanus toxoid,[24,26–28,52,55] 75% for conjugate Hib vaccine,[55] 46–92% for HBV vaccine,[35,37] 29–96% by serotype for pneumococcal vaccine,[29–32] and 50–100% by strain for influenza vaccine.[41–43,54] By contrast, studies of HAV involved vaccination of children for the first time after starting HAART, and 72–97% of children responded after receiving 2–3 doses.[28,38]

Several studies followed children after vaccination to evaluate the degree of waning immunity among children receiving HAART (table 3). In general, immunity declined but a high proportion of children maintained immunity about a year after vaccination. For tetanus toxoid, one study from the USA[24] reported a decline from 74% seropositive at 4 weeks to 38% by 32 weeks after vaccination,[24] although in three other studies 85–90% of children maintained immunity 1 year after vaccination.[26,28,55] For pertussis, antibody concentration declined from 22·3 EU/mL at 8 weeks to 10·1 EU/mL by 48 weeks and

6·8 EU/mL by 96 weeks after vaccination.[25] For Hib, three (75%) children had detectable antibodies 4 weeks after vaccination and two (100%) at 52 weeks.[55] For HBV, the proportion of seropositive children decreased from 46% 8 weeks after revaccination to 38% after 96 weeks and 25% after a median of 4·6 years.[37] All children remaining in the study after a median of 4·6 years were revaccinated a second time. Of the children who were seronegative within 1 week of the second revaccination, 37% seroconverted 4 weeks after vaccination. For influenza, protective responses tended to be lower for influenza B virus and decreased over time for all strains.[42,43,54] One

	Vaccine (number of doses)	Assay used; definition of immune response	New vaccine?*	Time after vaccination (short-term); response	Time after vaccination (long-term); response
Non-replicating vaccines					
Tetanus					
Melvin[55]	Tripedia or DT (one)	EIA; >0·1 IU/mL	No	4 weeks; 90% detectable	52 weeks; 86% detectable
Rigaud[28]	DTaP or Td or DT (three)	ELISA; >0·1 IU/mL	No	4 weeks after third dose; 94% responded	52 weeks after third dose; 90% responded
Ching[26]	Tetanus (one)	IgG EIA; ≥0·15 IU/mL	No	Median 2·3 months; 92% responded	Median 11·8 months; 85% responded
Farquhar[52]	TT (one)	ELISA; >0·01 IU/mL	No	4 weeks; 75% positive	..
Rosenblatt[24]	DTaP (one)	Red blood cell agglutination assay; Reciprocal titer >243	No	4 weeks; 74% positive 8 weeks; 67% positive 18 weeks; 53% positive	32 weeks; 38% positive
Ghosh[22]	TT (one)	ELISA; ..	No	After booster; mean 3·3 IE/mL (SD 1·8)	..
Pensieroso[53]	Hexavac or Infanrix-hexa plus Boosterix (··)	ELISA; >0·15 units/mL	..	..	Early:† mean 5·3 years; 92% protected Late control: mean 7·4 years; 38% protected Late failure: mean 9·7 years; 25% protected
Rigaud[28]	DTaP or Td or DT (three)	LPA; SI >3	No	4 weeks after third dose; 73% responded	52 weeks after third dose; 61% responded
Ching[26]	Tetanus (one)	LPA; SI >3	No	Median 2·3 months; 47% responded	..
Essajee[23]	DT (one)	LPA; SI ≥3	No	1–2 months; 67% positive	..
Ghosh[22]	TT (one)	LPA; SI ≥3	No	After booster; 86% response	..
Luzuriaga[27]	TT (··)	LPA; SI ≥3	No	LPA closest to 16 months of age; 100%	..
Diphtheria					
Essajee[23]	DT (one to two)	LPA; SI ≥3	No	1–2 months; 17% positive	..
Pertussis					
Abzug[25]	Infanrix (one)	ELISA; ..	No	8 weeks; mean 22·3 EU/mL, 95% CI 15·9–31·2	48 weeks; 10·1 EU/mL, 95% CI 7·1–14·4 96 weeks; 6·8 EU/mL, 95% CI 5·1–9·1
Haemophilus influenzae					
Melvin[55]	HibTiter (one)	EIA; >75 ng/mL	No	4 weeks; 75% detectable	52 weeks; 100% detectable
Hepatitis A virus					
Rigaud[28]	Havrix (three)	Quantitative ELISA; ≥20 mIU/mL	Yes	4 weeks after third dose; 72% responded	52 weeks after third dose; 66% responded
Siberry[40]	Havrix or Vaqta (one to two)	Microparticle EIA; ..	Yes	..	Median 42 weeks; 85% seropositive
Weinberg[38]	Havrix (two)	ELISA; ≥20 mIU/mL	Yes	8 weeks after second dose; 97% protected	18 months after second dose; 90% protected
Rigaud[28]	Havrix (three)	LPA; SI >3	Yes	4 weeks after third dose; 12% responded	52 weeks after third dose; 13% responded
Hepatitis B virus					
Pippi[36]	HBVAXPRO (three)	ELISA; ≥10 mIU/mL	..	..	5 months after third dose; 71% protected
Lao-araya[35]	HBV (three)	ELISA; ≥10 mIU/mL	No	2 months after first dose; 17% protected 4 months after second dose; 83% protected 1 month after third dose; 92% protected	..
Abzug[37]	Recombivax HB (one)	ETI-AB-AUK PLUS immunoassay; ≥10 mIU/mL	No	8 weeks; 46% seropositive	48 weeks; 38% seropositive 96 weeks; 38% seropositive Median 4·6 years; 25% seropositive
Influenza virus					
Tanzi[41]	Inflexal V (one)	HAI; ≥1:40	No (66% had prior vaccine)	30 and 90 days; A H1N1 100% protected, A H3N2 100%, B 76% at both time points	..
Montoya[42]	Imovax Gripe (one)	HAI; ≥1:40	..	1 month; A H1N1 75% protected, A H3N2 50%, B 56%	..
Vigano[43]	Inflexal V (one)	HAI; ≥1:40	Yes	1 month; A H1N1 79% protected, A H3N2 79%, B 75%	6 months; A H1N1 75%, A H3N2 54%, B 63%
Levin[54]	FluZone (one)	HAI; titre ≥40	No (prior TIV)	1 month; A H1N1 67% protected, A H3N2 96%, B 69%	6 months; A H1N1 55%, A H3N2 97%,B 63%

(Continues on next page)

	Vaccine (number of doses)	Assay used; definition of immune response	New vaccine?*	Time after vaccination (short-term); response	Time after vaccination (long-term); response
(Continued from previous page)					
Pneumococcus					
Tangsinmankong[29]	Pneumovax 23 (one)	ELISA; clinical protection‡	..	1–3 months; 35% protected	
Pensieroso[53]	Pneumo 23 (··)	ELISA; ..	..	..	Early: mean 3·8 years; med 95 IU/mL§ Late control: mean 3·8 years; med 50 IU/mL Late failure: mean 3·9 years; med 25 IU/mL
Tarrago[30]	Prev(e)nar (two)	ELISA/OPA; ≥2-fold increase in ELISA/OPA	No (prior PPV)	3 months after second dose; 44% responded (serotype 6B), 29% (14), 38% (24F)	..
Costa[31]	PCV-7 (two)	ELISA; ≥1·3 ug/mL	No (60% had prior PPV)	1–3 months after second dose; 65% responded to ≥4 serotypes (45% [serotype 4], 55% [6B], 58% [9V], 90% [14], 70% [18C], 80% [19F], 50% [23F])	..
Abzug[32]	Prevnar (2)+PNU-IMUNE 23 (one)	ELISA; ≥0·5ug/ml	No (75% had prior PPV)	8 weeks after third dose; 81% responded (serotype 1), 92% (6B), 92% (14), 96% (19F), 76% (24F)	80 weeks after third dose; 75% (serotype 1), 92% (6B), 88% (14), 95% (19F), 74% (24F)
Replicating vaccines					
Measles					
Melvin[55]	M-M-R II (one)	EIA; >1·10 ISR	No	4 weeks; 83% detectable	52 weeks; 73% detectable
Berkelhamer[45]	MMR (one)	ELFA; ≥0·7	No	1–4 months; 64% positive	..
Pensieroso[53]	Priorix (··)	ELISA; >0·2 units/mL	..	..	Early: mean 4·2 years; 82% protected Late control: mean 4·7 years; 39% protected Late failure: mean 3·7 years; 40% protected
Farquhar[52]	Measles (one)	ELISA; >1·1 antibody index	No	4 weeks; 78% positive	..
Aurpibul[47]	Priorix (one)	ELISA; ≥320 mIU/mL	No	4 weeks; 90% protected; 24 weeks; 80% protected	..
Bekker[48]	MMR (one)	EIA; ≥9·0 AU/mL	No	..	Median 48 weeks, IQR 19–93; 60% of negatives seroconverted
Mumps					
Bekker[48]	MMR (one)	EIA; ≥9·0 AU/mL	No	..	Median 48 weeks, IQR 19–93; 89% of negatives seroconverted
Aurpibul[47]	Priorix (one)	ELISA; titre >1:500	No	4 weeks; 78% protected; 24 weeks; 61% protected	..
Rubella					
Bekker[48]	MMR (one)	EIA; ≥10·0 IU/mL	No	..	Median 48 weeks, IQR 19–93; 80% of negatives seroconverted
Aurpibul[47]	Priorix (one)	ELISA; ≥10 mIU/mL	No	4 weeks; 100% protected; 24 weeks; 94% protected	..
Lima[46]	MMR (one)	ELISA; >10 IU/mL	Yes	3 months; 80% protected	..
Varicella zoster virus					
Levin[49]	Oka/Merck vaccine (two)	FAMA; titre ≥1:2	Yes	20 weeks (8 weeks after second dose); 71% positive	52 weeks; 65% positive 104 weeks; 47% positive 156 weeks; 38% positive
Levin[49]	Oka/Merck vaccine (two)	LPA; SI ≥3	Yes	20 weeks (8 weeks after second dose); 85% positive	52 weeks; 92% positive 104 weeks; 85% positive 156 weeks; 33% positive
Influenza virus					
King[50]	LAIV by Aviron (two)	HAI; ≥4-fold increase	..	28–70 days after first dose; 59% seroresponse to ≥1 strain (A H1N1 41%, A H3N2 32%, B 45%) 28–35 days after second dose; 77% seroresponse to ≥1 strain (A H1N1 69%, A H3N2 38%, B 62%)	..
Levin[54]	FluMist (one)	HAI; titre ≥40	No (prior TIV)	1 month; A H1N1 63% protected, A H3N2 92%, B 33%	6 months; A H1N1 45%, A H3N2 95%, B 32%

DT=diphtheria and tetanus vaccine. EIA=enzyme immunoassay. IU=international units. IE=internationale einheit. DTaP=diphtheria, tetanus, and acellular pertussis vaccine. Td=tetanus and diphtheria vaccine. TT=tetanus toxoid. LPA=lymphoproliferative assay. SI=stimulation indices. EU=ELISA units. HAI=haemagglutination inhibition assay. TIV=inactivated trivalent influenza vaccine. OPA=opsonophagocytic activity. ISR=immune status ratio. ELFA=enzyme-linked fluorescent assay. AU=arbitrary units. PPV=pneumococcal polysaccharide vaccine. MMR=measles, mumps, and rubella vaccine. FAMA=fluorescent antibody membrane assay. LAIV=live attenuated influenza vaccine. *Indicator for whether this was a new vaccine given to children for the first time while on HAART or revaccination for a vaccine given before started on HAART. †Early represents children who were started on HAART within the first year of life. Late control represents children who were started on HAART after age 1 year in whom viral suppression was achieved. Late failure represents children who were started on HAART after age 1 year in whom viral suppression was not achieved. ‡Calculated using specific IgG levels and incidence of invasive *Streptococcus pneumoniae* isolated in the USA and summed over all serotypes. §Values estimated from box plots.

Table 3: **Studies reporting immune response to vaccination while receiving HAART**

study also examined changes in influenza-specific antibodies and lymphocytes within the first 6 months after vaccination.[43] Increases were reported for IgG3 antibodies, CD8 interferon-γ-secreting T lymphocytes, and CD4 interleukin-2-secreting T lymphocytes 1 month after vaccination. There was a subsequent decline to almost baseline levels 6 months after vaccination. The increase at 1 month was only statistically significantly different from baseline for CD8 interferon-γ-secreting T lymphocytes. For pneumococcal vaccine, immune responses remained stable by serotype (81% to 75% from 8 weeks to 80 weeks for serotype 1, 92% to 92% for 6B, 92% to 88% for 14, 96% to 95% for 19F, and 76% to 74% for 24F).[32] For HAV, the proportion of children with an immune response declined from 72% at 4 weeks to 66% at 52 weeks after vaccination,[28] and from 97% at 8 weeks to 90% at 18 months in two studies from the USA.[38]

Lymphoproliferative responses were assessed in several studies (table 3). The proportion of children responding within 3 months of vaccination was 47–86% for tetanus toxoid,[22,23,26,28] and 17% for diphtheria toxoid.[23] For tetanus toxoid, Rigaud and colleagues[28] reported a decrease in lymphoproliferative responses 52 weeks after vaccination (73% at 4 weeks to 61% at 52 weeks). For HAV, the proportion of children responding at 4 weeks (12%) and 52 weeks (13%) was similar.[28]

Predictors of response to vaccination while receiving HAART were investigated in several studies. For tetanus toxoid, lymphoproliferative responses and antibody concentrations were higher for children who had undetectable viral load before study vaccination than for children who did not, although the proportions who were positive were not statistically different between the two groups.[26] Additionally, children with higher percentages of naive T cells (CD4+/CD62L+/CD45RA+) after study vaccination had better responses, although age was not associated with the response.[24] For pertussis, greater antibody concentration, greater proportions of CD4 T cells, and lower HIV viral load 24 weeks before study vaccination were associated with higher antibody concentrations after revaccination, whereas nadir proportions of CD4 T cells (ever or before HAART) were not associated.[25] For pneumoccocal vaccine, older age,[29] higher antibody concentration,[32] greater proportions of CD4 T cells[29,32] and lower HIV-1 viral load[32] at study vaccination, and longer duration of HAART,[32] were associated with better response in some studies, but not in others.[29,30] Other characteristics, such as race, sex, and clinical status before starting HAART or at study vaccination were not associated with response.[29,30,32] For HBV vaccine, lower HIV-1 viral load at the first dose of study vaccination[35,37] was associated with better response, although duration of HAART and viral load before HAART were not.[35,36] Inconsistent results were found for antibody concentration at vaccination, age, and immune status at revaccination.[35,37] For influenza, higher antibody concentrations at study vaccination were associated with better response,[54] although age, sex, and immunological status were not associated.[43,54] Lower HIV-1 viral load at study vaccination was associated with better response in one study[54] but not another.[42] For HAV, greater proportions of CD4[39,40] and CD8 T cells,[39] lower HIV-1 viral load,[38–40] greater proportions of B cells (CD19) at study vaccination (first dose),[39] detectable cell-mediated immunity[39] after vaccination, and proportion of CD4 T cells at second dose,[38] were associated with better response. Age, sex, and race were not associated with response in any study.[38–40] The proportion of naive and memory T cells were not associated with response to HAV vaccination.[39]

Two studies were specifically designed to assess the effects of duration of HAART and timing of HAART initiation in relation to vaccine responses. In the study by Rigaud and colleagues,[28] children starting HAART were randomised to receive vaccines at 8 weeks and 32 weeks after study enrolment. Children received either tetanus toxoid and then HAV or HAV and then tetanus toxoid to assess the effect of the level of immune reconstitution on vaccine responses. For tetanus toxoid in children who previously received vaccinations before starting HAART, lymphoproliferative responses, antibody concentrations, and serological response did not differ between the two groups (100% *vs* 89% 4 weeks after completing vaccine series; 100% *vs* 81% after 1 year). For HAV in children who received their first dose after starting HAART, children who received HAV vaccine at 32 weeks had substantially greater antibody concentrations than children who received the vaccine at 8 weeks. The proportion of responders was also greater (88% *vs* 60% 4 weeks after completing vaccine series; 86% *vs* 50% after 1 year), although not statistically different.

In the study by Pensieroso and co-workers,[53] immunity to childhood vaccines was assessed among children who started HAART at different ages (either early, within the first year of life, or late, after the first year of life) to establish the effect of the timing of HAART on vaccine responses. The investigators did not report whether children in the early group were given their primary vaccine series before or after starting HAART, therefore, whether the study assessed the timing of HAART in relation to age or vaccination is unclear. Antibodies to tetanus toxoid were higher in the early treatment group than in the late treatment group, with higher responses in the late treatment group that achieved HIV-1 suppression than in the late treatment group that did not (figure). A similar trend was noted for antibodies to pneumococcus, although these results were not statistically significant.

When compared with healthy, HIV-uninfected controls, children infected with HIV taking HAART tended to have lower antibody concentrations or lower protective immunity for pneumococcal antigens[29] and influenza (figure).[42,43] For influenza, children infected with HIV had lower concentrations of influenza virus-specific IgG3, but not IgG1, antibodies, CD8 interferon-γ-secreting

T lymphocytes and CD4 interleukin-2-secreting T lymphocytes than did healthy controls.[43] In the study by Pensieroso and colleagues,[53] children in the early treatment group had similar antibody concentrations to tetanus toxoid and pneumococcus as children in the control group, but those in the late treatment group tended to have lower antibody concentrations than did children in the control group. Children infected with HIV not receiving HAART had lower antibody concentrations after vaccination against HBV than did children receiving HAART;[36] however, this was not true for tetanus toxoid (figure).[53] Noted differences between children receiving and not receiving HAART likely depend on the immunological and virological status of children not receiving HAART at the time of vaccination.

Vaccine safety was reported in several studies. No serious, potentially life-threatening adverse events were reported for tetanus toxoid,[24,28,52] pertussis,[25] HAV,[28,38] HBV,[35–37] influenza,[42,43,54] or pneumococcal vaccine.[32] Mild adverse events were reported for HBV[35,36] and influenza vaccination, including pain or swelling at the injection site and fever. These events were reported by 16% of children infected with HIV, compared with 14% of uninfected children, receiving influenza vaccination in one study.[43] In another study,[54] 33% of children infected with HIV that received influenza vaccination reported grade 1 events (mild, no intervention required), 24% reported grade 2 (moderate, minimal intervention required), and 2% reported grade 3 (severe, medical care required). Grade 3 events included fever and injection site swelling. For pneumococcal vaccine, 5% of participants reported at least one vaccine-related grade 3 event, including localised or generalised erythema, induration, and pain.[32] An additional 1% of participants reported possible vaccine-related events, including fever, neutropenia, and pharyngitis. No studies reported adverse changes in CD4-T-cell counts or proportions, or in plasma HIV-1 viral loads, after vaccination.[24–26,28,32,35,41,42,54]

Live viral vaccines

For replicating vaccines, studies were available for MMR, varicella, and live attenuated influenza vaccines (table 3). For most vaccines, with the exception of varicella, children were revaccinated with vaccines first received before starting HAART. Within the first 3 months after vaccination, the proportion of children responding to vaccination, as defined by each study, was 64–90% for measles,[45,47,52,55] 61% for mumps,[47] 80–100% for rubella,[46,47] 71% for varicella,[49] and 33–92% by strain for influenza.[50,54] In studies of long-term responses to vaccination (>3 months), the proportion of children with an immune response was 39–82% for measles,[48,53,55] 89% for mumps,[48] 80% for rubella,[48] 65% for varicella,[49] and 32–95% by strain for influenza.[54] Several studies assessed both short-term and long-term immunity and generally found that immunity decreased with time. For measles, one study in the USA[55] reported that the proportion of children with detectable antibodies decreased from 83% at 4 weeks to 73% at 52 weeks after revaccination. For varicella, the proportion of children who were seropositive decreased from 71% at 8 weeks after vaccination to 65% at 52 weeks, 47% and 104 weeks, and 38% at 156 weeks.[49] For influenza, the proportion of children with protective immunity remained steady through 24 weeks after vaccination.[54]

Lymphoproliferative responses were only reported from one study assessing varicella vaccine in the USA.[49] The proportion of children with positive lymphoproliferative responses was 85% at 8 weeks after vaccination, 92% at 52 weeks, 85% at 104 weeks, and 33% at 156 weeks.

Risk factors for response to vaccination were inconsistent. Several studies found no association with the proportion of CD4 T cells and viral load at the start of HAART or revaccination against measles with MMR vaccine,[45,47] although one study reported these associations for rubella at the time of revaccination.[46] Age, sex, and duration of HAART were not associated with response to MMR vaccine.[47] In the study by Pensieroso and colleagues,[53] children who were started on HAART early had greater measles antibody concentrations and were more likely to have protective immunity compared with those who were started on HAART after the first year of life. For varicella, immune response after each dose was associated with HIV-1 viral load but not proportion of CD4 T cells at first dose.[49] Additionally, immune responses to subsequent doses were more likely to be positive if the prior response was positive.

When compared with a control group of people not infected with HIV, children infected with HIV on HAART had lower antibody concentrations for measles and rubella and were less likely to have protective immunity (figure).[46,53] However, these findings were limited to children who were started on HAART after the first year of life in the study by Pensieroso and colleagues,[53] and to children with evidence of moderate or severe immunosuppression in the study by Lima and co-workers.[46] For live intranasal influenza vaccine, children on HAART had similar responses to vaccination compared with uninfected people in the control group.[50] When compared with children infected with HIV not on HAART, a higher proportion of infected children on HAART developed protective immunity to varicella[49] and measles,[45,53] although in the study by Pensieroso and colleagues,[53] this was true only for children who were started on HAART in the first year of life.

Vaccine safety was assessed for measles, varicella, and influenza. For measles vaccine, no serious adverse events were reported;[47,52] mild events included pain at the injection site (23 patients; 45%).[47] For varicella vaccine, one (6%) patient reported reactions at the injection site (none were grade three) and two (12%) reported systemic reactions (none were grade three), including fever, otitis media or sinusitis, rash, and "viral syndrome", after the first dose of vaccine. Two (12%) children had local reactions after the second dose of vaccine (one [6%] grade three) and five (29%) had systemic reactions (one [6%]

grade three).[49] For the live intranasal influenza vaccine, 60 (49%) patients reported grade one events, 23 (19%) grade two, and three (2%) grade three. Grade three events included malaise, finger pain, and leg boils, only one of which was thought to be vaccine related (not specified).[54] A second influenza vaccine trial[50] reported that 14 (61%) children infected with HIV experienced reactogenicity events after the first dose of the vaccine and five (33%) after the second dose, including fever, cough, headache, and nausea or vomiting. Five (22%) children had possible vaccine-related adverse events after the first dose and two (13%) after the second dose, including otitis media, upper respiratory illness, sinusitis, wheezing, and coughing. Rates of events did not differ between infected and uninfected children.[50] No studies reported any adverse changes in CD4-T-cell counts or proportions, or in plasma HIV-1 viral loads, after vaccination.[47,49,50,54]

Discussion

The proportion of children with immunity after being started on HAART is low for most vaccines studied, but no characteristic consistently predicted immunity after starting HAART. In general, children infected with HIV on HAART developed immune responses within several months of vaccination, with no differences in the level of primary or secondary responses to new or previously received vaccines. However, immunity waned in some children. In some studies, children on HAART who had a higher CD4-T-cell count and lower plasma HIV-1 viral load at vaccination were more likely to develop immunity.

HAART is unlikely to restore memory T cells for vaccine antigens to which children were exposed before treatment, but should restore the ability of the immune system to respond to new antigens. Few studies measured immunity before and after the start of HAART. In studies that measured immune responses only after HAART, it was not possible to establish whether the noted low levels of immunity were due to a lack of primary response to vaccination before HAART or the inability of HAART to restore waning immunity. From the few studies that measured immune responses both before and after HAART, some children did regain immunity to vaccine-preventable diseases after being started on HAART. However, many children lost measurable antibody responses to vaccine antigens after being started on HAART, potentially as a result of the shorter lifespan of plasma cells and persistent B-cell abnormalities in children infected with HIV.[56] Consequently, levels of immunity to vaccine-preventable diseases in this population remained low, suggesting that the majority of children on HAART would benefit from revaccination. Waning immunity after revaccination and vaccination with new vaccines was greater and more rapid than in children not infected with HIV, who typically maintain high antibody concentrations years after vaccination.[57] Waning immunity among children infected with HIV on HAART can be explained by persistent B-cell abnormalities in children[22,58] and adults[56,58,59] despite increases in the number and function of CD4 T cells. Most notable are the loss of memory B cells and a decrease in memory B cell function in treatment-naive patients that are not fully reversed after starting HAART. These losses are associated with defects in antigen-specific memory-B-cell responses to both T-cell-dependent and T-cell-independent antigens,[56,58] and might affect long-term responses to vaccination in children on HAART.

The best timing of vaccination after starting HAART is not known, both for revaccination and primary vaccination with new vaccines, and few studies address this important question. Most studies found that higher CD4-T-cell counts and lower HIV-1 viral loads were crudely or independently associated with higher levels of immunity after vaccination on HAART, suggesting sufficient time should be allowed to restore immune function and suppress viral replication. Only one published study[28] was specifically designed to examine this issue. Children who received HAV vaccine for the first time more than 6 months after being started on

Panel: Study findings and implications for revaccination of children infected with HIV on HAART

Findings

- Children on HAART generally have low immunity to childhood vaccines received before starting HAART
- Children on HAART generally mount good antibody and lymphoproliferative responses to revaccination during therapy
- Children vaccinated while on HAART can lose protective immunity over time
- Timing of HAART, in relation to age, degree of immunosuppression, and primary vaccination status can influence response to vaccination
- Gaps in knowledge:
  - The best timing of revaccination after starting HAART
  - The effect of age at the start of HAART on response to revaccination
  - Responses to primary vaccination after starting HAART
  - Necessity for and timing of repeat doses after revaccination while on HAART
  - Relation between antibody concentrations and protective immunity

Implications

- HAART does not restore immunity to prior vaccination
  - Children on HAART would probably benefit from revaccination against childhood diseases
- HAART might not ensure long-lasting immunity
  - Repeat or higher doses might be needed for some children
- Children who start HAART in infancy might retain functional immunity and have better responses to vaccination
  - Continued efforts are needed to identify and treat HIV-infected children at younger ages and earlier stages of disease
- Initial vaccination or revaccination after viral suppression and immune reconstitution might improve immune responses to vaccination
  - Children with poor treatment responses might remain susceptible to vaccine-preventable diseases and might need to be monitored for adequate levels of protective immunity and possibly revaccinated when treatment responses improve

Search strategy and selection criteria

We searched PubMed for articles published in English before April 1, 2010, by use of the terms "HIV", "antiretroviral therapy", and "vaccine" (n=835), and also "HIV", "therapy", and "vaccine" in combination with "tetanus" (n=60), "pertussis" (n=11), "diphtheria" (n=16), "mumps" (n=21), "hepatitis" (n=229), "influenza" (n=89), "pneumococcal" (n=93), "measles" (n=35), "Haemophilus" (n=19), "varicella" (n=25), and "yellow fever" (n=4). Additionally, we reviewed citations within relevant studies. Studies were included if they involved children (mean or median age <16 years) in whom most (>95%) were receiving HAART or if the results were stratified by receipt of HAART; and they either described cellular or humoral immunity to at least one vaccine-preventable disease after the start of HAART but before revaccination, or they described cellular or humoral responses to vaccination after the start of HAART. Studies were excluded if pooled antigens were used or the type of antiretroviral therapy could not be established.

HAART had higher immunity than did those who received HAART for only 2 months.[28] However, duration of HAART was not associated with improved responses to tetanus toxoid, a vaccine that children first received before being started on HAART, suggesting a lower level of immune reconstitution might be sufficient to induce a memory response. Prospective studies of children infected with HIV are needed to establish the best timing of revaccination and whether this response differs by vaccine.

Age of the patient when started on HAART, particularly in relation to the timing of vaccination, might be important in enhancing vaccine responses. In one study,[53] children who were started on HAART in infancy (<12 months) had greater protective immunity than did children who were started on HAART later in childhood, and had similar levels of immunity with uninfected children of the same age. Early administration of HAART preserved the memory B-cell compartment. The restoration of immune function in infants on HAART might be similar to the immunological benefits noted among adults treated during acute infection.[60–63] Many children in the early treatment group probably received some of their primary vaccinations after being started on HAART. For all vaccines studied, immune reconstitution seems to have allowed this group to both preserve immune responses to previously received vaccines and successfully mount and maintain an immune response to new vaccines. These findings support recommendations for early administration of HAART among infants, which reduces HIV-related morbidity and mortality.[64]

Several issues limit study comparability and the inferences drawn from their review. First, although many studies were identified that assessed immunity or vaccine responses among children infected with HIV on HAART, few studies were identified for each vaccine, which limited comparisons. Second, great heterogeneity existed across studies in the type of study design, eligibility criteria on the basis of immunological and virological status, characteristics of the study population (including age, disease stage, and duration of HAART), assays used to measure immune responses, definition of immunity, and the presence of a comparison group. These factors also limited the comparability of study results. Third, vaccine-induced immunity could not be distinguished from immunity derived from natural infection. This problem is particularly relevant for studies with a long interval between the start of HAART, vaccination, and measurement of antibody levels, and obviously depends on the incidence of wild-type infection in the study population. Last, all identified studies used surrogate markers of protective immunity, specifically antibody concentrations and lympho-proliferative responses. How well these markers correlate with protective immunity in children infected with HIV on HAART is not known and data on vaccine efficacy in this population are lacking.

Despite these differences, the broad findings were consistent (panel). Most children treated with HAART remained susceptible to vaccine-preventable diseases, and in some children, immune responses to vaccines received before treatment were lost after the start of HAART. Most children receiving HAART, however, responded to vaccination but immune reconstitution was not sufficient to ensure long-term immunity for some children. Many children in low-resource settings start taking HAART at older ages, after having received their primary vaccine series.[16] As treatment programmes scale-up and more children receive HAART and live into adolescence and adulthood, a larger proportion of these children might be susceptible to vaccine-preventable childhood diseases. Levels of protective immunity in these children will need to be monitored, and some children might need additional doses of vaccines to maintain protective immunity. Vaccination policies and strategies for children infected with HIV on HAART should be developed in regions of high HIV prevalence to ensure adequate levels of population immunity. Starting HAART in infancy, before receipt of routine childhood vaccines, might preserve immunity to vaccine-preventable diseases. Consequently, efforts should continue to identify infants and children infected with HIV and start treatment as early as possible. Further studies are needed on the nature and longevity of immune responses among infants on HAART, and vaccination policies might need to be reviewed and revised as more children start treatment in infancy.

Contributors
All authors contributed equally to this paper.

Conflicts of interest
We declare that we have no conflicts of interest.

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Guillain-Barré syndrome after exposure to influenza virus

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Guillain-Barré syndrome (GBS) is an acute, acquired, monophasic autoimmune disorder of peripheral nerves that develops in susceptible individuals after infection and, in rare cases, after immunisation. Exposure to influenza via infection or vaccination has been associated with GBS. We review the relation between GBS and these routes of exposure. Epidemiological studies have shown that, except for the 1976 US national immunisation programme against swine-origin influenza A H1N1 subtype A/NJ/76, influenza vaccine has probably not caused GBS or, if it has, rates have been extremely low (less than one case per million vaccine recipients). By contrast, influenza-like illnesses seem to be relevant triggering events for GBS. The concerns about the risk of inducing GBS in mass immunisation programmes against H1N1 2009 do not, therefore, seem justified by the available epidemiological data. However, the experiences from the 1976 swine flu vaccination programme emphasise the importance for active and passive surveillance to monitor vaccine safety.

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Introduction

Infection with influenza virus affects a substantial proportion of the worldwide population each year.[1–3] In 2009, the perceived global threat of influenza reached an exceptional level after the emergence of a novel swine-origin influenza A H1N1 (so-called swine flu, subtype H1N1 2009), which was first isolated in local outbreaks in Mexico, Canada, and the USA.[4] The subsequent rapid global spread of this strain and concerns about its possible virulence led national and global health authorities to initiate countermeasures in early 2009, by means of mass immunisation programmes in several countries, including the USA and countries of the European Union. These vaccination campaigns were the cornerstone of public health measures to prevent the undesired consequences of a pandemic. They also served as a reminder (at least to the neurological community) of the 1976 national influenza immunisation programme against swine flu subtype A/NJ/76 in the USA, which was stopped because of the emergence of Guillain-Barré syndrome (GBS) in vaccine recipients.[5] GBS after vaccination is rare, and most studies have concluded that it is a chance event except in the 1976 programme. However, the small size of vaccine safety trials before licensing, the testing and licensing of vaccines for potential pandemic diseases before the start of the pandemic (so-called mock-up licensing), and the very low incidence of GBS mean that data could be insufficient to assess the risk reliably.[6] Conventional vaccine safety monitoring after licensing does not entirely eradicate this concern.

By contrast with vaccination, evidence is increasing that influenza infection and influenza-like illnesses can act as triggers for GBS. This important fact, which has been highlighted in epidemiological studies,[7,8] seems to be underappreciated in public and professional advisory interpretations of influenza vaccine adverse event data and subsequent risk–benefit assessments. The establishment of background rates for GBS will be very useful in this regard; this information has already been provided for several countries.[1]

We believe that, in addition to the prevention of multiple non-neurological diseases by influenza vaccination, awareness and correct interpretation of all available data about the relation of GBS, influenza infection, and influenza vaccination are a prerequisite for an objective risk–benefit analysis of current and future influenza vaccination campaigns. We review the existing data derived from studies about GBS after influenza infection and GBS after exposure to influenza vaccine. We also summarise the current information about the plausibility of influenza immunisation as a biological cause of GBS.

GBS

GBS is an acute, acquired, monophasic peripheral neuropathy.[9,10] It is now one of the most common acute paralytic neuromuscular disorders and is associated with substantial mortality and morbidity.[11] GBS has various subtypes that can be distinguished by electrophysiological and pathological criteria. The most common form (90–95% of cases) in Europe and North America is acute inflammatory demyelinating polyradiculoneuropathy, which is characterised by demyelinating changes that involve cellular infiltration of T cells, macrophages, and subsequent demyelination and additional axonal damage.[10] By contrast, the axonal forms of GBS (acute motor axonal neuropathy and acute motor sensory axonal neuropathy) are rare in Europe and North America, but in China, Japan, and South America can account for 30–40% of GBS cases.[12–14] The axonal variants of GBS are pathologically distinct and are characterised by primarily antibody-mediated damage to axons. Other rare atypical variants exist, such as Miller Fisher syndrome and a form characterised by oropharyngeal weakness.[15–18] Despite differences in clinical presentation, the overall annual incidence of GBS is similar across geographical regions, and ranges from 0·4 to 4·0 cases per 100 000 of the general population.[19–24] In about 60% of cases, GBS is preceded by an infection, of which the most commonly identified microbiological agent is *Campylobacter jejuni*.[12,25,26]

Substantial progress has been made in elucidating the pathological mechanisms underlying GBS, which is summarised in several excellent reviews.[9,10,17,27–29] GBS is

deemed to be an autoimmune disease, because of its pathological characteristics.[30–34] A similar disease, so-called experimental autoimmune neuritis (EAN), can be induced in animals by direct immunisation with nerve tissue or by passive transfer via specific T cells against myelin proteins.[28,35,36] Moreover, autoantibodies to gangliosides and related glycolipids (including GM1, GD1a, GD1b, GQ1b) are detected in many patients with axonal GBS variants and Miller Fisher syndrome.[37–43] Various in-vivo and in-vitro models provided evidence for a pathogenic role of these antibodies.[15,44–55] Molecular mimicry, invoking an immune response to antigenic targets that are coincidentally shared by an infectious organism and nerve tissue, has emerged as the leading hypothesis for the pathogenesis of GBS.[56–60]

Virology and vaccination of influenza

Influenza viruses are RNA viruses that belong to the family Orthomyxoviridae. Three types of influenza viruses are known (A, B, and C) of which A and B cause seasonal epidemics.[3] Influenza A is classified into different serotypes on the basis of epitope differences in two main types of envelope glycoproteins: haemagglutinin and neuraminidase. 15 haemagglutinin subtypes (H1–H15) and nine neuraminidase subtypes (N1–N9) are known. Influenza A strains that established stable lineage in humans contain only haemagglutinin subtypes H1–H3 and neuraminidase subtype N1 or N2.[3]

Influenza viruses use carbohydrate molecules expressed on glyoconjugates on the cell membrane to enter the host cell. These viral receptors encompass glycans with different sugar sequences. Of note, influenza A haemagglutinins bind preferentially to carbohydrates containing sialic acid, including gangliosides.[61] To release virions from the cell surface, this binding is subsequently cleaved by viral neuraminidase. Antiviral drugs such as zanamivir and oseltamivir are sialic-acid analogues that can block viral neuraminidase.[62]

Most influenza vaccines are made from virus grown in chicken eggs. They are inactivated by formaldehyde and contain whole virus, split virus, or purified haemagglutinin and neuraminidase protein of virus strains that are recommended annually by WHO.[3]

GBS after influenza infection

Polyneuritis during the influenza pandemic of 1918–20

The influenza pandemic of 1918–20 (so-called Spanish influenza) was the most devastating in recorded history, affecting 25–40% of the worldwide population and causing between 15 million and 40 million deaths.[63–65] The pandemic was caused by an abnormally virulent strain of H1N1 influenza A virus and spread in several waves from the USA to Europe and other regions of the world. Data from old serum banks have shown that the influenza outbreaks were caused by an antigenic shift and subsequent antigenic drift that resulted in an antigenically new virus haemagglutinin to which most people in 1918 were susceptible.[63] Other data indicate that the virulence of this strain was caused by a mutation in non-structural segment protein 1 (NS1), which prevents the production of human type 1 interferon, one of the most important mediators of antiviral immune response.[63,66]

If influenza is a triggering event for GBS, the incidence of this complication should have been increased during the 1918–20 Spanish flu pandemic compared with non-pandemic rates. However, epidemiological data for the period are not compatible with modern scientific standards, and the term Guillain-Barré syndrome was not introduced until about 11 years after Guillain, Barré, and Strohl published their observations;[67,68] therefore, this hypothesis is difficult to prove.

Some reports of case series from the USA, Canada, and Sweden, as well as those for British troops serving in Egypt and the Euphrates–Tigris region mentioned postinfectious polyneuritis.[69–71] Other investigators, however, express doubt about or do not mention an increased occurrence of

	Vaccination seasons	Study location	Study design	Outcomes and conclusions
Sivadon-Tardy et al[76]	1996–2004	Paris, France	Time-series method single-centre study based on reports of influenza-like illnesses by surveillance networks to assess correlation between monthly incidence of GBS	Association between monthly incidence of GBS and influenza-like illnesses. 10 (14%) of 73 GBS patients had serological evidence of recent influenza A and four (5%) of 73 had serological evidence of influenza B
Sivadon-Tardy et al[77]	1996–2001	Paris, France	Single regional reference centre case series	GBS cases after unidentified infections characterised by respiratory disorders and influenza-like syndromes (60%) peaked in winter months
Tam et al[8]	1991–2001	UK	Case-control study of data from the UK General Practice Research Database	18-fold increased risk of GBS after influenza-like illnesses
Tam et al[78]	1993–2002	England	Time-series method to study correlation between weekly incidence of laboratory-confirmed influenza reports and hospital admissions for GBS	Association between weekly numbers of laboratory-confirmed influenza A cases and hospital admissions
Stowe et al[7]	1990–2005	UK	Self-controlled case series method to assess data from primary-care database	Increased relative incidence of GBS within 90 days of influenza-like illnesses, greatest within 30 days

GBS=Guillain-Barré syndrome.

Table 1: **Guillain-Barré syndrome after influenza infection**

polyneuritis after influenza infection.[72,73] After 1920, several small case series and anecdotal case reports continued to document the occurrence of polyneuritis during outbreaks of other influenza subtypes.[74,75]

Current association data

Upper respiratory tract infection precedes GBS in many patients, but in most (around 60%) the infectious cause remains unidentified. The microbes most commonly isolated are *C jejuni, Haemophilus influenzae, Mycoplasma pneumoniae*, and cytomegalovirus.[26] By contrast, infection with influenza virus has only been reported anecdotally and has not traditionally been thought to be a common triggering event of GBS. However, the view has been challenged by the findings of several studies that explored causal relations between influenza or influenza-like illnesses and GBS (table 1).[7,8,76–78] These studies suggest that influenza viruses may have important but previously underestimated roles as triggering factors for GBS during major influenza outbreaks. Influenza A and B have both been reported to precede cases of GBS.[75,76] In a single-centre French study from the greater Paris area, Sivadon-Tardy and colleagues[76] compared serological evidence for influenza infection in 405 patients with GBS with seasonal peaks of influenza-like illness in the same region, as provided by two regional surveillance networks. The monthly incidence of GBS with an unidentified cause was positively correlated with the numbers of reported influenza-like illnesses. Although influenza serology is less sensitive and specific for diagnosis than other methods, such as viral culture or PCR,[79] a notable proportion of patients with GBS had serological evidence of influenza A (13·7%) or B (5·5%).

A UK study investigated the temporal relation between GBS, influenza vaccination, and the occurrence of influenza-like illnesses between 1990 and 2005, by assessment of data from a countrywide primary-care database.[7] This study used the self-controlled case series method, which compares the incidence of an adverse event in a defined risk period after a given exposure with that during other periods. The investigators found an increased relative incidence of GBS in the periods 0–30 (16·64, 95% CI 9·37–29·54) and 0–90 days (7·35, 95% CI 4·36–12·38) after consultation for influenza-like illness. By contrast, no increased risk was observed in the same time period after immunisation with influenza vaccine. Likewise, Tam and colleagues[8] assessed the same UK database to investigate the risk of GBS after influenza infection in 1990–2001. They found an increased risk of GBS within 2 months of an influenza-like illness or an acute respiratory infection, but not after influenza immunisation. These results were consistent with previous observations of a positive association between weekly reports of laboratory-confirmed influenza infections and weekly data for hospital admissions for GBS in England in 1993–2002.[78]

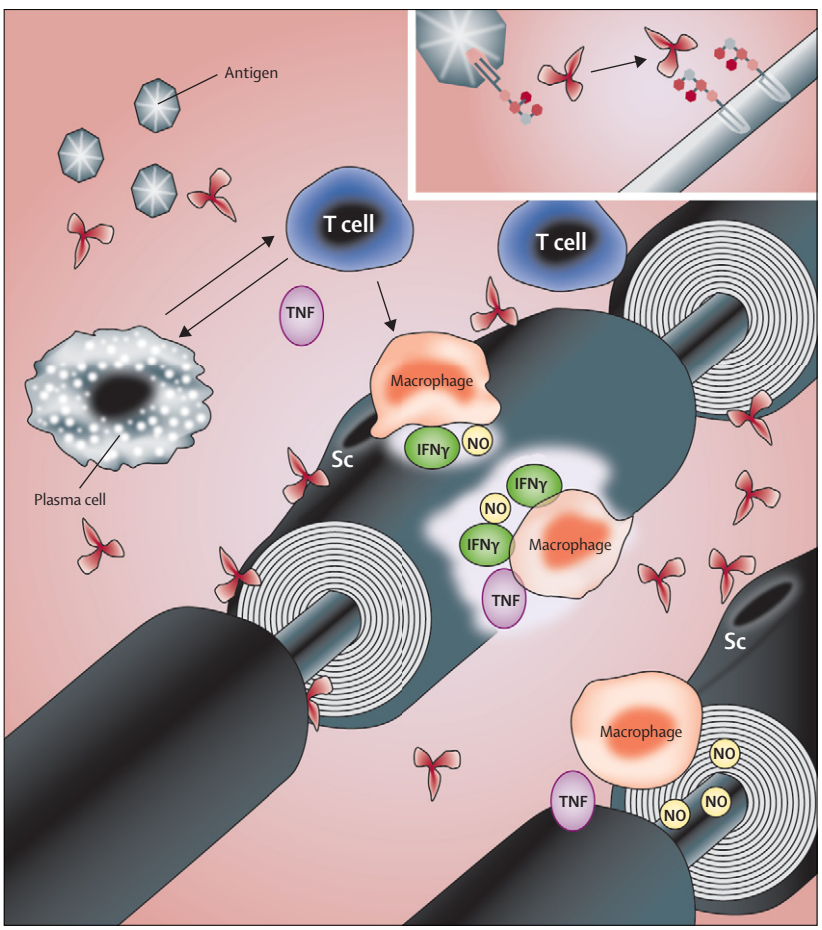

Figure: **Hypothetical mechanism of immune response in Guillain-Barré syndrome**
Inflammatory infiltrates, which contain T lymphocytes and macrophages, cause demyelination and subsequent axonal degeneration by direct cytotoxic effects and via release of inflammatory mediators, such as cytokines and nitric oxide. Insert: autoantibodies (in red) are produced by plasma cells after exposure to an antigen that cross-react with autoantigens expressed on the axolemma and mediate injury to axons (molecular mimicry). IFNγ=interferon γ. NO=nitric oxide. Sc=Schwann cell. TNF=tumour necrosis factor.

A major drawback of these epidemiological studies is the lack of diagnostic certainty supported by microbiological evidence from patients with suspected influenza. Respiratory disease caused by pathogens with seasonal peaks similar to those for influenza virus might cause symptoms that cannot be differentiated from influenza virus infection, for example the respiratory syncytial virus.[80]

Whether the H1N1 2009 pandemic was associated with an increased number of GBS cases is unknown. However, sporadic cases of GBS after H1N1 infection have been reported.[81,82]

Clinical presentation and pathogenesis

The few available case reports of GBS after influenza suggest that the overall clinical presentation is not different from that for GBS subsequent to other viral infections,[75,83–85] except that the involvement of cranial nerves was more common in one case series.[75] Most

patients are severely affected for a period, leading to inability to walk even with aid and the need for ventilation. Although some patients die, the long-term outcome seems to be favourable in most patients.[76] The time from influenza infection to GBS onset varies, but is generally between 3 days and 30 days, with an average of about 15 days.[75,76] This timing to onset is similar to that reported in GBS after other infections.

Molecular mimicry is the leading pathogenetic concept in postinfectious GBS (figure), and peripheral-nerve cross-reactive epitopes having been found in microbes isolated from patients, especially *C jejuni*, that can carry ganglioside-like moieties.[56,58,86] By contrast, data that support the molecular mimicry hypothesis in GBS after viral infections (including influenza) are sparse, with many questions remaining unanswered. Even though there are known aminoacid sequence homologies between the myelin protein P0 that can induce EAN and viral peptides derived from cytomegalovirus or varicella zoster virus, these peptides are insufficient to induce EAN in rats.[87] One study has demonstrated that influenza A/NJ/1976 H1N1 virus grown in chicken eggs can carry epitopes that are recognised by antibodies to GM1. The relevance of these findings, however, remains unclear, since this outcome might be caused by an artificial retention of host ganglioside on viral haemagglutinin.[88] Anti-GD1b antibodies have been reported in one GBS case after influenza A infection,[89] but whether GBS after influenza infection is generally associated with ganglioside antibodies is not known. Prospective studies with larger cohorts than used so far will be needed to establish (or to rule out) a specific ganglioside antibody profile in postinfluenza GBS cases.

GBS after influenza immunisation

1976 US national influenza immunisation programme

In 1976, US health authorities initiated a mass immunisation programme against H1N1 influenza strain A/NJ/76 to prevent an anticipated epidemic after an outbreak among US Army recruits in Fort Dix, NJ, USA.[2,90] The programme was launched to immunise the entire adult population in the USA, but was suspended prematurely after reports of GBS among vaccine recipients.[2,5,91] Between Oct 1 and Dec 16, 1976, 40 million doses of the vaccine were distributed. In the same period a total of 532 cases of GBS after vaccination were reported to the Centers for Disease Control and Prevention.[5] Analysis of nationwide surveillance data confirmed that the rate of GBS (the attack rate) was high in vaccine recipients within 6 weeks of immunisation in all age groups: 7·2 (7·4 in adults) cases per million recipients nationwide, compared with 0·79 (0·97 in adults) cases per million in unvaccinated people.[5] The vaccine-attributable risk for GBS within the same time period was 8·8 cases per million recipients.[5] These data were confirmed in two epidemiological studies that used surveillance data from Ohio or from Michigan and Minnesota.[92,93]

Seasonal immunisation

After 1976, several studies from different countries explored a possible causal association between GBS and immunisation against seasonal influenza (table 2).[7,91,94–100] Despite striking differences in time periods investigated, population sizes, and methodological approaches, most of these studies did not establish or confirm a significantly increased risk of GBS after influenza vaccination.

During the 1993–94 influenza season, the US postmarketing national vaccine safety surveillance programme, Vaccine Adverse Event Reporting System, noted an increase in the numbers of reported GBS cases for that and the previous season. However, risk of GBS during either season was not increased, and only a slight rise in risk of one additional case per one million vaccinated people was calculated for the two seasons combined.[100]

Haber and colleagues[91] published data from the passive surveillance system of the Vaccine Adverse Event Reporting System covering 13 seasons (1990–2003). The reported rates for adult GBS cases after vaccination decreased from 0·17 per 100 000 vaccinations in 1993–94 to 0·04 per 100 000 in 2002–03. This change is in contrast to rates for other postvaccination events, which remained unchanged. These findings were confirmed in an extension study of data for influenza seasons in 1990–2005.[96] The interpretation of data from passive surveillance systems, such as those from the Centers for Disease Control and Prevention, have some limitations, since they are subject to under-reporting, variable quality and completeness, reporting bias, and absence of control background rates in unvaccinated individuals, and by the degree of public awareness and media attention.[2]

In the study from the UK, Stowe and colleagues[7] explored the risk of GBS after seasonal influenza immunisation in 1990–2005, by use of the self-matched case series method. This method relies only on cases with a linked vaccination record. Like the previous studies from the USA, no increase in risk of GBS was seen within 90 days after immunisation. Likewise, a general practice study in the UK showed no increase in relative risk of GBS within 42 days after any immunisation (1·03, 95% CI 0·48–2·18) or after seasonal influenza immunisation (relative risk 0·99, 95% CI 0·32–3·12).[97]

By contrast with the above findings, a study from Ontario, Canada, demonstrated a slight increase in the incidence of GBS 2–7 weeks after vaccination (relative risk 1·45, 95% CI 1·05–1·99). The rate of hospital admissions for GBS, however, did not increase after a universal influenza immunisation programme was introduced to the province in 2000.[98]

The question arises as to what period after vaccination is reasonable to suggest a causal association. Most studies have used a period of 6 weeks, which is based on the significantly increased risk attributed to immunisation in the 1976 US national influenza immunisation programme. The onset of GBS after infection is usually seen at around 2 weeks, which

	Vaccination seasons	Study location	Study design	Vaccine or WHO recommended composition[101]	Outcomes and conclusions
Schonberger et al[5]	1976–77	USA	Nationwide active surveillance programme	A/NJ/76 (H1N1)	Significantly elevated rates of GBS within 6 weeks of vaccination (8·8 additional GBS cases per million vaccines)
Marks and Halpin[93]	1976–77	OH, USA	State-wide active surveillance programme	A/NJ/76 (H1N1)	Higher rate for GBS among vaccine recipients than among unvaccinated individuals (13·3 per million *vs* 2·6 per million). Fewer people with GBS had a history of antecedent infection
Safranek et al[92]	1976–77	MI and MN, USA	Review of state-wide active surveillance data (case records) and acute-care databases	A/NJ/76 (H1N1)	Increased relative risk of GBS within 6 weeks of vaccination (8·6 excess cases of GBS per million recipients in MI, 9·7 per million in MN), but not beyond 6 weeks
Hurwitz et al[95]	1978–79	USA (except MD)	Nationwide surveillance programme	A/Texas/1/77 (H3N2), A/USSR/90/77 (H1N1), and B/Hong Kong/05/1972	No increased risk for GBS
Kaplan et al[94]	1979–80, 1980–81	USA	Nationwide surveillance programme	A/Texas/1/77 (H3N2), A/USSR/90/77 (H1N1), A/Bangkok/01/1979 (H3N2), A/Brazil/11/78 (H1N1), and B/Singapore/222/79	No increased risk of GBS
Roscelli et al[99]	1980–88	USA*	Self-controlled case series method to assess data from US Army health statistics database	Various	No increased risk of GBS
Haber et al [91]	1990–2003	USA	Nationwide passive surveillance programme	Various	Decrease in yearly reporting rates for GBS after influenza vaccination from 1990 to 2003. Possible causal association
Stowe et al[7]	1990–2005	UK	Self-controlled case series method to assess data from primary-care database	Various	No increased risk of GBS within 90 days
Hughes et al[97]	1992–2000	UK	Self-controlled case series method to assess data from primary-care database	Various	No or minimally increased risk of GBS within 42 days†
Juurlink et al[98]	1992–2004	ON, Canada	Data from health-insurance database for a universal influenza immunisation programme	Various	Significantly increased relative incidence of GBS within 2–7 weeks, but no significant increase in hospital admissions
Vellozzi et al[96]	1990–2005	USA	Nationwide passive surveillance programme	Various	No increased risk for GBS associated with influenza vaccine
Lasky et al[100]	1992–94	IL, MD, NC, WA, USA	Hospital discharge summaries, telephone interview	Various	One additional case of GBS per million vaccinees

GBS=Guillain-Barré syndrome. *US Army. †For influenza and other vaccines.

Table 2: **Guillain-Barré syndrome after influenza immunisation**

suggests a primary immune response to that infection.[102] Causality does not, therefore, seem a reasonable assumption when symptoms of GBS occur within a very small number of days after vaccination, but there is no consensus on how small that number should be. Furthermore, the pathogenesis of postvaccination GBS is still unclear and more-rapid and qualitatively different secondary immune responses might contribute to GBS after vaccination.

Although GBS is a monophasic disease, up to 6% of patients have recurrent episodes or relapse.[103,104] Recurrent GBS episodes have been linked to influenza vaccination in two patients, who had had GBS before receiving the A/NJ/76 vaccine.[105] No risk of relapse or recurrence after immunisation (including that for seasonal influenza) has, however, been reported elsewhere.[103,106] Wijdicks and colleagues[107] reported a patient who received influenza vaccination annually for 15 years but never experienced a relapse of GBS.

Despite the overwhelming evidence from epidemiological studies that immunisation against seasonal influenza is probably not associated with an increased overall risk of developing GBS, some observations suggest that the GBS cases reported after vaccination are not entirely coincidental. Apart from case reports that suggest a temporal association with the exposure to the vaccine,[108,109] a history of antecedent infection is less common in patients who develop GBS after influenza vaccination than in patients with GBS not associated with immunisation. This pattern was first noted in the 1976 US immunisation programme (32·8% of vaccinated individuals *vs* 61·8% of unvaccinated individuals had preceding infection)[5] and confirmed in the study by Haber and colleagues[91] that covered subsequent vaccination periods.

Pathogenesis

Possible biological explanations for the occurrence of GBS after immunisation have been put forward in association with the A/NJ/76/H1N1 vaccine. Epidemiological data collected during the 1976 mass immunisation programme suggest that the vaccine itself, rather than specific formulations or lots, caused associated GBS.[5] This conclusion is based on observations that the risk did not differ according to vaccine type,

Search strategy and selection criteria

References for this review were identified through searches of PubMed for articles published from January, 1971, to June, 2010, by use of the terms "Guillain-Barré syndrome", "influenza", "H1N1", "immunization", and "vaccination". Relevant articles published between 1918 and 1920 were identified through searches in the authors' personal files, in Google Scholar, and Springer Online Archives Collection. Articles resulting from these searches and relevant references cited in those articles were reviewed. Articles published in English, French, and German were included.

manufacturer, period of vaccination, or regional distribution, despite the fact that monovalent or bivalent inactivated vaccines were used with more than 100 different lots.[5]

The conceptual framework of molecular mimicry has also directed attention to the pathogenetic role of antibodies to gangliosides in GBS after influenza immunisation. This view is supported by anecdotal reports showing that cases of GBS and Miller Fisher syndrome after immunisation with influenza vaccine can be associated with antibodies against gangliosides GD1b[110] and GQ1b.[108] Nachamkin and colleagues[88] explored the potential of influenza vaccines to elicit a pathogenic humoral immune response in mice. The researchers immunised mice with original A/NJ/1976 vaccines from the 1991–92 and 2004–05 influenza seasons. All these vaccines were able to induce antibodies to haemagglutinin and, more importantly, to the monosialoganglioside GM1. Furthermore, thin-layer chromatography showed that the A/NJ/1976 and later vaccines contained glycans. In at least one of the contemporary influenza vaccines (2004–05) these glycans could be immunostained with antibodies to GM1-like moieties.

Although these data provide an important link between a pathological autoimmune response (against GM1), which replicates some pathological features of GBS in passive transfer models, some important issues remain unresolved. All the vaccines tested induced antibodies to GM1, which clearly argues against a specific role of these antibodies in the induction of GBS by the A/NJ/1976 vaccine. Furthermore the antibody titres were low and many immunisation regimens can induce antibodies to gangliosides in some mouse strains.[111] Moreover, the study does not answer the questions of how and under which circumstances vaccines are able to gain glycans with carbohydrate epitopes that can mimic ganglioside moieties. Nachamkin and colleagues[88] proposed that viral haemagglutinin, which binds under normal conditions to carbohydrates containing sialic acid, can form stable complexes of sialic acid and haemagglutinin. These complexes are generally dissolved by viral neuraminidase that catalyse the hydrolysis of terminal sialic acid residues. Low viral neuraminidase activity might be insufficient to remove sialic acid from viral haemagglutinin, which could result in a molecular structure that mimics GM1 epitopes. This hypothesis is supported by reports that the A/NJ/1976 vaccine contained only low or even no detectable neuraminidase activity.[112]

The A/NJ/1976 vaccine has been purported to have been contaminated with bacterial antigens, less vigorously tested than other vaccines (in terms of adverse events before licensing), or both.[90] The methods for production and prelicence testing of seasonal influenza vaccines were less thorough in 1976 than they are today,[113] which suggests that the contamination hypothesis is feasible. Additionally, the 1976 vaccines were produced from virus grown in chicken eggs, which might have acted as a reservoir for microbes that could survive the vaccine manufacturing process, especially those from *C jejuni*. However, experimental data from Nachamkin and colleagues' study[88] do not support this theory, since tests on the original A/NJ/1976 vaccines from three different manufacturers showed no *C jejuni* DNA, and serum samples from mice immunised with those vaccines were negative for antibodies to *C jejuni*.

Conclusion

Although mass immunisation programmes against H1N1 2009 have finished in many countries, vaccine safety remains a concern for patients, carers, health authorities, and the public. The relation between GBS, influenza vaccine, and influenza infection is of special interest, owing to the negative experiences that were gained more than 30 years ago during the mass immunisation for swine influenza in the USA and informal media reports from the USA and France of suspected GBS after H1N1 2009 influenza vaccination.[114–116] To counteract these concerns, several countries (including the USA, the UK, and Germany) have initiated active surveillance studies to detect potential rare adverse events after H1N1 2009 vaccination, including GBS. The full results are awaited, but preliminary data from the USA show a slightly raised incidence of GBS, similar to that seen for seasonal influenza vaccination in 1992–94 in the USA.[100,117] Despite this rise, however, influenza infection seems to be a relevant trigger for GBS and, therefore, lowering the incidence of influenza by vaccination might protect against GBS.

Overall risk–benefit assessments must be interpreted very carefully, since the composition of annual influenza vaccines varies each season and the risk of GBS might depend on the subtype. Likewise, antigenic drift of seasonal influenza viruses is likely to alter risk of GBS after infection. Another way to shed further light on this important issue is to elucidate the relevant pathogenic steps that are crucial to the development of GBS after vaccination and after infection with influenza and other organisms.

Contributors
HCL and HPH designed the study. HCL collected data. HCL and HPH drafted the first version. BCK and RACH provided critical input and revised the manuscript. HPH provided organisational support.

Conflicts of interest
RACH has given expert testimony in cases of GBS after influenza immunisation. The other authors declare that they have no conflicts of interest.

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Cutaneous septic emboli from *Candida tropicalis*

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An 11-year-old girl with acute lymphoblastic leukemia presented 1 month after induction chemotherapy with febrile neutropenia, a new diastolic murmur, and a subtle asymptomatic persistent rash.

The patient had seven asymmetrically distributed 1–6 mm erythematous macules and papules with dusky centres on her palms, cheek, and abdomen (figure). Histopathology of a punch biopsy from the patient's abdomen revealed an ulcerated superficial abscess with yeast forms and pseudohyphae compatible with a candidal septic embolus (figure).

CT scans of the patient's abdomen and pelvis showed multifocal areas of hypoattenuation in the liver, spleen, and kidneys, suggestive of infectious abscesses (figure). Echocardiogram revealed echogenic foci on the mitral valve suspicious for fungal involvement. *Candida tropicalis* was subsequently isolated from her blood and splenic tissue.

The patient was given liposomal amphotericin B 5 mg/kg per day for 19 days, after which all cutaneous lesions resolved with hyperpigmentation. Neutrophil counts recovered, the patient remained afebrile, and blood cultures were negative for *C tropicalis*. The patient was discharged from the hospital and prescribed daily oral fluconazole. She developed spiking fevers 1 week after discharge and was noted to have persistent stable visceral abscesses. Triple therapy was begun with liposomal amphotericin B 5 mg/kg per day, fluconazole 10 mg/kg per day, and flucytosine 1000 mg per day for 30 days. At 1 month follow-up, cutaneous lesions were absent and visceral lesions were reduced in size.

Systemic candidiasis is associated with a high mortality rate and is usually caused by *Candida albicans*. However, clinicians should be aware that in cases of disseminated disease with cutaneous involvement and in patients with leukaemia, systemic candidiasis could be caused by *C tropicalis*. Because blood cultures might be negative or result in prolonged time to diagnosis, cutaneous histopathology and tissue culture are alternative options that can accelerate diagnosis and allow treatment to be started promptly.

Conflicts of interest

We declare that we have no conflicts of interest.

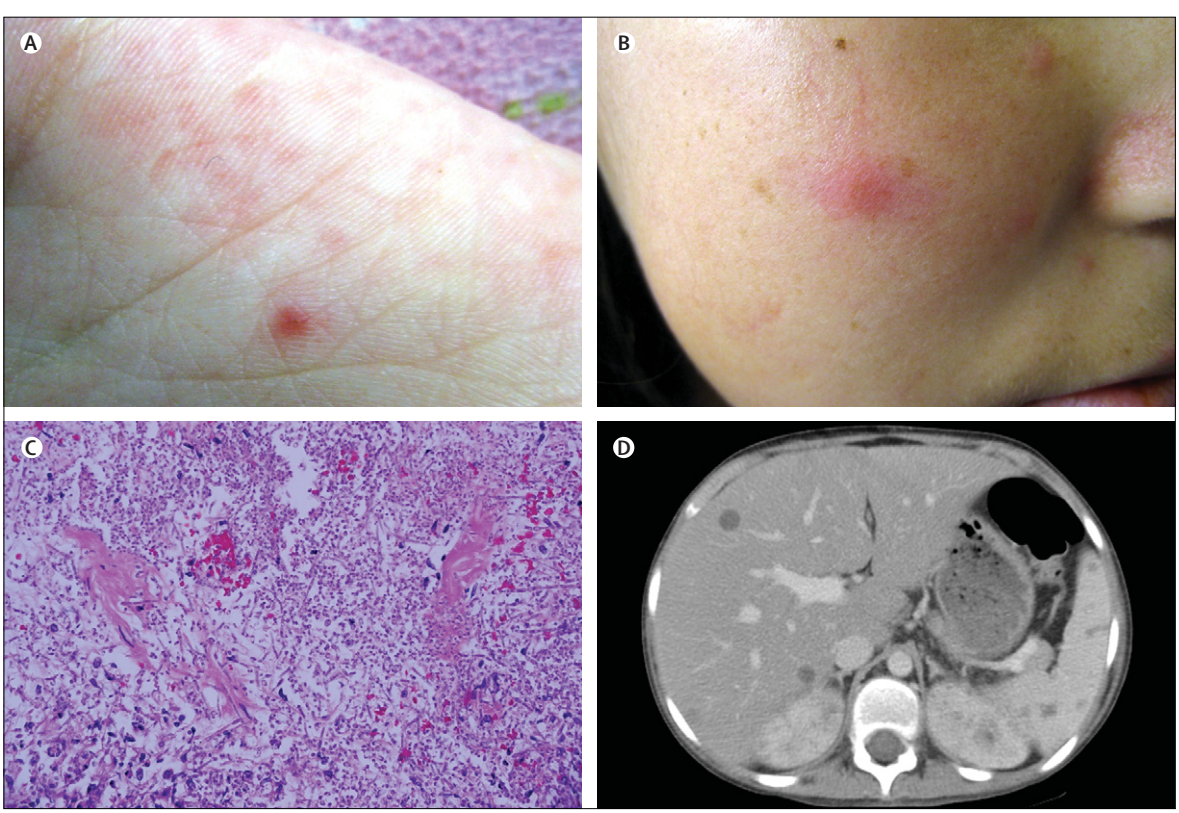

Figure*: 11-year-old girl with cutaneous septic emboli from *Candida tropicalis
Erythematous macules and papules on the patient's palm (A) and cheek (B). Histopathological image of a punch biopsy from the patient's abdomen (C). CT scan of the patient's abdomen and pelvis (D).