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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

PERFORMANCE EVALUATION OF BACTERIOLOGICAL CULTURE MEDIA

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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degree of

DOCTOR OF PUBLIC HEALTH

BY

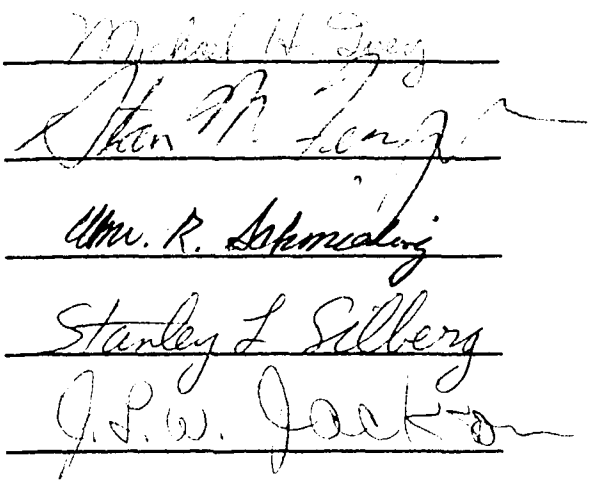
HAROLD KENT MALONE

Oklahoma City, Oklahoma

1975

PERFORMANCE EVALUATION OF BACTERIOLOGICAL CULTURE MEDIA

APPROVED BY



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PERFORMANCE EVAULATION OF BACTERIOLOGICAL CULTURE MEDIA

CHAPTER I

INTRODUCTION

Evaluations of diagnostic laboratories through proficiency testing programs have revealed unsatisfactory performance in microbiology (Barnett et al., 1972). Analysis of the data suggested that the substandard performance may be partially attributed to inferior culture media. This conclusion is supported by studies showing that culture media used by diagnostic microbiology laboratories display a wide range of response when inoculated with standardized cultures (Barry and Feeney, 1967).

Most laboratories now use commercial dehydrated preparations and packaged enrichments for the preparation of culture media rather than the basic ingredients specified in the original formulas. While the major manufacturers of media components have active, in-plant quality control programs, distribution of unsatisfactory products has occurred (Read and Reyes, 1968). Furthermore, products of satisfactory quality may be mishandled by the laboratory, resulting in a defective finished product (Vera, 1971a).

Several sources of error in culture media preparation were identified and other variables believed to affect media quality

suggested by Vera (1971a) and Difco (1974). These variables have been assembled as checklists for use by media preparation facilities. Barry and Fay (1972) validated some media preparation guidelines but others remained unproven.

Methods for measuring the suitability of prepared media were described by Stokes (1968), Cowan and Steel (1965, p. 133) and others. These methods usually included both observation of the response when the media were inoculated with known cultures and physical and chemical measurements. However, the relationship between the physical and chemical measurements and growth response was not indicated.

The present research was undertaken to: (1) develop and evaluate methods for measurement of the quality of bacteriological culture media suitable for use by small laboratories, (2) use these methods to measure the variability of media in use by several area microbiology laboratories, (3) establish standards of acceptance for variables measured, and (4) examine the data for correlation between growth response and other objective measurements.

CHAPTER II

REVIEW OF THE LITERATURE

Effects of Defective Culture Media on Laboratory Proficiency

Although wide variation in both skills and facilities in applied microbiological laboratories probably exists, documentation of differences in the performance levels between laboratories requires the use of objective proficiency measurement systems. Such systems have now been instituted by the Center for Disease Control (CDC) for state and federal laboratories and laboratories licenses under the Clinical Laboratory Improvement Act, by the Food and Drug Administration for milk and food laboratories, the College of American Pathologists for subscribing diagnostic laboratories and others (CDC, n.d.). Responses by the participating laboratories indicate that performance in microbiology has been grossly inadequate. For example, a survey by the Laboratory Quality Committee of the Oklahoma State Medical Association (Barnett et al., 1972) found 33.3 per cent of microbiological test values from small hospital laboratories technically unacceptable and 29.2 percent medically misleading. The quality of performance was even less satisfactory in office laboratories with 43.5 percent of 23 values both technically unacceptable

and medically misleading.

Critiques of proficiency test specimen performance have frequently incriminated defective culture media as a major source of error. Lack of success in the recovery of certain enteric pathogens and Corynebacterium diphtheriae was attributed by the CDC (1969a) to defective agar plate isolation media. They pointed out that improper or prolonged storage of selective and differential agar plates could result in evaporation of the aqueous phase, causing a change in the delicate balance of inhibitors. Such changes would shift the limits of differentiation and affect the recovery rate. In a similar report discussing the isolation of beta hemolytic streptococci on blood agar, CDC (1970) noted that an improper hydrogen ion concentration and/or excessive dextrose concentration in the blood agar could alter the expected hemolytic response.

Differentiation of pure cultures by the use of tubed biochemical test media may also fail due to defective media. The CDC (1969c; 1969d) reported that 5 percent of the participating laboratories failed to detect the production of indole which resulted in erroneous classification of isolates. Use of media deficient in tryptophane and/or containing excessive fermentable carbohydrates were mentioned as possible causes, as was failure to allow a free exchange of gases by use of a gas permeable closure. Several laboratories were unsuccessful in the identification of Clostridium perfringens in another series of samples (CDC, 1969b). Forty-three percent of the participating laboratories failed to observe stormy

fermentation of milk by this organism. Variation between lots of milk was cited as a possible source of error. To confirm proper media function, pretesting of each media production batch was suggested.

The CDC (1969c) attributed some problems with the identification of bacteria in proficiency test specimens to lack of uniformly accepted standard conditions for differential tests. Heat tolerance of the enterococci appeared to vary directly with the hydrogen ion concentration of the media in which they were grown, but the pH of the test media was not specified.

Properly formulated media may fail due to improper packaging. The CDC (1969d) reported numerous instances of false positive utilization of citrate test results in proficiency test specimens. Contamination of the media with carbon through the use of unclean glassware was suggested as a possible cause. Erroneous configuration of the butt and slant of triple sugar iron agar and lysine iron agar were also mentioned as possible sources of identification errors.

However, media performance may be unjustly blamed for laboratory problems. Two laboratories failed to detect Hemophilus influenzae in proficiency test specimens prepared by Barry and Bernsohn (1968). In contrast, the strain grew well on samples of chocolate agar from the two laboratories in media quality control tests performed by the same authors.

Preparation of Culture Media

Before development of commercial sources, all culture media

were prepared from ingredients by each separate laboratory. The formulations in use frequently specified fresh infusions and extracts which were prepared in the laboratory "kitchen." The nutritive value of these materials varied from batch to batch due to variation in the quality of the meat used (Wadsworth, 1947, p. 168). While such ingredients continued to be specified for some applications, many formulations began to call for more precisely defined components such as peptones or completely synthetic ingredients (Vera and Dumoff, 1974, p. 883). Regardless of the refinement of the individual media components used, preparation of culture media from ingredients still had the disadvantages of requiring extensive labor in preparation and poor replicability in culture results (Smith, 1973b).

Frost (1910) suggested the marketing of dehydrated culture media as a means to reduce labor and improve replicability. Commercial production of such preparations was pioneered by Difco Laboratories in 1915 (Difco, 1953, p. 15). Other companies followed and in 1974 the CDC listed 6 producers of dehydrated bacteriological culture media (Lewis, 1974a). The advantages of these products have become so widely accepted that Okey and Walter (1972, p. 48) specifically recommended that no media be prepared from basic ingredients. The use of commercial dehydrated media became, in effect, compulsory for Standard Methods Agar since the productivity tests required by the American Public Health Association (Okey and Walter, 1972, p. 64) are only practical for extremely large production lots. Mechanization of production methodology has further reduced the labor of preparation (Hadder, 1964;

Libman et al., 1972).

While dehydrated media have proven their worth, they may, on occasion, be defective. Read and Reyes (1968) found substantial variation between several manufacturers' lots of brilliant green agar in the ability to support the growth of Salmonella. Branson (1971) reported the isolation of Bacillus from dehydrated selenite broth. Furthermore, reconstituted brain heart infusion media used in her laboratory to culture spinal fluid was found to contain significant numbers of non-viable gram-negative staining rods. A direct smear from a spinal fluid culture in this media could have easily led to an erroneous report.

Many laboratories now purchase a part or all of their culture media as completely prepared tubes or plates. The CDC listed 33 known major producers of prepared media in 1974 (Lewis, 1974a). Other vendors operating on a small scale were reportedly proliferating.

Prepared media has several advantages, particularly for the small laboratory. Dehydrated products that are readily reconstituted into finished media are not available for some media formulations or reconstitution may be difficult. Vera (1971a) pointed out that improper reconstitution from satisfactory materials may result in an unsatisfactory finished product. This risk is compounded by the low skill level of many media production personnel (Fodor, 1968).

Barry and Fay (1972) measured the quality of media produced when commonly accepted guidelines of media preparation were intentionally violated. They reported that dehydrated Salmonella Shigella (SS) media stored in inadequately sealed containers gained 4.4 percent in weight due to absorption of moisture. Media prepared from this material

was inoculated with multiple dilutions of salmonellae. The yield was 22 percent of the number which grew on SS plates prepared from properly stored dehydrated media. Dehydrated media that was well sealed but exposed to light was also found to deteriorate; only 47 percent of the salmonellae were recovered compared to the controls. A similar reduction in the growth of Streptococcus pyogenes on blood agar was observed to result from excessive heating during solvation of dehydrated blood agar base. In recognition of such problems, Blair (1970, p. 10) recommended that laboratories should use prepared media unless they had sufficient personnel and facilities for proper preparation and quality control testing.

As laboratories began to expand their use of prepared media, questions were raised regarding the quality of the purchased products. Nagel and Kunz (1973) performed quality control tests on 900 lots of 46 different prepared media representing 350,000 units. Only 1.9 percent of the lots were found to be defective, and most of the defects would have been visibly apparent to the analyst at the bench. They concluded that pretesting of quality controlled commercially prepared media was not required.

In contrast, Bartlett (1973) reported several instances of defective prepared media purchased by his laboratory. He recommends using the results of media quality control testing generated over an extended period of time in the laboratory to define a frequency and scope of testing for each type of medium. Using this information, media historically shown to be of consistently good quality would not be tested as rigorously as media found to have a high rate of defects.

Defective prepared media has been reported by the CDC (1974a). Several lot numbers of blood culture medium in vacuum bottles produced by Difco Laboratories were found to be contaminated with Bacillus spp. These might have escaped the notice of the using laboratory since some bottles found to contain contaminating organisms showed no visible turbidity.

Methods for Monitoring Media Quality

In accordance with the general trend toward application of quality control principles to microbiological analyses, recent procedures manuals written for the diagnostic microbiologist usually suggest some form of media monitoring in their recommendations for quality control (Vera and Dumoff, 1974, p. 886; Bartlett, 1968). These recommendations usually provide for inoculation with known cultures and other tests such as measurement of hydrogen ion concentration. However, specific procedures for performance of such testing are rarely included.

Although several authors have suggested methods for challenging agar plate media with live organisms, significant differences exist between the methods suggested. Some indicate a preference for qualitative testing.

Halstead et al. (1971), Russell et al. (1969), Smith and Sandlin (1969) and Vera (1971a) described qualitative methods wherein selected organisms were inoculated and the results recorded as growth or absence of growth. Halstead et al. recommended inoculation with mixtures of strains while the others implied the use of pure cultures. Since pathogens are usually present in small quantities, Russell et al. recommended light inoculation with these organisms. Griffin and Vera (1969) specifically commended the qualitative approach. They asserted

that qualitative inoculation was superior since it realistically duplicated the actual use of media, it would better differentiate acceptable from unacceptable lots and was fast and practical. It was their further opinion that since there were few standards for quantitative data, interpretation of colony counts as a measure of media quality would be difficult.

Conversely, Stokes (1968, p. 300) suggested that qualitative testing would detect only gross difference and thus favored quantitative inoculation and interpretation. She recommended the use of the method of Miles and Misra (1938) for making surface viable counts of serial dilutions of the test organisms, citing advantages of accuracy, simplicity and economy of media. In this procedure, up to eight calibrated drops of the diluted suspensions were allowed to impinge on different areas of the surface of the plate to be tested. Colony counts were then made on each of the areas containing discrete colonies from which viable counts of the inoculum could then be computed.

For quality control of Transgrow and Thayer Martin culture media, Lewis (1971a, 1971b, 1972) recommended inoculation with a single optimum dilution of each of several organisms. These optimum dilutions were made so that a loop of the suspension would yield a countable number of colonies when spread over the surface of a plate of unhibitory media. Samples from both prechecked and unknown lots of the selective media were then implanted with a similar inoculum and the resulting colony counts recorded and compared. However, no specific standard of acceptance was indicated. Comparison of new lots with accepted lots is also recommended by Harding (1965) and Vera (1971a).

Several recent studies in which agar plate culture media were either evaluated or compared also utilized quantitative methods which are adaptable for use in quality control efforts. In their study of variation between manufacturers' production lots of brilliant green agar, Read and Reyes (1968) surface plated measured aliquots of serial dilutions of pure cultures. Following incubation, the resultant colonies were counted and the production lots compared statistically. In their report on media monitoring procedures, Barry and Feeney (1967) found the surface plate count to be a suitable technique for assay of media quality. Wun et al (1972) successfully utilized the surface plate count for assay of serial dilutions of the growth from enrichment broths in their evaluation of new plating media.

Further quantitation of the growth resulting from the above procedures can be obtained by measurement of colony diameter. Such measurements were employed by Stokes (1971) and Barry and Feeney (1967). These authors also recorded colony characteristics and changes in the surrounding medium.

Agar plates are ordinarily judged by the ability or inability of a single bacterium to thrive and multiply, usually accompanied by characteristic traits in the resulting colony and/or its immediate surroundings. Colony development is frequently influenced by the presence of nearby competing colonies of the same or different species. The situation is much different with tubed biochemical test media wherein the activity derives from inoculation with multiple, presumably identical, organisms. Here, the response is the sum of the entire inoculum population operating collectively and observed as a single qualitative or

semi-quantitative event. For this reason, methods suitable for monitoring the quality of agar plate media are inappropriate for evaluating tubed biochemical test media.

Several investigators have studied the quality of tubed biochemical test media. All inoculated the tubes were evaluated with known species in the same manner in which they are customarily used. However, there was some variation in the number of test strains used. Smith and Sandlin (1969) used four cultures for testing Kligler's iron agar and lysine iron agar while only one, Proteus, was utilized for urea media. Here, the results were recorded as the time required for initiation of the hydrolysis of urea as evidenced by development of a red color rather than simply positive or negative after a fixed incubation time. The CDC (1972b) and Russell et al. (1969) recommended two different cultures be used with each type of media in order to provide both positive and negative controls. In their evaluation of biochemical media, Halstead et al. (1971) recorded one of three findings on each sample; i.e., acceptable, unacceptable and intermediate to which they assigned values of 2, 0 and 1, respectively. These values were accumulated to give a quantitative total score for that media. The scores could then be graphically displayed or otherwise collated so as to reveal quantitative trends.

Media Evaluation by Monitoring the Hydrogen Ion Concentration

Need for pH Control

The relationship between hydrogen ion concentration (pH) and

growth of bacteria was first elucidated by Sorenson in his studies of factors limiting bacterial growth in culture media (cited by Davis et al., 1967, p. 136). Clark and Lubs (1917) showed that pH changes which occur in a maturing culture could be controlled by adding buffering salts to the medium.

Media pH values may be manipulated so as to favor a specific growth response. Since optimal pH varies with the species, selection of a pH more favorable to one has been used to enhance isolation from mixed cultures. For example, acid tolerant fungi are cultured on low pH media while alkaline media are used for the vibrio group which thrive at a high pH. Metabolic end products useful for characterization of a strain, such as toxin production by Corynebacterium diphteriae, may require careful pH control (Hermann and Bickham, 1974, p. 130).

Although the need for pH control is well established, effective pH management has been a continuing problem. Early difficulties centered around problems in measurement since only colorimetric methods were available to the microbiologist. Progressively greater acceptance of commercial dehydrated culture media was accompanied by an improvement in quality, including a greater uniformity in the pH of the media. However, statements by media manufacturers such as "Adjustment of the reaction of the medium is not required." found in the opening pages of the ubiquitous Difco manual (Difco, 1953, p. 22) undoubtedly leads to an unwarranted acceptance by many microbiologists of stated reconstituted product pH values.

Failure to maintain proper pH control may have many serious consequences. In her discussion of factors influencing zone size in

antibiotic sensitivity tests, Stokes (1968, p. 181) indicated that zone diameter varied directly with pH with some antibiotics and inversely with others. Faulty pH adjustment was said to be one of the eight major causes of defective media by the CDC (1969a). Smith and Sandlin (1969) found that Salmonella would produce atypical reactions on xylose lysine dulcitol (XLD) agar unless the pH was carefully controlled. In studies of variation in the growth of Escherichia coli on eosin methylene blue (EMB) agar, Parisi and Marsik (1969) attributed within plate variation in colony characteristics to variation in pH. They believed that the observed variation was caused by uneven cooling or inadequate mixing during preparation of the plates. While studying the effects of light on culture media, Waterworth (1969) found that the degree of inhibitory effects induced by light was functionally related to the pH of the media. Samples of oxidative-fermentative medium, noted to give false negative results with Pasturella multocida, were found to have an excessively high pH (Glasser et al., 1971). Following adjustment of the pH, the media performed as expected. Karlstrom et al. (1960) indicated that adequate pH monitoring would have prevented the low pathogen isolation rate they experienced using defective SS and bismuth sulfite agars. Even the aforementioned Difco manual pointed out that an improper pH may inhibit critical metabolic processes even though growth may occur (Difco, 1953, p. 17).

Physical changes may also follow failure to maintain a proper pH. A decrease in pH may hasten the heat destruction of the jellifying properties of agar (Perkins, 1954, p. 698).

These reports serve to point out the diversified, somewhat

unpredictable consequences of failure to control the pH of bacteriological culture media.

Causes of Incorrect pH

The most frequently cited sources of pH error are those deriving from changes occurring during solvation of dry ingredients by heating and sterilization. Overheating is reported to result in a reduction in media pH (Vera, 1971b) although Blair (1970, p. 806) pointed out that media containing bicarbonate may become more alkaline due to liberation of carbon dioxide. Stewart (1974) reported that heating media with a pH of less than 6.0 or greater than 8.0 will drive the pH toward neutrality. In the Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1971) it is noted that changes during autoclaving vary with the indivdual sterilizer and with the buffering capacity of the media but ordinarily range from 0.1 to 0.2 pH units. Burnett et al. (1957, p. 39) reported that the pH may drop as much as 0.4 pH units with some media. Re-autoclaving will result in a further pH shift (Harding, 1965).

Errors in pH may be introduced by materials used in media preparation. Blair (1970, p. 807) pointed out the error in media pH introduced by the use of distilled water which had become acid due to absorption of atmospheric carbon dioxide. Chambers et al. (1962) reported that the pH of distilled water prepared from tap water averaged 6.2. The Oxoid manual (Oxoid, 1971, p. 17) indicates that alkaline glass containers used in the preparation of media may be responsible for a drift in pH.

Some carbohydrates and amino acids induce changes in pH when

added to basal media (Difco, 1953, p. 187) (Edwards and Ewing, 1972, p. 344). Therefore, the final media pH must be adjusted for each additive.

Vera and Dumoff (1974, p. 884) pointed out that media prepared from dehydrated media stored in containers that have been opened for some time may exhibit a pH different from media prepared from freshly opened containers. Furthermore, after media are prepared, pH changes may occur during storage, particularly if the storage temperature is above 25°C (Bunker and Schuber, 1922; Oxoid, 1971, p. 17).

Measurement Methods

Effective monitoring of pH as an index of media suitability requires an accurate method of pH measurement. Of the several methods available to the microbiologist, the colorimetric and the electrometric are the most commonly used.

The colorimetric method takes advantage of the propensity of a class of chemical compounds to vary in color when in solutions of different hydrogen ion concentrations in a predictable way. This method was first presented as a practical tool by Clark and Lubs (1917) and refined by Medalia (1920). Bunker and Schuber (1922) utilized these methods to develop recommended pH levels for selected media based on evaluation of growth at different pH levels. Clark's 1928 publication which reviews the entire subject of pH measurement and control remains the definitive work on colorimetric methods. Subsequent authors have described methods for colorimetric measurement in less detail (Wadsworth, 1947, p. 103; Cohen, 1957, p. 72; Okey and Walter, 1972, p. 50) but

their descriptions may be of greater value to the practicing microbiologist due to their relative currency.

Several considerations have led to the declining use of colorimetric procedures. Variation in protein and salt concentrations in the media are known to produce variant pH values (Lamanna and Mallett, 1959, p. 134). Furthermore, use of the technique presents particularly difficult problems with colored media and media containing solidifying agents and the indicator itself may alter the pH of poorly buffered samples. The commonly used color block technique contains an inherent, unavoidable error due to the requirement for subjective interpretation by the human eye. Partly for these reasons, colorimetric methods have been deleted as an acceptable method of pH measurement in the 13th edition of Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1971). The advent of reliable, reasonably priced electrometric devices now provides an acceptable and, in many ways superior, alternative.

Many authoritative compendia of methods for culture media preparation indicate a preference for electrometric measurement of pH (Blair, 1970, p. 806; Vera, 1971b; Vera and Dumoff, 1974). Despite increasing use, pH meters are not devoid of pitfalls for the user. Operator error can be minimized by careful attention to directions accompanying the equipment coupled with a familiarity with the principles and application of the method such as is found in Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1971, p. 276). For example, Okey and Walter (1972, p. 149) pointed out that the temperature compensator feature of some

pH meters does not compensate for differences in temperature between sample and buffer.

Failure to properly standardize the pH meter is a common problem. Willard et al. (1965, p. 599) indicated that two buffers should be used in meter standardization to ensure proper functioning of the cell assembly and to verify conformity with the theoretical Nernst slope. The CDC (1972a) recommends that buffers be used at pH 4.0 and 7.0. Conversely, instruction manuals enclosed with IBC and Corning pH meters recommend only one. The buffers used for standardization are, themselves, potential sources of error. In addition to the need for protection of buffers from chemical and microbial contamination, Ellis (1973, p. 12) recommended checking buffers monthly with a second pH meter.

A third method of measuring pH of culture media is to use pH sensitive paper. While determinations are easy to perform with such products, they possess an inherent imprecision to an even greater degree than the colorimetric methods from which they derive. Blair (1970, p. 806) pointed out the possibility of inaccurate pH measurement due to decolorization of the indicator when used with media containing reducing agents. Selective sorption from the sample by the paper may result in erroneous values (Cohen, 1957, p. 87).

Monitoring of pH of media may be indirectly accomplished by examination of the color of media containing indicators. The BBL Quality Control Procedures Manual (Bioquest, 1971) listed color as one of the parameters for acceptance of finished (prepared) media for marketing.

Factors Influencing Measurement

Vera (1971b) pointed out that a variation in pH readings may be observed if measurements are made at different temperatures. In a study of media used in dairy microbiology, Walter et al. (1970) found variation ranging from 0.14 to 0.24 pH units between measurements made at 25°C and those at 45°C for four media tested. The recommended pH value is normally understood to be the pH after all processing is completed. Blair (1970, p. 806) suggested that measurements could be made prior to autoclaving once the pH changes had been determined by experience. Woods and Byers (1973) recommended checking each production batch. Geldreich (1972) indicated that after determination of the pH adjustment required for a batch prepared from a particular bottle or lot number, the same adjustment could be applied to media prepared from the balance of the bottle or lot without further pH measurement, provided that the other conditions of preparation were not changed.

Correction of pH

Media are commonly adjusted to the desired pH by the addition of sodium hydroxide or hydrochloric acid. This correction process itself may introduce undesirable effects. Wadsworth (1947, p. 174) cautioned that interaction of acid and media may cause local denaturization during the mixing process. Media requiring massive additions of acid or alkali may be unsuitable after correction due to an increase in salt content (Vera, 1971a).

Formulation for culture media frequently indicate an acceptance range or tolerance around the optimum pH value. The range of acceptance

for media listed in the 13th edition of Standard Methods for the Examination of Dairy Products (Okey and Walter, 1972, p. 57) varied from ±0.0 to ±0.2. Some formulations did not include any range of acceptance. Recently, some authors have suggested a generalized tolerance of ±0.2 pH units for all media (Blair, 1970, p. 806; Vera, 1971b; Woods and Byers, 1973). The tolerance of ±0.1, formerly specified by the United States Pharmacopeia (United States Pharmacopeial Convention, 1965) was changed in the 1970 edition to ±0.2. Vera (1971a) concluded that ±0.2 allows for reasonable variation in technique and products while still eliminating unsatisfactory media. The American Public Health Association (1971, p. 279) reported that when thirty laboratories performed pH determinations on a synthetic unknown of pH 7.3, the standard deviation was 0.13 pH units. They concluded that ±0.1 units represents a reasonable limit of accuracy under normal conditions.

Water Used in the Preparation of Culture Media

Distilled water has been specified for preparation of culture media by compilers of "standard methods" for 50 years or more (Wadsworth, 1927; APHA, 1925). However, tests to establish its suitability for microbiological applications are of relatively recent origin.

Tests designed to measure the suitability of distilled water for chemical determinations have been described by the APHA (1971, p. 323), Smith (1973a) and others. While such tests are useful, they are incomplete for microbiological applications. Preparation of quality culture media requires assurance that the distilled water is free of

significant toxic residues as well as low in total and specific ions and conforms to other physical and chemical standards. Price and Gare (1959) reported that traces of bacteriostatic, volatile, short chain fatty acids can occur in distilled water. Such contaminants may be caused by degradation of bacteria found in distilled water (Strange, et al., 1961).

A simple test to monitor the suitability of distilled water was described by the American Public Health Association (1948, p. 89) in the 9th edition of Standard Methods for the Examination of Dairy Products. In this procedure, milk samples are diluted 1:100 in the unknown water that has been weakly buffered followed by pour-plating immediately, and after 10, 20 and 30 minutes to measure the viable bacterial count. Water was considered toxic if a definite trend toward lower counts was seen as the holding time increased. This test was observed to have at least two limitations. First, the milk sample itself and the media into which it was placed provided an unknown, probably significant, neutralizing effect on toxic constituents. Second, the test would not readily detect nutritive contaminants in the water. A more sensitive test was desirable.

In 1965, Geldreich and Clark reported on a method which they called the distilled water suitability test. In their procedure, Enterobacter aerogenes was grown in a minimal medium dissolved in ultra pure water and water of unknown quality and the ratio of organism proliferation in the two media computed. Thus, if the organism grew more abundantly in the unknown water, it was said to contain nutrients. Conversely, if less growth was noted, the water was declared toxic.

Studies by Ronald and Morris (1967) indicated that water containing copper approximately equal or greater than 10 parts per billion would be classified as toxic by this test.

The distilled water suitability test was reported to be overly sensitive by some authors (Ronald and Morris, 1967). For this reason, the American Public Health Association lessened the sensitivity of the test by increasing the nitrogen source when it was published in the 12th edition of Standard Methods for the Examination of Dairy Products (1967).

Water purified by passage through demineralizing resins has been used as a substitute for distilled water for some time. Concern about organic contaminants in such water prompted warnings regarding the free substitution of deionized for distilled water in the 10th edition of Standard Methods for the Examination of Dairy Products (American Public Health Association, 1953, p. 257). In the 12th edition (American Public Health Association, 1967, p. 41), only distilled water was permitted for the preparation of dilution blanks.

Cleanliness of reservoirs used for storage of purified water must also be given proper attention. Inadequately cleaned glassware is a potential source of contamination with microorganisms and bacteriostatic detergent residues (Truant, 1974, p. 5).

Studies of "In Use" Culture Media

Although limited studies have been made of culture media in use by microbiological laboratories, deficiencies in response have been reported by several investigators. Stokes (1967) found marked differences in the ability of desoxycholate citrate agar obtained from five large

hospitals to support the growth of Shigella sonnei. Similar results were reported by Barry and Feeney (1967) in a study of samples of blood agar from several small hospital laboratories. They noted dramatic differences between laboratories in the degree of hemolysis elicited by beta hemolytic streptococci. In the same report, they noted that differences in the performance of Simmons' citrate agar and triple sugar iron agar slants were correlated with the conformation of the slants.

In a study conducted by the Oklahoma State Department of Health (Parsons, 1974) significant differences were noted in the recovery rate of Neisseria gonorrhoeae from clinical specimens inoculated on trans-grow culture media produced by two different media preparation facilities. Quality control studies performed by Lewis (1974b) on samples from the two sources revealed that one source prepared bottles containing inadequate CO_2 while the other prepared media which failed to properly inhibit the growth of Staphylococcus epidermidis.

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains

The eight bacteria used in these studies were Enterobacter cloacae (ATCC 13047), Escherichia coli (ATCC 11775), Proteus vulgaris (ATCC 6380), Pseudomonas aeruginosa (ATCC 9721), Salmonella typhimurium (ATCC 13311), Staphylococcus aureus (ATCC 12600), Staphylococcus epidermidis (ATCC 14990), and Streptococcus pyogenes (ATCC 10384). These organisms, marketed under the trade-mark BACT-CHEK,[1] were provided in the form of soluble discs, three to five millimeters in diameter. The discs were packed in screw capped, resealable vials containing thirty discs, each capable of initiating growth. Each production lot was assigned an expiration date. Growth and morphological characteristics, biochemical activity and antibiotic susceptibility data described by the manufacturer for each strain have been confirmed in studies performed at the CDC and a private laboratory (Douglass et al., 1973).

Reagents

The distilled water used for the preparation of all culture media and reagents was tested for freedom from both toxic and growth promoting agents as described later in this chapter. Reagent grade

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[1]Roche Diagnostics, Nutley, New Jersey

chemicals were used in the preparation of solutions unless otherwise stated. Culture media used for baseline purposes were prepared from dehydrated commercial products according to directions stated in the accompanying product literature.

Culture Media Examination - Preliminary Considerations

Selection of Participating Laboratories

The laboratories selected for this study agreed to participate over a period of 8 weeks, be accessible for regular sampling and use acceptable brands and formulations of media. The selected laboratories employed a wide range of management and production practices that might affect media quality such as scale of operation, training of personnel, facilities available, interest in and sensitivity to quality control, participation in external and internal proficiency testing programs and morale and interest of personnel. Laboratories were also selected so as to provide a variety of types of sponsorship (private, federal, state, military, church); nature of operation (hospital, independent, public health) and types of microbiological activity (diagnostic and agricultural). Descriptions of the twelve laboratories which were ultimately selected for participation are listed in Table 1.

Media Studied

Media with similar names and purposes but with significantly different formulations were examined separately in order to minimize the influence of formulation on test results. Peptones from different manufacturers of accepted equivalence were considered equal for purposes of this study.

TABLE 1

TYPE OF LABORATORY FACILITIES SAMPLED

Laboratory Code[a]	Type of Ownership and Management	Type of Operation	Size Class
1	Corporation	Independent Clinical	
2	Federal	General Hospital	400-500 Bed
3	State	Public Health	
4	State	General Hospital	200-300 Bed
5	State	General Hospital	100-200 Bed
6	Military	General Hospital	100 Bed
7	Corporation	General Hospital	100-200 Bed
8	Church	General Hospital	100-200 Bed
9	Church	General Hospital	300-400 Bed
10	Corporation	Independent Clinical	
11	Corporation	General Hospital	100-200 Bed
12	Private	Independent Agricultural	

[a]Random numerical designation in lieu of actual laboratory name

Further limitations were imposed by the types of organisms available. Omission of the fastidious bacteria by the manufacturer of the bacterial discs precluded the inclusion of media designed for their isolation. Blood containing agars were not included due to the heterogeneity of agar bases and bloods employed by the various laboratories thus preventing meaningful comparison of growth response. Since statistical analysis is potentiated by an increase in the sample size, preference was given to media in use by several laboratories. For these reasons, media considered to be most suitable for testing were those used for study of the enterobacteriaceae and selective media for the primary isolation of Staphylococci. Media meeting these criteria and thus included for testing were as indicated in Tables 2 and 3 for agar plate media and biochemical test media respectively. Nomenclature used is that most commonly employed by diagnostic laboratories.

Sampling

The number of plates and tubes tested was necessarily a compromise between ideal experimental design and the economic limits imposed by participating laboratories. Sample size selected was five units of each type of agar plate medium and three to four units of each type of tubed biochemical test medium per sample.

Samples were collected and tested over a period of 8 weeks. Two laboratories were sampled each week and the others bi-weekly. The number of types of media obtained varied somewhat from week to week depending on availability at the time of collection. Samples were picked up on Tuesday of each week.

TABLE 2

AGAR PLATE MEDIA SAMPLED

Common Medium Name	Catalog Number[a]		
	Difco	BBL	Pfeizer
Eosin Methylene Blue Agar (EMB)	0076	11214/5	
Eosin Methylene Blue Agar, Levine (LEMB)	0005	11220/1	
Xylose Lysine Decarboxylase Agar (XLD)	0788	11827/8	
MacConkey Agar (MAC)	0075	11386/87	
Desoxycholate Agar (DC)	0273	11149/50	
Salmonella Shigella Agar (SS)	0074	22596/7	
Brilliant Green Agar (BG)	0285	11072/3	
Bismuth Sulfite Agar (BS)	0073	11030/1	
Hektoen Enteric Agar (HE)	0853		243 B
Staphylococcus Medium 110 (S110)	0297	11646/7	
Mannitol Salt Agar (MSA)	0306	11406/7	

[a]Manufacturers' stock numbers accepted as equivalent formulations for this study

TABLE 3

BIOCHEMICAL TEST MEDIA SAMPLED

Medium	Catalog Number[a] Difco	BBL
Triple Sugar Iron Agar (TSI)	0265	11748/9
Kligler Iron Agar (KIA)	0086	11316/7
Lysine Iron Agar (LIA)	0849	11362/3
SIM Medium (SIM)	0271	11577/8
Motility Test Medium (MOT)	0105	11435/6
Urea Agar (UA)	0283	11794/5
Simmons Citrate Agar (SC)	0091	11619/20
Moeller Decarboxylase, Base (MB)	0890	11429/30
Moeller Decarboxylase, Lysine (ML)	0890	11429/30
Moeller Decarboxylase, Arginine (MA)	0890	11429/30
Moeller Decarboxylase, Ornithine (MO)	0890	11429/30
Phenylalanine Agar (PA)	0745	11536/7

[a]Manufacturers' stock numbers accepted as equivalent for purposes of this study

Efforts were made to insure that each sample consisted of media from the same production batch. Production data requested for each sample included formulation, brand, manufacturer's lot number and the date of preparation of finished media.

The styrofoam box used for sample transport was constructed with 0.75 inch thick walls and a tight fitting lid. Temperature was controlled by using four frozen Sno-Gel type reusable freeze pads[2] with paper insulation between samples and pad. Product temperature was thus maintained between 1 and 6 degrees centigrade during the approximately 0.5 to 3 hours in transit. Samples were refrigerated at 1 to 4 degrees centigrade until testing was initiated later the same day.

Performance Evaluation of Agar Plate Media

Preparation of Agar Plates

Growth of discrete colonies required the development of methods to prevent coalescence of inoculation fluid dropped on the plate surface. Physiological saline was tinted with methylene blue and dropped on to the surface of three types of agar plates containing varying amounts of wetting agents. Plates were inoculated both directly from the refrigerator and after preconditioning by warming and drying the surface of the agar. The latter was accomplished by placing the plates, inverted and with tops ajar, in a gravity convection incubator, equipped with a conventional humidifier and maintained at a temperature of 35°C. Results showed that preconditioning for a minimum of one hour was required to prevent coalescence of the drops. Furthermore, 8 different

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[2]Plastic Ice Co., Lodi, California

inoculation sites could be accommodated by careful ruling of the bottom of the plate into a rectangular grid pattern.

Assembly of Culture Dispenser

An apparatus for dispensing suspensions of organisms was assembled as shown in figure 1. An autoclaved polypropylene precision dropping pipet[3] fitted with a metal tip, and designed to deliver drops containing 0.025ml ±2.0 percent of a standard 0.85 percent saline solution, was used to dispense each suspension. The pipet was suspended from a ring stand using a buret clamp so that the tip was 7 inches above a beaker containing disinfectant. A 4 inch ring was attached to the stand 2.5 inches below the tip of the pipet to support the agar plates. Control of the drop delivery rate was maintained by connecting the pipet mouth to a Sage model 351 syringe pump[4], fitted with a 10ml plastic syringe, with amber latex rubber tubing. By use of an adjustable rate control, the apparatus could be made to deliver at any desired rate from less than 10 to greater than 60 drops per minute. The pump was equipped with a power switch used to interrupt drop delivery while maintaining the bacterial suspension in the pipet.

Inoculation of Agar Plates

Bacterial suspensions were drawn into the pipet by manually operating the plastic syringe. The dispensing assembly was adjusted to deliver drops of culture suspensions at a convenient rate. Plates

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[3]Cooke Engineering Company, Alexandria, Virginia

[4]Scientific Products Division of American Hospital Supply, McGaw Park, Illinois

inoculation sites could be accommodated by careful ruling of the bottom of the plate into a rectangular grid pattern.

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An apparatus for dispensing suspensions of organisms was assembled as shown in figure 1. An autoclaved polypropylene precision dropping pipet[3] fitted with a metal tip, and designed to deliver drops containing 0.025ml ±2.0 percent of a standard 0.85 percent saline solution, was used to dispense each suspension. The pipet was suspended from a ring stand using a buret clamp so that the tip was 7 inches above a beaker containing disinfectant. A 4 inch ring was attached to the stand 2.5 inches below the tip of the pipet to support the agar plates. Control of the drop delivery rate was maintained by connecting the pipet mouth to a Sage model 351 syringe pump[4], fitted with a 10ml plastic syringe, with amber latex rubber tubing. By use of an adjustable rate control, the apparatus could be made to deliver at any desired rate from less than 10 to greater than 60 drops per minute. The pump was equipped with a power switch used to interrupt drop delivery while maintaining the bacterial suspension in the pipet.

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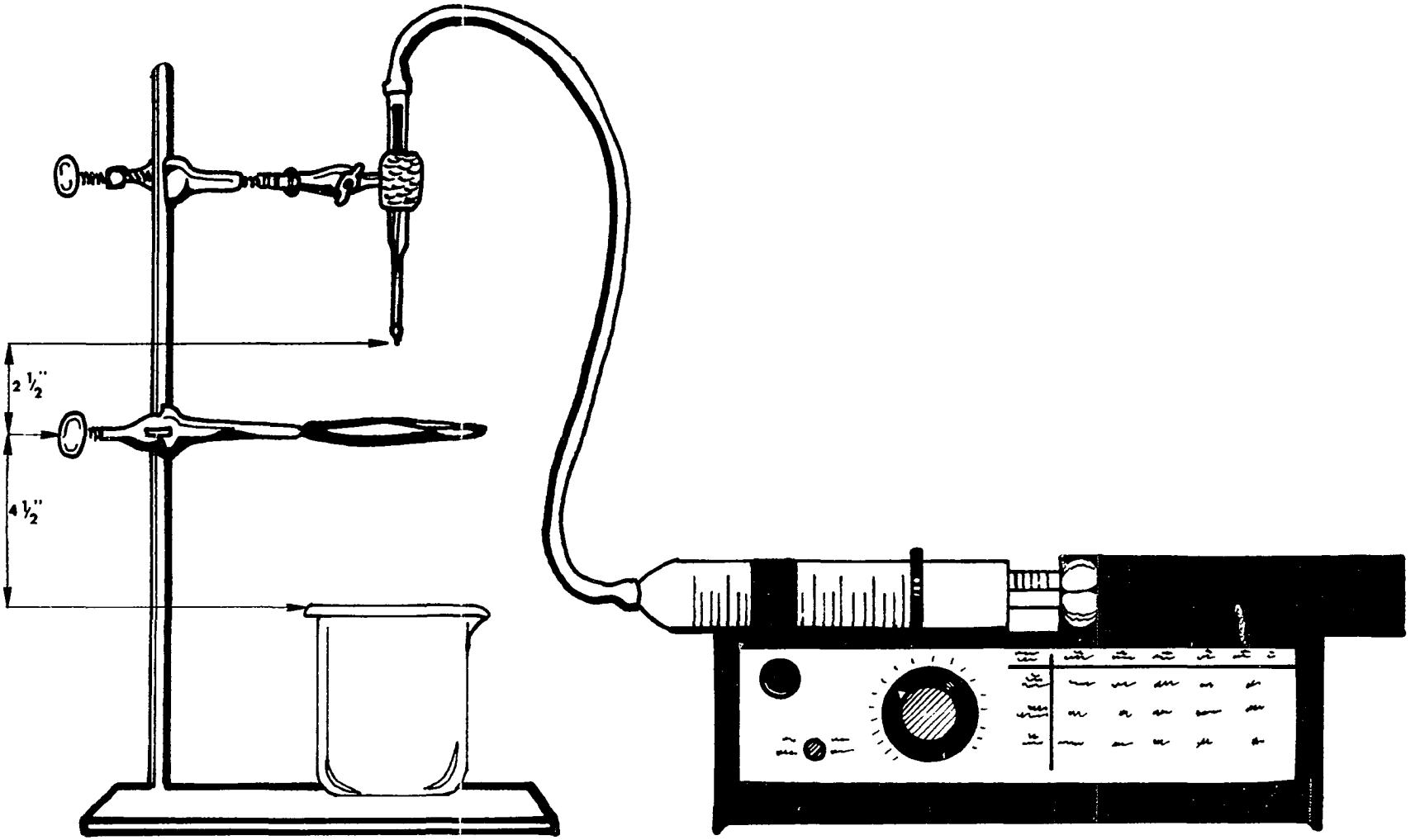

Figure 1. Assembled culture dispenser.

were then placed on the ring below the pipet. A drop of suspension was allowed to impinge on the surface of the agar at the appropriate grid location. Duplicate inoculations of each suspension were made at distant points on the place to minimize bias due to location and interaction with adjacent inoculation sites. After allowing one hour for absorption into the media, the plates were inverted. Dispensing was accomplished within two hours after preparation of the suspensions.

Incubation and Examination of Agar Plates

Inoculated agar plates were incubated for 24 hours at 35°C except that bismuth sulfite agar, mannitol salt agar and Staphylococcus medium 110 were examined after 48 hours at 35°C. Inoculation sites were examined using an American Optical Cycloptic stereoscopic microscope, model 58M-1[5], fitted with ten power ocular lenses and 0.7, 1.1, 1.5, 2.0 and 2.5 power objective lenses. An American Optical eyepiece reticle, style 1406A[5], was installed in the right ocular lens. The reticle was calibrated for each of the five objective lenses to convert reticle units to millimeters. Colony counts were made where discrete colonies were observed. The colony diameters were recorded in millimeters. Other colony characteristics such as morphology, color, changes in the surrounding medium, absence of growth, confluent growth and atypical responses were recorded.

Preliminary Studies

Studies were made to establish the optimum method for reconstituting the test organisms so that the resulting suspension would yield

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[5]American Optical Company, Buffalo, New York

the maximum density of organisms in the minimum time. Eight discs of each test organism were transferred aseptically to separate screw capped 13 x 100 mm tubes containing 2ml of Trypticase ™ soy broth (TSB)[6] and incubated at 35°C. for 4 hours. Two tubes of each organism were removed at 10, 30, 60 and 120 minutes. One of the pair was agitated for 10 seconds and the other for 30 seconds on a Scientific Industries Vortex-Genie mixer.[7] Following agitation, the tubes were returned to the incubator for the balance of the incubation period. The resulting growth was examined visually following agitation and also after the incubation was completed.

Since some knowledge of the probable density of the reconstituted organisms was required for selection of dilutions, studies were made to determine the population of the bacterial suspensions. Five discs of each test organism were deposited in separate tubes containing 2ml of TSB and placed in the incubator. Each suspension was agitated after 10 minutes and again following 2.5 hours incubation. Ten fold serial dilutions of the organisms ranging from 10^{-1} to 10^{-6} were prepared in sterile 0.85 percent NaCl solution. Drops of each dilution were inoculated on to tryptic soy agar (TSA)[8] plates and incubated for 24 hours before the colonies developing in each inoculation site were counted.

The reproducibility of the drop colony count was studied in

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[6]BBL, Division of Bioquest, Cockeysville, Maryland

[7]Scientific Products Division of American Hospital Supply, McGaw Park, Illinois

[8]Difco Laboratories, Detroit, Michigan

order to measure the degree of variation introduced by the methodology. One disc of each test organism was reconstituted and diluted as described above. Ten replicate drop inoculations of each of the 10^{-4}, 10^{-5} and 10^{-6} dilutions were made on TSA and the resulting colonies counted.

Sample size limitations required that the maximum amount of information be obtained from a small number of plates. Pilot samples of each of the media to be studied were furnished by 2 of the participating laboratories. These plates were inoculated with 10^{-1} through 10^{-6} dilutions of the 8 test organisms and the growth following incubation observed as described above. Test organisms and dilutions used for testing were selected based on the character of growth or absence of growth observed compared with the expected response of the media as regards promotion and inhibition of growth.

Examination of Samples

Agar plate media collected from participating laboratories were inoculated the same day collected using the techniques developed as described above. TSA plates were prepared by the writer and included with each series of samples to provide a reference count.

Evaluation of the Effects of Aging on Agar Plate Media

Six different types of agar plate media were examined for changes in response to test organisms following prolonged storage. Samples of media that had been stored for 7 and 14 days in a refrigerator at 1 to 4°C. without wrapping were tested simultaneously along with freshly prepared media. Both the fresh and aged media were furnished by a participating laboratory. Assay was performed as described above

for agar plate media.

Percentage weight loss was calculated for the aged plates to provide an index of dehydration during storage. The weight of each aged agar plate was determined immediately following preparation and when examined. After testing, the agar was removed and the empty plate weighed to provide a tare.

Performance Evaluation of Biochemical Test Media

Selection of species for inoculation into biochemical test media was based on recommendations appearing in the product literature accompanying the organisms. Where possible, organisms expected to yield positive, negative and intermediate reactions were included.

Eighteen hour subcultures on TSA slants were prepared from the reconstituted bacterial suspensions. Growth from the agar slant was emulsified in sterile 0.85 percent NaCl solution when suspensions were specified as the inoculum. Biochemical test media collected from the participating laboratories were inoculated after overnight storage at 1 to 4°C. Media were brought to room temperature and inoculated and incubated as shown in Table 4. Culture results were interpreted as indicated in Table 5 and the results recorded. Deviations from expected alternative patterns of media response were also noted. In general, methods for the inoculation, incubation and examination of biochemical test media suggested by Edwards and Ewing (1972) were employed in this study.

Evaluation of a Modified Decarboxylase Medium

A modified decarboxylase medium proposed by a participating

TABLE 4

INOCULATION AND INCUBATION PROTOCOL FOR BIOCHEMICAL TEST MEDIA

Medium	Inoculum[a]	Method	Hours Incubated at 35°C.
TSI, KIA and LIA	A	Platinum wire stab twice to the base of the butt and the slant streaked.	22
SIM	A	Platinum wire stab two-thirds of the depth of the medium.	22
MOT	A	Platinum wire stab 5mm into the top of the medium.	22 and 48
UA	AH	Platinum wire streak over the entire surface of the slant.	2, 4 and 22
SC	S	Platinum wire stab to the base of the butt and the slant streaked.	22,48 and 120
MB, ML, MA and MO	AL	Platinum wire swirled in broth followed by mineral oil overlay.	22,48 and 120
PA	AH	Platinum wire streak over the entire surface of the slant.	22

[a]A indicates from agar slant, S indicates from suspension, H indicates heavy, L indicates light.

TABLE 5

INTERPRETATION OF BIOCHEMICAL TEST MEDIA RESPONSE

Medium	Characteristic Observed	Interpretation
TSI and KIA	Butt and Slant[b]	Yellow color indicates acid (A) reaction. Red color indicates alkaline (K) reaction. Blackening indicates H_2S production.
LIA	Butt[b]	Yellow color indicates acid reaction. Purple color indicates alkaline reaction. Blackening indicates H_2S production. Red color indicates oxidative deamination (R).
SIM	Motility[b] H_2S Indol	Turbidity indicates motility. Blackening indicates H_2S production. Red color following addition of Kovacs reagent indicates indol production.
MOT	Motility[b]	Turbidity indicates motility.
UA	Butt and Slant[b]	Red color indicates production of urease.
SC	Growth, color[b]	Growth, with or without blue color, indicates utilization of citrate.
MB, ML, MA and MO	Color[a]	Yellow color indicates acid reaction. Purple color indicates alkaline reaction.
PA	Phenylalanine[b]	Green color following addition of 0.5M ferric chloride indicates deamination of phenylalanine.

[a]Included inoculated negative control

[b]Included uninoculated negative control

laboratory was evaluated. This medium was prepared similar to Moeller's decarboxylase medium (1955) except that it contained 1 percent agar. This modification, according to the originator, eliminated the requirement for a mineral oil overlay. Accordingly, the medium was inoculated and incubated similar to the standard medium as described in Table 4 except the mineral oil overlay was omitted. Similar media of standard composition with oil overlay were examined simultaneously.

Culture Media Examination-In vitro Measurements

Hydrogen Ion Determination

The pH of agar plate media was determined within ± 0.01 pH unit accuracy using a Beckman Expandomatic pH meter[9] equipped with Beckman 39170 reference and 41263 glass electrodes. Agar plates with agar sheets at least 2.5 mm thick, were measured by direct insertion of the electrodes into the agar. For thinner plates, a wooden applicator stick was used to lift one side of the agar sheet and fold it over until an adequate thickness was obtained. Repeat measurements were made at several points about the plate after thorough rinsing of the electrodes with distilled water between measurements. Hydrogen ion concentrations were not determined for biochemical media due to inadequate sample size.

Other Observations

Tubed biochemical test media prepared as agar slants were measured as to depth of media at the highest and lowest point. Liquid

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[9]Beckman Instruments, Inc., Fullerton, California

media were measured as to depth. The tube style, size, type of glass and type of closure were recorded. The general appearance of both uninoculated plate and tube media including color, texture and variations from the norm, were recorded.

Evaluation of Water Used to Rehydrate Media

Samples of distilled or demineralized water used to rehydrate culture media were obtained from five participating laboratories and examined for toxic and growth promoting residues. Water was collected in screw capped, borosilicate glass flasks which had been carefully washed, rinsed three times with glass distilled water and thoroughly drained. Flasks were then sterilized by autoclaving and dried. After collection, samples were stored overnight and then tested using the distilled water suitability test described by the American Public Health Association (1971). This test is based on a comparison of the growth of a bacterium in a minimal medium prepared in the unknown water with growth in the same medium dissolved in double glass distilled water.

Procedures employed in these studies were as described in the cited reference except as follows. The Enterobacter cloacae strain used for performance evaluation was employed as the test organism. All solutions were prepared in double glass distilled water and sterilized by filtration through a Millipore type HAWG 0.45 micron pore size membrane filter supported by a polycarbonate filter holder.[10] All water samples were similarly sterilized. TSA was used as slants for

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[10]Millipore Corporation, Bedford, Massachusetts

initial growth of the test organism and as a plating medium.

Following the referenced procedure, the test organism was added in a final concentration of 50 cells per ml to flasks of the pure and unknown waters containing the minimal medium. Viable counts were made of the growth in the flasks following 24 hours incubation at 35°C. by making ten fold serial dilutions in phosphate buffered water (Butterfield, 1932) and inoculating 1ml portions in TSA as pour plates. The resulting colonies were counted after 48 hour incubation at 35°C. The ratio of the count from each unknown water to the pure water was computed.

Hydrogen ion measurements were made on each sample by use of a color block with bromthymol blue as an indicator rather than a pH meter because the low ion content of purified water precludes accurate electrometric measurement.

CHAPTER IV

EXPERIMENTAL RESULTS

Preliminary Studies

Various methods for rapid reconstitution of organisms used for testing media were investigated. Discs of each organism were deposited in tubes of TSB and incubated for 4 hours. Tubes were removed at various intervals during the incubation period, agitated on a vortex mixer, then returned to the incubator. Visual examination revealed the most rapid increase in density occurred when the broths containing the discs were incubated for ten minutes, removed and agitated for 30 seconds, then returned to the incubator for an additional 2 hours and 20 minutes.

The probable densities of the suspensions following this reconstitution procedure were measured by determining the number of viable cells in 5 replicate reconstituted suspensions of each organism and calculating the variation between counts. Standard deviations were calculated from the logarithms of the number of viable organisms per drop since the logarithms of such counts are found to be normally distributed (Public Health Service, 1965, p. 85). These data are presented in Table 6. Discs containing E. cloacae gave the most uniform organism recovery. In contrast, the discs containing the two strains of staphylococci gave variable recovery rates.

TABLE 6

VARIATION IN BACTERIAL ORGANISM RECOVERY
FROM BACTERIA-IMPREGNATED DISCS

	Count Per Calibrated Drop					
Trial	*E. cloacae*[a]	*E. coli*[b]	*P. vulgaris*[b]	*S. typhiumuium*[b]	*S. aureus*[b]	*S. epidermidis*[c]
1	23	34	28	2	70	60
2	15	81	4	7	12	310
3	20	56	50	44	39	18
4	17	52	5	8	200	40
5	19	60	210	45	50	400
Mean	18.8	56.6	59.4	21.2	74.2	165.6
Standard Deviation	$\pm$2.71	$\pm$15.09	$\pm$77.18	$\pm$19.03	$\pm$65.63	$\pm$157.80

[a] count x 10^5

[b] count x 10^4

[c] count x 10^3

Interpretation of sample test results required knowledge of the degree of variation in colony counts introduced by the methodology. Suspensions of the test organisms were diluted and 10 replicate drop inoculations of TSA plates were made from each of three 10 fold dilutions. After 24 hours incubation at 35°C., plates were selected which yielded between 8 and 80 colonies per drop and colony counts were made. Results of these counts and their degrees of variation are presented in Table 7. Standard deviations calculated for each strain revealed reproducibility ranging from ±12 percent for E. cloacae to ±66 percent for S. aureus at the 95 percent confidence level. Viable counts of suspensions of the two strains of staphylococci were generally less reproducible than counts of the gram negative rods.

Inoculation of pilot samples of agar plate media with multiple dilutions of standardized suspensions indicated that inoculation with duplicate undiluted and 10^{-3}, 10^{-4} and 10^{-5} dilutions would yield the maximum useful information from the eight inoculation sites available on each plate. The 10^{-3} and 10^{-4} dilutions permitted counts to be made on plates exhibiting up to 90 and 99 percent repression of the inoculum respectively, thus revealing moderate variation between samples. Gross inhibition of the test organism would be revealed by failure of the undiluted suspension to initiate growth while growth at the 10^{-5} dilution would permit counts where little or no repression occurred. The two strains of staphylococci were regularly inhibited by the gram negative media while the E. cloacae strain consistently grew if inoculated undiluted. Therefore, the media were inoculated with duplicate undiluted and 10^{-3}, 10^{-4} and 10^{-5} dilutions of E. coli, P. vulgaris

TABLE 7

REPRODUCIBILITY OF DROP COUNT METHOD

Replication	Count per Calibrated Drop					
	E. cloacae	*E. coli*	*P. vulgaris*	*S. typhimurium*	*S. aureus*	*S. epidermidis*
1	33	24	21	62	13	41
2	35	24	15	58	14	34
3	30	23	23	53	14	36
4	32	19	16	61	11	20
5	36	21	21	53	10	28
6	31	17	20	62	15	33
7	32	19	17	64	2	33
8	32	22	20	53	8	38
9	33	18	19	59	12	27
10	29	18	19	56	11	32
Mean	32.3	20.5	19.1	58.1	11.0	32.2
Standard Deviation	±2.00	±2.50	±2.34	±3.96	±3.61	±5.69

and S. typhimurium; 10^{-3}, 10^{-4} and 10^{-5} dilutions of E. cloacae; and single undiluted drops of S. aureus and S. epidermidis. The MSA and S110 plates, designed to be selective for staphylococci, were inoculated with duplicate undiluted and 10^{-3}, 10^{-4} and 10^{-5} dilutions of S. aureus and S. epidermidis to check their ability to support growth. The same dilutions of E. coli and P. vulgaris were used to test the ability of these media to inhibit such organisms.

Performance Evaluation of Agar Plate Media

Examination of Growth Response on Sampled Plates

Agar plates from participating laboratories and TSA control plates were inoculated with 10 fold dilutions of the test strains, in duplicate, as described above. Inoculation sites were examined and the colonies counted where coalescence of colonies had not occurred. Growth was recorded as the average of the two duplicate counts times the reciprocal of the dilution. Where possible, sites containing between 8 and 80 colonies were selected for computation as recommended by Stokes (1968).

The growth on the sample plates was compared to the growth on the TSA plates by calculation of the number of logarithms of difference between the counts. This is a modification of the scheme described by Stokes (1968). For example, if 24 colonies were counted at the 10^{-6} dilution on TSA, then counts on the media sampled of less than 24×10^{-5} were recorded as 1 log repression, less than 24×10^{-4} as 2 logs of repression, etc. Repression exceeding 4 logs was recorded as "greater than 4". This difference, which reflects the degree of repression of

inoculum growth by the media, is called the "Apparent Reduction in Inoculum Titer".

Classification of Results

Values representing the degree of repression for a particular media type, expressed in logs, were analyzed and the most frequent (mode) value found was designated as the "Standard of Acceptance". This modal growth pattern is presented in Table 8 for each organism inoculated. Failure of a sample medium to meet this standard with respect to any of the strains inoculated was considered grounds for an unsatisfactory classification for that production batch of medium.

Response of the media produced by the different laboratories is summarized in Table 9. Overall, 63 percent of the agar plates sampled were classified as acceptable. Table entries for performance of each laboratory's media were corrected for differences between media to reduce the bias introduced by the unequal sample sizes. The averages of both the uncorrected and corrected media acceptability rates within each laboratory are given in Table 10. Overall media acceptability ranged from 42.8 percent for laboratory number 12 to 81 percent for laboratory number 7. Each of the table entries were similarly corrected for differences between laboratories. Both the uncorrected and corrected values were averaged for each media and the means entered in Table 11. Corrected average media acceptability ranged from 19.6 percent for SS to 98 percent for MAC.

Table entries were corrected for differences between media by the formula:

TABLE 8

STANDARD OF ACCEPTANCE FOR
AGAR PLATE MEDIA

Media	Apparent Reduction of Inoculum Titer[a]					
	E. cloacae	E. coli	P. vulgaris	S. typhimurium	S. aureus	S. epidermidis
EMB	0	0	0	0	≥4	≥4
LEMB	0	0	≥4	0	≥4	≥4
XLD	0	≥4	0	0	≥4	≥4
MAC	0	0	0	0	≥4	≥4
DC	0	0	0	0	≥4	≥4
SS	≥0	≥4	≥3	≤2	≥4	≥4
BG	0	≥3	≥3	0	≥4	≥4
BS	≥3	≥4	≥4	≤2	≥4	≥4
HE	≥0	≥4	≥0	0	≥4	≥4
S110	—[b]	≥4	≥3	—	0	0
MSA	—	≥4	≥3	—	0	0

[a]expressed as the difference between the exponents of the maximum dilution at which growth may occur on the medium listed and on TSA plates.

[b]dash means not tested.

TABLE 9

ACCEPTABILITY OF AGAR PLATE MEDIA SAMPLED

Media	Laboratory Number												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
EMB	7/8[a]	___	___	4/4	3/4	3/4	3/3	___	4/4	3/3	3/4	___	30/34
LEMB	___	___	4/7	___	___	___	___	2/4	___	___	___	___	6/11
XLD	___	0/3	___	1/3	___	4/4	___	___	___	___	___	___	5/10
MAC	3/3	___	8/8	___	___	4/4	___	___	___	___	___	1/1	16/16
DC	6/7	2/2	___	___	___	___	___	___	___	___	___	___	8/9
SS	1/8	___	1/8	___	0/3	___	___	___	1/3	___	1/3	___	4/25
BG	___	___	6/8	___	___	___	___	___	___	0/1	___	1/2	7/11
BS	___	___	3/7	___	___	___	___	___	___	___	___	0/4	3/11
HE	5/8	2/3	___	1/2	___	___	3/3	3/3	2/2	___	___	___	16/21
S110	2/5	___	1/1	___	___	___	___	___	___	1/1	___	___	4/7
MSA	2/5	___	___	___	1/1	___	___	1/2	2/3	___	___	___	6/11
Total	26/44	4/8	23/39	6/9	4/8	11/12	6/6	6/9	9/12	4/5	4/7	2/7	105/166

[a]Number of acceptable samples/number of samples tested

TABLE 10

ACCEPTABILITY OF AGAR PLATE MEDIA SAMPLED,
VARIATION BETWEEN LABORATORIES

Laboratory Number	Percentage of Samples Classified Acceptable	
	Uncorrected[a]	Corrected[b]
1	59.1	54.5
2	50.0	43.7
3	59.0	68.9
4	66.7	57.1
5	50.0	56.3
6	91.7	75.5
7	100.0	81.0
8	66.7	68.2
9	75.0	78.5
10	80.0	63.9
11	57.1	63.1
12	28.6	42.8

[a]calculated from data in Table 9.

[b]corrected for differences between media.

TABLE 11

ACCEPTABILITY OF AGAR PLATE MEDIA SAMPLED,
VARIATION BETWEEN MEDIA

Media	Percentage of Samples Classified Acceptable	
	Uncorrected[a]	Corrected[b]
EMB	88.2	81.6
LEMB	54.5	56.0
XLD	50.0	41.6
MAC	100.0	98.0
DC	88.9	95.1
SS	16.0	19.6
BG	63.6	71.5
BS	27.3	42.6
HE	76.2	72.5
S110	57.1	58.3
MSA	54.5	53.8

[a]calculated from data in Table 9.

[b]corrected for differences between laboratories.

$$\frac{A_i - B_i \quad (\bar{x}_i - \bar{\bar{x}})}{B}$$

where A_i = number of successes for each table entry

B_i = number of trials of each table entry

$\bar{x}_i$ = average success for all samples of that media from all laboratories

$\bar{\bar{x}}$ = grand average success for all media (= 105/166 = 0.6325301)

Table entries were similarly corrected for differences between laboratories except that $\bar{x}_i$ equals the average success for all samples of different media from that laboratory.

An arithmetic average of the number of logarithms of repression was calculated for all samples of each media as shown in Table 12. Unlike the other parameters studied such as pH and biochemical test media quality, there was no standard or "accepted" response. These data present a general picture of the average response of each of the 11 media when tested by these methods. For example, MAC plates sampled showed no discernible repression of growth with any of the four gram-negative rods. These findings are compatible with the expectations for this media as it is used in the diagnostic laboratory. Conversely, SS demonstrated a greater repression of Salmonella than either P. vulgaris or E. cloacae even though SS was expressly designed to be selective for Salmonella.

Measurements of colony diameters were made where discrete colonies were observed. Analysis of the data revealed wide variation in colony diameters between crowded and sparsely populated inoculation sites. These differences masked any variation between media samples since exact predictability of site populations could not be obtained

TABLE 12

AVERAGE REPRESSION OF GROWTH OF
AGAR PLATE MEDIA SAMPLED

Media	Number of Labs	Number of Samples	Apparent Reduction in Inoculum Titer[a]					
			E. cloacae	*E. coli*	*P. vulgaris*	*S. typhimurium*	*S. aureus*	*S. epidermidis*
EMB	8	34	0	0	0.5	0	>4.0	>4.0
LEMB	2	11	0.2	0.1	3.2	0	>4.0	>4.0
XLD	3	10	0	4.0	0	0.4	>4.0	>4.0
MAC	4	16	0	0	0	0	>4.0	>4.0
DC	2	9	0	0.1	0	0.1	>4.0	>4.0
SS	5	25	0.5	4.0	2.2	3.4	>4.0	>4.0
BGA	2	11	0.6	3.4	3.5	0.2	>4.0	>4.0
BS	2	11	>4.0	>4.0	2.7	2.6	>4.0	>4.0
HE	6	21	0.1	>4.0	0.3	0.2	>4.0	>4.0
S110	3	7	___	>4.0	2.7	___	0	0.1
MSA	4	11	___	>4.0	2.1	___	0	0

[a]expressed as the logarithmic mean of reduction in titer below that observed with the TSA plate for all samples tested.

with the method used.

Observations made of the color, appearance and texture of the uninoculated media and description of the colonies and their interaction with the media were compared between laboratories and between weeks. Lack of objective classification criteria made meaningful interpretation difficult. However, the degree of nucleation of colonies observed macroscopically on SS, HE, EMB, LEMB and XLD showed promise as an objective index. Discernible variation in the size, tint and opacity of colony nuclei was noted between samples of these media. This parameter is especially valuable as a quality control index since nucleation is a differential characteristic for selecting a colony for further study. Occasional frank deviations were observed such as opacity in normally translucent XLD agar from laboratory 2 in week 4.

Although sample size limitations did not permit separate sterility testing, occasional contamination was noted on uninoculated media. Lack of sterility appeared to be randomly distributed except that contamination was found in all 3 samples of SS agar from laboratory 11.

Performance Evaluation of Biochemical Test Media

Acceptance Criteria

Biochemical test media were inoculated with appropriate test strains according to the protocol described in Chapter III. While the manufacturer's literature accompanying the organisms described expected biochemical reactions for most medium/organism combinations, these were expanded to fit the conditions of this study by construction

of internal standards based on the data obtained. Criteria used to define acceptability are indicated in Table 13. Failure of a particular sample unit to respond acceptably with respect to any of the organisms inoculated resulted in an unacceptable classification for the production lot from which it was drawn.

Sample Test Results

Each lot of media sampled was classified as to acceptability. A summary of these findings is presented in Table 14. Table entries were corrected for differences between media and between laboratories as previously described for agar plates. Means of these corrected values are presented in Tables 15 and 16. Acceptability between laboratories varied from 38.4 to 97.1 percent. Percentage acceptability calculated for each media varied from 37.7 to 93.7 percent. Overall, 75 percent of the samples tested were classed as acceptable.

Specific reactions of samples from the several production lots of each media tested are presented in Tables 17 through 33. Intermediate or incomplete reactions are recorded separately in some cases.

The reactions of test strains on TSI, KIA and LIA are presented in Tables 17 through 19. Presence or absence of gas production was recorded for TSI, KIA and LIA. However, since gas production as evidenced by splitting of the agar is not consistent in satisfactory media, it was not considered in the acceptance or rejection of a production lot (Wadsworth, 1947, p. 302; Edwards and Fife, 1961). Failure to produce a distinctive acid butt occurred only 3 times in 165 TSI and KIA slants inoculated with strains expected to produce an acid reaction. In all three cases, the tubes of media were prepared

TABLE 13

STANDARD OF ACCEPTANCE FOR BIOCHEMICAL TEST MEDIA

Media		E. cloacae	E. coli	P. vulgaris	P. aeruginosa	S. typhimurium	S. aureus
TSI	Butt		A or AG	A or AG	N	A or AG	
	Slant		A	A	N or K	K	
	H_2S		-	+ or $^{+}_{w}$	-	+ or $^{+}_{w}$	
KIA	Butt		A or AG	A or AG	N	A or AG	
	Slant		A	K	N or K	K	
	H_2S		-	+ or $^{+}_{w}$	-	+	
PA		-		+			
LIA	Butt	A	N or K	A		K	
	Slant	K	K	R		K	
	H_2S	-	-	+ or -		+ or $^{+}_{w}$	
UA	2 hr.	-	-	+ or -			
	4 hr.	-	-	+ or $^{+}_{w}$			
	22 hr.	+ or $^{+}_{w}$	-	+			
MOT	22 hr.		+ or $^{+}_{w}$	+ or -			-
	48 hr.		+	+ or $^{+}_{w}$			-
SC	22 hr.		-		+ or $^{+}_{w}$	+ or $^{+}_{w}$	
	48 hr.		-		+	+	
	120 hr.		-		+	+	
SIM	H_2S		-	+		+ or $^{+}_{w}$	-
	Indole		+	+		-	-
	Motility		+	+ or $^{+}_{w}$		+	-
ML	22 hr.	-	+ or $^{+}_{w}$	-			
	48 hr.	-	+	-			
	120 hr.	-	+	-			
MA	22 hr.	+ or $^{+}_{w}$		-		+ or -	
	48 hr.	+ or $^{+}_{w}$		-		+ or -	
	120 hr.	+ or $^{+}_{w}$		-		+ or $^{+}_{w}$	
MO	22 hr.	+		-		+	
	48 hr.	+		-		+	
	120 hr.	+		-		+	

TABLE 14

ACCEPTABILITY OF BIOCHEMICAL TEST MEDIA SAMPLED

Media	Laboratory Number												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
TSI	10/10[a]	___	6/6	___	2/3	1/4	1/2	4/4	___	1/1	0/1	___	25/31
KIA	10/10	3/3	5/6	3/4	___	___	___	___	___	___	1/1	___	22/24
PA	5/5	___	4/4	___	1/1	3/4	___	___	___	___	___	___	13/14
LIA	___	2/2	5/5	___	0/1	___	1/4	0/1	___	2/2	___	1/1	11/16
UA	7/7	2/2	3/11	3/3	1/3	___	0/1	___	___	1/2	___	___	17/29
MOT	7/9	___	___	___	___	3/3	___	___	___	___	___	___	10/12
SC	8/10	3/3	8/11	4/4	4/4	4/4	3/4	4/4	___	___	___	___	38/44
SIM	10/10	1/3	2/6	2/4	1/4	___	1/1	2/4	___	3/3	___	___	22/35
ML	1/7	___	3/3	___	___	___	___	___	___	___	___	___	4/10
MA	4/7	___	2/2	___	___	___	___	___	___	___	___	___	6/9
MO	3/7	___	2/2	___	___	___	___	___	___	___	___	___	5/9
Total	65/82	11/13	40/56	12/15	9/16	11/15	6/12	10/13		7/8	1/2	1/1	174/233

[a]Number of acceptable samples/number of samples tested.

TABLE 15

ACCEPTABILITY OF BIOCHEMICAL TEST MEDIA SAMPLED, VARIATION BETWEEN LABORATORIES

Laboratory Number	Percentage of Samples Classified Acceptable	
	Uncorrected[a]	Corrected[b]
1	79.3	81.0
2	84.6	84.3
3	71.4	73.2
4	80.0	78.6
5	56.3	57.4
6	73.3	61.9
7	50.0	50.0
8	76.9	75.6
10	87.5	97.1
11	50.0	38.4

[a]calculated from the data in Table 14.

[b]corrected for differences between media.

TABLE 16

ACCEPTABILITY OF BIOCHEMICAL TEST MEDIA SAMPLED, VARIATION BETWEEN MEDIA

Media	Percentage of Samples Classified Acceptable	
	Uncorrected[a]	Corrected[b]
TSI	80.6	83.3
KIA	91.7	89.4
PA	92.9	93.7
LIA	66.7	72.3
VA	58.6	59.3
MOT	83.3	80.1
SC	86.4	88.7
SIM	62.9	62.0
ML	40.0	37.7
MA	66.7	63.7
MO	55.6	52.6

[a]calculated from the data in Table 9.

[b]corrected for differences between laboratories.

TABLE 17

REACTIONS OF TEST STRAINS WITH TRIPLE SUGAR IRON AGAR (TSI) SAMPLED

REACTION[a]			NUMBER OF SAMPLES			
Butt	Slant	H_2S	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*S. typhimurium*
AG	A	-	28	0	0	0
A	A	-	2	(5)	0	0
N	A	-	(1)[b]	0	0	0
AG	A	+	0[c]	12	0	0
A	A	+	0	5	0	0
AG	A	$+_w$	0	3	0	0
A	A	$+_w$	0	5	0	0
N	A	$+_w$	0	(1)	0	0
N	K	-	0	0	31	0
AG	K	+	0	0	0	20
A	K	+	0	0	0	3
AG	K	$+_w$	0	0	0	4
A	K	$+_w$	0	0	0	3
A	N	-	0	0	0	(1)
Total Acceptable			30	26	31	30
Total Sampled			31	31	31	31
Percentage Acceptable			97	84	100	97

[a]A means acid, G means gas, K means alkaline, N means neutral, $+_w$ means blackening less than one-third of the distance between the base of the slant and the bottom of the tube, + means blackening greater than $+_w$, - means negative.

[b]Numbers in parenthesis indicate unacceptable results.

[c]No samples gave the reaction specified.

TABLE 18

REACTIONS OF TEST STRAINS WITH KLIGLERS IRON AGAR (KIA) SAMPLED

Reaction[a]			Number of Samples			
Butt	Slant	H_2S	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*S. typhimurium*
AG	A	-	23	0	0	0
A	A	-	1	0	0	0
AG	K	$+_w$	0	0	0	1
A	K	$+_w$	0	3	0	4
AG	K	+	0	12	0	15
A	K	+	0	8	0	3
N	K	-	0	0	24	0
N	K	$+_w$	0	(1)[b]	0	0
AG	A	+	0	0[c]	0	(1)
Total Acceptable			24	23	24	23
Total Sampled			24	24	24	24
Percentage Acceptable			100	96	100	100

[a]A means acid, G means gas, K means alkaline, N means neutral, $+_w$ means blackening less than one-third of the distance between the base of the slant and the bottom of the tube, + means blackening greater than $+_w$, - means negative.

[b]Numbers in parenthesis indicate unacceptable results.

[c]No samples gave the reaction specified.

TABLE 19

REACTIONS OF TEST STRAINS WITH LYSINE IRON AGAR (LIA) TESTED

Reaction[a]			Number of Samples			
Butt	Slant	H_2S	*E. cloacae*	*E. coli*	*P. vulgaris*	*S. typhimurium*
A	K	-	11	(1)[b]	0	0
N	K	-	0[c]	5	(1)	0
K	K	-	(5)	10	(1)	0
A	R	-	0	0	13	0
K	R	-	0	0	(1)	0
A	K	^{+}w	0	0	0	(1)
K	K	^{+}w	0	0	0	5
K	K	+	0	0	0	10
Total Acceptable			11	15	13	15
Total Sampled			16	16	16	16
Percentage Acceptable			69	94	81	94

[a]A means acid, K means alkaline, N means neutral, R means red, ^{+}w means blackening less than one-third of the distance between the base of the slant and the bottom of the tube, + means blackening greater than ^{+}w, - means negative.

[b]Numbers in parenthesis indicate unacceptable results.

[c]No samples gave the reaction specified.

so as to have less than the recommended 1/2 inch deep butt thereby permitting a more alkaline final pH (Kligler, 1918). Aberrant H_2S reactions were noted more frequently. Thirteen of the 110 slants inoculated with H_2S producing strains exhibited blackening less than one third of the distance from the base of the slant to the bottom of the tube. Since a taxonomic error would not have been made, this reaction was classified as acceptable. Proteus vulgaris produced detectable H_2S on only 1 of 16 samples of LIA inoculated but this result was not unexpected (Edwards and Fife, 1961). Two of these 16 tubes did not yield the typical red slant expected with Proteus. Incorrect decarboxylase reactions, expressed by the color of the butt, were noted in 10 of 64 LIA tubes inoculated with strains capable of producing characteristic lysine decarboxylase reactions.

The reactions of 4 test strains on 35 lots of SIM medium are presented in Table 20. No evidence of H_2S production was noted on 3 of 70 tubes inoculated with H_2S positive strains. These 3 tubes also showed a false negative motility. No false positive H_2S reactions were seen in 70 tubes inoculated with H_2S negative strains. Indol was detected in all 70 tubes inoculated with indol positive strains and in none of the 70 tubes inoculated with indol negative strains. The motility reactions were positive for 34 of the 35 tubes inoculated with the strongly motile S. typhimurium and none of the 35 tubes inoculated with S. aureus appeared to show motility. The weakly motile strain of P. vulgaris gave variable reactions on SIM. Of 35 tubes inoculated, 16 showed strong motility, 7 weak motility and 12 tubes showed no evidence of motility. This pattern correlated well with the source of

TABLE 20

REACTIONS OF TEST STRAINS WITH
SIM MEDIUM (SIM) TESTED

Reaction[a]			Number of Samples			
H_2S	Indol	Motility	E. coli	P. vulgaris	S. typhimurium	S. aureus
-	-	-	0[b]	0	0	35
-	+	-	0	(3)[c]	0	0
+	+	-	0	(9)	0	0
-	+	$+_w$	0	(1)	0	0
+	+	$+_w$	0	6	0	0
-	+	+	35	0	0	0
+	+	+	0	16	0	0
+	-	+	0	0	33	0
+	-	$+_w$	0	0	(1)	0
+	-	-	0	0	(1)	0
Total Acceptable			35	22	33	35
Total Sampled			35	35	35	35
Percentage Acceptable			100	63	94	100

[a] H_2S + means any degree of blackening, Indol + means any degree of color, Motility $+_w$ means growth does not extend to side of tube, Motility + means motility greater than $+_w$, - means negative

[b] No samples gave the reaction specified.

[c] Numbers in parenthesis indicate unacceptable results.

the media, implying a real difference between laboratories.

This difference in motility of P. vulgaris was also seen in MOT media data presented in Table 21. Motility was observed in 2 samples but only after 48 hours incubation. The other 10 samples were negative at both 24 and 48 hours. The E. coli strain appeared uniformly motile while no motility was observed in tubes inoculated with S. aureus.

The response of UA when inoculated with three test strains is presented in Table 22. Media from all 29 production samples gave positive urease reactions within 4 hours following inoculation with P. vulgaris. However, 12 of 29 samples did not give the expected weak positive reaction at 22 hours with E. cloacae. While UA is used primarily for Proteus detection, a delayed reaction on UA is expected with some members of the Klebsiella-Enterobacter-Serratia group (Christensen, 1946). There were no false positives; all 29 samples inoculated with E. coli were negative at 22 hours.

Reactions of 3 strains inoculated on SC agar are given in Table 23. Three of 44 tubes inoculated with E. coli showed visible growth although the color of the indicator was not changed. Since the inoculum was diluted in saline prior to inoculation to preclude the possibility of carryover of nutrients (Edwards and Ewing, 1972, p. 343), the media apparently incorrectly contained some nutrients for the E. coli. Failure to produce color change cannot be interpreted as a negative reaction since some organisms which utilize citrate as a sole source of carbon do not produce such a change (Simmons, 1926). One tube of 44 inoculated with P. aeruginosa and one of 44 inoculated with S. typhimurium were classified unacceptable

TABLE 21

REACTIONS OF TEST STRAINS WITH MOTILITY TEST MEDIUM (MOT) SAMPLED

Reaction[a]		Number of Samples		
24 hr.	48 hr.	E. coli	P. vulgaris	S. aureus
+	+	10	0	0
$+_w$	+	2	0	0
-	+	0[b]	2	0
-	-	0	(10)[c]	12
Total Acceptable		12	2	12
Total Samples		12	12	12
Percentage Acceptable		100	17	100

[a]$+_w$ means growth extends from stab line but does not touch side of tube, + means growth extends to side of tube, - means negative.

[b]No samples gave the reaction specified.

[c]Numbers in parenthesis indicate unacceptable results.

TABLE 22

REACTIONS OF TEST STRAINS WITH
UREA AGAR (UA) TESTED

Reaction[a]			Number of Samples		
2 hr.	4 hr.	22 hr.	E. cloacae	E. coli	P. vulgaris
-	-	-	(12)[b]	29	0
-	-	$^{+}_{w}$	17	0	0
-	$^{+}_{w}$	+	0[c]	0	7
$^{+}_{w}$	$^{+}_{w}$	+	0	0	21
$^{+}_{w}$	+	+	0	0	1
Total Acceptable			17	29	29
Total Sampled			29	29	29
Percentage Acceptable			59	100	100

[a] $^{+}_{w}$ means red color does not extend beyond one-third of the distance from the base of the slant to the bottom of the tube, + means red color greater than $^{+}_{w}$, - means negative.

[b] Numbers in parenthesis indicate unacceptable results.

[c] No samples gave the reaction specified.

TABLE 23

REACTIONS OF TEST STRAINS WITH SIMMONS CITRATE AGAR (SC) SAMPLED

Reactions[a]			Number of Samples		
24 hr.	48 hr.	120 hr.	E. coli	P. aeruginosa	S. typhimurium
-	-	-	41	0	0
-	-	$+_w$	(2)[b]	0	0
-	$+_w$	+	(1)	0	0
-	+	+	0[c]	(1)	(1)
$+_w$	$+_w$	$+_w$	0	0	(1)
$+_w$	$+_w$	+	0	0	(1)
$+_w$	+	+	0	0	3
+	+	+	0	43	38
Total Acceptable			41	43	41
Total Sampled			44	44	44
Percentage Acceptable			93	98	93

[a] $+_w$ means growth observed; blue color, if any, does not extend beyond one-third of the distance from the base of the slant to the bottom of the tube; + means blue color in excess of $+_w$; - means negative.

[b] Numbers in parenthesis indicate unacceptable results.

[c] No samples gave the reaction specified.

due to failure to show any growth at 24 hours. Three tubes of 44 inoculated with S. typhimurium showed some growth at 24 and 48 hours but no apparent color change within that time. Simmons (1926) indicates that a deep prussian blue tint should be evident on the slant within 48 hours after inoculation with this organism.

Decarboxylase reactions are presented in Tables 24 through 26. Negative reactions with these media should appear as a rapid (less than 20 hours) color change from purple to yellow while a decarboxylase positive organism will elicit a similar rapid change from purple to yellow, followed by a reconversion to purple within 4 days (Moeller, 1955). A control tube, without added amino acid, was also inoculated as a control.

The 10 samples of lysine media responded correctly, with E. coli and P. vulgaris but 6 of the 10 tubes gave false positive reactions with E. cloacae. This may be a result of the substitution by Difco of the peptone specified in the Falkow (1958) formula for the special peptone of the Moeller (1955) formula. Ewing et al (1960) reported that the Falkow formula gave equivocal results with the genera Klebsiella and Enterobacter.

A correctly negative response was observed with all 9 samples of arginine containing decarboxylase media inoculated with P. vulgaris but 2 of 9 samples gave a false negative with E. cloacae and a similar portion were falsely positive with S. typhimurium. One tube was correctly positive with this organism at 22 and 48 hours but inexplicably turned negative at the 120 hour reading.

TABLE 24

REACTIONS OF TEST STRAINS WITH MOELLERS LYSINE DECARBOXYLASE MEDIA (ML) SAMPLED

Reactions[a]			Number of Samples		
22 hr.	48 hr.	120 hr.	E. cloacae	E. coli	P. vulgaris
-	-	-	4	0	10
-	$+_w$	+	(1)[b]	0	0
$+_w$	$+_w$	$+_w$	(2)	0	0
$+_w$	$+_w$	+	(3)	0	0
$+_w$	+	+	0[c]	2	0
+	+	+	0	8	0
Total Acceptable			4	10	10
Total Sampled			10	10	10
Percentage Acceptable			40	100	100

[a] $+_w$ means brown color, + means purple color, - means negative.

[b] Numbers in parenthesis indicate unacceptable results.

[c] No samples gave the reaction specified.

TABLE 25

REACTIONS OF TEST STRAINS WITH MOELLERS ARGININE DECARBOXYLASE AND DIHYDROLASE MEDIA (MA) SAMPLED

Reactions[a]			Number of Samples		
22 hr.	48 hr.	120 hr.	E. cloacae	P. vulgaris	S. typhimurium
-	-	-	(2)[b]	9	(2)
-	-	$+_w$	0[c]	0	1
-	-	+	0	0	1
-	$+_w$	+	0	0	4
$+_w$	$+_w$	$+_w$	2	0	0
+	+	+	5	0	0
+	+	-	0	0	(1)
Total Acceptable			7	9	6
Total Sampled			9	9	9
Percentage Sampled			78	100	67

[a] $+_w$ means brown color, + means purple color, - means negative.

[b] Numbers in parenthesis indicate unacceptable results.

[c] No samples gave the reaction specified.

TABLE 26

REACTIONS OF TEST STRAINS WITH MOELLERS ORNITHINE DECARBOXYLASE MEDIA (MO) SAMPLED

Reactions[a]			Number of Samples		
22 hr.	48 hr.	120 hr.	E. cloacae	P. vulgaris	S. typhimurium
-	-	-	(2)[b]	7	(3)
-	-	+	(1)	0	0
+	+	+	6	(1)	6
+	-	-	0[c]	(1)	0
Total Acceptable			6	7	6
Total Sampled			9	9	9
Percentage Sampled			67	78	67

[a] +w means brown color, + means purple color, - means negative.

[b] Numbers in parenthesis indicate unacceptable results.

[c] No samples gave the reaction specified.

Examination of the data for the 9 samples of ornithine media revealed the greatest number of discrepancies. Three of 9 samples gave false negative results with both E. cloacae and S. typhimurium suggesting the amino acid may have been left out of these production lots. One tube inoculated with P. vulgaris was initially positive at 22 hours but converted to the correct negative reaction at 48 hours. This result could be explained by a delay in the glucose fermentation required by all test organisms in order to obtain meaningful results (Moeller, 1955).

Analysis of the results of the sample of modified decarboxylase media produced by a participating laboratory revealed that the lysine, arginine and ornithine containing media all produced expected reactions with each of the three test organisms. In comparison, only 8 of 21 samples of conventional decarboxylase media produced by this laboratory gave expected reactions when tested with the same strains. The hypothesis that the added agar in the modified media would obviate the need for a mineral oil overlay was not rejected by these tests.

Results of inoculation of 14 samples of PA medium with E. cloacae and P. vulgaris are presented in Table 27. Thirteen samples gave positive tests with P. vulgaris as evidenced by development of a deep blue-green color following addition of the indicator solution. One sample, while positive, gave an atypical, very weak blue reaction although a generous growth was evident on the surface of the slant (Ewing et al, 1957). Subsequent examination of a gram stain of the growth from the weak as well as one of the strong reacting tubes revealed no apparent morphological differences suggestive of a mixed

TABLE 27

REACTIONS OF TEST STRAINS WITH PHENYLALANINE AGAR (PA) SAMPLED

Reaction[a]	Number of Samples	
	E. cloacae	*P. vulgaris*
-	14	0
$+_w$	0[b]	(1)[c]
+	0	13
Total Acceptable	14	13
Total Sampled	14	14
Percentage Acceptable	100	93

[a] $+_w$ means color developed significantly less than normal, + means full color developed.

[b] No samples gave the reaction specified.

[c] Numbers in parenthesis indicate unacceptable results.

culture. All 14 samples inoculated with phenylalanine deaminase negative E. cloacae gave a negative test.

Hydrogen Ion Concentration of Agar Plate Media

Hydrogen ion concentrations were determined for agar plate samples representing 174 production lots of media. Determinations were made on all samples examined for growth response except where the required fifth plate was not available because of contamination, breakage, etc. Determinations were also made for samples from several additional lots prepared by the participating laboratories which were too small (less than 4) for growth response testing.

Preliminary studies were conducted to ascertain suitable mechanics for measurement of pH of agar plate media. Direct insertion of the electrodes was found to be acceptable for agar sheets approximately 2.5mm thick. Thinner sheets provided inadequate contact of the electrodes with the media but by folding the agar sheet, the required depth was obtained. Multiple pH determinations were made of both thick and thin agar plates poured from the same production batch. No differences were noted between thick and folded thin agar sheets.

Mean pH values of multiple samples of 11 different agar plate media collected over the eight weeks of the study from 12 laboratories are shown in Table 28. The number of samples of each media tested ranged from 1 to 12. The manufacturers' recommended final pH is also indicated.

Overall averages for each media expressed as the weighted mean and the simple mean revealed that the target pH was exceeded in 5 cases, not reached in 3 cases and equivocal in 3 cases. The three media with

TABLE 28

MEAN HYDROGEN ION CONCENTRATION FOR AGAR PLATE MEDIA SAMPLED

Media Tested	Target[a] pH	Laboratory Number												Weighted[b] Mean	Mean[c] of Means
		1	2	3	4	5	6	7	8	9	10	11	12		
EMB	7.2	7.14[d] / 10	—	—	7.09 / 4	6.99 / 3	7.23 / 3	7.30 / 3	—	7.02 / 4	7.11 / 3	7.26 / 3	—	7.14 / 33	7.14 / 8
LEMB	7.1	—	—	6.93 / 6	—	—	—	—	7.61 / 4	—	—	—	—	7.21 / 10	7.27 / 2
XLD	7.4	—	7.68 / 3	—	7.46 / 3	—	7.48 / 4	—	—	—	—	—	—	7.53 / 10	7.54 / 3
MAC	7.1	6.87 / 4	—	6.99 / 8	—	—	7.15 / 4	—	—	—	—	—	6.97 / 1	7.00 / 17	7.00 / 4
DC	7.3	6.88 / 8	7.26 / 1	—	—	—	—	—	—	—	—	—	—	6.92 / 9	7.07 / 2
SS	7.0	7.51 / 10	—	7.22 / 8	—	7.43 / 3	—	—	—	6.93 / 3	7.39 / 1	7.06 / 3	—	7.31 / 28	7.26
BG	6.9	—	—	7.31 / 8	—	—	—	—	—	—	6.60 / 1	—	6.94 / 12	7.16 / 12	6.95 / 3
BS	Varied[e]	—	—	7.69 / 8	—	—	—	—	—	—	—	—	7.33 / 4	7.57 / 12	7.51 / 2
HE	Varied[f]	8.05 / 10	7.59 / 3	—	7.64 / 2	—	—	7.78 / 3	7.54 / 3	7.58 / 2	—	—	—	7.48 / 23	7.70 / 6
S110	7.0	7.13 / 10	—	6.58 / 1	—	—	—	—	—	—	—	—	—	7.08 / 11	6.86 / 2
MSA	7.4	7.48 / 9	—	—	—	7.34 / 1	—	—	7.34 / 2	7.56 / 3	—	—	—	7.47 / 15	7.43 / 4

[a]Final pH value recommended by manufacturer (Difco and BBL).

[b]Mean of all the individual observations.

[c]Mean of the means for each laboratory.

[d]Mean of samples/Number of samples.

[e]Difco = 7.7; BBL and Special formulation used in Lab number 3 = 7.5

[f]Pfeizer = 7.6; Difco and BBL = 7.5.

below minimum pH are of low selectivity while five media with excessive pH were, with the exception of Levine EMB, of a moderate to highly selective character. Additionally, the highly selective media appeared to exhibit a greater divergence in mean pH between laboratories.

Accuracy data presented in Table 29 show the degree of success by the participating laboratories in producing agar plate media within the limits $\pm$0.2 of the manufacturer's recommended final pH. Percentage of success between laboratories contributing 6 or more samples ranged from 17 to 100 percent. The number of samples submitted, approximately proportional to the number of different media produced, appeared not to be functionally related to the success in maintaining proper pH. Within laboratories, the variation between media was quite pronounced. This is particularly apparent in laboratory number 1. Of the seven media produced by this laboratory, less than 25 percent of the samples of 4 media were acceptable while 88 percent of 3 media were within limits.

Accuracy findings for different media ranged from 36 to 86 percent for media received from two or more laboratories. Data presented revealed no apparent association between accuracy of pH and selectivity of the media.

Findings were also collated to show the reproducibility attained by each laboratory. A sample mean was computed for each medium from laboratories submitting two or more samples. Each pH measurement was then catagorized as meeting or not meeting the standard of sample mean pH$\pm$0.2. The degree of success found for each sample and the overall success by laboratory and by medium is presented in Table 30. Eighty-seven percent of the total of 174 samples tested met the criteria sample

TABLE 29

ACCURACY OF HYDROGEN ION CONCENTRATION IN AGAR PLATE MEDIA

Media	Number Acceptable/Number Tested[a] Laboratory Number												Totals	Percentage
	1	2	3	4	5	6	7	8	9	10	11	12		
EMB	10/10	-	-	4/4	1/3	3/3	2/3	-	3/4	0/3	2/3	-	25/33	76
LEMB	-	-	5/6	-	-	-	-	2/4	-	-	-	-	7/10	70
XLD	-	0/3	-	3/3	-	4/4	-	-	-	-	-	-	7/10	70
MAC	1/4	-	5/8	-	-	3/4	-	-	-	-	-	-	9/16	56
BG	-	-	1/8	-	-	-	-	-	-	-	-	3/3	4/11	36
BS	-	-	7/8	-	-	-	-	-	-	-	-	0/4	7/12	58
SS	2/10	-	5/8	-	0/3	-	-	-	3/3	-	3/3	-	13/27	48
DC	1/8	-	-	-	-	-	-	-	-	-	-	-	1/8	13
HE	0/10	3/3	-	2/2	-	-	2/3	3/3	2/2	-	-	-	12/23	52
MSA	8/9	-	-	-	-	-	-	2/2	2/3	-	-	-	12/14	86
S110	9/10	-	-	-	-	-	-	-	-	-	-	-	9/10	90
Totals	31/61	3/6	23/38	9/9	1/6	10/11	4/6	7/9	10/12	0/3	5/6	3/7	106/174	
Percentage	51	50	61	100	17	91	67	78	83	0	83	43		61

[a]Shows number of samples falling within the limits: Manufacturers' target pH ±0.2 out of the total number sampled.

TABLE 30

REPRODUCIBILITY OF HYDROGEN ION CONCENTRATION IN AGAR PLATE MEDIA

Media	Number Acceptable/Number Tested[a] Laboratory Number												Totals	Percentage
	1	2	3	4	5	6	7	8	9	10	11	12		
EMB	10/10	-	-	4/4	3/3	3/3	2/3	-	4/4	1/3	1/3	-	28/33	85
LEMB	-	-	6/6	-	-	-	-	1/4	-	-	-	-	7/10	70
XLD	-	3/3	-	3/3	-	4/4	-	-	-	-	-	-	10/10	100
MAC	4/4	-	7/8	-	-	3/4	-	-	-	-	-	-	14/16	88
BG	-	-	7/8	-	-	-	-	-	-	-	-	3/3	10/11	91
BS	-	-	7/8	-	-	-	-	-	-	-	-	4/4	11/12	92
SS	5/10	-	5/8	-	3/3	-	-	-	3/3	-	3/3	-	19/27	70
DC	7/8	-	-	-	-	-	-	-	-	-	-	-	7/8	88
HE	10/10	3/3	-	2/2	-	-	3/3	3/3	2/2	-	-	-	23/23	100
MSA	9/9	-	-	-	-	-	-	2/2	1/3	-	-	-	12/14	86
S110	10/10	-	-	-	-	-	-	-	-	-	-	-	10/10	100
Total	55/61	6/6	32/38	9/9	6/6	10/11	5/6	6/9	10/12	1/3	4/6	7/7	151/174	
Percentage	90	100	84	100	100	91	83	67	83	33	67	100		87

[a]Shows number of samples falling within the sample mean ± 0.2 out of the total number sampled.

mean pH ±0.2. Overall success for laboratories submitting 2 or more different media ranged from 67 to 100 percent. These data show that, overall, the laboratories produced media of rather consistent quality whether they were acceptable or not. No particular patterns with respect to media selectivity or other known variables were noted. However, examination of the mean acceptability between media revealed apparent differences. Percentage success varied from 70 percent for Levine EMB and SS agar to 100 percent for XLD, HE and S110.

Comparison of the differences between media with respect to accuracy and reproducibility indicated no general pattern of correlation between these two variables.

Evaluation of Water Used to Rehydrate Media

Waters used for preparation of culture media in four media preparation facilities were tested for suitability. The results are shown in Table 31. Classification as to acceptability was based on a toxicity ratio. This ratio is derived by dividing the viable plate count of *E. cloacae* grown in a minimal medium dissolved in the unknown water by the count of the same strain grown in the minimal medium prepared with double glass distilled water. This procedure is further described in Chapter III. The toxicity ratio is deemed acceptable by the APHA if it falls within the range 0.8 to 1.2. Waters having a ratio less than 0.8 are considered excessively toxic while a ratio in excess of 1.2 is indicative of the presence of growth promoting substances. Hydrogen ion determinations were also performed on the samples.

Viable plate counts were made of the water samples. While the organisms which grew out were not identified, macroscopic examination of

TABLE 31

QUALITY OF WATER USED TO REHYDRATE MEDIA IN SELECTED LABORATORIES

Processing System	Fabrication	Sampling Point	PRODUCT WATER			
			APC[a]	pH	Toxicity Ratio	Acceptable
A	Stainless Steel Still	Still Effluent	<1	6.6	1.06	Yes
B	Tin Lined Copper Still	Distribution Line	440	5.1	0.82	Yes
C	Tin Lined Copper Still	Still Effluent	<1	6.8	0.41	No
D	Borosilicate Glass Still	Still Effluent	2	6.2	0.88	Yes
E	Resin Demineralizer	Demineralizer Effluent	10,000	7.4	1.42	No

[a]Aerobic Plate Count per ml on TSA

the plates revealed the growth was of a uniform colony type within each sample but different between samples.

Processing system C which produced excessively toxic water and processing system E which contained growth promoting substances were both located in laboratory number 12.

Correlation of Media Performance with Other Factors Studied

Agar Plate Performance vs Hydrogen Ion Concentration

Data for several agar plate media were statistically analyzed for correlation between pH and productivity. A summary of these findings is presented in Tables 32 and 33. The "theoretical probability" ($\bar{y}$), derived from Li (1964, p. 443), was generated by the formula

$$\bar{y} = \frac{(n_1)(n_2)+(N-n_1)(N-n_2)}{N} = \frac{N^2 n_1 N - n_2 N + 2 n_1 n_2}{N}$$

where n_1 = number of samples with acceptable pH, n_2 = number of samples with acceptable growth and N = total number of samples.

The "experimental probability" (μ) was computed by the formula

$$\mu = \frac{n_i + n_j}{N}$$

where n_i = number of samples with both acceptable pH and productivity, n_j = number of samples with both unacceptable pH and productivity and N = total number of samples.

These statistics were analyzed for significant correlation by the method of Li (1964, p. 456) where the statistic

$$u = \frac{\bar{y} - \mu}{\sqrt{\frac{\mu(1-\mu)}{N}}}$$

and the probabilities $\bar{y}$ and μ are significantly different if u is less than minus 1.96 or greater than 1.96.

TABLE 32

CORRELATION BETWEEN FAILURE TO CONTROL HYDROGEN ION CONCENTRATION AND ABERRANT GROWTH OF *E. CLOACAE*, *E. COLI* OR *P. VULGARIS*

Media	Sample Size	Theoretical Probability	Experimental Probability	Significant Correlation at 5% Level
LEMB	11	0.512	0.909	Yes
BG	11	0.397	0.182	No
SS	27	0.498	0.852	Yes

TABLE 33

CORRELATION BETWEEN FAILURE TO CONTROL HYDROGEN ION CONCENTRATION AND ABERRANT GROWTH OF S. TYPHIMURIUM

Media	Sample Size	Theoretical Probability	Experimental Probability	Significant Correlation at 5% Level
XLD	10	0.540	0.900	Yes
BG	11	0.397	0.182	No
SS	27	0.512	0.704	Yes

Data was not analyzed for each case where $N(\mu)$ and $N(1-\mu)$ were not both greater than 5 (Li, 1964, p. 449) and where absence of correlation was noted by inspection.

Media Performance versus Brand of Media

Results of performance evaluations of both agar plate and biochemical test media detailed earlier in this chapter were segregated by manufacturer and examined for correlation between manufacturer and productivity. Data was corrected for differences between laboratories as previously described. No significant differences were detectable between media prepared from Difco and BBL dehydrated products. A similar examination was made for correlation between hydrogen ion concentration of agar plate media and brand of media. Again, no correlation between these factors was evident. Seventy-two percent of media samples which were identifiable by source were of Difco origin while 26 percent were BBL products. Pfeizer brand of dehydrated HE media, representing 2 percent of media sampled, was used in 3 laboratories.

Agar Plate Performance vs Age of Media

Age of agar plate media was obtained on 31 percent of samples. Declared age ranged from 0 to 35 days with a mean of 7.1. No significant correlation was observed between age and productivity except that the few samples of very old media (greater than three weeks) were decidedly inferior. However, it should be noted that, where age was given, 94 percent exceeded the three days (36 hours for Bismuth Sulfite) recommended by Edwards and Ewing (1972, p. 12) although 88 percent did not exceed 8 days.

Samples of 6 different media 7 and 14 days old and freshly prepared media were inoculated with the test strains. Differences in productivity between the fresh and aged media are listed in Table 34 together with the percent weight loss during storage. Greatest repression of growth occurred with S. typhimurium where 3 of the 5 media sampled showed reduced growth with age. The maximum repression of growth due to age occurred with SS agar where 1 week old media demonstrated a 1 log greater repression of both E. cloacae and S. typhimurium. After 2 weeks storage, S. typhimurium was repressed 2 full logs greater than the fresh media. No changes in productivity due to aging were seen with EMB, MAC, and S110. Percent weight loss was similar for all media and averaged 2 percent.

Media Performance vs Other Factors

Biochemical test media sampled were identified as to physical characteristics of the tube and contents. Eight laboratories used screw capped tube closures. Media from these laboratories scored an aggregate 76 percent satsifactory. This compares favorably with the overall average (75 percent). Eleven of the 655 tubes (2 percent) from these laboratories displayed visible changes such as cracking or discoloration of the agar slant and noticeable reduction in volume. Four of these eleven tubes (36 percent) were unsatisfactory.

Laboratory numbers 5 and 6 used foam and cotton plug closures respectively. These laboratories scored an aggregate 65 percent satisfactory which suggests some overall deterioration due to dehydration. Twenty-eight of the 125 tubes from laboratories 5 and 6 (22 percent) had visible changes compatible with dehydration. Twelve of these 28

TABLE 34

REPRESSION OF GROWTH ON ONE AND TWO WEEK OLD MEDIA IN EXCESS OF REPRESSION OBSERVED WITH FRESH MEDIA

Media	Age in Days	Percentage Weight Loss	Apparent Reduction in Inoculum Titer[a]			
			E. cloacae	*E. coli*	*P. vulgaris*	*S. typhimurium*
EMB	7	1	0[b]	0	0	0
	14	2	0	0	0	0
MAC	14	4	0	0	0	0
DC	7	2	0	1	0	1
SS	7	2	1	0	0	1
	14	2	1	0	0	2
HE	7	2	0	0	0	0
	14	4	0	0	0	1
			S. aureus	*E. coli*	*P. vulgaris*	*S. epidermidis*
S110	7	2	0	0	0	0
	14	2	0	0	0	0

[a]expressed as the difference between the exponents of the maximum dilution at which growth occurred with aged and with fresh media.

[b]no significant difference between aged and fresh media.

tubes (43 percent) were unsatisfactory. Laboratory number 7 was using Lysine Iron Agar without a slant on three of the four sampling dates. As expected, these samples did not yield acceptable results.

Water used to rehydrate media in four laboratories was tested for toxic and growth promoting agents. Data was examined for correlation between water toxicity and media performance. Three laboratories using acceptable water produced 77, 81 and 83 percent acceptable agar plate and biochemical test media respectively while laboratory number 12 which used one water source showing toxic residues and another containing nutrients produced acceptable media only 43 percent of the time.

CHAPTER V

DISCUSSION

Quality control is equally important for the small as well as the large laboratory. Therefore, techniques for measurement of media quality that are within the capability of modestly endowed laboratories must be devised. Economic considerations preclude the employment of elaborate equipment usable only for media testing, large quantities of expendables (including the media to be tested), sophisticated statistics and extensive labor. The basic techniques employed in this study were selected for their suitability for use in the small laboratory. The few deviations from this philosophy were permitted only to facilitate the handling of the large numbers of samples tested and are not required for routine testing.

Seed cultures suitable for use in quality control are now available from several vendors[11] but they are inadequate for comprehensive media testing. All share the deficiency of not including the more fragile and fastidious genera. Furthermore, the complex growth requirements of the omitted organisms make them particularly susceptible to defects in media quality. At this time, the only method for maintaining

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[11]Difco Laboratories, Detroit, Michigan.
Hyland Division, Travenol Laboratories, Costa Mesa, California

ready availability of these organisms is to establish a stock culture collection using ultra low temperature freezing or lyophilization techniques. Since such capability is not feasible for the small laboratory, complete quality control procedures must await the development of innovations in packaging technology that could permit the marketing of seed cultures at a low unit cost (Dumoff and Isenberg, 1967). This problem is under study by the laboratory product industry (CDC, 1974c).

It was necessary to use ten fold dilutions of test organisms to inoculate agar plates due to the wide variation observed in the density of the reconstituted suspensions (Table 6). Inoculation of three consecutive 10 fold dilutions insured that the viable count end points would be measured accurately. If bacterial discs with a more reproducible viable organism density were commercially available, one of the following improvements in methodology could be instituted. Fewer dilutions could be used which would require a smaller number of test plates or intermediate --for example 2 fold-- dilutions could be employed. The latter would insure drop counts of adequate uniformity to allow the measurement of colony diameters as an additional index uninfluenced by the number of colonies developing from the drop. The greater variation observed with replicate drops of the staphylococci may have been due to a natural propensity to clump and/or a greater resistance to breaking of aggregates (Stanier et al, 1957, p. 106).

Standards of acceptance for the degree of growth or repression of test strains on agar plates may be based on collated findings from media "in use" by multiple laboratories. However, the reliability of

such standards is directly related to the numbers of replicate samples tested. The standards derived from the data generated in this study are limited by the small numbers of samples but it is submitted that they are valid in principle.

Standards of acceptance (modal growth patterns) were difficult to define in some cases. For example, after collation of the data for the growth of S. typhimurium on SS agar samples, growth repression was found to be distributed almost equally between 2, 3 and 4 logs of repression resulting in a somewhat arbitrary selection of a standard. A modal degree of repression was nevertheless identifiable for most media (Table 8).

The basic premise of utilization of the drop count method as a quality assay may be questioned. The hypothesis that changes in the growth or repression of pure cultures of test organisms on agar plates is an appropriate index of the media's ability to enhance the detection of selected organisms from clinical material has not been proven. For example, media intended for the isolation of enteric pathogens are inoculated with feces which is a heterogeneous suspension of undefined solids including a mixed population of bacteria in a menstrum of unknown composition. Interaction of this material with the media, including possible synergism and/or antagonism between pathogens and commensals, may influence the success of the medium in an unknown way. However, pure culture test methods have gained wide acceptance because some knowledge of media growth and/or repressive ability was deemed essential in comprehensive quality control monitoring procedures. Confidence in the suitability of pure culture test methods

could be strengthened by examination for correlation between media test results and pathogen isolation rates.

Application of the standards of acceptance listed in Table 8 resulted in rejection of 37 percent of the agar plate media production samples (Table 9). The validity of these findings as a measure of suitability must be tempered by the artificiality of the measurements as noted above. However, they do represent a measure of reproducibility between batches. Efforts made by the media preparation personnel to minimize deviations in production methods which result in variable response to the test inoculum should produce media of a more consistent quality. In this study, marked variation was observed in the quality of media produced in different laboratories (Table 10).

Biochemical test media quality is more easily appraised than agar plate media for several reasons. First, inoculation with pure culture strains resembles the "in use" situation in a way not possible with agar plates. Second, tables of expected results accompany the test organisms. Third, differences in response are ordinarily qualitative rather than quantitative.

Despite these apparent advantages, several difficulties were encountered in these studies. Tables of expected results provided with the bacterial discs did not precisely define the conditions of the tests. Expected reactions may not occur unless the exact manner of inoculation, incubation, time and temperature, reagents and interpretation are stated (Sulkin and Willett, 1940). Furthermore, while a test for H_2S may be said to be qualitative, the degree of positivity must be defined. A production batch of SIM medium may show weak H_2S

production with P. vulgaris and thereby be classed acceptable while a wild, weak H_2S producing strain, normally positive in this medium, may appear as negative when inoculated into this abnormal batch of SIM.

Similar problems were encountered by failure of the seed culture manufacturer to specify the composition of the media used to compile the tables of expected results. The strain of P. vulgaris, declared by the manufacturer to be weakly motile, was inoculated into samples of SIM medium containing 0.3 percent agar and motility test medium containing 0.4 percent agar. Results ranged from no observable motility to apparent full motility. In order to interpret such results, the quality control analyst must know: first, what constitutes a weakly motile reaction; second, the concentration of agar and other constituents of the medium; third, what results may be expected with different concentrations of agar and other constituents; and fourth, a standard method for inoculation and incubation time and temperature.

A modified decarboxylase medium proposed by a participating laboratory was compared with conventional media using the same methods employed for testing performance of "in use" media and found to yield acceptable results. These studies were included to ascertain how the test strains would respond in a modified medium. Additionally, it would test the suitability of these techniques for evaluation of new formulations. New biochemical test media, particularly those provided as kits designed so that multiple parameters can be determined, become commercially available each year. Evaluations of such products usually appear in the literature after some time lapse. Criticisms result when the product does not yield results equivalent to "conventional" media when tested with several stock cultures. For example, Edwards and

Ewing (1972, pp. 16 and 344) reported that the Falkow (1958) formulation for decarboxylase media yields reactions for some genera different from the Moeller (1955) formulation which they assert is the standard. In reality, this is simply a statement of preference. If the results with the new method were reproducible, the conditions of the tests well defined and tables of expected reactions constructed from studies with multiple strains available, then it should be acceptable for use. Rejection of new products for failure to agree with established methods may retard the development and acceptance of innovations in applied microbiology.

Performance evaluation of agar plate and biochemical test media is the final step prior to acceptance or rejection of a particular production batch. However, it is by nature retrospective. If the produce does not meet specifications, the entire production batch must be rejected since it would ordinarily be impossible to correct the defective units. The solution to production of satisfactory products is to carefully control each step in the production process. One such point of control is the hydrogen ion concentration.

Capability for accurate electrometric measurement of pH is a basic necessity in the media production facility. For these studies, a pH precision of ± 0.01 was utilized in order to detect small differences. However, most published tolerances are expressed in tenths of a pH unit for which even modestly priced instruments are adequate.

In an effort to simplify the mechanics of pH measurement, it was found possible to make measurements of agar plate media directly in the plate rather than first transferring the media to a separate

container, thus saving both analyst time and glassware. However, configuration of some electrodes may require insertion to a depth greater than that obtained with a single or folded agar sheet. If replacement of the electrodes is not feasible, the agar can be macerated in a suitable cup and the required depth thereby obtained.

Hydrogen ion concentration measurements were collated so as to reveal the accuracy and reproducibility attained. Overall accuracy with respect to manufacturers' recommended final pH of the media tested was 61 percent (Table 29) which indicated a general failure of the participating latoratories to control this parameter. However, analysis of the results for individual media revealed marked variation in pH accuracy between media; the quality was found to be either rather uniformly good or abysmally bad. Conversely, reproducibility studies showed generally good results for each media (with the exception of SS agar) resulting in an overall acceptability of 87 percent (Table 30). These data indicate production techniques are consistent with respect to variables affecting pH but that production personnel are apparently unaware of the pH of their products.

The quality of purified water depends, in part, on the nature of the water processed. While all water processed for use in media originated from the same municipal supply, the degree of mineralization differed with the geographic area from which it was drawn and from day to day within each area. Samples of water incorporated into culture media by four participating laboratories were examined for several parameters. The results of these studies are presented in Table 31. The low pH found in water from system B was not unexpected since a

reservoir and distribution system would allow for absorption of CO_2 thus driving the pH down.

System C, a forty-year-old tin lined copper still, was found to produce decidedly toxic water. Examination of the still construction revealed some tin lined copper tubing had been replaced with unlined copper and other surfaces were suspect regarding the integrity of the tin lining. Resulting contamination of the water with copper could be responsible for the toxicity detected since the testing method is exquisitely sensitive to copper (Ronald and Morris, 1967).

System E, a resin demineralizer, produced water yielding a toxicity ratio indicating the presence of growth promoting substances. This could be caused by a build-up of bacterial mass on the resin bed sloughing off as water is passed through. Physical examination showed a gelatinous material on the surface of the resin and the resin had a noticeable odor.

Aerobic plate counts of the water revealed an extremely high plate count for the product from the resin demineralizer which is consistent with the observations noted above. A moderately high count was noted in the water from system B compared to the other three stills. Since this sample was taken from the distribution system, this elevated count may be explained by the time delay between production and sampling which provided time for some bacterial growth.

The distilled water suitability test employed in these studies is a rather time consuming method of quality assay. However, it does not require sophisticated instrumentation and is quite sensitive. Acceptable alternatives are not known to be available. Since time

spent in the performance of the test would not be appreciably increased with an increase in the number of samples, it could be feasible for several laboratories to share the responsibility for this determination.

Laboratory number 12 utilized water from two processing systems; one found to be toxic and one found to have a high bacterial count and evidence of growth promoting substances. The agar plate media produced by this facility was of the lowest quality found in the twelve laboratories sampled with only 43 percent of the plates sampled showing acceptable growth of the test organisms. This type of correlation emphasizes the necessity for documentation of production practices and materials concurrently with performance testing.

Faulty pH has been incriminated as a cause of media failure but direct effects on the productivity of agar plate media have not been well documented. These studies revealed significant correlation between pH and productivity of LEMB, SS and XLD agar plate media. Examination for correlation between pH and growth of S. typhimurium was computed separately since all of the types of agar plate media tested should support the growth of this organism. Conversely, E. cloacae, E. coli and P. vulgaris are variably inhibited by the several types of media. Both repression of commensals and growth of S. typhimurium appeared to be related to fluctuations in pH. It should be noted that the inaccuracies in pH may have been caused by the physical and chemical factors enumerated in Chapter II, such as overheating and reconstitution with acid water, or by other factors not yet identified. These factors may well have induced other changes in the media affecting its ability to support growth while pH changes may

be only an innocuous side effect. Therefore, studies designed to measure the effect of pH on productivity of culture media by adding acid or base would not necessarily permit valid inferences to be made regarding the implications of pH changes caused by careless production methods.

Unsatisfactory media performance may be caused by defective commercial dehydrated media or media components. However, analysis of the data generated in this study revealed no evidence linking deviations in productivity or pH to the brand of manufacturer. Increasing pressure from regulat agencies has undoubtedly had a positive influence on quality control in the manufacturing plant. For example, Wallace (1972) indicated that most of the Transgrow culture media being marketed at that time failed quality control tests performed at the CDC. The quality of commercial media subsequently improved until the CDC listed 17 manufacturers submitting gonorrhea culture media which passed their quality control tests (1974b).

Acceptance of a production batch based on successful response to quality control tests should not be interpreted as an indefinite guarantee of quality of that batch. Stored media may become defective in several ways. Defects may be readily apparent such as changes induced by freezing. However, problems attributable to aging may develop long before visible changes occur. These studies did not reveal significant differences between sample lots that could be attributed to age. However, 94 percent of the agar plate samples tested were over 3 days old and therefore not within optimum age limits. Conclusions regarding the effects of age would require comparison with

fresh media. An abbreviated study was conducted to test the effects of aging under controlled conditions. While the number of samples was small, agar plates were observed to diminish in quality after storage for 7 days and further deteriorate after an additional 7 days. The largest differences were observed with SS agar in which substantial repression of S. typhimurium was observed to occur as storage time increased. Coincidentally, this media was found to exhibit the lowest percent acceptability of any agar plate media routinely sampled for performance evaluation. Deterioration of media due to age is at least partially attributable to dehydration although deterioration may be delayed by use of air tight packaging (Horan, 1959). While primarily considered a problem with agar plates, dehydration also occurs in tubed media with permeable closures.

Participating laboratories produced biochemical test media with cotton plug, foam plug, loose fitting metal cap and screw cap closures. Permeable closures, in general, performed less well than media in screw capped tubes. As expected, greater dehydration was observed in tubes with permeable closures, a factor which was shown to result in poor media performance. While tubed media is believed to have a shelf life of six months to one year (Bioquest, 1973; Difco, 1974), this is predicated on maintenance of an airtight seal. Permeable closures may be satisfactory if the media is used promptly.

The microbiologist is confronted with pressure from both regulatory agencies and peer groups to institute quality control procedures in the laboratory (CDC, 1967). Study of the literature reveals a plethora of recommendations by authoritative sources which,

if fully implemented, would be of prohibitive cost and, in any case, of dubious value. Therefore, the microbiologist must establish priorities and use those most essential procedures that are within his resources.

For culture media, these procedures can be grouped as: (1) control of raw materials (e.g., dehydrated media, water, enrichments and glassware); (2) control of production (e.g., heating and sterilization processes) and (3) control of finished product (e.g., productivity testing and pH measurement).

The analyst should consider the quality of raw materials and perform quality testing where feasible. Such testing could include sterility testing of enrichments and checking cleanliness of glassware with pH indicator solutions as an index of residual detergents. Dehydrated media and media components should be obtained from sources which perform quality control testing on their products and will provide results of such testing to the consumer.

Production control can be enhanced by training media preparation personnel in proper media production techniques and accurate record keeping. These records should identify production lots, document temperature and time and list lot numbers of ingredients and other measureable variables suspected to influence media quality.

Finally, the finished product should be tested for its ability to give acceptable results when challenged with appropriate test organisms. Knowledge of test organism characteristics must be ascertained and criteria established to evaluate their response on the media tested. Inclusion of a portion of media from a previous batch will help detect and neutralize deviations in appraisal techniques.

Periodic comparison with quality media from an outside source would enhance the reliability of these tests.

Other tests, such as pH measurements, would provide additional indices of quality. However, an aberrant pH should be considered as a sign that preparation techniques or components may be out of control and not simply as an indication that acid or alkali should be added to the next batch. Finished product testing is useful only if it is followed by examination and correction of production methods.

Where capability for testing of a parameter is not feasible, assistance from other laboratories on a share or fee basis should be considered. If adequate testing cannot be performed or obtained, consideration should be given to purchase of quality checked, prepared media.

Once checked, media should be stored so as to minimize deterioration if not used immediately. Airtight packaging should be used for agar plate media and screw capped closures for tubed media. Time limits for storage can be established by study of the literature but such recommendations should be confirmed by testing of media stored at the maximum age suggested.

Manufacturers can be expected to respond to marked demands for quality control testing aids. Technology permitting, industry will probably market a wide range of test organisms, perhaps designed so as to be used in a system similar to antibiotic sensitivity testing, at a low unit cost. Other labor saving innovations in supplies and equipment may be expected to reduce the overall costs of quality control.

Future research will have to provide more definitive data as to what criteria should be met and their relative importance. Minimum standards and tolerances may not necessarily be the same for all parameters with different media. For example, one medium may permit a wide pH tolerance and have a short shelf life while another may be the reverse. Furthermore, more work needs to be done to tie production practices to media quality.

A prerequisite to such studies is agreement on standardized productivity tests. This will require definition of standardized (e.g., American Type Culture Collection) strains to be used, standard conditions of testing and standard interpretation of results. Most importantly, standards for media used with mixed cultures and clinical specimens will have to be linked to tests with actual specimens to insure acceptance criteria correlate accurately with effectiveness of the media.

CHAPTER VI

SUMMARY

Methods for quality control of bacteriological culture media were evaluated for their suitability for use by small laboratories. Several procedures were selected which were believed to combine effectiveness, simplicity and low cost. Omission of the more fragile and fastidious bacterial species from currently marketed convenience packaged test organisms was observed to constitute a significant barrier to general implementation of effective media evaluation by the small laboratory.

Methods selected were applied to samples of agar plate and biochemical test culture media obtained from twelve area microbiology laboratories over a period of eight weeks. This permitted evaluation of the methods' suitability for use as a quality control procedure and, at the same time, produced quantitative measurements of culture media in use by the laboratories. Media parameters measured included response to inoculation with test organisms, pH, physical characteristics and quality of water used in the preparation of media.

Performance evaluation of agar plate media sampled required the establishment of standards of acceptance for response following inoculation of test species. Using these standards, 37 percent of the

agar plates sampled were rejected. However, interpretation of productivity tests using pure cultures may be tenuous until it can be demonstrated that such tests parallel results with actual specimens. Media productivity varied substantially between laboratories.

Biochemical test media sampled were similarly evaluated by inoculation with the test organisms. The media response was compared to standards of acceptance constructed from tables of expected biochemical activity enclosed with the bacterial discs, supplemented by internal data generated in the course of this study. Tables of expected reactions proved difficult to apply directly due to the omission of testing conditions such as media formula, incubation time and temperature and definitions of positive, negative and intermediate reactions. Application of the standards of acceptance resulted in rejection of 25 percent of the production lots sampled.

Hydrogen ion determinations were performed on agar plate media sampled. The data showed irregular compliance with the standards set by dehydrated media manufacturers. Forty nine percent of the plates sampled were outside the preferred limits. Results of pH measurements were also examined for reproducibility. Good agreement was found between samples collected from each laboratory. Overall, 87 percent of the samples were within ± 0.2 pH units of the mean of the samples from the same laboratory (Table 30).

Water used to rehydrate media was tested for toxic or growth promoting residues and pH and aerobic plate count determinations were made. One laboratory using water of unsatisfactory quality was found to produce the lowest quality agar plate media among the laboratories sampled.

Performance of media with test organisms was measured for correlation with pH, brand of media, age and physical observations. Statistically significant correlation was observed between performance of agar plate media and pH. Performance of biochemical test media was found to be lower quality when dispensed in tubes with permeable closures.

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