NEW METHODS FOR PATHOGENS: A REVIEW

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Introduction

Interests in new methods for identification of bacterial pathogens have increased greatly in recent years due to the awareness of, the need for, and the potential of rapid methods and automation in Microbiology. In 1971 a report by the U.S. National Institute of General Medical Sciences presented the status of mechanization and automation in the clinical laboratory; automation in microbiology was one of the topics reviewed (Kinney, 1971). Richardson (1972) summarized the developments of automation in the dairy laboratories. These topics have been discussed in detail in the International Symposium on Rapid Methods and Automation in Microbiology first held in Stockholm, Sweden, 1973, and again in Cambridge University, England, 1976. Another symposium was held in Kiel, Germany, in 1974 with the title Automation of Microbiological Food Analysis. The proceedings of the First International Symposium were published in two volumes: Automation in Microbiology and Immunology (Heden and Illeni, 1975a) and New Approaches to the Identification of Microorganisms (Heden and Illeni, 1975b). The purpose of this article is to summarize the highlights of some automated microbiological tests and rapid methods relevant to the food industry. Hopefully some of these methods could be adapted and used routinely by the food industry.

New Approaches and Automation in Microbiology

Generally the approach to developing new methods for pathogens involved either a search for new ideas and related instrument development or improvement of existing bacteriological methods. The impetus for the development of new ideas and instruments mainly comes from clinical needs for rapid detection of organisms and antibiotic sensitivity testing. There are a number of new approaches to studying pathogens which involve measuring growth of organisms in the presence of various antibiotics.

One way of improving antibiotic sensitivity testing in liquid medium is to miniaturize the entire procedure and design ways to dilute or dispense varying concentrations of antibiotics into miniaturized wells; into these wells a standardized number of the pathogens under investigation is inoculated by a convenient multipoint inoculator. After growth period the minimal inhibition concentration (MIC) of various

antibiotics could be ascertained by reading the endpoint of no growth in each series of wells. A machine called the Dptiter (Dynatech Lab. Inc., Alexandria, VA) can automatically dilute antibiotics and add nutrient and test organisms into the wells for rapid MIC studies in the Microtiter plates (a plastic plate with 8 x 12 wells). This machine can perform a variety of immunological and serological tests also. A machine named the Autotiter (Ames Company, Elkhart, IN) can also perform similar features. Recently the Dynatech Lab. Inc. designed a machine that can dispense varying concentrations of antibiotics into the Microtiter plate for MIC determinations. Along with an automatic multipoint inoculator system and a convenient reading aid this system offers a convenient package for the determination of MIC; the machine is called MIC-2000. Various other dilution machines and procedures exist for rapid dilution of antibiotic and the addition of other liquids to the dilution series (e.g., Medimixes by Linbro Chemical Co. Inc., New Haven, CT; Titertek by Flow Lab., Rockville, MD; Bioreactor by Biomeca, Geneva, Switzerland; etc.).

Another way of improvement of antibiotic sensitivity test is to design methods to quickly detect light scattering changes in nutrient solution with and without antibiotics under rigidly controlled condition. An elaborate system, the AutoBac 1 system was designed for this purpose (Praglin et al., 1975; McKie et al., 1975). Bacterial isolates are first dispensed into liquid and adjusted to the correct turbidity before introduced to a specially designed 13-cell chamber (a cuvette module) with 12 of the cells containing the desired antibiotic disc in each; one cell serves as the growth control. The cuvette module is then incubated in the machine. After 3-4 hrs, light scattering ability of each cell is monitored and matched against the control cell. A computer printout from the machine indicates the antibiogram of the organism under study. When prolonged incubation time is required, the module is reincubated and scanned at a later time. Laboratory evaluation for the AutoBac 1 system versus the conventional Kirby-Bauer method showed a 90% agreement for Gram-negative organisms and a 93% agreement with Staphylococcus aureus (Stubb and Wicker, 1976). However some major discrepancies were observed for some organisms on certain antibiotics by these authors as well as by Greenberry, et al. (1976). Due to the efficiency of the system, an attempt was made to use antimicrobial susceptibility profiles generated by the AutoBac 1 system in cooperation with a suitable computer program to rapidly identify bacterial isolates. A 95-97% accuracy of identification were reported (Sielaff et al., 1976) when 481 clinical isolates were studied using this system as compared with conventional identification procedures.

1 Naming of machine and manufacturer in this article is for reference only.
An ambitious system--The AutoMicrobic System--which can detect, identify and enumerate bacteria and yeast in urine samples was recently (1976) announced by McDonnel Douglas Astronautic Co. (East St. Louis, MD). This system consists of a small cuvette with 20 tiny culture wells; 16 of which contain freeze-dried, highly selective media (patent pending). Introduction of dilute urine into the cuvette was done by a pneumatically controlled system. The cuvettes are then placed in a reader-incubator. Final printed results are obtained in 12 hrs after automatic measurement of the change in optical absorption of each well from the initially obtained valve (Jones et al., 1976). Microbiological evaluation of the system showed an overall high accuracy (96% or above) compared to conventional methods for most test organisms (Algridge et al., 1976; Sommenwirth, 1976). This system seems to have good promise as a high capacity (240 clinical specimens) automated microbiological identification system.

Radiometry and microcalorimetry are two newer approaches to rapidly determine growth of bacteria in clinical and food samples. The radiometric method relies on the fact that in a suitable medium containing radioactive glucose (or other sugars) and bacteria, radioactive CO₂ will be liberated due to the utilization of sugar by most common pathogens. The liberated radioactive CO₂ are then collected and allowed to pass through an ionization chamber. The degree of ionization depends on the amount of radioactive CO₂ collected and this is translated to "Growth index" on the machine. Bactec, designed and marketed by Johnston Lab. Inc. (Cockeyville, MD) is a completely automated system for radiometric determination of microbial growth. A large body of literature exists for radiometry detection of bacterial growth in clinical and environmental specimens (Schort et al., 1973; Rosner, 1975; Buda and Bromman, 1975; Foley and Bromman, 1975; Previte et al., 1976; etc.). This technique is basically designed for fermentative bacteria. In order to make use of this procedure for "non-fermentors," a special radiometric medium was developed which allowed the rapid detection of Pseudomonas and Alcaligenes (Previte et al., 1975). Bacterial activities in liquid and food systems can also be measured by small changes in heat production due to bacterial growth (Sachs and Menefee, 1972). When more refined instruments are used, microcalorimetry can even be used to identify bacteria (Staples et al., 1973; Russel et al., 1975). An attempt to use radiometry and microcalorimetry for the rapid detection of food borne microorganisms was made by Lampi et al. (1974). Beside studying pure cultures, they reported that there is a high degree of correlation between viable cell count and radiometric measurements as meat loaf underwent spoilage; they did not report practical usefulness of microcalorimetry in food systems.

When bacteria grow in a liquid medium there is a change of electrical conductivity; this can be measured by a procedure called electrical impedance measurement due to microbial activity. This principle has been exploited for the studying of microbial growth in media with and without antibiotic for antibiotic sensitivity testing (Cady, 1975; Ur and Brown, 1975). Detection of bacteria in blood and spinal fluids in
children by this machine (Bactometer 32, Bactomatic Inc., Palo Alto, CA) was reported by Khan, et al. (1976) who claimed that average detection time was 8.5 hrs compared with 24 hrs needed for the conventional method. Other applications of Bactometer 32 include monitoring of anaerobic bacteria (Hardy et al., 1976) and detection of fecal coli (Mischak et al., 1976).

Measure of the degree of light generated by the luciferin-luciferase system in the presence of bacterial ATP offers another procedure to detect microbial growth in liquid samples (Chappella and Levin, 1968). The method has been used to detect bacterial growth in blood cultures and urine (Schtock et al., 1976; Gutekunst et al., 1976). One important aspect of this assay is the need to destroy ATP of nonbacterial origin in the biological samples before performing the bacteria ATP measurement. Lab-Line ATP Photometer (Lab-Line Plaxa, Melrose Park, IL) and DuPont 760 Luminescence Biometer (DuPont, Wilmington, DE) are two instruments designed for this test.

Identification of bacteria, especially anaerobic organisms, by gas chromatography is well known (Holdermen and Moore, 1972; Dowell and Hawkin, 1972; Mitruka, 1975). Application of gas-liquid chromatography to biological samples to ascertain microbial presence and activities have also been reported, e.g., the measurements of lactic acid in spinal fluid of bacterial meningitis (Brook et al., 1976; Bricknell et al., 1976). Pyrolysis in combination with gas-liquid chromatography provides another means of rapid and automated bacterial identification (Meuzelaar et al., 1975; Kistemaker et al., 1975; Oxborrow et al., 1976). Making use of this combination procedure an instrument called Bacterial ID system 700 series is marketed by Chemical Data System, Inc., Oxford, PA.

An ingenious and quite unique way of identification of microorganisms is provided for by a technique called differential light scattering. In this system a laser beam is applied to a bacterial suspension; the scattered light (due to the various components of the bacterial cell) is collected by a rotating detector. The intensity of light collected at various angles provides a specific pattern. Since different bacteria scatter light differently, the patterns can be used to identify the organisms when compared to standard "prints" of known cultures. Moreover, when the bacterial cells are challenged with certain antibiotics the cell structures undergo changes, this can readily be detected by this system. Using this technique antibiotic sensitivity testing can also be performed. For greater detail of the principles and applications of this method, the article by Wyatt should be consulted (Wyatt, 1973). The commercial products for this test are Differential I, II and III marketed by Science Spectrum, Santa Barbara, CA.

There are no doubt many other ideas and techniques for rapid and novel ways to identify bacteria (Goldschmidt, 1970). Most of these tests are designed for pure culture studies and antibiotic sensitivity tests. More work are needed to determine the usefulness of these methods in food systems where many other interfering compounds are present to mask the identify of pathogens in the natural habitats.
Another area of research and development of new methods for pathogens centers on convenient and rapid diagnostic kits. The one closest to the conventional test is the R/B system (Diagnostic Research Inc., Roslyn, NY) which consists of the basic 2 tube R/B system using 8 agar-base reactions and the 2 tube Expanders system which utilizes another 6 solid media tests. A special sterile needle is used to stab-streak the suspect colony into the tubes. Reading of reactions are made after 18 to 24 hrs at 35 to 37 C incubations. The results can be compared with the Enteric Analyzer for ease of interpretation. Reliability of this system compared with conventional system in the identification of enteric was cited at 90-96% (Isenbery and Painter, 1971; Smith et al., 1971a and 1971b). Studies by O'Donnel et al. (1970) and Brown (1973) indicated a high 99-100% accuracy when certain species were examined while Martin et al. (1970) could only ascribe a poor 45% accuracy. (In citing accuracy, the author only used accurate identification of the organisms under study. A discussion on the accuracy of individual tests between systems is not made due to limitation of space; the readers are referred to the original articles for such informations).

The Enterotube system consists of 11 agar-base tests housed in a compartmented plastic tube (Roche Diagnostics, Nutley, NJ). Inoculation is done by touching an isolated bacterial colony with the tip of a special needle of the kit and pulling the needle through the kit. As the needle is pulled through the chambers bacteria are deposited into various media. After incubation (18-24 hrs) appropriate reagents are added to some chambers and reactions are recorded. To facilitate identification of unknown, a convenient booklet (ENCISE) with special codes and keys is available for keying out the enterics. A computer consultation service is available for helping technicians to identify cultures. This system is easy to use and seems to have wide acceptance. The disadvantage is that only 11 tests are available and the short shelf-life. The original system was designed for Enterobacteriacea; recently (1976) a new system is marketed for the identification of non-fermentors. Compared with conventional tests the Enterotube in combination with ENCISE ID system did very well (ca. 91-98% accuracy) in providing rapid and efficient identification of Enterobacteriaceae (Grunbery et al., 1969; Elston et al., 1971; Tomfohrde et al., 1973; Painter and Isenbery, 1973; Wust and Kayser, 1974; Isenberg et al., 1975). Both the R/B system and the Enterotube suffer from short shelf-life because of dehydration of agar and the lack of flexibility of application.

Dehydrated media or paper discs impregnated with media will prolong shelf-life of the diagnostic kits. The Auxotab system (Wilson Diagnostic Inc., Glenwood, IL) consists of a series of carded bacterial identification systems each containing ten reagent filled capillary chambers. After the addition of cell suspension, results of reactions can be read and common pathogens identified within 7 hrs. These tests depend on enzymatic reactions of the cell suspension; therefore aseptic
technique is not necessary since bacterial cell division does not occur in this system. Stability of this system is about 18 months. This system has been used to study species of Enterobacteriaceae (Cottereau et al., 1967; Barbe, 1969) and a wide range of aerobic and anaerobic organisms (Puissièret et al., 1967; Joubert et al., 1968). More recent evaluations indicate that the Auxotab system is accurate at 87% (Rhoden et al., 1973a) and after improvements, advanced to 96% (Rhoden et al., 1973b).

Another dehydrated system is the API system (Analytab Product, Inc., Carle Place, NY) which consists of the API 20 system and the API 50 system; the former has 20 tests and the latter has 50 tests. The dehydrated media are housed in small plastic chambers in a convenient plastic card. Suspensions of the organisms are introduced to the individual chambers. After 18-24 hrs at 37°C and appropriate reagents introduced, the reactions are read. Data are matched with the API Coder and API Profile Register for identification. To facilitate accurate identification, the company has a computer service to help technicians to interpret data either by direct usage of the computer bank or utilization of telephone service for information. Overall accuracy of this system was cited at 93-96% (Washington et al., 1971; Smith et al., 1972). A new anaerobic system was recently introduced (API 2OA): the accuracy was cited at 90% (Hauser and Zabransky, 1976). The API 50 system is useful for studying Salmonella biotypes at intrasubspecific level (Tomfohred and D'Amato, 1976). The API system is also adapted for the rapid identification of yeast (Burgard and Sommenwirth, 1976). This system is easy to use and has long shelf-life (2 years) but the system is not sterile, therefore some difficulty may develop when tests are incubated for 48 hrs.

The next two systems to be described have stability in storage (1 to 2 years) as well as flexibility. The PathoTec system (General Diagnostic, Morris Plain, NJ) is sold separately as 12 different paper strips each impregnated with appropriate substrates. An isolate is first suspended in liquid and then pipetted into a series of 12 tubes each containing one paper strip. Reactions are allowed to occur and changes of color on the strips are recorded. Since only enzymatic reactions are utilized, the results can be read in 4 hrs and aseptic techniques are not necessary. Also since the strips are sold separately the user can chose the strips they need for testing. The accuracy of the system is approximately 95% (Blazeic et al., 1973; Rosner, 1973). The advantage of PathoTec system is speed (4 hrs) and flexibility; however, the need to set up the tests and subsequent cleaning of test tubes make this system less desirable for technicians in terms of operational convenience. It seems easy enough to make a kit for this system but in so doing flexibility of the system will have to be sacrificed.

The newest entry into the market is the Minitek system of BBL (Baltimore, MD). It consists of a 12-well plastic plate, a dispenser (which allows ease and flexibility of dispensing substrate impregnated paper discs into the plastic plate), and a convenient plunger to
introduce 0.1 ml of bacterial suspension into the small wells. After incubation time the reactions are recorded and results compared with a color Coder to aid identification of unknowns. At least 35 different substrates are available for use in the system; thus providing the user with a good choice of tests. The accuracy of this system is about 97% compared to the conventional method (Hansen et al., 1974; Kiehn et al., 1974; Hansen and Stewart, 1976). The Minitek system can also be used for anaerobic organisms; of 108 clinical isolates tested all were correctly identified compared with the more tedious conventional anaerobic cultivation techniques (McCarthy et al., 1976).

All these commercial kits have been studied extensively both by the manufacturers and impartial investigators as to the ability to identify unknowns. Depending upon the investigators, many showed reliability of 90% or above after improvements are made. (One commercial product not mentioned in this review was withdrawn from the market due to consistency poor market testing and evaluations). A comprehensive study of five test kits--API, Enterotube, Auxotab, Pathotec, and R/B--versus the conventional tests was made by Nord et al. (1974). They tested 329 strains of Enterobacteriaceae with all six methods and concluded that as far as accurate identification of unknown is concerned the ranking was as follows: Enterotube (97%), API (94%), R/B (91%), Pathotec (77%), and Auxotab (42%). In another study, Moussa (1975) using API, Pathotec, and Enterotube compared with conventional tests reported the following results: Enterotube (99.2%), API (92.2%), and Pathotec (80.5%). It should be emphasized that these studies were done a few years ago before many important improvements and modifications were made by Pathotec and Auxotab. A recent comparison between API and Minitek showed that Minitek gave 97.6% accuracy and API 95.3% (Aguino and Dowell, 1975). A comparison of these 2 methods against conventional methods for identification of 175 anaerobes showed that the percentage correlation of both positive-negative reactivities with the API anaerobic system ranged from 70-99.4% and with the Minitek from 97.1-100% (Hansen and Stewart, 1976). When R/B and Minitek systems were compared to conventional techniques for 294 isolates, an accuracy of 85% for both methods was reported (Shayegani et al., 1975).

An interesting "double blind" identification study was made by three hospital laboratories each contributed 130 isolates and each tested 100 randomly chosen "blinds" from the pool of cultures, using the conventional method, R/B system and API 20 system. The results showed that all three methods provided identical genus and species identification with more than 90% of the isolates (Ayres et al., 1976).

It is of interest to know also the relative costs of these systems compared with conventional procedures in the identification of isolates. The only data available are for the identification of isolates in the family Enterobacteriaceae. For the conventional 7-tube (10 test) setup and 17-tube (20 test) setup, the cost per isolate identification is $3.60 and $7.98, respectively. For a similar identification using API 10E and API 20E the cost is $2.33 and $3.02, respectively (Robertson, et al., 1976). Estimation of costs for each identification by N. A. Cox (personal communication) is as follows: API 20 ($2.17), API 50 ($4.38), Enterotube ($1.85), Minitek 12 test ($1.83), Minitek 34 test
(4.74), and R/B 4 tube system ($2.03). The values are obtained for purchasing of lots of 100 for each system. It seems that these commercial diagnostic kits are less expensive and more convenient than the conventional procedures while offering accurate identification of bacteria isolates.

**Miniaturized Microbiological Techniques**

Many of the recently developed diagnostic kits can be called miniaturized microbiological techniques. Summaries of miniaturized microbiological methods have been made by Hartman (1968), and Fung and Hartman (1972, 1975).

The system developed by Fung (1969) for the identification of bacterial isolates involves miniaturization of tests as well as multiple inoculation of many organisms to a variety of test substrates. The general procedure of this system involves obtaining pure cultures, preparation of a master plate, aseptic multiple inoculation of cultures into liquid or onto solid media, observation of biochemical changes and collection of data and interpretation.

Microtiter plates (96 wells, each holds 0.35 ml) or vessels with many wells can be used for cultivation of organisms. Sterilization of the plates can be achieved by soaking the plates in 500 ppm hypochlorite solution (1 hr) and rinsing the plates with sterile distilled water. Ultraviolet and Co-60 irradiation (Jayne-William, 1975) are also convenient ways to sterilize the plates. Liquid, semi-solid, and solid media can be introduced into the wells of the plate by a hand pipette or by automatic pipetting machine. A large petri dish (15 x 150 mm) can also be used for solid media.

In order to facilitate mass transfer of test cultures into substrates in the Microtiter plate or onto solid media in petri dishes a multiple inoculator can be constructed by fixing 96 stainless steel pins (27 mm long) into woodblocks or other materials; the design of the 96 pins is patterned after the 8 x 12 configuration of the Microtiter plate. Pin heads are suitable for inoculation into liquid media and solid agar surfaces while pin points are suitable for stabbing into agar or semi-solid agar. Sterilization of the device is by alcohol-flaming. The sterile device can be used to change inocula from a Master plate containing test organisms (maximum of 96 unknown) to either Microtiter plates containing suitable substrates or petri dishes containing solid agar.

Routine use of this procedure usually involves collection of a large number of unknown cultures. The cultures are grown overnight in broth and a few drops of the culture are introduced into the wells of a Microtiter plate to form a master plate. From the master plate the organisms are transferred to suitable solid or liquid media by use of the multiple inoculation device. Incubation time can be shortened to 6-8 hrs for many tests. Interpretation of results is by use of pre-designed charts and tables of individual investigators.
The author has used this procedure for carbohydrate tests (Fung and Miller, 1970), IMViC tests (Fung and Miller, 1972), litmus milk test (Fung and Hartman, 1975) effect of dyes on bacterial growth (Fung and Miller, 1973), and others. Organisms tested included a variety of Gram-positive and Gram-negative organisms of Public Health significance. The use of this system for rapid and convenient mass identification of Streptococci (Hahn, 1974), Enterobacteriaceae (Newsom, 1973), fish pathogens (Chen, 1976), Streptococci, Lactobacilli, and Gram-negative rods from animal sources (Jayne-Williams, 1975 and 1976), anaerobic bacteriology (Wilkins et al., 1975a and 1975b) etc. have been reported. The type of tests described by these authors covered the whole gamut of diagnostic microbiology indicating that most microbiological tests can be miniaturized.

The advantage of this system are low cost of operation, flexibility of tests, mass production of data for many unknowns, speed of reactions and savings of space and time. The disadvantages of this system include the need to have manual dexterity, experience, and training. Also for laboratories testing limited numbers of organisms at one time, setting up a complete system is inconvenient. An automatic machine can reduce the need for dexterity and the design of "kits" geared for the specific laboratories will reduce the number of complete plates to be prepared, i.e., design 8 sets of 12 tests in one Microtiter plate for studying 8 unknowns at a time.

Conclusion

A variety of methods are discussed in this review on the search of new methods for the identification of pathogens. Some of these methods are applicable to the Food Industry laboratories and others have some potential for adaptation to these laboratories. It is up to the laboratory directors to decide which procedure(s) or method(s) to use in place of or in conjunction with the conventional techniques. To venture into a new method, a director must be well informed and trained concerning the new method and should know about the advantages and disadvantages of the method as well as the knowledge of the potential and limitations of the system in relation to his particular needs and concerns.

References


Bruce Langlois: Do we have any questions?

Bruce Tompkin, Swift and Company: Where do you place the relative importance of the kits that you have listed there as opposed to serological testing for stating whether or not a sample is Salmonella positive or negative?

D.Y.C. Fung: I cannot tell you that definitely. All that I can tell you is, first of all, the kits have been tested and many of them have an accuracy of about 95 percent when compared to conventional methods. Each one of these probably will cost $2 per identification, where the conventional method is about $8 per complete identification. So there is a relative difference in terms of money. I can't tell you which is better for Salmonella. Maybe you know.

Bruce Tompkin: Well, which kit you use is immaterial in terms of what I'm asking, since they all are based upon biochemical reactions?

D.Y.C. Fung: Yes.

Bruce Tompkin: Now, a serological reaction is more specific, so what would I use when I fall apart on serological tests. I don't know what it is. In other words, that would be a backup system. That would not be the primary identification system in terms of finding whether or not it is Salmonella or not.

D.Y.C. Fung: Well, a lot of people have done a lot of comparative work and study on that. As far as I can tell, the API system is the most complete in terms of defending whether the organism is Salmonella or not. Now, there has been big competition in the past between API and Minitek in terms of the completeness of the biochemical test. They all say that you have to back up with physiological tests for Salmonella and Shigella.

Bruce Langlois: Thank you, Dan. Our next speaker, the final speaker for this session, is a native of Minnesota. He got his B.A. and M.S. at the University of Minnesota and his Ph. D. at Illinois. He served a tour of duty at North Carolina State at Raleigh for about four years, before he went back to Minnesota, where he is now Professor of Food Microbiology. Dr. Busta will be covering "Bacterial Injury and Recovery."

F. F. Busta: I'd like to express my appreciation to be invited to speak to your association today. It's an honor to talk to the Reciprocal Meat Conference, especially when I'm going to talk about one of my best and favorite loves: "Bacterial Injury and Recovery."