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Rapid Methods and Automation in Microbiology

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Introduction

Rapid methods and automation in microbiology is a dynamic area in applied microbiology dealing with the study of improved methods in the isolation, early detection, characterization, and enumeration of microorganisms and their products in clinical, food, industrial, and environmental samples. In the past 15 years this field has emerged into an important subdivision of the general field of applied microbiology and is gaining momentum nationally and internationally as an area of research and application to monitor the numbers, kinds, and metabolites of microorganisms related to food spoilage, food preservation, food fermentation, food safety , and foodborne pathogens. This article traces the historical development of this field and provides information and discussions on major developments in many aspects of this rapidly changing discipline mainly from the standpoint of food microbiology and food safety. The purpose is to provide an overview of the current status of the total field and predict the developments in the near future.

History and Key Developments

Medical microbiologists started to be involved with rapid methods around mid-1960s and started to accelerate in the 1970s and continue developments in the 80s, 90s and up to the present day. Food microbiologists were lagging about 10 years behind the medical microbiologists for about 20 years but in the past decade they have greatly increased their activities in this field. Fung (1995) on Figure 1 estimated the trends of rapid methods and automation in microbiology by medical microbiologists and food microbiologists from 1965 to 2000. From 1965 to 1975 it can called the age of miniaturization and diagnostic kit developments. From 1975 to 1985 it was the age of immunological test kits developments. From 1985 to 1995 it was the age of genetic probes, molecular testing systems, and polymerase chain reaction (PCR) applications. Currently, we are in the biosensor, computer chip technology, and microarray system development era in response to human genome projects and the evolving field of "Proteomics" and related fields. Spanning across these major areas was the development of instrumentations and automation for mechanization of these methods and technologies and in biomass monitoring. Bailey (2000) revised Fung's information by superimposing his curves of the trends on Fung's curves. He indicated that starting from mid-1980s, activities of medical microbiologists and food microbiologists moved in a parallel fashion (Figure 2). Both Figures are personal observations of Fung and Bailey after following trends in this field for about three decades.

Fung (1992) made a comprehensive review of the historical development of rapid methods and automation in microbiology and published the paper in the inaugural issue of the *Journal of Rapid Methods and Automation in Microbiology*. Many methods and procedures currently in use in food microbiology laboratory were developed more than 100 years ago. The "conventional" methods used by many regulatory laboratories around the world are based on these laborious, large volumes usage of liquid and solid media

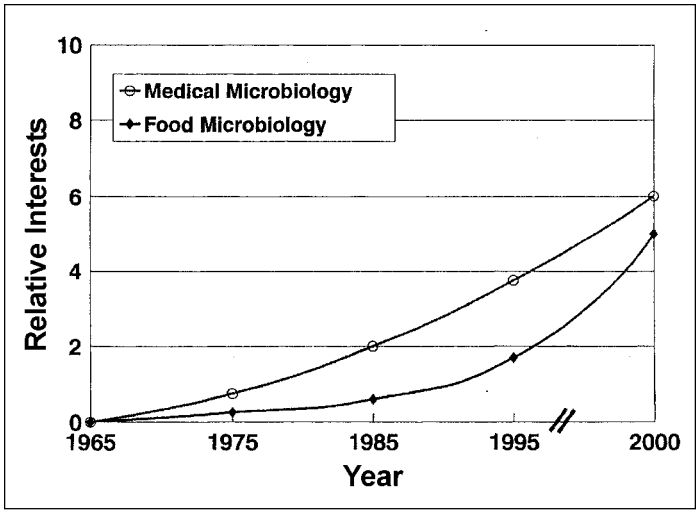

Figure 1—Development in interest in rapid methods

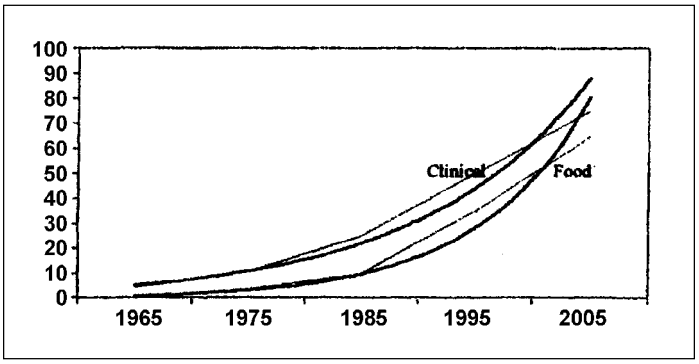

Figure 2—Evolution of rapid methods for pathogen detection

and reagents, and time consuming procedures both in operation and data collection. However, these methods remain as the "Gold Standards" in applied microbiology and in the books of regulatory agencies nationally and internationally to the present time.

At the dawn of the development of bacteriology and microbiology, scientists must have slowly tried to improve efficiency in the operation of bacteriological procedures. A look into older bacteriology text books and laboratory manuals revealed some form of improved operations for handling large numbers of cultures tubes, test tubes, plates, and inoculation procedures. The most important book dealing with *Miniaturization of Microbiological Methods* was written by Paul A. Hartman in 1968. The book provided over 1,200 citations of techniques for the cultivation of bacteria, fungi, protozoa, and other plants and animal cells. The purpose of the book was to describe techniques which were more rapid, convenient, or reliable than conventional laboratory methods by miniaturization. Another very useful book was compiled by Skerman (1969) on *Abstracts of Microbiological Methods* with almost 900 pages of valuable information on methodologies in applied microbiology. These 2 books are classics and should be read by all students of rapid methods and automation in microbiology.

From the beginning, bacteriologists attempted to improve efficiencies of methods

Several interesting articles appeared in early 1970's concerning this field. Goldschmidt (1970) had a chapter in the book *Rapid Diagnosis Methods in Medical Microbiology* by Gerber (1970) entitled "Instrumentation for microbiology: Horizons unlimited". Richardson (1972) had an article on "Automation in the Dairy Laboratory" in the *Journal of Milk and Food Technology* and Trotman (1973) on "The Philosophy of the application of automatic methods to hospital diagnostic bacteriology" in *Biochemical Engineering*.

One of the earliest symposia on this topic was organized by Daniel Amsterdam in 1971 at the Annual Meeting of the American Society for Microbiology in Minneapolis, Minnesota with the title " Rapid Methods for Detection and Characterization of Microorganisms". Although many smaller meetings, seminars, and symposia were held on this topic, the key identifiable start of the field was the first *International Symposium on Rapid Methods and Automation in Microbiology* held in Stockholm, Sweden, under the chairmanship of Carl-Goran Heden in 1973. There were about 500 to 600 people at that meeting. Subsequently, symposia of this series were held in Cambridge, UK (1976), Washington, DC (1981), Berlin (1984), Florence, Italy (1987), and Helsinki, Finland (1990). The series has changed its name to *International Congress on Rapid Methods and Automation in Microbiology and Immunology.* The last meeting of this series was held in London, UK in 1993. The planned meetings for Florence in 1996 and Beijing in 1999 were not held and thus ending this illustrious series after 20 years. The proceedings of these meetings were published after the meetings. The most valuable ones were the 2 books edited by Heden and Illeni (1975a,b): *Automation in Microbiology and Immunology* (1975a) and *New Approaches to the Identification of Microorganisms* (1975b). These 2 books, although outdated by now, contain the basic approaches and philosophies of the field of rapid methods.

In between these major meetings, international conferences also were held on similar topics in Kiel, Germany (1974); Ottawa, Canada (1975); Dallas, Texas (1978); Liblice Castle, Czechoslovakia (1980), Ploufragan, France (1983), Lille, France (1983), Anchorage, Alaska (1986), Taipei, Taiwan (1987), Nancy, France (1988) and Singapore (1989). In the 1990s an explosion of meetings and symposia occurred in USA, Europe and around the world organized by professional societies such as *Institute of Food Technologists, American Society for Microbiology, National Environmental Health Association, Biodeterioration Society*, AOAC *International*, and so on, and universities, governmental agencies, and industrial companies. An interesting development occurred in recent years when professional conference managing companies and trade magazine companies started to organize these meetings and attracted quite a diversified audience. A few examples include the *1999 Food Safety Summit and Expo*, Washington DC organized by Eaton Hall Exposition, the *Food Quality and Expo 2000* organized by Food Quality Magazine in Philadelphia and the *2000 Rapid Methods and Automation in Microbiology for Pharmaceutical, Biotechnology, and Device Applications* organized by Barnett International in Washington DC. Barnett International conducted the same conference in San Juan, Puerto Rico in February 2001 and again in Philadelphia, Pa., U.S.A. in January 2002.

Another important development was the initiation of "hands-on" workshops concerning these rapid methods. Some of the workshops were 1 or 2 days with lectures and limited demonstrations and "hands-on" experiences of various systems. The most comprehensive program was developed by the author in 1981 at Kansas State University, Manhattan, Kansas. The program lasted 8 days. The first workshop had 16 participants from several countries. The number of participants steadily increased through the years to about 60 participants. Total number of participants exceeded 2,500 scientists from 55 countries and 46 States. In July 2000, 185 people from 20 countries and 25 states came to celebrate the 20th Gala Anniversary of this long running series. A comprehensive 750 page *Handbook on Rapid Methods and Automation in Microbiology* edited by the author (Fung 2000) was published for the participants to use in the intensive workshop. The 22nd Symposium/Workshop will commence in July 2002. Similar symposia and workshops also were held on a smaller scale in Singapore, Taiwan, Australia, Brazil, Chile, Argentina, Japan, Zimbabwe, Bangkok, France, the Philippines, Hong Kong, and so on. Many more of these meetings will certainly occur in the near futures.

Hands-on workshops provide practical training . . .

All these conferences, workshops, symposia, and meetings greatly heightened the interests and participation of an ever-expanding audience on a global scale. The author has been privileged to be the keynote speaker, presenter, organizer, director and promoter of many of these national and international meetings and thus is able to keep close watch of the developments in the field.

Advances in Sample Preparation and Treatments

One of the most important steps for successful microbiological analysis of any material is sample preparation. Without proper sampling procedures the data obtained will have limited meaning and usefulness. With the advancement of microbiological tech-

niques and miniaturization of kits and test systems to ever-smaller sizes, proper sample preparation becomes critical. Statistic sampling plans for various foods for microbiological analysis is beyond the scope of this review. The following discussions are sample preparation methods for applied microbiological analysis for foods and food plant environments.

Microbiological samples can be grouped as solid samples, liquid samples, surface samples, and air samples. Each type of sample has its unique properties and concerns in sample preparation and analysis. This section discusses the improvement of methods for solid, liquid, surface, and air sampling procedures. These procedures are important for both conventional microbiological techniques as well as new and sophisticated rapid methods.

Solid Samples

Common laboratory procedures for solid samples include aseptic techniques to collect sample, rapid transport (less than 24 h) to laboratory site in frozen state for frozen foods, and chilled state for most other foods. The purpose is to minimize growth or death of the microorganisms in the food to be analyzed. The next step is to aseptically remove a subsample such as 5 g, 10 g, 25 g or more for testing. Sometimes samples are obtained from different lots and composited for analysis. In food microbiology, almost always the food is diluted to 1:10 dilution (that is, 1 part of food in 9 part of sterile diluent) and then homogenized by a variety of methods. It should be noted that 1 g of food sample is equivalent to 1 ml of diluent (based on the specific gravity of water) for ease of calculation of dilution factors in microbiological manipulations. To make a 1:10 dilution, the procedure is simple, but when an analyst has to make 10 or more samples this becomes laborious and time-consuming. An instrument called Gravimetric Diluter marketed by Spiral Biotech (Bethesda, Md., U.S.A.) can automatically perform this function. The analyst simply puts an amount of food (for example, 10.5 g) into a sterile Stomacher bag, set the desired dilution (1:10) and the instrument will deliver the appropriate amount of sterile diluent (for example, 94.5 g). Thus, the dilution operation can be done automatically and efficiently. The dilution factor can be programmed to deliver other factors, such as 1:25, 1:50, and so on. Manninen and Fung (1992a) found this system to be efficient and accurate over a wide range of dilutions. A product named Diluflo has been in use satisfactorily in the author's laboratory for about 10 years. A similar system called Dilumacher is marketed by PBI of Milan, Italy for dispersing diluents to samples automatically.

After dilution, the sample needs to be homogenized. Traditionally, a sterile blender or Osterizer is used to homogenize the food suspension for 1 to 2 min before further diluting the sample for microbiological analysis. The disadvantages of using a blender include: (1) the blender must be cleansed and re-sterilized between each use; (2) aerosols may be generated and contaminate the environment; and, (3) heat may be generated mechanically and may kill some bacteria. In the past 25 years the Stomacher invented by Anthony Sharpe has become standard equipment in food analysis laboratories. About 40,000 Stomacher units are in use worldwide. The sample is placed in a sterile plastic bag and an appropriate amount of sterile diluent is added. The sample in the bag is then "massaged" by 2 paddles of the instrument for 1 to 2 minutes and then the content can be analyzed with or without further dilution. The advantages of the Stomacher include: (1) no need to re-sterilize the instrument between samples because the sample (housed in a sterile plastic bag) does not come in contact with the instrument; (2) disposable bags allow analysis of large number of samples efficiently; (3) no heat or aerosols will be generated; and, (4) the bag with the sample can serve as a container for time course studies. A similar instrument called Masticator is marketed by IUL Instruments (Erlanger, Ky., U.S.A.). Recently, Anthony Sharpe invented the Pulsifier for dislodging microorganisms from foods without excessively breaking the food structure. The Pulsifier has an oval ring which can house a plastic bag with sample and diluent. When the instrument is activated the ring will vibrate vigorously for a predetermined time (30 to 60 s). During this time microorganisms on the food surface or in the food will be dislodged into the diluent with minimum destruction of the food. Fung and others (1998) evaluated the Pulsifier against the Stomacher with 96 food items (included beefs, pork, veal, fish, shrimp, cheese, peas, a variety of vegetables, cereal, and fruits) and found that the systems gave essential the same viable cell count in the food but the "Pulsified" samples were much clearer than the "Stomached" samples. A more recent report by Kang and others (2001) found that the Pulsifier and Stomacher had a correlation coefficient of 0.971 and 0.959 for total aerobic count and coliform count, respectively, with 50 samples of lean meat tissues. The "Pulsified" samples, however, contained much less meat debris than "Stomached" samples. In the case of Stomached samples, much meat debris occurred which interfered with plating samples on agar. The superior quality of microbial suspensions with minimum food particles from the Pulsifier has positive implications for general analysis as well as for techniques such as ATP bioluminescence tests, DNA/RNA hybridization, PCR amplifications, enzymatic assays, and so on.

40,000 stomacher instruments in use worldwide

Hands-free 'pop-up' adhesive tape sampling method developed

Liquid Samples

Liquid samples are easier to manipulate than solid samples. After appropriate mixing (by vigorous hand shaking or by instrument), one only needs to aseptically introduce a known volume of liquid sample into a container and then add a desired volume of sterile diluent to obtain the desired dilution ratio (1:10, 1:100, and so on). Further dilutions can be made as necessary. There are now many automated pipetting instruments available for sample dilutions such as the Rapid Plate 96 Pipetting Workstation marketed by Zymark Corp., Hopkinton, Mass., U.S.A. Viscous and semisolid samples need special considerations such as the use of large month pipettes during operation. Regardless of the consistency of the semisolid sample, 1 ml of sample is considered as 1 ml of liquid for ease of making dilution calculations. It should also be noted that in a dilution series there are dilution errors involved, thus, the more dilutions one makes the more errors one will introduce.

Surface Samples

Sampling of surfaces of food or the environment presents a different set of concerns. The analyst needs to decide on the proper unit to report the findings, such as number of bacteria per inch square, per cm square, or other units. One can analyze different shapes of the surface such as a square, rectangle, triangle, or circle, and so on. A sterile template will be useful for this purpose.

Figure 3—Pulling sterile "pop-up" tape for sampling

Figure 4—Application of tape to meat surface for 15 sec

Occasionally, one has to analyze unusual shapes such as the surface of an egg, apple, or the entire surface of a chicken, and so on. The calculation of these areas becomes quite complex. For intact meat or other soft tissues, one can excise an area of the food by use a sterile knife assuming that all the organisms are on the surface and that the meat itself is sterile. Often a sterile moisten cotton swab is used to obtain microbes from the surface of a known area and then the swab is placed into a diluent of known volume (for example, 5 ml), shaken and then plated on a general purpose agar or a selective agar. Instead of a cotton swab, one can use contact materials to sample surfaces. This include selective and nonselective agar in Rodac plate, adhesive tape, sterile gauge, sterile sponge, and so on.

The nature and characteristics of the surfaces are also very important. Obtaining microbiological samples from dry surfaces, wet surfaces, oily surfaces, slimy surfaces, meat, chicken skin, orange skin, stainless steel, concrete, rocks, hair nets, and so on, are very different. A lot of microorganisms will remain on the surface even after repeatedly sampling the same area. Biofilms are very hard to completely remove from any surface. This, however, should not be a deterrent to use surface sampling techniques if one can relate the numbers obtained to another parameter such as cleanliness of the surface or quality of a food product. Lee and Fung (1986) made a comprehensive review on surface sampling techniques for bacteriology.

Recently Fung and others (2000) developed a convenient method to obtain surface samples called "Hands-free, 'Pop-up' adhesive tape method for microbial sampling of meat surfaces". In this procedure the 3M "Pop-up" tape unit is placed on the wrist of an analyst, while both hands can be free to manipulate experimental materials such as obtaining the meat sample, arranging agar plates, labeling samples, and so on. When the time is ready, the analyst simply pulls 1 tape out of the unit from the wrist and uses the tape to obtain microbial sample from the meat surface (15 s) and then transfer the tape to an agar surface (15 s) and finally incubate the plate for viable cell count of the meat surface. The correlation coefficient of the "Pop-up" tape method and the more cumbersome conventional swab/rinse method for obtaining viable cell counts was 0.91. Thus the simple "Pop-up" tape method is a viable alternative to other methods for estimating microbial surface contamination. Figures 3, 4, and 5 illustrate the use of the "Pop-up" tape method for surface sampling of meat.

Air Sample

Air sampling in food microbiology received much less attention compared with other sample techniques already discussed. Due to recent concerns of environmental air pollution, indoor air quality, public health, and the threat of bio-terrorism there is a renewed interest in rapid techniques to monitor microbes and their toxins in the air. The most common way to estimate air quality is the use of "air plates" where the lid of an agar plate is removed and the agar surface exposed to air of the environment for a determined time such as 10 minutes, 30 minutes, or a couple of hours. The plate is then covered and incubated and later colonies are counted. If the colony numbers exceed a certain value, for example 15 per plate, the air quality may be considered as unacceptable. However, this simple method is "passive" and the information is not too quantitative. A much better way is to "actively" pass a known volume of air through an instrument to measure biological particles over an agar surface (impaction) to obtain viable cell numbers after incubation of the agar, or trap microorganisms a liquid sample (impingement) and then analyze the liquid for various viable cells. There are a variety of commercially available air samplers. Some of them are quite sophisticated such as the Anderson Air Sampler, which can separate particle sizes from the environment in six stages from large particles (more than 5-mm dia) to small particles (0.2 mm). The author has used the SAS sampler (PBI, Milan, Italy) for many years with good results. With this instrument, a Rodac plate or an ordinary plate with a suitable agar is clipped in place. A cover with precision pattern of holes (to direct air flow precisely) is then screwed on. After activating the instrument, a known volume of air is sucked through the holes and the particles will hit and be lodged onto the surface of the agar. After operation (for example, 60 liters of air in 20 s) the air sampler cover is removed and the lid of the agar plate is replaced and the plate is incubated. The number of colonies developed on the agar can be converted to Colony Forming Units (CFU) per cubic meter. A similar system, named MAS 100 Air Sampler, is marketed by EM Science, Darmstadt, Germany. Al-Dagal and Fung (1993) suggested that for food processing plants 0 to 100 CFU/cubic meter is considered clean air, 100 to 300 CFU/cubic meter is acceptable air, and over 300 CFU/cubic meter is considered not acceptable. As a comparison more than 500 CFU/cubic meter of air is unacceptable according to the standard used in Singapore for food plants. .

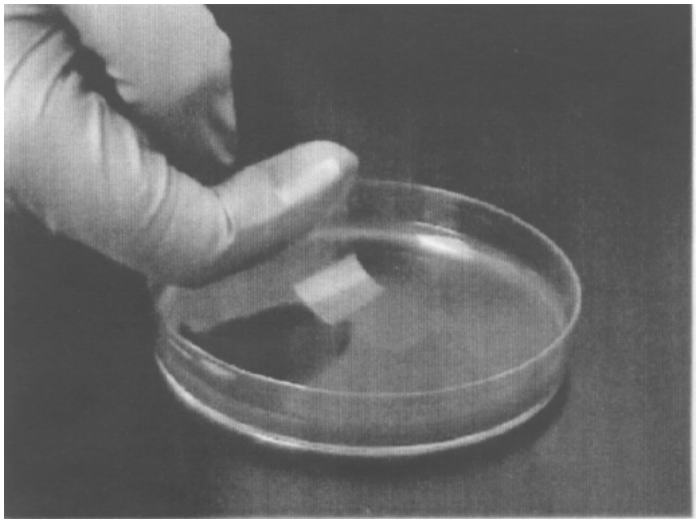

Figure 4—Application of tape with microbial sample from meat to agar for 15 sec

Applied food microbiologists are constantly searching for better sample preparation methods to improve recovery of microbes from foods and the environment. This section only dealt with improvements related to solid and liquid foods, surfaces of food and food contact areas, and air samples. A great variety of physical, chemical, physicochemical, and biological sampling methods used in clinical sampling, industrial sampling, and environmental sampling can also be explored by food microbiologists to make sampling of microorganisms in foods more precise and accurate.

Advances in Total Viable Cell Count Methodologies

One of the most important information concerning food quality, food spoilage, food safety, and potential implication of foodborne pathogens is the total viable cell count of food, water, food contact surfaces, and air of the food plants. The conventional "Standard Plate Count" method has been in use for the past 100 years in applied microbiology. The method involves preparing the sample, diluting the sample, plating the sample with a general nonselective agar, incubating the plates at 35 °C and counting the colonies after 48 h (there is a great variety of combinations of volumes to be plated; the use of nonselective and selective agars, incubation times, incubation temperatures, incubation gaseous environments, and so on). The operation of the conventional "Standard Plate Count" method, although simple, is time-consuming both in terms of operation and data collection. Also, this method utilizes a large number of test tubes, pipettes, dilution bottles, dilution buffer, sterile plates, incubation space and the related disposable and clean up of reusable materials and re-sterilizing them for further use.

Several methods have been developed, tested, and used effectively in the past 20 years as alternative methods for viable cell count. Most of these methods were first designed to perform viable cell counts and relate the counts to "Standard Plate Counts". Later coliform count, fecal coliform count, yeast and mold counts were introduced in these systems. Further developments in these systems include differential counts, pathogen counts and even pathogen detection after further manipulations. Many of these methods have been extensively tested in many laboratories throughout the world and went through AOAC International collaborative study approvals. The aim of these methods is to provide reliable viable cell counts of food and water in more convenient, rapid, simple, and cost effective alternative formats compared to the cumbersome "Standard Plate Count" method.

Table 1—Comparison of pour plate and spiral plated counted manually and by laser for bacterial cultures

	Pour plate		Spiral plate	
Test cultures	Manual	Laser	Manual	Laser
		Log_{10} cfu/ml		
Escherichia coli	8.9	8.9	8.7	8.9
Salmonella enteritidis	8.8	8.7	8.8	8.9
Pseudomonas aeruginosa	8.0	8.0	8.0	8.0
Staphylococcus aureus	8.0	7.8	8.2	8.2
Lactobacillus plantarum	9.5	9.4	9.6	9.7
Streptoccus sp.	7.7	7.7	8.0	8.1
Bacillus cereus	7.3	7.2	7.2	7.3
Micrococcus luteus	7.4	7.3	7.5	7.6

The Spiral Plating Method is an automated system to obtain viable cell count (Spiral Biotech, Bethesda, Md., U.S.A.). By use of a stylus, this instrument can spread a liquid sample on the surface of a pre-poured agar plate (selective or nonselective) in a spiral shape (the Archimedes spiral) with a concentration gradient starting from the center and decreasing as the spiral progresses outward on the rotating plate. The volume of the liquid deposited at any segment of the agar plate is known. After the liquid containing microorganisms is spread, the agar plate is incubated overnight at an appropriate temperature for the colonies to develop. The colonies appearing along the spiral pathway can be counted either manually or electronically. The time for plating a sample is only several seconds compared to minutes used in the conventional method. Also, using a laser counter an analyst can obtain an accurate count in a few second as compared with a few minutes, in the tiring procedure, of counting colonies by the naked eye. The system has been used extensively in the past 20 years with satisfactory microbiological results from meat, poultry, seafood, vegetable, fruits, diary products, spices, and so on. Manninen and others (1991) evaluated the spiral plating system against the conventional pour plate method using both manual count and laser count and found that the counts were essentially the same (**Table 1**) for bacteria and yeast. Newer versions of the spiral plater are introduced as "Autoplater" (Spiral Biotech, Bethesda, Md., U.S.A.) and Whitley Automatic Spiral Plater (Microbiology International, Rockville, Md., U.S.A.). With these automatic instruments an analyst needs only to present the liquid sample and the instrument completely and automatically processes the sample, including resterilizing the unit for the next sample.

The ISOGRID system (QA Laboratories Ltd., San Diego, Calif., U.S.A.) consists of a square filter with hydrophobic grids printed on the filter to form 1600 squares for each filter. A food sample is first weighted, homogenized, diluted, and enzyme treated then passed through the filter assisted by vacuum. Microbes are trapped on the filter and into the squares. The filter is then placed on pre-poured nonselective or selective agar and then incubated for a specific time and temperature. Since a growing microbial colony cannot migrate over the hydrophobic material, all colonies are localized into a square shape. The analyst can then count the squares as individual colonies. Since there is a chance that more than 1 bacterium was trapped in 1 square the system has a Most Probable Number (MPN) conversion table to provide statistically accurate viable cell counts. Automatic instruments are also

available to count these square colonies in seconds. Again this method has been used to test a great variety of foods in the past 20 years.

Petrifilm (3M Co., St. Paul, Minn., U.S.A.) is an ingenious system with appropriate rehydratable nutrients embedded in a series of films in the unit. The unit is a little larger than the size of a credit card. To obtain viable cell count the protective top layer is lifted and 1 ml of liquid sample is introduced to the center of the unit and then the cover is replaced. A plastic template is placed on the cover to make a round mold. The rehydrated medium will support the growth of microorganisms after suitable incubation time and temperature. The colonies are directly counted in the unit. This system has a shelf life of over 1 year in cold storage. The attractiveness of this system is that it is simple to use, small in size, long shelf life , no need to prepare agar, and ease to read results. Recently the company also introduced a Petrifilm counter so that an analyst only needs to place the Petrifilm with colonies into the unit and the unit will automatically count and record the viable cell count in the computer. The manual form of the Petrifilm has been used for many food systems and is gaining international acceptance as an alterative method for viable cell count.

Alternative viable cell count methods successfully tested

Redigel system (3M Co., St. Paul, Minn., U.S.A.) consists of tubes of sterile nutrient with a pectin gel in the tube but no conventional agar. This liquid system is ready for use and no heat is needed to "melt" the system since there is no agar in the liquid. After an analyst mixes 1 ml of liquid sample with the liquid in the tube, the resultant contents are poured into a special petri dish coated with calcium. The pectin and calcium will react and form a gel which will solidify in about 20 min. The plate is then incubated at the proper time and temperature and the colonies will be counted the same way as the conventional standard plate count method.

The 4 methods described above have been in used for almost 20 years. Chain and Fung (1991) made a comprehensive evaluation of all 4 methods against the conventional standard plate count method on seven different foods, 20 samples each, and found that the alternative systems and the conventional method were highly comparable at an agreement of r = 0.95. In the same study they also found that the alternative systems cost less than the conventional system for making viable cell counts.

A newer alternative method, the SimPlate system, (BioControl, Bellevue, Wash.) has 84 wells imprinted in a round plastic plate. After the lid is removed, a diluted food sample (1 ml) is dispensed onto the center landing pad and 10 ml of rehydrated nutrient liquid provided by the manufacturer is poured onto the landing pad. The mixture (food and nutrient liquid) is distributed evenly into the wells by swirling the SimPlate in a gentle, circular motion. Excessive liquid is absorbed by a pad housed in the unit. After 24 h of incubation at 35 °C, the plate is placed under UV light. Positive fluorescent wells are counted and the number is converted in the MPN table to determine the number of bacteria present in the SimPlate. The method is simple to use with minimum amount of preparation. A 198 well unit is also available for samples with high counts. Using different medium, the unit can also make counts of total coliforms and *E. coli* counts, as well as yeast and mold counts and even *Campylobacter*.

The above methods are designed to count aerobic microorganisms. To count anaerobic microorganisms, one has to introduce the sample into the melted agar and after solidification the plates need to be incubated in an enclosed anaerobic jar. In the anaerobic jar, oxygen is removed by the hydrogen generated by the "Gas Pack" in the jar to create an anaerobic environment. After incubation, the colonies can be counted and reported as anaerobic count of the food. The method is simple but requires expensive anaerobic jars and disposable "Gas Packs". Also, it takes about 1 h before the interior of the jar becomes anaerobic. Some strict anaerobic microorganisms may die during this 1 h period of reduction of oxygen. The author developed a simple anaerobic double tube system which is easy to use and provides instant anaerobic condition for the cultivation of anaerobes from foods (Fung and Lee 1981). In this system, the desired agar (ca. 23 ml) is first autoclaved in a large test tube (OD 25 × 150 mm). When needed, the agar is melted and tempered at 48 °C. A liquid food sample (1 ml) is added into the liquid. A smaller sterile test tube (OD 16 X 150 mm) is inserted into the large tube with the food sample and the melted agar. By so doing, a thin film is formed between the 2 test tubes. The unit is tightly closed by a screw cap. The entire unit is placed into an incubator for the colonies to develop. No anaerobic jar is needed for this simple anaerobic system. After incubation, the colonies developing in the agar film can be counted and provide an anaerobic count of the food being tested. This simple method has been used extensively for applied anaerobic microbiology in the author's laboratory for about 20 years. (Ali and Fung 1991; Schmidt and others 2000). A commercial system, Lee tube (Ogg and others 1979), utilizes a fused inverted small tube inside a larger glass tube is also available for effective anaerobic cultivation of bacteria. The system is very fragile and difficult to clear and to obtain cultures for further confirmation of isolates.

The above mentioned methods are designed to grow colonies to visible sizes for enumeration and report the data as CFU per gram, ml or centimeter squares of the food being tested.

A few "real time" viable cell count methods have been developed and tested in recent years. These methods rely on using "vital" stains to stain "live" cells or ATP detection of live cells. All these methods need careful sample preparation, filtration, careful selection of dyes and reagents, and instrumentation. Usually the entire system is quite costly. However, they can provide 1-shift results and can handle large number of samples.

'Real-time' viable cell count methods proposed

The Direct Epifluorescent Filter Techniques (DFET) method has been tested for many years and is in use in the United Kingdom for raw milk quality assurance programs. In this method, the microorganisms are first trapped on a filter and then the filter is stained with acridine orange dye. The slide is observed under UV microscopy. "Live" cells usually fluoresce orange-red, orange-yellow, or orange-brown whereas "dead" cells fluoresce green. The slide can be read by the eye or by a semi-automated counting system marketed by Bio-Foss. A viable cell count can be made in less than an hour.

The Chemunex Scan RDI system (Monmouth Junction, N.J., U.S.A.) involves filtering cells on a membrane and staining cells with vital dyes (Fluorassure) and after about 90 min incubation (for bacteria), the membrane with stained cell is read in a scanning chamber which can scan and count fluorescing viable cells. This

system has been used to test disinfecting solutions against such organisms as *Pseudomonas aeruginosa*, *Serratia marcescens*, *Escherichia coli*, and *Staphylococcus aureus* with satisfactory results.

The MicroStar System developed by Millipore Corp. (Benford, Mass., U.S.A) utilizes adenosine triphosphate (ATP) bioluminescence technology by trapping bacteria in a specialized membrane (Milliflex). Individual live cells are trapped in the matrix of the filter and grow into microcolonies. The filter is then sprayed with permeablizing reagent in a reaction chamber to release ATP. The bioluminescence reagent is then sprayed onto the filter. Live cells will give off light due to the presence of ATP and the light is measured by a CCD camera and fluorescent particles (live cells) are counted .

These are new developments in staining technology, ATP technology and instrumentation for viable cell counts. The application of these methods for the food industry is still in the evaluation stage. The future looks promising.

Advances in Miniaturization and Diagnostic Kits

Identification of normal flora, spoilage organisms, foodborne pathogens, starter cultures, and so on, in food microbiology is an important part of microbiological manipulations. Conventional methods, dating back to more than 100 years ago, utilize large volumes of medium (10 ml or more) to test for a particular characteristic of a bacterium (for example, lactose broth for lactose fermentation by *Escherichia coli*). Inoculating a test culture into these individual tubes one at a time is also very cumbersome. Through the years many microbiologists have devised vessels and smaller tubes to reduce the volumes used for these tests (Hartman 1968). This author has systematically developed many miniaturized methods to reduce the volume of reagents and media (from 5 to 10 ml to about 0.2 ml) for microbiological testing in a convenient Microtiter plate which has 96 wells arranged in a 8 X 12 format. The basic components of the miniaturized system are the commercially sterilized Microtiter plates for housing the test cultures, a multiple inoculation device, and containers to house solid media (large Petri dishes) and liquid media (in another series of Microtiter plates with 0.2 ml of liquid per well). The procedure involves placing liquid cultures (pure cultures) to be studied into sterile wells of a Microtiter plate (ca. 0.2 ml for each well) to form a master plate. Each Microtiter plate can hold up to 96 different cultures, 48 duplicate cultures, or various combinations as desired. The cultures are then transferred by a sterile multipoint inoculator (96 pins protruding from a template) to solid or liquid media. Sterilization of the inoculator is by alcohol flaming. Each transfer represents 96 separate inoculations in the conventional method. After incubation at an appropriate temperature, the growth of cultures on solid media or liquid media can be observed and recorded, and the data can be analyzed. These methods are ideal for studying large numbers of isolates or for research involving challenging large numbers of microbes against a host of test compounds. Through the years using the miniaturized systems the author has characterized thousands of bacterial cultures isolated from meat and other foods, studied the effect organic dyes against bacteria and yeasts, and performed challenge studies of various compounds against microbes with excellent results. Many useful microbiological media were discovered through this line of research. For example, an aniline blue *Candida albicans* medium was developed and marketed by DIFCO under the name of Candida Isolation Agar. The sensitivity and specificity were 98.0 and 99.5%, respectively, with a predictive value of 99.1 % (Goldschmidt and others 1991).

Other scientists also have miniaturized many systems and developed them into diagnostic kits around late 1960s to 1970s. Currently, API systems, Enterotube, Minitek, Crystal ID system, MicroID, RapID systems, Biolog, and Vitek systems are available. Most of these systems were first developed for identification of enterics (*Salmonella, Shigella, Proteus, Enterobacter*, and so on). Later, many of the companies expanded the capacity to identify nonfermentors, anaerobes, gram positive organisms and even yeast and molds. Most of the early comparative analyses centered around evaluation of these kits for clinical specimens. Comparative analysis of diagnostic kits and selection criteria for miniaturized systems were made by Cox and others (1984) and Fung and others (1989). They concluded that miniaturized systems are accurate, efficient, labor saving, space saving and cheaper than the conventional methods. Originally, an analyst needs to read the color reaction of each well in the diagnostic kit and then use a manual identification code to "key" out the organisms. Recently diagnostic companies have developed automatic readers phasing in with computer to provide rapid and accurate identification of the unknown cultures.

The most successful and sophisticated miniaturized automated identification system is the VITEK system (bioMerieux, Hazelwood, Mo., U.S.A.) which utilizes a plastic card which contains 30 tiny wells in which each has a different reagent. The unknown culture in a liquid form is "pressurized " into the wells in a vacuum chamber and then the cards are placed in an incubator for a period of time ranging from 4 to 12 h. The instrument periodically scans each card and compared the color changes or gas production of each tiny well with the data base of known cultures. Vitek can identify a typical *Escherichia coli* culture in 2 to 4 h. Each Vitek unit can handle 120 cards or more automatically. There are a few thousand Vitek units in use currently in the world. The data base is especially good for clinical isolates.

Miniaturized microbiological methods widely used

Biolog system (Hayward, Calif., U.S.A.) is also a miniaturized system using the Microtiter format for growth and reaction information. Pure cultures are first isolated on agar and then suspended in a liquid to the appropriate density (approx. Log 6 cell/ml). The culture is then dispensed into a Microtiter plate containing different carbon sources in 95 wells and 1 nutrient control well. The plate with the pure cultures is then incubated overnight after which the Microtiter plate is removed and the color pattern of the wells with carbon utilization is observed and compared with profiles of typical patterns of microbes. This manual evaluation is too tedious to perform and the company developed a software system for the users to enter the data in a computer and then receive the identification. A more convenient mode is to put the Microtiter plate in an instrument which can scan the pattern of the positive wells and conduct a match with known cultures to make an identification. This systems is easy to operate and with the use of the automatic data analysis the instrument is a useful tool to characterize and identify unknown cultures. This system is very ambitious and tries to identify more than 1,400 genera and species of environmental, food and medical isolates from major groups of gram-positive, gram-negative and other organisms. The database of many cultures is still limited and it needs further development to identify cultures from food and the environment. Nevertheless this system provides a simple operational format with good identification for typical isolates.

There is no question that miniaturization of microbiological

methods have saved much materials and operational time and has provided needed efficiency and convenience in diagnostic microbiology. The flexible systems developed by the author and others can be used in many research and development laboratories for studying large number of cultures. The commercial systems have played key roles in diagnostic microbiology and have saved many lives due to rapid and accurate characterization of pathogenic bacteria. These miniaturized systems and diagnostic kits will continue to be very useful and important in the medical and food microbiology arenas.

Another area in improving the viable cell count procedure is miniaturization. This is possible in 2 areas. The first area is to actually miniaturize the conventional viable cell count procedure, which involves growing bacteria on agar after dilution of the sample. The second area is miniaturization of the entire Most Probable Number (3 or 5 tube MPN) procedure used extensively for water testing for almost 100 years in public health laboratories.

Automated MPN procedures perfected

In the previous section, the discussion on viable cell count involving conventional and alternative methods to manipulate the standard plate count method did not describe miniaturization of the procedures. More than 30 years ago the author and colleagues (Fung and Kraft 1968; Fung and LaGrange 1969) miniaturized the viable cell count procedure by diluting the samples in the Microtiter plate using 0.025 ml size calibrated loops in 1:10 dilution series. One can simultaneously dilute 12 samples to 8 series of 1:10 dilutions in a matter of minutes. After dilution, the samples can be transported by a calibrated pipette and spot plating 0.025 ml on agar. One conventional agar plate can accommodate 4 to 8 spots. After incubation, colonies in the spots can be counted and the number of viable cells in the original sample can be calculated since all the dilution factors are known. The accepted range of colonies to be counted in 1 spot is 10 to 100. The conventional agar plate standard is from 25 to 250 colonies per plate. This procedure actually went through an AOAC International collaborative study with satisfactory results (Fung and others 1976). However, the method has not received much attention and is waiting to be "rediscovered" in the future.

In a similar vein, the author also miniaturized the Most Probable Number method in the Microtiter plate by diluting a sample in a 3-tube miniaturized series (Fung and Kraft 1969). In 1 Microtiter plate one can dilute 4 samples each in triplicate (3-tube MPN) to 8 series of 1:10 dilution. After incubation, the turbidity of the wells are recorded and a modified 3-tube MPN table can be used to calculate the MPN of the original sample. This procedure recently received renewed interest in the scientific community.

Walser (2000) in Switzerland reported the use of an automated system for Microtiter plate assay to perform classical most probable number (MPN) of drinking water. He used a pipetting robot equipped with sterile pipetting tips for automatic dilution of the samples and after incubation placed the plate in a Microtiter plate reader and obtained MPN results with the use of a computer. The system can cope with low or high bacterial load from 0 to 20,000 colonies per ml. The system takes out the tedious and personnel influence of routine microbiological works and can be applied to determine MPN of fecal organisms in water as well as other microorganisms of interest in food microbiology.

Irwin and others (2000) in the USA also worked on a similar system by using a modified Gauss-Newton algorithm and 96 well microtechnique for calculating MPN using Microsoft EXCEL spreadsheets. These improvements are possible in 2000 compared with the original work of the author in 1969 because: (1) automated instruments are now available in many laboratories to dispense liquid into the Microtiter plate. Automated dilution instruments are also available to facilitate rapid and aseptic dilutions of samples; (2) Automated readers of Microtiter wells are now common place to efficiently read turbidity, color, and fluorescence of the liquid in the wells for calculation of MPN; and, (3) elegant mathematical models, computer interpretations and analysis, and printout of data are now available which the author could not have envisioned back in 1969.

The future is very bright for miniaturized viable cell count procedures in food and water microbiology.

Advances in Immunological Testings

Antigen and antibody reaction has been used for decades for detecting and characterizing microorganisms and their components in medical and diagnostic microbiology. Antibodies are produced in animal systems when a foreign particle (antigen) is injected to the system. By collecting the antibodies and purifying the antibodies one can use these antibodies to detect the corresponding antigens. Thus when a *Salmonella* or a component of *Salmonella* is injected into a rabbit the animal will produce antibodies against *Salmonella* or the component (for example, somatic antigen). By collecting and purifying these antibodies, one can use these antisera to react with a culture of suspected *Salmonella*. When a positive reaction commences, agglutination of antigens (*Salmonella*) and antibodies (antibodies against *Salmonella) will* occur and can be observed on a slide by a trained technician. This is the bases for serotyping bacteria such as *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and so on. These antibodies can be polyclonal (a mixture of several antibodies in the antisera which can react with different sites of the antigens) or monoclonal (there is only 1 pure antibody in the antiserum which will react with only 1 epitope of the antigens). Both polyclonal antibodies and monoclonal antibodies have been used extensively in applied food microbiology. There are many ways to perform antigen-antibody reactions but the most popular format in recent years is the "Sandwiched" Enzyme Linked Immunosorbant Assay or popularly known as the ELISA test.

Briefly, antibodies (for example, anti-*Salmonella* antibody) are fixed on a solid support (for example, wells of a Microtiter plate). A solution containing a suspect target antigen (for example, *Salmonella*) is introduced to the Microtiter well. If the solution has *Salmonella* the antibodies will capture the *Salmonella*.

ELISA tests have been completely automated

After washing away food debris and excess materials another anti-*Salmonella* antibody complex is added into the solution. The second anti-*Salmonella* antibody will react with another part of the trapped *Salmonella*. This second antibody is linked with an enzyme such as horseradish peroxidase. After another washing to remove debris, a chromagen complex such as tetramethylbenzidine and hydrogen peroxide is added. The enzyme will react with the chromagen and will produce a color compound, which will indicate that the first antibody has captured *Salmonella*. If all the reaction procedures are done properly and the liquid in a Microtiter well exhibits a color reaction then the sample is considered positive

for *Salmonella*.

This procedure is simple to operate and has been used for decades with excellent results. It should be mentioned that these ELISA tests need about 1 million cells to be reactive and therefore before performing the ELISA tests the food sample has to go through an overnight incubation so that the target organism has reach a detectable level. The total time to detect a pathogen by these systems should include the enrichment time of the target pathogens.

Many diagnostic companies (such as BioControl, Organon Teknika, Tecra, and so on) have marketed ELISA test kits for foodborne pathogens and toxins such as *Salmonella*, *Escherichia coli*, staphylococcal enterotoxins, and so on. However, the time involved in samples addition, incubating, washing and discarding of liquids, adding of another antibody complex, washing, and finally adding of reagents for color reaction all contribute to inconvenience of the manual operation of the ELISA test. Recently some companies have completely automated the entire ELISA procedure.

The VIDAS system (bioMerieux, Hazelwood, Mo., U.S.A.) is an automated system which can perform the entire ELISA procedure automatically and can complete an assay from 45 min to 2 h depending on the test kit. Since VIDAS utilizes a more sensitive fluorescent immunoassay for reporting the results, their system is named ELFA. All the analyst needs to do is to present to the reagent strip a liquid sample of an overnight enriched sample. The reagent strip contains all the necessary reagents in a ready-to-use format. The instrument will automatically transfer the sample into a plastic tube called the SPR (Solid Phase Receptacle), which contains antibodies to capture the target pathogen or toxin. The SPR will be automatically transferred to a series of wells in succession to perform the ELFA test. After the final reaction, the result can be read and interpretation of positive or negative test will be automatically determined by the instrument. Presently, VIDAS can detect *Listeria*, *Listeria monocytogenes*, *Salmonella*, *E. coli* O157, staphylococcal enterotoxin, and *Campylobacter*. They also market an immuno-concentration kit for *Salmonella* and *E. coli* O157. More than 13,000 units of VIDAS units are in use in 2002.

BioControl (Bellevue, Wash., U.S.A.) markets an Assurance EIA system which can be adapted to automation for high-volume testing. Assurance EIA is available for *Salmonella*, *Listeria*, *E. coli* O157:H7, and *Campylobacter*.

Diffchamb (Hisings Backa, Sweden) has a high precision liquid delivery system that can be used to perform a variety of ELISA tests depending on the pathogens to be tested. Tecra OPUS (International BioProducts, Redmond, Wash., U.S.A.) and Bio-Tek Instrument (Highland Park, Vt., U.S.A.) can also perform ELISA test automatically as long as the proper reagents are applied to the system.

One of the newest entries into this field is the Detex system by Molecular Circuitry Inc. (King of Prussia, Pa., U.S.A.). This is an automated ELISA system with all reagents in ready-to-use format. The test sample is introduced into the reagent cassette and the sequence of reagent for immunological reaction is automatically done. The reaction is reported by impedance changes in the instrument. In-house test results of meat and culture samples for *Salmonella* indicated a 100% sensitivity and 99% specificity and for *Campylobacter*, 99% sensitivity and 99% specificity.

The message of the above discussion is that many ELISA test kits are now highly standardized and can be performed automatically to increase efficiency and reduce human errors.

Another exciting development in immunology is the use of Lateral Flow Technology to perform antigen-antibody testes. In this system, the unit has 3 reaction regions. The first well contains antibodies to react with target antigens. These antibodies have color particles attached to them. A liquid sample (after overnight enrichment) is added to this well and if the target organism (for example, *E. coli* O157:H7) is present it will react with the antibodies. The complex will migrate laterally by capillary action to the second region, which contains a second antibody designed to capture the target organism. If the target organism is present, the complex will be captured and a blue line will form due to the color particles attached to the first antibody. Excess antibodies will continue to migrate to the third region, which contains another antibody, which can react with the first antibody (which has now become an antigen) to the third antibody and will form a blue color band. This is a "control" band indicating that the system is functioning properly. The entire procedure takes only about 10 min. This is truly a rapid test!

Lateral migration immunoassays provide answers in 10 minutes

Reveal system (Neogen, Lansing, Mich., U.S.A.) and VIP system (BioControl, Bellevue, Wash. U.S.A.) are the 2 main companies marketing this type of system for *E. coli* O157, *Salmonella* and *Listeria*. The newest entry to this field is Eichrom Technologies which markets a similar lateral migration system called Eclipse for the detection of *E. coli* O157:H7. Merck KGaA (Darmstadt, Germany) is also working on a similar lateral migration system for many common foodborne pathogens using a more sensitive gold particle system to report the reactions.

A number of interesting methods utilizing growth of the target pathogen are also available to detect antigen-antibody reactions.

The BioControl 1-2 test (BioControl, Bellevue, Wash., U.S.A.) is designed to detect motile *Salmonella* from foods. In this system, the food sample is first pre-enriched for 24 h in a broth and then 0.1 ml is inoculated into 1 of the chambers in an L-shaped system. The chamber contains selective enrichment liquid medium for *Salmonella*. There is a small hole connecting the liquid chamber with a soft agar chamber through which *Salmonella* can migrate. An opening on the top of the soft agar chamber allows the analyst to deposit a drop of polyvalent anti-H antibodies against flagella of *Salmonella*. The antibodies move downward in the soft agar due to gravity and diffusion. If *Salmonella* is present, it will migrate throughout the soft agar. As the *Salmonella* and the anti-H antibodies meet, they will react and form a visible V-shaped "immunoband". The presence of the "immunoband" indicates the presumptive positive for *Salmonella* in the food sample. This reaction occurs after overnight incubation of the unit. This system is easy to use and interpret, and has gained popularity because of its simplicity.

Tecra (Roseville, Australia) developed a Unique *Salmonella* detection system that combines immuno-capturing and growth of the target pathogen and ELISA test in a simple to use self- contained unit. The food is first pre-enriched in a liquid medium overnight and an aliquot is added into the first tube of the unit. Into this tube a dipstick coated with *Salmonella* antibodies is introduced and left in place for 20 min at which time the antibodies will capture the *Salmonella,* if present. The dipstick with *Salmonella* attached will then be washed and placed into a tube containing growth medium. The dipstick is left in this tube for 4 h. During this time if *Salmonella* is present, it will start to replicate and the newly produced *Salmonella* will automatically be trapped by the coated antibodies. Thus, after 4 h of replication, the dipstick will be saturated with trapped *Salmonella*. The dipstick will be transferred to another tube containing a second antibody conjugated to enzyme and allowed to react for 20 min. After this second antigen-anti-

body reaction the dipstick is washed in the 5th tube and then placed into the last tube for color development similar to other ELISA tests. A purple color developed on the dipstick indicates the presence of *Salmonella* in the food. The entire process from incubation of food sample to reading of the test results is about 22 h making it an attractive system for detection of *Salmonella*. The system can now also detect *Listeria*.

The BioControl 1-2 test and the Unique *Salmonella* test are designed for laboratories with a low volume of tests. Thus, both the automatic systems and the hands-on unit systems have their place in different food testing laboratory situation.

A truly innovative development in applied microbiology is the immuno-magnetic separation system. Vicam (Somerville, Mass., U.S.A.) pioneered this concept by coating antibodies against *Listeria* on metallic particles. Large numbers of these particles (in the millions) are added into a liquid suspected to contain *Listeria* cells. The antibodies on the particles will capture the *Listeria* cells after rotating the mixture for about an hour. After the reaction, the tube is placed next to a powerful magnet, which will immobilize all the metallic particles to the side of the glass test tube regardless of whether the particles have or do not have captured the *Listeria* cells. The rest of the liquid will be decanted. By removing the magnet from the tube, the metallic particles can again be suspended in a liquid. At this point, the only cells in the solution will be the captured *Listeria*. By introducing a smaller volume of liquid (for example, 10% of the original volume), the cells are now concentrated by a factor of 10. Cells from this liquid can be detected by direct plating on selective agar, ELISA tests, PCR reaction or other microbiological procedures in almost pure culture state. Immuno-magnetic capture can save at least 1 day in the total protocol of pre-enrichment and enrichment steps of pathogen detection in food.

Dynal (Oslo, Norway) developed this concept further by use of very homogeneous paramagnetic beads which can carry a variety of molecules such as antibodies, antigens, DNA, and so on. Dynal has developed beads to capture *E. coli* O157, *Listeria*, C*ryptosporidium*, *Giardia*, and so on. Furthermore, the beads can be supplied without any coating materials and scientists can tailor to their own needs by coating the necessary antibodies or other capturing molecules for detection of target organisms. Currently many diagnostic systems (ELISA test, PCR, and so on) are combining immuno-magnetic capture step to reduce incubation and increase sensitivity of the entire protocol.

Immuno-magnetic separation technologies save time

Fluorescent antibody techniques have been used for decades for the detection of *Salmonella* and other pathogens. Similar to the DEFT test designed for viable cell count, fluorescent antibodies can be used to detect a great variety of target microorganisms. Tortorello and Gendel (1993) used this technique to detect *E. coli* O157:H7 in milk and juice.

Umedik, Inc. (Stamford, Conn., U.S.A.) recently introduced the DIA/PRO™ system that includes 3 components, an on-chip separation unit-ICEflo™ Technology (patent-pending). A Biochip on which ICEflo™ Technology is used, along with a hand-held electronic reader. An enriched sample with a suspected pathogen (for example, *E. coli* 0157) first reacts with a fluorescent antibody in a proprietary easy-to-use sample applicator, DIA/PREP™. A small drop of the DIA/PREP™ processed sample is applied directly to the Biochip where the ingenious separation barrier–ICEflo™ unit is held. Only bacteria can flow through the barrier into a detection area while the food particles of larger size are prevented from flowing into the detection area of the Biochip. The Biochip is then inserted into the electronic reader to detect pathogen-fluorescent antibody complexes, and in this example, the presence of *E. coli* 0157. This system provides a "real-time" detection of target pathogens. It detects 1 CFU of *E. coli* 0157 per 25 gram of food within 15 min following a certain enrichment period. Depending on the enrichment media chosen, the enrichment time can be either 6 h (using Umedik's patent-pending *E. coli* fast medium) or 18 h (using conventional broth). The electronic reader is able to enumerate about 3 to 4 log CFU/ml of the bacteria on the Biochip. This system is at the verge of being on the market for the detection of *E. coli* 0157. Detection of other pathogens such as *Salmonella, Listeria*, and so on, will be sure to follow.

Biochips able to analyze samples effectively

The DIA/PRO™ System can also detect the presence of a variety of diseases and other conditions including Alzheimer's disease, various cancers, bacterial and viral infections, heart attacks and strokes. According to the manufacturer, "The inexpensive self-contained plastic Biochip is discarded after use following standard hazardous waste guidelines. The DIA/PRO™ system provides a rapid, safe, and inexpensive alternative to today's labor-intensive diagnostic techniques".

Antigen-antibody reactions is a powerful system for rapid detection of all kinds of pathogens and molecules. This section describes some of the more useful methods developed for applied food microbiology. Some systems are highly automated and some systems are exceedingly simple to operate. It should be emphasized that many of the immunological tests described in this section provide presumptive positive or presumptive negative screening test results. For negative screening results, the food in question is allowed to be shipped for commerce. For presumptive positive test results, the food will not be allowed for shipment until confirmation of the positive is done by the conventional microbiological methods.

This field of immunological testing will continue to evolve as detection methodologies are being explored.

Advances in Instrumentation and Biomass Measurements

As the field of rapid methods and automation develops, the boundaries between instrumentation and diagnostic tests began to merge. As mentioned in the Miniaturization and Diagnostic Kit section, instrumentation is now playing an important function in diagnostic kit systems and the trend will continue. The following discussions are mainly on instrumentation related to signal measurements of microbial growth.

Instruments are needed to monitor changes in a population such as ATP levels, specific enzymes, pH, electrical impedance, conductance, and capacitance, generation of heat, radioactive carbon dioxide, and so on. It is important to note that for the information to be useful, these parameters must be related to viable cell count of the same sample series. In general, the larger the number of viable cells in the sample, the shorter the detection time of these systems. A scattergram is then plotted and used for further comparison of unknown samples. The assumption is that

as the number of microorganisms increases in the sample, these physical, biophysical, and biochemical events will also increaser accordingly. When a sample has 5 log or 6 log organisms/ml, detection time can be achieved in about 4 h.

All living things utilize Adenosine Triphosphate (ATP). In the presence of a firefly enzyme system (luciferase and luciferin system), oxygen, and magnesium ions, ATP will facilitate the reaction to generate light. The amount of light generated by this reaction is proportional to the amount of ATP in the sample. Thus, the light units can be used to estimate the biomass of cells in a sample. The light emitted by this process can be monitor by a sensitive and automated fluorimeters. Some of the instruments can detect as little as 100 to 1000 femtogram (1 femtogram, fg. is -15 log in gram). The amount of ATP in 1 colony-forming unit has been reported as 0.47 fg with a range of 0.22 to 1.03 fg. Using this principle, many researchers have used ATP to estimate microbial cells in solid and liquid foods.

At the beginning, scientists attempted to use ATP to estimate the total number of viable cell counts in foods. The results are inconsistent due to: (1) different microorganisms have different amount of ATP per cell. For example a yeast cell can have 100 times more ATP than a bacterial cell; (2) even for the same organism the amount of ATP per cell is different at different growth stages; and, (3) background ATP from other biomass such as blood and biological fluids in the foods interferes with the target bacterial ATP. Only after much research and development scientists can separate nonmicrobial ATP from microbial ATP and obtain reasonable accuracy in relating ATP to viable cell counts in foods. Since obtaining a ATP reading takes only a few minutes, the potential of exploring this methods further exists. To date, not much routine work has been applied using ATP to estimate viable cell counts in food microbiology laboratories.

Real-time hygiene monitoring systems widely used

From another viewpoint, the presence of ATP in certain food such as wine is undesirable regardless of the source. Thus monitoring ATP can be a useful tool for quality assurance in the winery.

There is a paradigm shift in the field of ATP detection in recent years. Instead of detecting ATP of microorganisms, the systems are now designed to detect ATP from any source for hygiene monitoring. The idea is that a dirty food processing environment will have a high ATP level and a properly cleansed environment will have low ATP level regardless of what contributed to the ATP in these environments. Once this concept is accepted by the food industry, there will be an explosion of ATP systems on the market.

In all of these systems, the key is to be able to obtain a ATP reading in the form of Relative Light Units (RLUs) and relate these units to cleanliness of the food processing surfaces. Most systems design an acceptable RLU, unacceptable RLU and a marginal RLU for different surfaces in food plants. Since there is no standard in what constitutes an absolute acceptable ATP level on any given environment, these RLUs are quite arbitrary. In general, a dirty environment will have high RLUs and after proper cleaning the RLUs will decrease. Besides the sensitivity of the instruments, for an analyst to select a particular system the following attributes are considered: simplicity of operation, compactness of the unit, computer adaptability, cost of the units, support from the company, and documentation of usefulness of the system.

Besides the above mentioned issues, Dreibelbis (1999) in a study of five ATP instruments for hygiene monitoring of a food plant considered the following attributes to be important as selection criteria of the systems : the ability of the technicians in the microbiological laboratory to use the ATP bioluminescence hygiene monitoring system without supervision, the reputation of the ATP system in the industry, and the quality of services received from the manufacturer during the evaluation of the product.

Currently the following ATP instruments are available: Lumac (Landgraaf, the Netherlands), BioTrace (Plainsboro, N.J., U.S.A.), Lightning (BioControl, Bellevue, Wash., U.S.A.), Hy-Lite (EM Science, Darmstadt, Germany), Charm 4000 (Charm Sciences, Malden, Mass., U.S.A.), Celsis system SURE (Cambridge, UK) , Zylux (Maryville, TN), Profile 1 (New Horizon, Columbia, Md., U.S.A.), and others.

As microorganisms grow and metabolize nutrients, large molecules changed to smaller molecules in a liquid system and cause a change in electrical conductivity and resistance in the liquid as well as at the interphase of electrodes. These changes can be expressed as impedance, conductance, and capacitance changes. When a population of cells reach to about log 5/ ml it will cause a change of these parameters. Thus, when a food has a large initial population, the time to make this change will be shorter than a food that has a smaller initial population. The time for the curve change from the baseline and accelerates upward is the detection time of the test sample, which is inversely proportional to the initial concentration of microorganisms in the food. In order to use these methods a series of standard curves must be constructed by making viable cell counts of a series of food with different initial concentration of cells and then measuring the resultant detection time. A scattergram can then be plotted. Thereafter, in the same food system, the number of the initial population of the food can be estimated by the detection time on the scattergram.

The Bactometer (bioMerieux, Hazelwood, Mo., U.S.A.) has been in use for many years to measure impedance changes in foods, water, cosmetics, and so on, by microorganisms. Samples are placed in the wells of a 16-well module which is then plugged into the incubator to start the monitoring sequence. As the cells reach the critical number (5 log to 6 log /ml), the change in impedance increase sharply and the monitor screen shows a slope similar to the log phase of a growth curve. The detection time can then be obtained to determine the initial population of the sample. If one sets a cut off point of log 6 organisms/g of food for acceptance or rejection of the product and the detection time is 4 h $\pm$ 15 min then one can use the detection time as a criterium for quality assurance of the product. Food which exhibit no change of impedance curve more than 4 h 15 min in the instrument is acceptable while food which exhibits a change of impedance curve before 3 h 45 min will not be acceptable. For convenience, the instrument is designed such that the sample bar for a food on the screen will flash red for unacceptable sample, green for acceptable sample, and yellow for marginally acceptable sample. A similar system called RABIT (Rapid Automated Bacterial Impedance Technique), marketed by Bioscience International (Bethesda, Md., U.S.A.) is available for monitoring microbial activities in food and beverages. Instead of a 16 well module used in the Bactometer, individual tubes containing electrodes are used to house the food samples.

The Malthus system (Crawley, UK) uses conductance changes of the fluid to indicate microbial growth. It generates conductance curves similar to impedance curves used in the Bactometer. It uses individual tubes for food samples. Heated water to desirable temperature (for example, 35 °C) is used as the temperature control instead of heated air in the previous 2 systems. All these systems have been evaluated by various scientists in the past 10 to 15 years with satisfactory results. All have their advantages and dis-

advantages depending on the type of food being analyzed. These systems can also be used to monitor target organisms such as coliform, yeast, and mold by specially designed culture media. In fact, the Malthus system has a *Salmonella* detection protocol that has AOAC International approval.

BacT/Alert Microbial Detection System (Organon Teknika, Durham, N.C., U.S.A.) utilizes colorimetric detection of carbon dioxide production by microorganisms in a liquid system using sophisticated computer algorithms and instrumentation. Food samples are diluted and placed in special bottles with appropriate nutrients for growth of microorganisms and production of carbon dioxide. At the bottom of the bottle there is a sensor, which is responsive to the amount of carbon dioxide in the liquid. When a critical amount of the gas is produced, the sensor changes from dark green to yellow and this change is detected by reflectance colorimetry automatically. The units can accommodate 120 or 240 culture bottles. Detection time of a typical culture of *E. coli* is about 6 to 8 h.

Automated instruments can monitor microbial activities with ease

An instrument named Omnispec Bioactivity Monitor System (Wescor, Inc., Logan, Utah, U.S.A.) is a tri-stimulus reflectance colorimeter that monitors dye pigmentation changes mediated by microbial activity. Dyes can be used that product color changes as a result of pH changes, changes in the redox potential of the medium, or the presence of compounds of free amino groups. Samples are placed in the wells of a Microtiter plate (96 wells) or other types of containers and are scanned by an automated light source with computer interface during the growth stages (0 to 24 h). The change of color or hue (a*, b*, L*) can be monitored similar to impedance and conductance curve. Manninen and Fung (1992b) evaluated this system in a study of pure cultures of *Listeria monocytogenes* and food samples and found high correlation coefficients (r) of 0.90 to 0.99 for pure cultures and 0.82 for minced beef between the colony counts predicated by the colorimetric technique and the results of the traditional plate count method. They also showed that detection times for bacterial cultures were substantially (2 to 24 h) shorter using the instrument and concluded that the Omnispec system simplifies the analyses, saves labor and materials and provides a high sample capacity. Tuitemwong and others (1994) completed an extensive study using the Omnispec 4000 to monitor growth responses of food pathogens in the presence of membrane-bound enzymes (Oxyrase). This instrument is highly efficient in large-scale studies (up to about 400 samples at a time) of microbial interaction with different compounds in liquids and foods.

BioSys (BioSys, Inc., Ann Arbor, Mich., U.S.A.) utilizes color changes of media during the growth of cultures to detect and estimate organisms in foods and liquid systems. The uniqueness of the system is that the color compounds developed during microbial growth are diffused into an agar column in the unit and the changes are measured automatically without the interference of food particles. Depending on the initial microbial load in the food, same shift microbial information can be obtained. The system is easy to use and can accommodate 32 samples for 1 incubation temperature or 128 samples for 4 independent incubation temperatures in different models. The system is designed for bioburden testing and HACCP control and can test for indirect total viable cell, coliform, *E. coli*, yeast, mold , lactic acid bacteria counts, swab samples and environmental samples.

Basically, any type of instrument that can continuously and automatically monitor turbidity and color changes of a liquid in the presence of microbial growth can be used for rapid detection of the presence of microorganisms. There will definitely be more systems of this nature on the market in years to come.

Advances in Genetic Testings

Up to this point, all the rapid tests discussed for detection and characterization of microorganisms were based on phenotypic expresses of genotypic characteristics of microorganisms. Phenotypic expression of cells are subject to growth conditions such as temperature, pH , nutrient availability, oxidation-reduction potentials, environmental and chemical stresses, toxins, water activities, and so on.

Even immunological tests depend on phenotypic expression of cells to produce the target antigens to be detected by the available antibodies or vice verse. The conventional "gold standards" of diagnostic microbiology rely on phenotypic expression of cells and are inherently subject to variation.

Genotypic characteristics of a cell is far more stable. Natural mutation rate of a bacterial culture is about 1 in 100 million cells. Thus, there is a push in recent years to make genetic test results as the confirmatory and definitive identification step in diagnostic microbiology. The debate is still continuing and the final decision has not been reached by governmental and regulatory bodies for microbiological testing. Genetic based diagnostic and identification system are discussed in this section.

Hybridization of the deoxyribonucleic acid (DNA) sequence of an unknown bacteria by a known DNA probe is the first stage of genetic testings. Genetrak system (Framingham, Mass., U.S.A.) is a sensitive method and convenient system to detect pathogens such as *Salmonella, Listeria*, *Campylobacter* and *E. coli* O157 in foods. At the beginning, the system utilized radioactive compounds bound to DNA probes to detect DNA of unknown cultures. The drawbacks of the first generation of this type of probes are: (1) most food laboratories are not eager to work with radioactive materials in routine analysis; and, 2) there are limited copies of DNA in a cell. The second generation of probes uses enzymatic reactions to detect the presence of the pathogens and uses RNA as the target molecule. In a cell, there is only 1 complete copy of DNA; however, there may be 1,000 to 10,000 copies of ribosomal RNA. Thus, the new generation of probes are designed to probe target RNA using color reactions. After enrichment of cells (for example, *Salmonella*) in a food sample for about 18 h, the cells (target cells as well as other microbes) are lysed by a detergent to release cellular materials (DNA, RNA, and other molecules) into the enrichment solution. Two RNA probes (designed to react with 1 piece of target *Salmonella* RNA) are added into the solution. The capture probe with a long tail of a nucleotide (for example, adenine, AAAAA) is designed to capture the RNA onto a dipstick with a long tail of thymine (TTTTT). The reporter probe with an enzyme attached will react with another part of the RNA fragment. If *Salmonella* RNA molecules are present, the capture probes will attach to one end of the RNA and the report probes will attach to the other end. A dipstick coated with many copies of a chain of complementary nucleotide (for example, Thymine, TTTTT) will be placed into the solution. Since Adenine (A) will hybridize with Thymine (T), the chain (TTTTT) on the dipstick will reaction with the AAAAA and

Genetic methods came of age

thus capture the target RNA complex onto the stick. After washing away debris and other molecules in the liquid, a chromagen is added. If the target RNA is captured then the enzyme present in the second probe will react with the chromagen and will product a color reaction indicating the presence of the pathogen in the food. In this case, the food is positive for *Salmonella*. The Genetrak has been evaluated and tested for many years and has AOAC International approval of the procedure for many food types. More recently Genetrak has adapted a Microtiter format for more efficient and automated operation of the system.

Polymerase Chain Reaction (PCR) is now an accepted method to detect pathogens by amplification of the target DNA and detecting the target PCR products. Basically, a DNA molecule (double helix) of a target pathogen (for example, *Salmonella*) is first denatured at about 95 °C to form single strands, then the temperature is lowered to about 55 °C for 2 primers (small oligonucleotides specific for *Salmonella*) to anneal to specific regions of the single stranded DNA. The temperature is increased to about 70 °C for a special heat stable polymerase, the TAQ enzyme from *Thermus aquaticus*, to add complementary bases (A, T, G, or C) to the single-stranded DNA and complete the extension to form a new double strand of DNA. This is called a thermal cycle. After this cycle, the tube will be heated to 95 °C again for the next cycle. After 1 thermal cycle, 1 copy of DNA will become 2 couples. After about 21 cycles and 31 cycles, 1 million and 1 billion copies of the DNA will be formed, respectively. This entire process can be accomplished in less than an hour in an automatic thermal cycler. Theoretically, if a food contains 1 copy of *Salmonella* DNA, the PCR method can detect the presence of this pathogen in a very short time. After PCR reactions, one still needs to detect the presence of the PCR products to indicate the presence of the pathogen. The following are brief discussions of 4 commercial kits for PCR reactions and detection of PCR products.

PCR and related technologies revolutionized microbiology

The BAX® for Screening family of PCR assays for foodborne pathogens (Qualicon, Inc., Wilmington, Del., U.S.A.) combines DNA amplification and automated homogeneous detection to determine the presence or absence of a specific target. All primers, polymerase, and deoxynucleotides necessary for PCR as well as a positive control and an intercalating dye are incorporated into a single tablet. The system works directly from an overnight enrichment of the target organisms. No DNA extraction is required. Assays are available for *Salmonella* (Mrozinski and others 1998), *E. coli* 0157:H7 (Johnson and others 1998; Hochberg and others 2000), *Listeria* genus and *Listeria monocytogenes* (Steward and Gendel 1998; Norton and others 2000; Norton and others 2001; Hochberg and others 2001). The systems uses an array of 96 blue LEDs as the excitation source and a photomultiplier tube to detect the emitted fluorescent signal. This integrated system improves the ease-of-use of the assay. In addition to simplifying the detection process, the new method converts the system to a homogeneous PCR test. The homogenous detection process monitors the decrease in fluorescence of a double-stranded DNA (dsDNA) intercalating dye in solution with dsDNA as a function of temperature. Following amplification, melting curves are generated by slowly ramping the temperature of the sample to a denaturing level (95 °C). As the dsDNA denatures, the dye becomes unbound from the DNA duplex, and the fluorescent signal decreases. This change in fluorescence can be plotted against temperature to yield a melting curve waveform. This assay thus eliminates the need for gel-based detection and yields data amenable to storage and retrieval in an electronic database. In addition, this method reduces the hands-on time of the assay and reduces the subjectivity of the reported results. Further, melting curve analysis makes possible the ability to detect multiple PCR products in a single tube. The inclusivity and exclusivity of the BAX® system assays reach almost 100% meaning that false positive and false negative rates are almost zero. Additionally, the automated BAX® system can now be used with assays for the detection of *Cryptosporidium parvum* and *Campylobacter jejuni/coli* and for the quantitative and qualitative detection of genetically modified organisms in soy and corn.

The new BAX® system is far more convenient than the old system in which a gel electrophoresis step was required to detect PCR products after thermal cycling.

The following 2 methods have been developed also to bypass the electrophoresis step to detect PCR products:

TaqMan system of Applied Biosystems (Foster City, Calif., U.S.A.) also amplifies DNA by PCR protocol. However, during the amplification step a special molecule is annealed to the single stranded DNA to report the liner amplification. The molecule has the appropriate sequence for the target DNA. It also has 2 attached particles. One is a fluorescent particle and another one is a quencher particle. When the 2 particles are close to each other no fluoresce occurs. However when the TAQ polymerase is adding bases to the liner single strand of DNA, it will break this molecule away from the strand (like the PacMan in computer games). As this occurs, the 2 particles will separate from each other and fluorescence will occur. By measuring fluoresce in the tube, a successful PCR reaction can be determined. Note that the reaction and reporting of a successful PCR protocol occur in the same tube, thus eliminating the need to detect PCR products by electrophoresis in the old BAX® system.

A new system called Molecular Beacon Technology (Stratagene, La Jolla, Calif., U.S.A.) is developed and can be used for food microbiology in the future (Robinson and others 2000). In this technology, all reactions are again in the same tube. A Molecular Beacon is a tailor made hairpin-shaped hybridization probe. The probe is used to attach to target PCR products. On one end of the probe there is attached a fluorophore and on the other end a quencher of the fluorophore. In the absence of the target PCR products the beacon is in a hairpin shape and there is no fluoresce. However, during PCR reactions and the generation of target PCR products, the beacons will attach to the PCR products and cause the hairpin molecule to unfold. As the quencher moves away from the fluorophore, fluorescence will occur and this can be measured. The measurement can be done as the PCR reaction is progression thus allowing real-time detection of target PCR products and thus the presence of the target pathogen in the sample. This system has the same efficiency as the TaqMan system but the difference is that the beacons detect the PCR products themselves, while in the TaqMan system, it only reports the occurrence of a liner PCR reaction and not the presence of the PCR product directly. By using molecular beacons containing different fluorophores, one can detect different PCR products in the same reaction tubes thus, can be able to perform "multiplex" tests of several target pathogens or molecules. The use of this technology is very new and not well known in food microbiology areas.

One of the major problems of PCR systems is the problem of contamination of PCR products from one test to another. Thus, if any PCR products from a positive sample (for example, *Salmonella* PCR products in a previous run) enter the reaction system of the next analysis, it may cause a false positive result. Probelia system, developed by Institut Pasteur (Paris, France) attempts to eliminate

PCR product contamination by substituting the base uracil for the base thymine in the entire PCR protocol. Thus, in the reaction tube there are adenine, uracil, guanine, and cytosine and no thymine. During PCR reaction the resultant Probelia PCR products will be AUGC pairing and not the natural ATGC pairings.

The PCR products are read by hybridization of known sequences in a Microtiter plate. The report of the hybridization is by color reaction similar to an ELISA test in the Microtiter system.

After 1 experiment is completed, a new sample is added into another tube for the next experiment. In the tube there is an enzyme called Uracil-D-Glycosylase, which will hydrolyze any DNA molecules that contains a uracil. Therefore, if there are contaminants from a previous run, they will be destroyed before the beginning of the new run. Before a new PCR reaction, the tube with all reagents is heated to 56 °C for 15 min for UDG to hydrolyze any contaminants. During the DNA denaturation step the UDG will be inactivated and will not act on the new PCR products containing uracil. Currently, Probelia can detect *Salmonella* and *Listeria monocytogenes* from foods. Other kits under development include *E. coli* O157:H7, *Campylobacter* and *Clostridium botulinum* (Bio-Rad, Hercules, Calif., U.S.A.).

BIOTECON (BioteCon Diagnostics Inc., Hamilton Square, N.J., U.S.A.) is also a new PCR-ELISA type system designed to detect *Salmonella*, *Listeria monocytogenes* in food matrix. They also have kits to screen for genetically modified organisms (tomato and potato) and GMO for soy beans and Maize BT176).

Theoretically, PCR system can detect 1 copy of target pathogen from a food sample (for example, *Salmonella* DNA). In practice, about 200 cells are needed to be detected by current PCR methods. Thus, even in a PCR protocol the food must be enriched for a period of time, for example, overnight or at least 8 h incubation of food in a suitable enrichment liquid, so that there are enough cells for the PCR process to be reliable. Besides the technical manipulations of the systems which can be complicated for many food microbiology laboratories, 2 major problems need to be addressed: inhibitors of PCR reactions and the question of live and dead cells. In food, there are many enzymes, proteins and other compounds that can interfere with the PCR reaction and result in false negatives. These inhibitors must be removed or diluted. Since PCR reaction amplifies target DNA molecules, even DNA from dead cells can be amplified and thus food with dead *Salmonella* can be declared as *Salmonella* positive by PCR results. Thus, food properly cooked but contained DNA of dead cells may be unnecessarily destroyed because of a positive PCR test.

PCR can be a powerful tool for food microbiology once all the problems are solved and analysts are convinced of the applicability in routine analysis of foods.

Riboprinting and pulse net system can pin-point the source of contamination

The aforementioned genetic methods are for detection of target pathogens in foods and other samples. They do not provide identification of the cultures to the species and subspecies level so critical in epidemiological investigations of outbreaks or routine monitoring of occurrence of microorganisms in the environment. The following discussions will center around the developments in genetic characterization of bacterial cultures.

The RiboPrinter Microbial Characterization System (DuPont Qualicon, Wilmington, Del., U.S.A.) characterizes and identifies organisms to genus, species and subspecies levels automatically. To obtain a RiboPrint of an organism, the following steps are followed:

1. A pure colony of bacteria suspected to be the target organism (for example, *Salmonella*) from an agar plate is picked by a sterile plastic stick.
2. Cells from the stick are suspended in a buffer solution by mechanical agitation.
3. An aliquot of the cell suspension is loaded into the sample carrier to be placed into the instrument. Each sample carrier has space for eight individual colony picks.
4. The instrument will automatically prepare the DNA for analysis by restriction enzyme and lysis buffer to open the bacteria, release and cut DNA molecules. The DNA fragments will go through an electrophoresis gel to separate DNA fragments into discrete bands. Lastly, the DNA probes, conjugate and substrate will react with the separated DNA fragments and light emission from the hybridized fragments is then photographed. The data are stored and compared with known patterns of the particular organism.

The entire process takes eight h for eight samples. However, at 2 h intervals, another eight samples can be loaded for analysis.

Different bacteria will exhibit different patterns (for example, *Salmonella* versus *E. coli*) and even the same species can exhibit different patterns (for example, *Listeria monocytogenes* has 49 distinct patterns). Some examples of numbers of RiboPrint Patterns for some important food pathogens are: *Salmonella*, 145; *Listeria*, 89; *Escherichia coli*, 134; *Staphylococcus* , 406; and *Vibrio*, 63. Additionally, the database includes 300 *Lactobacillus*, 43 *Lactococcus,* 11 *Leuconostoc* and 34 *Pediococcus*. The current identification database provides 3267 RiboPrint patterns representing 98 genera and 695 species.

One of the values of this information is that in the case of a foodborne outbreak, scientists not only can identify the etiological agent (for example, *Listeria monocytogenes*) but can pinpoint the source of the responsible subspecies . For example, in the investigation concerning an outbreak of *Listeria monocytogenes*, cultures were isolated from the slicer of the product and also from the drains of the plant. The question is which source is responsible for the outbreak. By matching RiboPrint patterns of the 2 sources of *L. monocytogenes* against the foodborne outbreak culture, it was found that the isolate from the slicer matched the outbreak culture thus determined the true source of the problem. The RiboPrinter system is a very powerful tool for electronic data sharing worldwide.

These links can monitor the occurrence of foodborne pathogens and other important organisms as long as different laboratories utilize the same system for obtaining the RiboPrint Patterns.

Another important system is the Pulsed-Field Gel Electrophoresis Patterns of pathogens. In this system, pure cultures of pathogens are isolated and digested with restriction enzymes and the DNA fragments are subjected to a system known as pulsed-field gel electrophoresis which effectively separate DNA fragments on the gel (DNA fingerprinting). For example in a foodborne outbreak of *E. coli* O157:H7, biochemically identical *E. coli* O157:H7 cultures can exhibit different patterns. By comparing the gel patterns from different sources one can trace the origin of the infection or search for the spread of the disease and thereby control the problem.

In order to compare data from various laboratories across the country the Pulse Net System is established under the National Molecular Subtyping Network for Foodborne Disease Surveillance at the Centers for Disease Control and Prevention (CDC). An extensive training program has been established so that all the collaborating laboratories use the same protocol and are electronically linked to share DNA fingerprinting patterns of major pathogens. As soon as a suspect culture is noted as a possible source of

Section Five:
US Food Industry—Introduction

1. In 1999 there were at least 144.3 million microbiology tests performed in the US in the Food Industry by the 5,979 processing plants defined by company size and SIC Code.
2. Each of these plants will average 464 microbiology tests per week or over 24,000 tests per year.
3. Of these 144.3 million microbiology tests, 16.3% or 23.5 million tests were Pathogen/Specific Organism tests in 1999.
4. The total market size for pathogen tests performed in 1999 by the US Food Industry is estimated at $53.4 million. This estimate is based on the cost of purchased materials only, and results in $2.27 as the average price per test performed. Not included in this market value estimate is any of the labor or overhead that is directly associated with the microbiological testing.
5. For this Report, the US Food Industry is defined as the four market Sectors of Meat, Dairy, Fruit/Vegetable and Processed Food. Each of these Sectors is examined in greater detail in Section 6.

US Food Industry—Market Summary	
Target Food Processing Plants	5,979
Total Microbiology Tests Per Year	144.3 Million
Tests/Plant/Week	464
Tests/Plant/Year	24,128
Total Pathogen Tests Per Year	23.5 Million
Market Value of Pathogen Tests	53.4 Million
Average Sell Price/Test	$2.27

Figure 6—U.S. food industry market summary (Source: "Pathogen Testing in the U.S. Food Industry," Strategic Consulting, Inc., 2000)

US Food Industry-Sector Review

US Food Industry

Sector	## Plants	Micro Tests	Avg/Plant/Week
Meat	1,679	32,212,471	369
Dairy	1,388	45,887,576	636
Fruit/Veg	652	13,981,305	412
Processed	2,260	52,196,282	444
Total	5,979	144,277,634	464

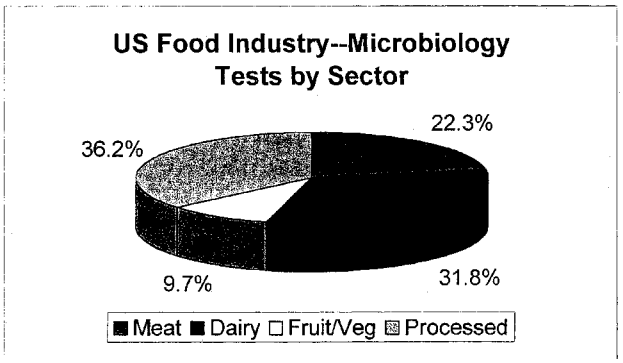

Figure 7—U.S. food industry microbiology tests by sector (Source: "Pathogen Testing in the U.S. Food Industry," Strategic Consulting, Inc., 2000)

an outbreak, all the collaborating laboratories are alerted to search for the occurrence of the same pattern to determine the scope of the problem and share information in real time.

There are many other genetic base methods but they are not directly related to food microbiology and are beyond the scope of this review. It is safe to say that many genetic base methods are slowly but surely finding their ways into food microbiology laboratories and they will provide valuable information for quality assurance, quality control and food safety programs in the future.

Advances in Biosensors

Biosensor is an exciting field in applied microbiology. The basic idea is simple but the actual operation is quite complex and involves much instrumentation. Basically, a biosensor is a molecule or a group of molecules of biological origin attached to a signal recognition material.

When an analyte comes in contact with the biosensor the interaction will initiate a recognition signal which can be reported in an instrument.

Many types of biosensors have been developed such as enzymes (a great variety of enzymes have been used), antibodies (polyclonal and monoclonal) , nucleic acids , cellular materials, and so on.

Sometime whole cells can also be used as biosensors. Analytes detected include toxin (staphylococcal enterotoxins, tetrodotoxins, saxitoxin, botulinum toxin, and so on) , specific pathogens (for example, *Salmonella*, *Staphylococcus*, *Escherichia coli* O157:H7, and so on.) , carbohydrates (for example, fructose, lactose, galactose, and so on.), insecticides and herbicides, ATP, antibiotics (for example, penicillins), and others. The recognition signals used include electrochemical (for example, potentiometry, voltage changes, conductance and impedance, light addressable, and so on.), optical (such as UV, bioluminescence and chemiluminescence, fluorescence, laser scattering, reflection and refraction of light, surface phasmon resonance, polarized light, and so on) and miscellaneous transducers (such as piezoelectric crystals, thermistor, acoustic waves, quartz crystal, and so on.)

An example of a simple enzyme biosensor is sensor for glucose. The reaction involves the oxidation of glucose (the analyte) by glucose oxidase (the biosensor) with the end-products of gluconic acid and hydrogen peroxide.

Biosenors may be the ultimate solution

The reaction was reported by a Clark Oxygen electrode, which monitors the decrease in oxygen concentration amperometrically. The range of measurement is from 1 to 30 mM, and response time of 1 to 1.5 min and the recovery time of 30 s. Lifetime of the unit is several months. Some of the advantages of enzyme biosensors are binding to the subject, highly selective, and rapid acting. Some of the disadvantages are expense, loss of activity when they are immobilized on a transducer, and loss of activities due to deactivation. Other enzymes used include galactosidase, glucoamlyase, acetylcholinesterase, invertase, lactate oxidase, and so on. Excellent review articles and books on biosensors are presented by Eggins (1997), Cunningham (1998), Goldschmidt (1999), and others.

The antibody-antigen reactions used in the DETEX system described in the immunological testing section of this article is in fact a biosensor since it reacts with the target cell components and reports the reactions by impedances changes. The advantages in-

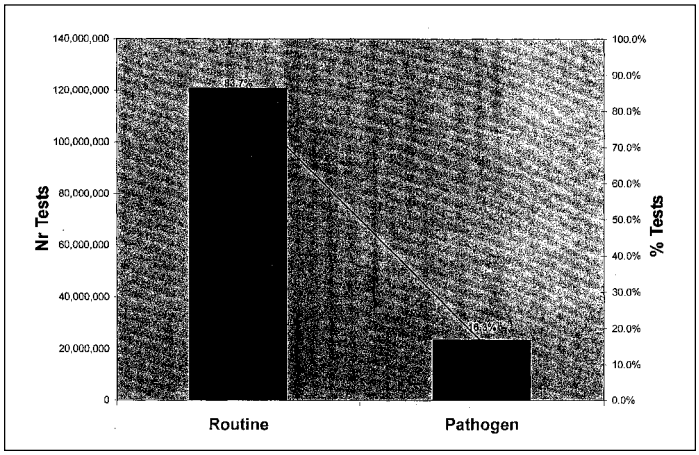

Figure 8—Number and percentage of routine tests compared with pathogen tests (Source: "Pathogen Testing in the U.S. Food Industry," Strategic Consulting, Inc., 2000)

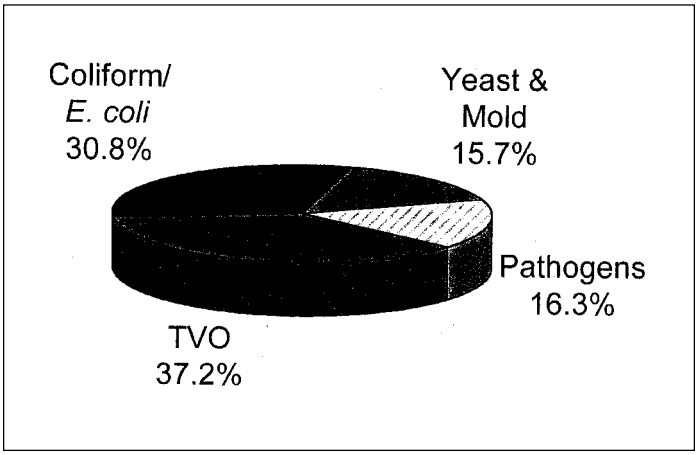

Figure 9—Percentage of total viable organism counts, coliform/*E. coli* counts, yeast and mold counts, and pathogen tests performed (Source: "Pathogen Testing in the U.S. Food Industry," Strategic Consulting, Inc., 2000)

clude being very selective , ultra-sensitive and bind very powerfully.

Recently, much attention has been directed to the field of "biochips" and "microchips" developments to detect a great variety of molecules including foodborne pathogens.

Due to the advancement in miniaturization technology as many as 50,000 individual spots (for example, DNA microarrays) with each spot containing millions of copies of a specific DNA probe can be immobilized on a specialized microscope slide. Fluorescent labeled targets can be hybridized to these spots and be detected. An excellent article by Deyholos and others (2001) described the application of microarrays to discover genes associated with a particular biological process such as the response of the plant (*Arabidopsis*) to NaCl-stress and detailed analysis of a specific biological pathway such as 1-carbon metabolism in maize.

Biochips can also be designed to detect all kinds of foodborne pathogens by imprinting a variety of antibodies or DNA molecules against specific pathogens on the chip for the simultaneous detection of pathogens such as *Salmonella, Listeria, Escherichia coli, Staphylococcus aureus,* and so on, on the same chip. According to Elaine Heron of Applied Biosystems of Foster City, Calif., U.S.A. (Heron 2000), biochips are an exceedingly important technology in life sciences and the market value is estimated to be as high as $5 billion by the middle of this decade. This technology is especially important in the rapidly developing field of proteomics, which requires massive amount of data that generate valuable information.

Certainly, the developments of these biochips and microarray chips are impressive for obtaining a large amount of information for biological sciences. As for foodborne pathogen detection, there are several important issues to consider. These biochips are designed to detect minute quantities of target molecule. The target molecules must be free from contaminants before being applied

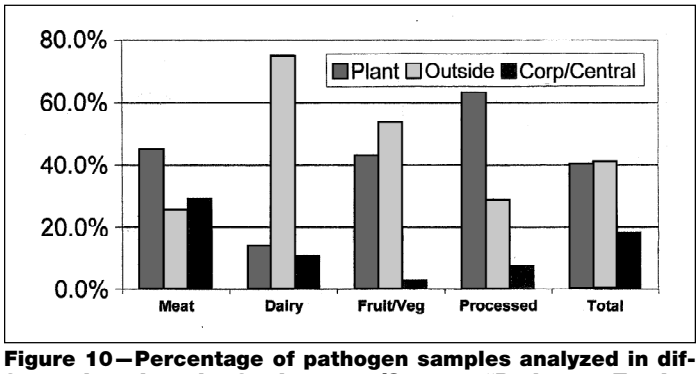

Figure 10—Percentage of pathogen samples analyzed in different locations by food sector (Source: "Pathogen Testing in the U.S. Food Industry," Strategic Consulting, Inc., 2000)

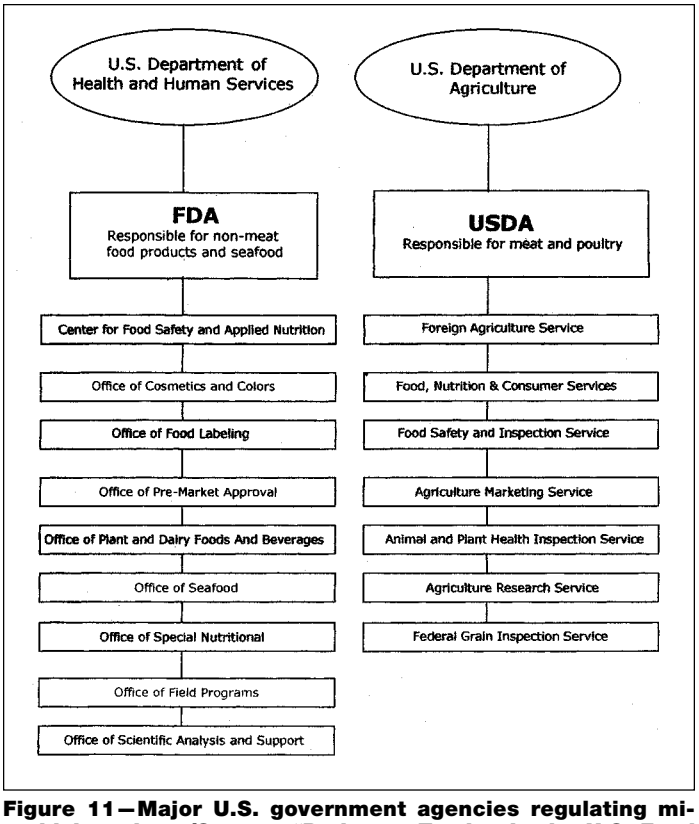

Figure 11—Major U.S. government agencies regulating microbial testings (Source: "Pathogen Testing in the U.S. Food Industry," Strategic Consulting, Inc., 2000)

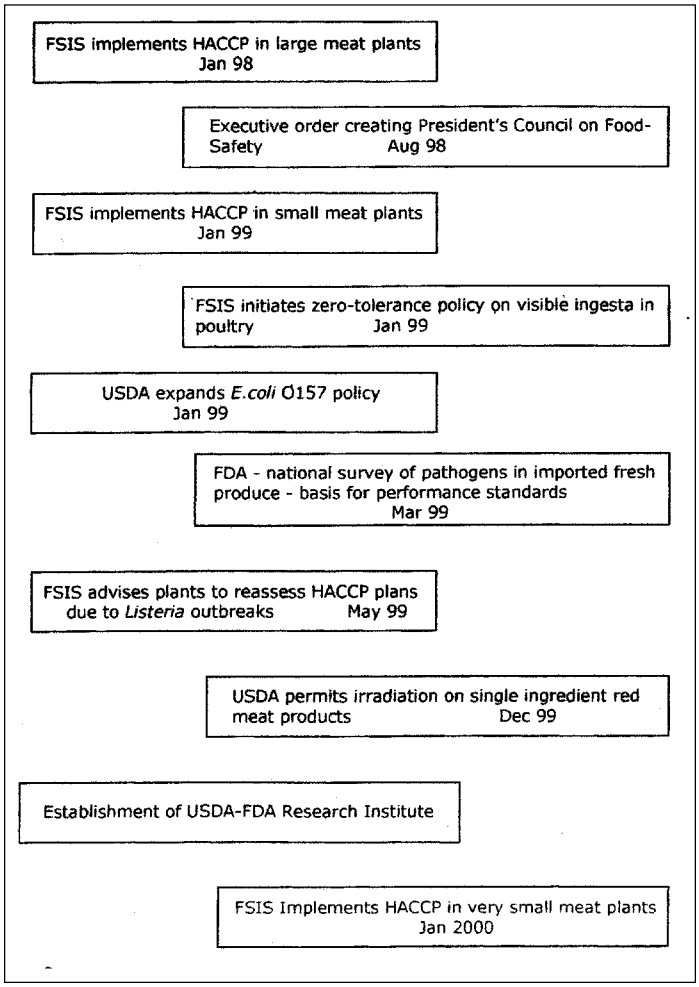

Figure 12—Key developments in food safety regulations in U.S. (Source: "Pathogen Testing in the U.S. Food Industry," Strategic Consulting, Inc., 2000)

to the biochips. In food microbiology, the minimum requirement for pathogen detection is 1 viable target cell in 25 g of a food such as ground beef. A biochip will not be able to seek out such a cell from the food matrix without extensive cell amplification (either by growth or PCR) or sample preparation by filtration, separation, absorption, centrifugation and so on, as described in this article. Any food particle in the sample will easily clot the channels used in biochips. These preparations will not allow the biochips to provide "real-time" detection of pathogens in foods.

Another concern is viability of the pathogens to be detected by biochips. Monitoring the presences of some target molecule will only provide the presence or absence of the target pathogen and will not provide viability of the pathogen in question. Some form of culture enrichment to ensure growth is still needed in order to obtain meaningful results. It is conceivable that biomass of microbes can be monitored by biochips but instantaneous detection of specific pathogens such as *Salmonella*, *Listeria*, *Campylobacter*, and so on, in food matrix during food processing operation is still not possible. The potential of biochip and microarrays for food pathogen detection is great but at this moment much more research is needed to make this technology a reality in applied food microbiology.

U.S. and World Market and Testing Trends (1999-2003)

There is no question that many microbiological tests are being conducted nationally and internationally in food, pharmaceutical products, environmental samples, and water. The most popular tests are total viable cell count, coliform/*E. coli* count and yeast and mold counts. A large number of tests are also performed on pathogens such as *Salmonella*, *Listeria* and *Listeria monocytogenes*, *E. coli* O157:H7, *Staphylococcus aureus, Campylobacter* and other organisms.

Applied microbiologists working in medical, food, environmental, and industrial settings in government, industries, academia, and the private sector are interested in the numbers and kinds of microbiological tests being done annually at local, regional, national and international scales.

However, there are no real statistics on these numbers and only estimates are available from various groups in the past couple of decades. The author at Kansas State University has been conducting a workshop on rapid methods and automation in microbiology since 1981 and has been collecting statistics on the number of tests performed by participants in their work environment. Summary of data from 1981 to 1991 indicated that the average food microbiology laboratory performed about 20,000 total viable cell count, 13,000 coliform count and about 2,000 specific pathogens (*Salmonella*, *Listeria*, and so on) tests per year. An average laboratory serving a medium size food company would therefore perform about 35,000 tests per year. Assuming that there are 20,000 reasonable large microbiological laboratories worldwide (a conservative estimate) the number of tests per year would be a staggering 700 million tests! Even at a modest estimated cost of US$2 per test the market will be US$1.4 billion per year.

Informal estimation for *Salmonella* testing from food samples per year for United States and Europe was 6 to 8.4 million and for *Listeria* 3 to 4.2 million in 1989. More recent estimates for United States alone in 1996 was 6 to 7.5 million for *Salmonella* and 2.5 to 3.5 million for *Listeria*. The latest estimates for total viable cell count was 60 million; coliform, 50 million; yeast and mold, 10 million; *Salmonella*, 8 million; *Listeria*, 5 million; *E. coli* O157:H7, 1 million; generic *E. coli* 3 to 5 million, *Campylobacter* and Staphylococcal enterotoxin, less than 1 million, each (Bailey 2000). The aforementioned numbers were pure estimation by observers of the development in this field.

More than 750 million microbiological tests performed

Strategic Consulting, Inc. (tel: 802-457-9944; Woodstock, Vt., U.S.A.) produced 2 major reports on the market of microbiological testing: *Industrial Microbiology Market Review* (IMMR 1998) and *Pathogen Testing in the US Food Industry* (US Food Tests 2000). This group researched diagnostic testing companies through public records and interviews of hundreds of practitioners of applied microbiology by phone or other means to obtain estimated data to compile the reports. Readers are advised to contact Strategic Consulting, Inc. for details of these reports. Below are information that the author received permission to use for this article.

In 1998, the worldwide industrial microbiological tests was estimated to be 755 million tests, quite similar to the estimation of the author mentioned above. The total market value was US$1.1 billion assuming the average price per test to be US$1.47. They also estimated that 56% of the tests were for food; 30% for pharmaceutical; 10% for beverages; and 4% for environmental water tests (IMMR 1998). Of these tests, 420 millions tests were done in

food laboratories with 360 million for "routine tests" (total viable cell counts, coliform counts and yeast and mold counts) and 60 million for "specific pathogen tests" (*Salmonella*, *Listeria*, *Staphylococcus aureus*, *E. coli* O157:H7 tests). Approximately one third of all the tests were done in the United States, another third in Europe, and the rest were performed by the rest of the world.

They projected that from 1998 to 2003 there will be a 24.6% increase in number of tests; 17% increase in price per test, and 45.8% increase in total revenue of the testing market by 2003. Of the 50 or so diagnostic companies reviewed, there seems to be no absolute dominance of the field by any one company although there are clear leaders in the area (IMMR 1998). The situation is quite fluid since some companies are constantly acquiring products from other companies. Many new companies are also emerging in this area as new technologies are developed.

The 2000 US Food Industry Market study indicated that the total per year microbiological test were 144.3 million, total pathogens were 23.5 million, with a market value of pathogen tests valued at US$53.4 million and the average selling price per test as $2.27. These data were obtained from survey of 5,979 food processing plants with an average of 464 test per plant per week and 24,128 test per plant per year (Figure 6). A more detailed breakdown of microbiological tests of meat, dairy, fruit/vegetable and processed food plants is presented in Figure 7. Processed foods constituted 36.2%; dairy, 31.8%; meat, 22.3% and fruit/vegetable, 9.7% (Figure 7). The number of test to be done in the future for fruit and vegetable will certainly increase due to recent foodborne outbreaks related to these food commodities.

Another valuable set of data is the proportion of routine test version pathogen tests which is 83.7% versus 16.3% (Figure 8). Further break down of the routine versus pathogen test revealed the total viable count consisted 37.2% of all tests, coliform/ *E. coli*, 30.8%, yeast and Mold, 15.7% and Pathogens, 16.3% (Figure 9). This is an increase from 15% of pathogen tests reported in the 1998 IMMR. It is projected that this number will increase further in the years to come.

There is a question on the location where the pathogen samples are analyzed due to considerations of possible contamination of the food plant environments. Figure 10 provides the locations of the pathogens test being performed by food sectors. In meat plants about 45% of the samples were analyzed in the plant, about 25% from outside laboratories, and 30% in corporate and Central laboratories. In the dairy industry, about 75% of the samples were analyzed by outside laboratories probably due to safety considerations and the lack of trained microbiologists in the plants. Microbial samples analyzed locally in plant and analyzed in central laboratories was 15% and 10%, respectively. For fruits and vegetable samples about 55% were analyzed by outside laboratories and 43 % analyzed in plant. Very few samples were analyzed by corporate and central laboratories. This is probably because many corporations, until recently, were not aware of the problems of foodborne pathogens in these products and do not have corporate laboratory facilities. This may change as the demand for safer fruit and vegetable products increase in the coming years. For processed food, more than 60 % were analyzed in plant, 35% in outside laboratories and less than 10% in corporate laboratories. The reason for this food sector to perform their own in plant testing is probably because processed food has relatively low microbial contamination and thus easier to handle the procedures. As a whole for all food sector, about 40% were done in plant, 40% were in outside laboratories and 20% done in corporate central laboratories.

Estimation of the use of "rapid methods" versus "conventional method" is even harder to obtain.

From the author's experiences, at this moment, about 70% of the microbial tests are done using manual or "conventional" methods and 30 % using "rapid methods". By 2005, for total test, about 50% will be using "conventional" methods and 50 will be using "rapid" tests. However, for pathogen tests 60 to 70 % of the test will be in some form of "rapid tests" and 30 to 40% will be using the "conventional" tests. This is because of great improvement of rapid methods are under development and will accelerate in the near future.

The subject of regulations of microbial standards, microbial food testing protocols, foodborne outbreak investigations, litigation, food laws, food recall protocols, and so on, are very complex and beyond the scope of this review. In the USA the 2 major agencies regulating food products and microbial problems in food products are United States Department of Agriculture for meat and poultry and Food and Drug Administration for non-meat food products and seafood. The various departments under these agencies are listed in Figure 11. Figure 12 provides some current legislation and initiative action related to food safety which will impact on microbiological testings.

In conclusion, it is safe to say that the field of rapid methods and automation in microbiology will continue to grow in number and kinds of tests to be done in the future due to the increase concern on food safety.

Prediction of the Future

It is always difficult to predict the future development in any field of endeavor. In 1995 the author was honored to present a lecture at the annual meeting of the American Society of Microbiology as the Food Microbiology Divisional Lecturer concerning the current status and the future outlook of the field of rapid methods and automation in microbiology. Figures 1 and 2 illustrate the trend of development in medical and food microbiology. The following are synopsis of the ten predictions with a look into the future made in 1995. A more detail description of the predictions can be found in the paper by Fung in 1999. Many approaches and technologies have already been described in various sections of this paper and will not be repeated here.

1. Viable cell counts will still be used. It is the firm belief of the author that viable cell counts (total aerobic count, anaerobic count, coliform/*E. coli* count, differential count, and pathogenic count) will remain an important parameter to assess the potential safety and hygiene quality of food supplies. Although, the current methods are cumbersome and labor-intensive, many improvements have been made and will continued to be made to improve the viable cell count procedure as described in section 4 of this paper. Special developments in early sensing of viable colonies on agar, electronic sensing of viable cells under the microscope, improvements of vital stains to count living cells and more effective sensing of most probable number (MPN) of samples will greatly improve the viable cell count procedure.

Fung: Ten predictions of the future . . .

2. Real-time monitoring of hygiene will be in place. Several exciting developments in this area have occurred such as ATP bioluminescence, catalase measurement, and instant protein detection kits. In the past several years, many ATP systems have been marketed and used. These kits are easy to use and provide useful information in a few minutes. Catalase is a very reactive enzyme. The author has worked on this enzyme for a number of years to monitor hygiene of surfaces, microbial spoilage potential of aero-

bic cold stored food, and endpoint temperature monitoring of cooked foods. The test is simple, inexpensive, and rapid (a few seconds to minutes). This system deserves more research and development. Monitoring of the presence of protein fat, carbohydrate and so on, on food contact surfaces can also provide rapid and meaningful information concerning the hygiene quality of the surfaces. Recently, BioControl (Bellevue, Wash., U.S.A.) introduced a protein testing kit called FLASH which can detect the presence of protein level on food contact surfaces almost instantaneously. Positive surfaces change the color of the swab from yellow to green/blue color in 5 seconds. This type of real-time monitoring systems will be developed for other compounds in the future.

3. Polymerase Chain Reaction (PCR), Ribotyping, and genetic tests will become reality in food laboratories. This was a bold prediction in 1995 but now there are food companies, pharmaceutical companies, and related industries routinely using these advanced technology to monitor the presence of normal flora, spoilage flora and pathogens in food and other materials. This trend will advance rapidly in the near and far future.

4. Enzyme-Linked Immunosorbent Assay (ELISA) and Immunological Tests will be completely automated and widely used. This prediction certainly came true. After pre-enrichment of food samples (overnight incubation or eight h incubation) an analyst can place the sample into an automated system and monitor the presence of the target pathogen in a matter of 1 to 2 h. Automated systems will continue to be developed and used in the future.

5. Dipstick Technology will Provide Rapid Answers. Many forms of dipsticks are available for screening of pathogens by "lateral" migration of antigen-antibody complex. These kits can detect target organisms in about 10 minutes after enrichment of the cultures overnight. This type of technology will continued to be developed and used in the future.

6. Biosensors will be in place for HACCP Programs. This prediction is still for the future. A variety of biosensors are now available on the market to monitor microbes but they are not yet suitable to use in routine monitoring of pathogens in the food industry. More research and development will be needed to have this technology in use for the food industry.

7. Instant Detection of Target Pathogens will be possible by computer-generated matrix in response to particular characteristics of pathogens. This prediction depends on the development of a much more in-depth understanding of the microbial cells and the pathogenic traits of pathogens before it can become a reality. By the completion of the mapping of the human genome and the development of proteomics (identification and quantitation of proteins and elucidation of their functions) this field is rapidly moving into practical use in the near future for food microbiology.

8. Effective Separation, Concentration of Target Cells will greatly Assist in Rapid Identification. A variety of approaches have been mentioned in this paper. These developments have been and will continue to improve detection sensitivity and increase speed of detection of target pathogens

9. A Microbiological Alert System will be in food packages. This prediction is certainly within the reach of modern technology. During growth and spoilage, microbial cells will generate a variety of compounds that can be detected by ingenious devices such as gas and pH indicators. It is conceivable that a series of reagents in the form of "bar codes" be placed inside the packaging materials and will change color due to the development of gas (ammonia, hydrogen sulfide, hydrogen, carbon dioxides, and so on) acid or temperature abuse to indicate a potential spoilage problem.

10. Consumers will have Rapid Alert Kits for Pathogens at Home. Nowadays there are urine tests, blood glucose test, pregnancy test, and even AIDS test kits available for the consumer to use at home. It is possible that rapid alert kits for food spoilage and even food pathogen detection available for home use will be developed. More development in this area is needed and a lot of consumer education will have to come along with these kits to make them useful and meaningful.

Along with the prediction of the future of rapid method testings , it is useful to describe the ten attributes for an ideal automated microbiology assay system as follows:

1. Accuracy for the intended purposes. Sensitivity, minimal detectable limits, specificity of test system, versatility, potential applications, comparison to reference methods.

2. Speed in productivity. Time in obtaining results, number of samples processed per run, per hour, per day.

3. Cost. Initial, per test, reagents, labor

4. Acceptability by scientific community and regulatory agencies

5. Simplicity of operation. Sample preparation, operation of test equipment, computer versatility

6. Training. On site, length of time, qualification of operator.

7. Reagents. Preparation, stability, availability and consistency

8. Company reputation

9. Technical services. Speed, availability, cost and scope

10. **Utility and space requirement**.

The future looks very bright for the field of rapid methods and automation in microbiology. The potential is great and many exciting developments will certainly unfold in the near and far future.

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