RAPID METHODS AND AUTOMATION IN MICROBIOLOGY: 25 YEARS OF TRENDS AND PREDICTIONS OF THE FUTURE

Daniel Y. C. Fung, MSPH, Ph.D.
Professor of Food Science and Animal Sciences and Industry, Kansas State University
University Distinguished Professor, Universitat Autonoma de Barcelona, Spain.
207 Call Hall, Kansas State University. Manhattan. Kansas 66506-1600.
Tel: USA (785) 532-1208. Fax: USA (785) 532-5681 e-mail: dfung@ksu.edu

Special Article for KSU XXX International Workshop on Rapid Methods and Automation in Microbiology, June 11 to 18, 2010. Manhattan, Kansas USA 12:00 Midnight June 10, 2010.

Key words : Rapid microbiological methods, sampling, viable cell count, diagnostic kits, instrumentation. biomass, advances in immunology, genetic testings, biosensors, and nanotechnology, US and World market and testing trends (1995 to 2020).

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. Fung (2000) made a comprehensive review of the subject in the article "Rapid Methods and Automation in Microbiology" in the Inaugural Issue of *Comprehensive Reviews in Food Science and Food Safety.* Many national and international meetings on the subject were held regularly. A "hands-on" International Rapid Methods and Automation in Microbiology Workshop was developed by the author in 1981 at Kansas State University. About 4,000 scientists from 60 countries participated in the workshop in the past 29 years. The XXX th Workshop was held from June 11 to 18, 2010 at Kansas State University. This article summarized the major developments of this field in the past 30 years in Applied Microbiology and Food Microbiology. These concepts, techniques, and instrumentation can be used in many areas of biological sciences.

Sample preparation is very important in any field of applied microbiology. Traditionally a solid or liquid food sample is placed in a sterile blender with sterile diluents (usually 25 gram of food

or 25 ml of liquid food in 225 ml of sterile diluents) for 60 seconds or more. After each use the blender has to be cleansed and re-sterilize making this a very inconvenient procedure when large number of samples are to be processed. The **Stomacher** (Seward Instruments), invented by Tony Sharpe more than 35 years ago masticates a food sample housed in a sterile bag usually with 25 g of food and 225 ml of sterile diluents for one minute or more has become a standard Food laboratory instrument around the world with 40,000 units in use. After each use the analyst only needs to remove the bag with processed sample from the instrument and then another sample in another bag can be operated. There is no contact between the food and the instrument. Similar instruments such as **Smasher** (AEC-CIIEMUNEX) and **BioMixer** (Interscience) have also been marketed. A new instrument called the **Pulsifier** (Microgen Bioproducts), also invented by Sharpe, vigorously vibrates the food and diluent in a sterile bag by an elongated ring to dislodge microorganisms into the diluent with minimum destruction of the food. Both methods provide results with essentially the same viable cell counts but the "Pulsified" samples were much clearer and have less debris than the "Stomached" samples and are advantageous for filtration, instrumental, PCR, immunology and other subsequent microbiological tests.

Recently. the author's team (Caballero et al., 2010) extensively evaluated all four instruments on total viable cell counts and *Escherichia colt* counts of **ten foods** (25 g of food in 225 ml of sterile 0.1% Peptone water for 60 second in each treatment: ground beef, hot dog, spinach leaves, alfalfa sprouts, chicken sticks, chicken wings. fish meat, milk, tofu, and peanuts) in terms of **Noise level** of each instrument perceived by four Laboratory' personnel at five feet distance (1.Very quiet, 2.Quiet, 3. Nearly Quite, 4. Acceptable Noise, and 5. Loud) and a **decibel meter** reported in decibels (dB) at five feet distance. **Ease of Cleaning** (1. Very Easy. Complete and Quick Cleaning, 2, Acceptable Cleaning, and 3. Difficult) , and **Ergonomics** (1. Very User-friendly and Ergonomic, 2. Acceptable, or 3. Low Ergonomics). The experiment was repeated three times and data were statistically analyzed.

The results indicated that all four instruments provided no statistical differences between and among the four instruments regarding to total viable cell counts and *E. coil* counts of the ten foods tested. Thus, either one of these instruments is acceptable for routine preparation of food samples for viable cell count and *E. coli* counts in Food Laboratories. As to noise level by objective evaluation (1. being very quiet and 5. being loud) in ascending order the Bagmixer (1.56), Smasher (1.95), are the quietest, followed by the Stomacher (3.97) and the Pulsifer (5.00). In terms of decibel units, a subjective evaluation in ascending order Bagmixer (64.50 dB), Smasher (64.60 dB) Stomacher (68.00dB), and Pulsifer (82.86dB). According to United States Department of Labor the acceptable noise exposure levels range for exposures to workers are: 90 dB for 8 h, 100 dB for 2 hr, 105 dB for 1 h, and 115 dB for 15 min exposure. Since the exposure time in our study was I minute and all instruments had lower than 90 dB we concluded that all instruments used in this study posed no noise health hazards to the operator.

Viable Cell Count remains an essential method to ascertain the quality and potential spoilage problems in air, food, and water. The **conventional method** has been in use for more than 100 years. In the past 25 years the following methods have been tested and used effectively for viable cell counts and differential counts : **Spiral Plating, Petrifilm, Isogrid, and Redigel.** In the author's laboratory a comparative analysis of all four systems on seven different foods, 20 samples each, was made and found that these systems and the conventional method were highly comparable at an agreement of r=0.95. **Simplate** is also a convenient system for viable cell counts. All these methods require 24 hours of incubation of sample to obtain definitive viable cell counts.

The author developed a simple, inexpensive and effective anaerobic system, the **Fung**

Double Tube system (FDT) which can provide *Clostridium perfringens* colony counts from food and water in 5 hrs without the use of any anaerobic chambers such as Brewers anaerobic jar or expansive anaerobic laboratory units. *Clostridium perfringens is* an important food- and water -borne pathogen and also is the fastest growing bacterium known to microbiologists as it has a generation time of only 9.1 min at 42C and will form visible black colonies in SFP agar in the FDT system in 5 hours from the time of sample preparation to obtaining results. Conventional method takes more than 24 hours to have clear-cut results. The FDT system is very simple to use. One needs only to prepare sterile melted appropriate agar such as SFP agar (12 ml at 2X strength) into a larger sterile glass tube (outer diameter 25 x 150mm) and add 10 ml of water sample to be tested into the tube. After mixing then insert a smaller sterile tube (OD16 X 150 mm) into the larger tube. This allows the mixed sample with SFP agar to fill the space between the two tubes. Then incubate the system *at* 42 C for 5 hour. *Clostridium perfringens* will form visible black colonies in about 5 hours The Water Authority of the State of Hawaii in USA is evaluating the use of viable C. *perfringens* number/ 100 ml of fresh or sea water as indicator of fecal contamination in recreational water to determine opening or closing of the beaches to protect public health. Current methods take more than 24hrs and involves many steps and equipments to provide the same data (Fung et al., 2007). Other real time viable cell systems include **Direct Epifluorescent Filter Techniques (DEFT test), Chemunex Scan, Millipore MicroStar System,** etc. can provide viable cell counts in about 4 to 6 hrs with the use of much manipulations and instruments.

Aeromicrobiology has received less attention in applied microbiology. The **SAS Air Sampler(pbi, Milan, Italy), Anderson Air Sampler** and other similar units can provide viable cell count obtained from air effectively. A known volume of air (e. g. 60 Liters) is sucked into the system and impact on an agar surface such as standard Plate agar) housed in the unit.

After the appropriate exposure time (15 to 20 mins) the unit will stop and the agar plate can be removed and incubated for viable cells to develop. This is the **air impaction technique.** To obtain air sample with lesser force the **impingement technique** is used when a known volume of air is bubbled into a sterile liquid and later obtain the viable cell numbers on agar plates and report the data as CFU/M^3. A variety of **Surface Viable Cell methods** are also available such as **Rodac Contact Plate, Swabs, Adhesive Tapes, Microbial-Vac Systems,** etc. to obtain viable cell counts per cm^2 of area. For convenience of utilizing Viable Cell Count (Colony Forming Units CFU) information for food, water, air, and surfaces the following **Fung Scales** have been developed:

For Food, Water, and Surfaces

0 to 2 Log CFU/gm. ml, and cm^2	Low Count (No Particular Concern)
3 to 4 Log CFU/gm, ml and cm^2	Intermediate Count (Slight Concern)
5 to 6 Log CFU/gm. ml and cm^2	High Count (Serious Concern)
7 Log CFU/gm, ml and cm^2	Index of Spoilage (Not Acceptable)
8 to 10 Log CFU/gm. ml and cm^2	Odor and slime (Discard)

For Air Quality in Food Plants

0 to 100 CFU/ M^3	Low Count (No Particular Concern)
100 to 300 CFU /M'3	Intermediate Count (Slight Concern)
>300 CFU /M^3	High Count (Needs Corrective Action)

Note: Singapore has a higher count limit of >500 CFU/M^3 for Food Plants

For Food Contact Surfaces (Food Serving Counters, Chop Boarads, Spoons, Knives, Forks, etc.)

0-10 CFU/cm^2	Low Count (No Particular Concern)
10-100 CFU/cm^2	Intermediate Count (Slight Concern)
>100 CFU/cm^2	High Count (Needs Corrective Action)

These scales are for general total counts. **No pathogens** such as *Salmonella, Staphylococcus aureus, Escherichia coli* 0157:H7, *Listeria monocytogenes,* etc. are allowed in food and water samples for human and animal consumption. On the other hand in food fermentation arena high counts such as 7-10 Log CFU/ml and gm are desirable. User discretions are expected for these convenient scales.

Most Probable Number (MPN) has been used for more than 100 years especially in public health and water microbiology. Statistically MPN (3, 5, or 16 tube), a dilution to extinction method, was determined to be more accurate than Colonies Forming Units for estimating the number of microorganism in water. This method however is very labor intensive, expensive, and utilizes large number of test tubes and media for each sample. The author developed a miniaturized 3 tube MPN system housed in a Microtiter plate 40 years ago. (Fung and LaGrange, 1969). Recently in 2007 TEMPO, a truly innovative and miniaturized MPN system was introduced and marketed by **bioMerieux.** The analyst first makes a 1:10 dilution of the food or water and then places 1 ml into a bottle containing the appropriate dehydrated nutrient such as for coliform test. *Salmonella* test, etc. Another 3 ml of sterile liquid are added to make a final 1:40 dilution of the sample. The heart of TEMPO is a sealed plastic card with 3 series of 16 tubes MPN card. The first series has 16 tiny wells, the second series has 16 larger holes (10 X the volume of the 2nd series) and the third series has again 16 larger holes (10X times larger than the second series). All 48 wells (16 x 3 series) are connected. A dispensing tube, attached to the card, is placed into the sample bottle.

Six of these cards with nutrient broth can be put into a holding unit and the unit is placed into a vacuum/pressure chamber. By activating the vacuum/pressure chamber exactly 4 ml of the sample is pressurized and distributed into all 48 wells. After the sample is prepared, the dispensing tube is cut off and the card is then incubated overnight at the correct temperature for the intended test. Lastly, the cards are placed into a dedicated reader which scans every well of the card for fluorescence or lack of fluorescence and calculates the MPN of the sample automatically. Very little labor is needed to obtain MPN of many food and water samples. Positive correlation of the conventional method and TEMPO system for viable cell counts have been reported in USA as well as in Europe. Currently Fung's laboratory is initiation a detailed study of TEMPO for food microbiology.

The author, through the years, have **miniaturized** many biochemical tests for **diagnostic microbiology** as well as **viable cell counts.** Other scientists also have miniaturized many systems and developed them into diagnostic kits. Currently. **API systems, Enterotube, Minitek, Crystal ID system, MicroID, Rap1D, Biolog, VITEK,** etc. systems are available. Most of these systems were first developed for identification of enterics *(Salmonella, Shigella. Proteus, Enterohacter,* etc.) for clinical microbiology. Later, these systems can identify nonfermentors, anaerobes, Gram positives, and even yeast and molds. There is no question that miniaturization of microbiological methods have saved much materials and operational time and have provided needed efficiency and convenience in diagnostic microbiology and saved many lives in clinical settings. The flexible systems developed by the author and others can be used in many research and development laboratories for studying large number of bacteria, yeasts, and molds cultures.

Antigen and Antibody (Monoclonal and Polyclonal) reactions have been used for decades for detecting and characterizing microorganisms and their components in medical and diagnostic microbiology. Some companies completely automated the entire **ELISA** (enzyme linked immunosorbant assay) procedure. The VIDAS system can automatically perform many units of

ELISA kits in 45 mins to 2 hrs after enrichment of target cultures in a broth. Since this system uses fluorescence to report results it is 10 time more sensitive than using light units to report reactions used in many other systems. There are about 15,000 VIDAS units in use worldwide.

Another ingenious development in immunology is the use of **Lateral Flow Technology** to perform antigen-antibody tests. In a unit about the size of a microscope slide there are three regions. The first region houses specific antibodies (in excess) tacked with a color marker (blue) designed to capture a specific antigen such as against *Escherichia coli* O157 cells. The second region toward the right has antibodies against another part of *E. coli* O157 cells. Liquid from the first region will migrate to the second region by diffusion or capillary action. If the sample has *E. coli* O157 cells from the first regions it will be captured by the antibodies housed at the **second region** and a blue band will form. This will indicate an antigen-antibody reaction has occurred and a presumptive *E. coli* O157 culture is present in the test sample. If there are no *E. coli* O157 cells in the liquid all the antibodies will continue to migrate to a third control region. At the third region the antibody for *E. coli* O157 will now act as **an antigen** and will react with **specific antibody** fixed in the third region. In the third region a blue band will form which will indicate the test was **performed properly**. In this unit after performing the lateral flow test if one sees a blue band in the **second region** and a blue band in the **third region** than **there is** *E. coli* O157 in the original sample. If the second region has **no color** and the third region has **blue color** than the test **is negative** for *E. coli* O157. If the third region has **no color,** the test is invalid and will have to be repeated with another new unit. The entire procedure takes only about 10 minutes after overnight enrichment of target pathogens such as *E. coli* O157 in this example. Confirmation of the presence of E. *coil* O157 from the overnight culture is needed to complete the test. This is truly a rapid test! Currently **Reveal system, VIP system, Merck-KGaA,** etc. can detect *E. coli* 0157, *Salmonella, Listeria* and other organisms of interests.

Immuno-magnetic Separation (IMS) is a very powerful tool to captured target molecules. **Dynal** markets IMS beads for molecules such as antibodies, antigens, DNA, etc. to detect target organisms. Many diagnostic systems (ELISA test, PCR, etc.) are combining IMS step to reduce incubation time and increase sensitivity. **Pathathrix** combines IMS and a step when the entire 4 hour pre-enriched sample (25 food in 225 ml selective broth) is circulated and re-circulated over the immobilized (by a magnet) paramagnetic beads with specific antibodies (e.g. against *E. coli* O157) for 30 minutes to capture as many target organisms as possible. After which the entire liquid content will be washed away and the beads with the captured target organisms are released by removal of magnet which was used to react and capture the beads with the antibodies. The beads with the captured pathogens are collected and the presence of target pathogens can be can be detected by ELISA, PCR, cultural methods, etc. With a short pre-enrichment step, Pathatrix circulation system, and ELISA test, 1 to 10 *E.coli* O157:H7/ 25 gram of ground beef was detected in **5.25 hrs** in the author's laboratory which is the fastest method to detect *E. coli* O157:H7 in ground beef to date (Wu et al., 2004). Pathathrix system has been used to detect a variety of pathogens from a large number of food systems and is used by many food companies as well as US government laboratories. Its potential in applied food microbiology is great.

Instruments can be used to monitor changes in a population such as **ATP levels, color, turbidity, specific enzyme activities, pH, electrical impedance, conductance, and capacitance, generation of heat, radioactive carbon dioxide,** etc. 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.

Monitoring Adenosine Triphosphate **(ATP)** in cultures and foods has been studied for more than 40 years. Currently this system is designed to detect ATP from **any source** (bacteria, yeast and molds, food debris containing living matters, blood, etc.) for **hygiene monitoring.** There are at least 11 companies marketing ATP detection systems and these systems are being used around the world. **Polymerase Chain Reaction (PCR)** is now an accepted method to detect pathogens by amplification of the target DNA (such as *Salmonella)* and detecting the target PCR products. Conventionally the target PCR products was detected by the time consuming gel-electrophoresis process. Currently ingenious fluorescence techniques are being used. The **BAX®, TaqMan, Molecular Beacon, Invitrogen, Warnex Diagnostics, Cephid , Qiagen, Applied BioSystems, LifeTech systesm** etc. are on the market for rapid and accurate detection of a variety of PCR systems. There are two ways to detecting fluorescence in PCR procedures: 1) Measure **release of the fluorophores** from the quencher during the PCR amplification step (Tagman) or 2) Interaction with the **amplified PCR products** with **molecular beacons** and measure fluorescence of separated fluorophore from quencher (Warnex Diagnostics). By use of fluorophores of different colors some systems can detect several pathogens in one tube **(multiplex** test). Also the larger the number of target DNA (e.g. more *Salmonella* DNA) in the sample, the sooner the instrument will report the fluorescence **(real time PCR). RiboPrinter System** and **Pulsed-Field Gel Electrophoresis (PFGE)** are powerful systems to separate bacteria within the same genus due to minute difference in ribosomal DNA. For example the number of RiboPrint Patterns *for Salmonella* is 97, *E. coli* O157 is 65, *Staphylococcus is* 252, *Listeria* is 80, etc. **PFGE** is being used in US Centers for Disease Control and Prevention **(CDC) PulseNet** system where about 40 laboratories are using the same procedure to detect target pathogens. They can share information rapidly and can trace and locate outbreaks around the country. These information are important for epidemiology investigations and can pin-point the culprit (e.g. spinach) when one bacterium *(e. g. Listeria monocytogenes)* was isolated from several sources (such lettuce, onion, spinach, cucumber, etc.) in a mixed vegetable salad outbreak involving patients with *L. monocytogenes* infection.

Biosensor is receiving much attention lately. The basic idea is simple but the actual operation is quite complex. The potential of **biochips, microarrays** and **nanotechnology** for food pathogen detection is great but at this moment much more research, especially in separation and concentration methods are needed to make this technology a reality in food microbiology. Also

food microbiologists need information concerning the number of organisms in a sample and whether the microorganisms detected are **alive or dead, injured or not injured**, etc. Many of these methods cannot provide this type of important information.

There is no question that many microbiological tests are being conducted nationally and internationally in foods, pharmaceutical products, environmental samples, and water. According to **Strategic Consulting, Inc**. (Woodstock, Vermont) the number of microbiological tests in the world has been increasing steadily from about **700 million** tests valued at **US$1.8 billion** in 1993 to projected **1.5 billion tests valued at US$ 5 billion in 2015.** Currently approximately **33%** of all the tests were done in **North America (USA and Canada), 33%** in **Europe,** and **33%** the **rest of the World.** This author predicts that in the next **15 to 20 years** there will be **25%** testing in **North America, 25%** in **Europe** and **50%** in the **rest of the world** because of improved living standards and awareness of detecting and controlling of microbial pathogens around the world!

Fung's Ten Predictions for the future. The following are the ten predictions made by the author in 1995 as the Key-note speaker of the Food Microbiology Division of American Society for Microbiology from **1995 to 2020.** Many predictions have been correct in **2010.**

(+) is a good prediction.

(?/+) is moderately good.

(?) is an uncertain prediction.

Fung's Prediction in 1995 and the current status in 2010.

1. Viable Cell Counts will still be widely used. (+)
2. Real-time monitoring of hygiene by ATP and other methods will be in place (+)
3. PCR, Ribotyping, and genetic tests will become reality in food laboratories (+)
4. ELISA and Immunological tests will be completely automated and widely used (+)
5. Dipstick Technology will provide rapid answers (+)
6. Biosensors will be in place for HACCP Programs (?)
7. Biochips, microchips, and microarrays will greatly advance in the field (+)
8. Effective separation, concentration of target cells will greatly assist in rapid identification (+)
9. Microbiological alert systems will be in food packages (?/+)
10. Consumers will have rapid alert kits for pathogen detection at home (?)

The future of Rapid Methods and Automation in Microbiology is very bright indeed with many new developments and applications, internationally.

Key References

Caballero. C. G., D. Y. C. Fung, and B.A. Crozier-Dodson. 2010. Comparative Evaluation of the AES-Smacher, Seward Stomacher, Intern-science Bagmixer, and Pulsifier on Viable Cell Counts of Foods, Noise Level, Ergonomics, and Ease of Cleaning. *J of Food Protection* (Submitted).

Fung, D. Y. C. 2000. Rapid Methods and Automation in Microbiology. Inaugural Issue *Comprehensive Reviews in Food Science and Food Safety. Institute of Food Technologists Electronic Publication* Vol 1, Issue 1, pages 3 to 22. <www.ift.org>.

Fung, D. Y. C. Fung. 2007. Rapid Microbiological Methods in Food Diagnostics. In Advances in Food Diagnostics. Nollet, L.M.I. and F. Toldra. 2007. Blackwell Publishing. Ames Iowa. USA.

Fung, D. Y. C. 2008. Rapid Methods for Detecting Microbial Contaminants in Food: Past, Present, and Future. Wilson, C. L. and Droby. S. cd. Microbial Food Contamination. Chapter 7. P. 149-186.

Fung, D. Y. C. Fung. 2010. Microbial Hazards in Foods: Food-Borne Infections and Intoxications. Toldra. F., ed. Handbook of Meat Processing. Wiley-Blackwell. Ames Iowa USA.

Fung, D. Y. C. and W. S. LaGrange. 1060. Microtiter method for bacterial evaluation of milk. *J.Milk and Food Technology* 32:144-146.

Fung. D. Y. C., Fujioka, R. S. , Vijayavel, K. Sato. D. and Bishop, I. 2007. Evaluation of Fung Double tube test for *Clostridium perfringens* and Easyphage test for F-specific RNA Coliphages as a rapid screening test for fecal contamination in recreation waters of Hawaii. *J. Rapid Methods and Automation in Microbiology* 15:217-229.

Wu, V. C. H., V. Gill, R. Oberst, R. Phebus, and D. Y. C. Fung. 2004. Rapid protocol (5.25 hr) for the detection *of Escherichia coli* 0157:H7 *in raw* ground beef by an immuno-capture system (Pathatrix) in combination with Colortrix and CVT-SMAC . *J Rapid Methods and Automation in Microbiology.* 12(1):57-68.