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Methods for Rapid Detection of Foodborne Pathogens: An Overview

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ABSTRACT

Food borne pathogens are a growing concern for human illness and death. There is increasing demands to ensure safe food supply. There is continuous development of methods for the rapid and relible detection of food borne pathogens. Advent of biotechnology has greatly altered food testing methods. Improvements in the field of immunology, molecular biology, automation and computer technology continue to have a positive effect on the development of faster, more sensitive and more convenient methods in food microbiology. Further, development of on-line microbiology, including ATP bioluminescence and cell counting methods, is important for rapid monitoring of cleanliness in HACCP programs. One of the most challenging problems is sample preparation. More research is needed on techniques for separating microorganisms from the food matrix and for concentrating them before detection to ensure food safety, by immunological or nucleic acid-based assays. The possibilities of combining different rapid methods, including immunological and DNA based methods should be further exploited. Further developments in immunoassays and PCR protocols should result in quantitative detection of microorganisms and the simultaneous detection of more than one pathogen or toxin. Lastly, technology continuing to advance at a great pace, the next generation of assays currently being developed potentially has the capability for near real time and online monitoring of multiple pathogens. Modern methods are based on molecular biology techniques like PCR, RFLP, DNA microarray assay, immunological techniques like ELISA, biophysical and biochemical principles with the application of biosensers like bioluminescence sensor, bio-analytical sensors utilizing enzymes, electrical impedometry and flow cytometry. In this review we have tried to summarize the conventional methods and newly developed rapid pathogen detection techniques and the need for newer and rapid methods are discussed.

Key words: Food, pathogen, rapid detection, DNA based methods, bio-sensors

INTRODUCTION

Pathogens are virtually present everywhere, reaching every aspect of life. Potentially threatening bacteria in foods, soil and in water has historically outrun any detection efforts resulting in unwarranted deaths and illness. Current trends in nutrition and food technology are increasing the demands on food microbiologist to ensure a safe food supply. Bacterial pathogens encountered to human illness in the last decades are through consumption of undercooked or

minimally processed ready-to-eat meats (hotdogs, sliced luncheon meats and salami), dairy products (softcheeses made with unpasteurized milk, ice cream, butter, etc.), or fruits (apple cider, strawberries, cantaloupe, etc.) and vegetables (Bhunia, 2007; Altekruse *et al*., 2006; CDC, 2006; Doyle and Erickson, 2006; Lynch *et al*., 2006). However, the presence of pathogens in ready-to-eat products is a serious concern since these products generally do not receive any further treatment before consumption. Food animals and poultry are the most important reservoirs for many of the food borne pathogens (Biswas *et al*., 2008), while animal by-products, such as feed supplements, may also transmit pathogens to other animals. The application of untreated manure onto farmland may contaminate soil or water and eventually transmits microbes to fruits or vegetables (Brandl, 2006; Solomon *et al*., 2002). Seafoods are another potential source of pathogens, such as Vibrio, Listeria, Yersinia, Salmonella, Shigella, Clostridium, Campylobacter and Hepatitis A (Carter, 2005; Feldhusen, 2000). The infectious doses of many of these pathogens are very low (10-1000 bacterial cells). Further, consumers have become much more aware of food safety issues as a result of publicity given to food-borne diseases in the media (Griffiths, 1993; Chang, 2000; Park, 2001). Hence, we are in urgent need to implement programmes such as HACCP as a part of Good Manufacturing Practices (GMP) and Sanitary and Phytosanitary measures (SPS) to monitor the quality of the products produced for the presence of the pathogens and microbial toxins (APHA, 1987). This is an ideal situation wherein rapid methods such as online monitoring system can be useful to quickly screen large number of samples and thereby enhancing the processing efficiency. The analysis of food for the presence of both pathogenic and spoilage bacteria is a standard practice for ensuring food safety and quality (Doyle, 2001). However, the advent of biotechnology has greatly altered food testing methods and there are numerous companies that are actively developing assays that are specific, faster and often more sensitive than conventional methods in testing for microbial contaminants in food (De Boer and Beumer, 1999).

A rapid method can be an assay that gives instant or real time results, but on the other hand it can also be a simple modification of a procedure that reduces the assay time. These rapid methods not only deals with the early detection and enumeration of microorganisms, but also with the characterization of isolates by use of microbiological, chemical, biochemical, biophysical, molecular biological, immunological and serological methods (Boening and Tarr, 1995; Yongsheng *et al*., 1996; Westerman *et al*., 1997; Groisman and Ochman, 2000; Shah *et al*., 2003; Naravaneni and Jamil, 2005; Biswas *et al*., 2008). The degree to which rapid method and automation are accepted and used for microbiological analysis is determined by the range and type of testing required, volume throughput of samples to be tested, availability of trained laboratory staff and the nature of manufacturing practices (Vasavada, 1993).

Conventional methods: Conventional bacterial testing methods rely on specific media to enumerate and isolate viable bacterial cells in food. These methods are very sensitive, inexpensive and can give both qualitative and quantitative information on the number and the nature of microorganisms present in the food sample (Doyle, 2001). Traditional methods for the detection of bacteria involve the following basic steps: pre-enrichment, selective enrichment, selective plating, biochemical screening and serological confirmation (Vunrcrzant and Pllustoesser, 1987). Hence, a complete series of tests is often required before any identification can be confirmed (Invitski *et al*., 1999). These conventional methods require several days to give results because they rely on the ability of the organisms to multiply to visible colonies (Biswas, 2005). Moreover, culture medium preparation, inoculation of plates and colony counting makes these methods labor intensive.

Conventional methods generally regarded as the golden standard often takes days to complete the identification of viable pathogens. Any modification that reduces the analysis time can technically be called rapid method.

Constraints in food analysis: Microbiological analysis of food, especially for particular pathogenic species remains a challenging tusk for virtually all assays and technologies. The problems may be due to the fact (Doyle, 2001):

- Bacteria are not uniformly distributed in the food
- Heterogenicity of food matrices
  - Ingredients such as proteins, carbohydrates, fats, oil, chemicals, preservatives etc
  - Physical form of food (powder, liquid, gel, semisolid or other forms)
  - Difference in viscosity due to fats and oils, which may interfere in proper mixing
- Presence of indigenous microbes which do not cause health risk but their presence often interferes with the selective identification and isolation of specific pathogens, which are usually found in low numbers

Need for rapid method: The effective testing of bacteria requires methods of analysis that can meet a number of challenging criterions. Time and sensitivity of analysis (Table 1) are the most important limitation related to the usefulness of microbial testing. The food industry is in need of more rapid methods which are sensitive for the following reasons (Vunrcrzant and Pllustoesser, 1987):

- To provide immediate information on the possible presence of pathogen in raw material and finished products
- Low numbers of pathogenic bacteria are often present in complex biological environment along with many other non-pathogenic organisms
- The presence of even a single pathogenic organism in the food may be a infectious dose
- For monitoring of process control, cleaning and hygienic practices during manufacture
- To reduce human errors and to save time and labor cost

Separation and concentration techniques: In order to discriminate the target pathogen from other cells a separation steps is normally required as food samples are highly complex consisting of fats, proteins, minerals and even sometimes contains antimicrobial preservatives (Doyle, 2001). Further, they are numerically very low so, efficient pathogen separation and concentration techniques need to be evolved for specific detection of pathogens and to avoid false-negative results.

Table 1: Characteristics of some alternative and rapid methods

Method	Detection limit (cfu mL^{-1} or g)	Time before result	Specificity
Plating technique	1	1-3 days	Good
Bioluminescence	10^4	½ h	No
Flow cytometry	10^2-10^3	½ h	Good
DEFT	10^3-10^4	½ h	No
Impedimetry	1	6-24 h	Moderate/good
Immunological methods	10^4	1-2 h	Moderate/good
Nucleic acid based assays	10^3	6-12 h	Excellent

Several strategies including antibody-based and physical- and chemical-based separation and concentration methods have been developed for separation and concentration of pathogens from various sample matrices (Bhunia, 2007; Chen *et al.*, 2005; Stevens and Jaykus, 2004). This subsequently generates large quantity of material of which only a portion is used for further analysis unless a concentration step is also used. By concentrating the target organisms the detection period can be shortened and more efficient.

Membrane filtration- Direct Epiflourescent Technique (DEFT): Membranes can be made from nitrocellulose, cellulose acetate esters, nylon, polyvinyl chloride and polyesters. Membrane filters are used in modified conventional methods for a variety of purposes:

- To concentrate target organisms from a large volume to improve detection limit
- To remove growth inhibitors
- To transfer organisms between growth media without physical injury through resuspension

DEFT is a direct method used for enumeration of microbes based on binding properties of flurochrome acridine orange. In this food samples are pretreated with detergents and proteolytic enzymes, filtered on to a polycarbonate membrane stained with acridine orange and examined under fluorescent microscope. The number of viable cells is determined based on the count of orange cells on the filter and can be performed in 10 min.

Immunomagnetic Separation (IMS): The isolation stage can be shortened by replacing a selective enrichment stage with non growth related procedures. IMS uses super-paramagnetic particles, which are coated with antibodies against the target organisms to selectively isolate the organisms from a mixed population. IMS is analogues to selective cultural enrichment, whereby the growth of other bacteria is suppressed while the pathogen of interest is allowed to grow. The separation process is consists of two fundamental steps, where the suspension containing target cells is mixed with immunomagnetic particles for incubation no longer than 60 min and finally, they are separated using an appropriate magnetic separator. In the second step, the magnetic complex is washed repeatedly to remove unwanted contaminants and the target cells with attached magnetic particles can be used for the further experiments. Polystyrene beads coated with iron oxide and antibodies (DynabeadsÒ, Dynal, Inc., Oslo, Norway) are the most common magnetic carriers used for concentration and separation of selected microorganism from foods (Skjerve *et al.*, 1990). The Immunomagnetic beads have been used for capture of *E. coli* O157:H7 (Chapman and Ashton, 2003), Salmonella (Jordan *et al.*, 2004) and Listeria (Kaclikova *et al.*, 2001). In recent years, applications of IMS coupled with PCR assays are showing very promising results for the detection of *E. coli* O157:H7 (Fu *et al.*, 2005), Salmonella enterica (Mercanoglu and Griffiths, 2005) and *Listeria monocytogenes* (Amagliani *et al.*, 2006; Ueda *et al.*, 2006). The detection limit for IMS with PCR was 1 cfu/1-25 g of sample following enrichment for *L. monocytogenes* (Hudson *et al.*, 2001). The immune magnetic separation may be employed either directly or indirectly. However, in selective enrichment stage separation, chemical reagents are antibiotics are used to select pathogens,. Since reagents can be harsh and may cause cells stress are injury, LMS is a milder alternative to enrichment; also the elimination of selective enrichment step shortens analysis time. The major drawbacks of the IMS-based assays are the requirement of enrichment and a sample clean up step.

Rapid methods can be classified into the following categories:

- Modified and automated conventional methods
- Biosensor's
  - Bioluminescence biosensor
  - Impedimetry (electrical impedance)
  - Piezoelectric biosensors
    - Flow cytometry
    - Solid phase cytometry
    - Electronic nose
- Immunological methods
- Nucleic acid based assays
    - DNA hybridization
    - Polymerase chain reaction
    - DNA micro assay (Gene chip technology)

Modified and automated conventional methods: Conventional methods used traditionally for microbial analysis are regarded as gold standards except for time delay and labor involved. Many attempts have been made to improve laboratory efficiency by making the procedures for traditional agar based methods more convenient, user friendly and to reduce the cost of material and labor. Several modifications in sample preparation, plating techniques, counting and identification systems have made these conventional methods faster and easier (Vunrcrzant and Pllustoesser, 1987; Doyle, 2001).

Sample preparation: *Gravimetric diluters*-automatically adds the correct amount of diluents to the test sample before homogenization.

Stomacher: Massages samples in a sterile disposable bag eliminating need to sterilize and to use blender cups.

Pulsifier: This apparatus beats the outside of a sterile disposal bag at high frequency (3500 rpm) producing a combination of shock waves and intense stirring which drives the microbes into suspension.

Plating technique: There are several methods of adding sample homogenate to the agar plates. *Spiral plater*-this deposits a small volume on to the surface of the agar in a spiral fashion such that there is a dilution ratio of 10^4 from the centre to edge of the plate. The colonies appearing along the spiral pathway can be counted either manually or electronically. As the volume dispensed at any point is known, this technique eliminates the need for serial dilution before plating and less time requited for colony counting.

Dip slides: The agar slides containing selective or non selective media are pressed on to the surface to be examined and replaced within a sterile sleeve.

Use of flurogenic and chromogenic substrate: In selective media detection, enumeration and identification. This eliminates the use of subculture media and further biochemical tests. These

compounds yield bright color fluorescent products when reacting with specific bacterial enzymes or metabolites. Flurogenic enzyme substrates are derived from coumarin, such as 4-methylnubelliferone, while chromogenic enzymes compounds arc mainly phenol derivatives.

Petrifilms: Alternative to agar poured plates, which consist of rehydratable nutrients that are embedded into a film along with gelling agent, soluble in cold water. One milliliter of liquid sample is placed on the centre of film system and the rehydrated growth of microorganism. After incubation, the colonies can be counted directly from the film system as in conventional plates. These petrifilm products are available for yeast and mould counts, TVC, coliforms and *E. coli*, OI57.

Hydrophobic Grid Membrane Filter (HGMF): Works by confining colony growths 10 a set of 1600 grid cells. These techniques has the advantage of removing inhibitors or unwanted nutrients, concentrating organisms, as well as three log unit range. The food samples obtained are homogenized and pre filtered in nitrocellulose membrane filters, which trap food particles larger than micrometers. This filtrate is then filtered through HGMF, which traps target microbes. The inoculated HGMF are placed in suitable agars and colonies are counted after incubation.

Colony counting: This process is time consuming and several attempts have been made to automate this last step in enumeration of microbes to improve efficiency and labor cost. Image analysis systems have been shown to be useful and cost effective. An image of the plate is stored and can be viewed, printed or imported to other programs. The user can set variables such as top or bottom lighting and colony size limit to exclude spreaders or background particulars.

Identification systems: A variety of morphological, physiological and biochemical tests are used for identification of microorganisms in conventional methods. Now several commercially available kits have been developed to simplify and automate the identification of individual organisms, the result of which is comparable to that of conventional identification systems.

Biosensors: Biosensors are defined as indicators of biological compound that can be as simple as temperature sensitive paints or as complex as DNA-RNA probes. The science of biosensor is a multidisciplinary area. The potential application of biosensor technology to food testing offers several attractive features. Many of the system are portable and hence can be used for field testing or on the spot analysis and are rapid test which are capable of testing multiple analysis simultaneously. Biosensing methods for pathogen detection are centered on four basic physiological or genetic properties of microorganisms: metabolic patterns of substrate utilization, phenotypic expression analysis of signature molecules by antibodies, nucleic acid analysis and the analysis of the interaction of pathogens with eukaryotic cells. Many of today's popular commercially available rapid methods use culture-based methods coupled with automated or semi-automated nucleic acids, antibody, or substrate utilization-based methods to obtain results in 24-72 h. Interestingly, many of the modern-day biosensor-based methods are developed utilizing one of the above four principles or combinations of some sort. However, antibody-based methods are the most popular because of their versatility, convenience and relative ease in interpretation of the data. It is interesting to note that a majority of biosensors use antibody for capture and detection of the target analyte (Ritcher, 1993).

Bioluminescence sensors: Recent advances in bio-analytical sensors have led to the utilization of the ability of certain enzymes to emit photons as a byproduct of their reaction. This phenomenon is known as bioluminescence and may be used to detect the presence and biological condition of the cells. Among the emerging technologies for rapid microbiological analysis, this technique giving results in a short time. Two distinct areas of Bioluminescence are of use in food industry:

ATP bioluminescence: All living cells contain the molecule ATP. This molecule may be analyzed simply using an enzyme and coenzyme complex (Luciferase- Luciferin) found in the tail of fire fly (*Photinus pyralis)*. The total light output of the sample is directly proportional to the amount of ATP present and can be quantified by luminometers. At least 10^4 cells are required to produce a signal. This system lacks specificity, but because of rapid response time for obtaining results, this system is very suitable for on-line monitoring of HACCP programs. This technique has a detection limit of 1 pg ATP which is equivalent to 1000 bacterial cells. ATP is present in both non-microbial and microbial cells. To determine microbial ATP selective extraction is used. First, non-microbial ATP is extracted with non-ionic detergents and then destroyed with high levels of potato ATPase for 5 minutes. Subsequently, microbial ATP is extracted using either trichloro-acetic acid (5%) or an organic solvent (ethanol, acetone or chloroform).

Bacterial bioluminescence: The gene responsible for bacterial bioluminescence (lux gene) has been identified and cloned. The DNA carrying this gene can be introduced into host specific phages. These phages do not posses the intracellular biochemistry necessary to express this gene, hence they remain dark. However, on transfer of lux gene to the host bacterium during infection results in light emission that can be easily detected by luminometers. This technique can detect 1 x 10^2 cells in 60 min. The specificity of this assay depends on phage specificity e.g: Bacteriophage p22 is specific for *Salmonella typhimurium*.

Fiber optic biosensor: Fiber optic biosensor is one of the first commercially available optical biosensors, marketed by Research International (Monroe, WA) for the detection of foodborne pathogens. The basic principle of the fiber optic sensor is that when light propagates through the core of the optical fiber i.e. waveguide, it generates an evanescent field outside the surface of the waveguide. The waveguides are generally made up of polystyrene fibers or glass slides. When fluorescent labeled analytes such as pathogens or toxins bound to the surface of the waveguide, are excited by the evanescent wave generated by a laser (635 nm) and emit fluorescent signal (Bhunia, 2007; Taitt *et al*., 2005), the signal travels back through the waveguide in high order mode to be detected by a fluorescence detector in real time.

Surface Plasmon Resonance (SPR) sensor: SPR is a phenomenon that occurs during optical illumination of a metal surface and it can be used for biomolecular interaction analysis. Receptors or antibodies immobilized on the surface of a thin film of a precious metal (gold) deposited on the reflecting surface of an optically transparent waveguide are used to capture the target analyte. The sensing surface is located above or below a high index-resonant layer and a low index coupling layer. When a visible or near-infrared radiation (IR) is passed through the waveguide in such a way, it causes an internal total reflection on the surface of the waveguide. At a certain wavelength in the red or near-IR region, the light interacts with a plasma or cloud of electrons on the high-index metal surface and the resonance effect causes a strong absorbance. The exact wavelength

of this absorption depends on the angle of incidence, the metal, the amount of capture molecules immobilized on the surface and the surrounding material. The presence of ligands or antigens interacting with the receptor or antibody causes a shift in the resonance to longer wavelengths and the amount of shift can be related to the concentration of the bound molecules. SPR-based sensors are governed by two basic principles: wavelength interrogation and angle interrogation. Wavelength interrogation uses a fixed angle of incidence but measures spectral changes, while in angle interrogation, a fixed wavelength is used but the angle of reflectance is monitored. Most of the commercial SPR systems are operated based on the angle interrogation mode. SPR-based sensors allow real-time or near real-time detection of binding events between two molecules. The detection system is label free, thus eliminating the need for additional reagents, assay steps and time. The sensor can be reused for the same analyte repeatedly. It is highly sensitive and it can detect molecules in the femtomolar range (Bhunia, 2007; Rasooly and Herold, 2006).

Electrical impedance biosensor: Impedance microbiology detects microbes either directly due to production of ions from metabolic end products or indirectly from liberation of CO_2. Microbial metabolism usually results in an increase in both conductance and capacitance, causing a decrease in impedance. A bridge circuit usually measures impedance. This method is well suited for detection of bacteria in clinical samples and to monitor quality and detect specific food pathogens.

In this method, a population of microbes is provided with nutrients (non-electrolyte) like lactose and microbes may utilize that nutrient and convert it to lactic acid (ionic form) thus changing the impedance. This impedance is measured over a period of 20 h after inoculation in specific media. Since this does not involve serial dilution, this technique is simple to perform and faster than agar plate count. This system is capable of analyzing hundreds of sample at the same time since the instrument (Bactometer) is computer driven and automated to enable continuous monitoring. Typically most impedance analysis of food samples can be completed in 24 h. This technique is not suited for testing samples with low number of microorganisms and that the food matrix may interfere with the analysis.

Impedance-based biochip sensor: Though the concept of this detection method is old, now getting wider popularity. Impedance is based on the changes in conductance in a medium due to the microbial breakdown of inert substrates into electrically charged ionic compounds and acidic by-products. The principle of all impedance-based systems is that they measure the relative or absolute changes in conductance, impedance, or capacitance at regular intervals. So threshold value for the detection of target pathogens is mainly depends on initial inoculums and the physiological state of the cells. In media-based impedance methods, bacterial metabolism results in increased conductance and capacitance, with decreased impedance (Invitski *et al*., 1999). The major advantage of this system is that it allows the detection of only the viable cells, which is the major concern in food safety. The basic technical equipment required for performing impedance microbiology consists of special incubators and their culture vessels and an evaluation unit with computer, printer and appropriate software.

Piezoelectric biosensors: This system is very attractive and offers a real time output, simplicity of use and cost effectiveness. The general principle is based on coating the surface of piezoelectric sensor with a selective binding substance for example antibodies to bacteria and then placing it in a solution containing bacteria. The bacteria will bind to the antibodies and the mass of the crystal will increase while the resonance frequency of oscillation will decrease proportionally.

Cell based sensor: Cell-based assays (CBAs) continue to serve as a reliable method for detection of pathogens in food samples. The CBA systems can report perturbations in the normal physiological activities of mammalian cells as a result of exposure to an external or environmental challenge. For this, mammalian cells are used as electrical capacitors. Electrical Impedance (EI) uses the inherent electrical properties of cells to measure the parameters related to the tissue environment. The mechanical contact between cell-cell and cell-substrates is measured via conductivity or EI. The cell can be equated to a simple circuit since it is nothing more than conductive fluid encapsulated by a membrane surrounded by another conductive fluid. The conductive fluids make up the resistance elements of the circuit, while the membrane acts as a capacitor. Changes in impedance were able to detect changes in cell density, growth, or cellular behavior. These biosensors are able to provide detailed information about the growth characteristics of the tissue culture, including information on spreading, attachment and cellular morphology. Mammalian cells have been widely used for the analysis of the pathogenic potential of foodborne bacteria (Bhunia and Wampler, 2005; Gray, 2004).

FOURIER TRANSFORM INFRARED SPECTROSCOPY

Fourier transform infrared spectroscopy (FT-IR) is used to generate bacterial spectral scans based on the molecular composition of a sample and mainly consists of the infrared source, the sample and the detector. It is a nondestructive rapid method and sample identification depends on the available spectral library. When IR is absorbed or transmitted through the sample to the detector, it generates a scan or fingerprint profile. A library of spectral scans can be generated for different bacterial species and strains, which can be used for future comparison. This method requires transfer of cells (biomass) from the growth media to an IR reflecting substrate for spectral collection. FT-IR has been used for classification or identification of several foodborne pathogens: Yersinia, Staphylococcus, Salmonella, Listeria, Klebsiella, Escherichia, Enterobacter, Citrobacter, etc. (Gupta *et al*., 2005; Mossoba *et al*., 2005; Sivakesava *et al*., 2004). FT-IR photoacoustic spectroscopy was used for the identification of spores of several Bacillus species with 100% accuracy (Thompson *et al*., 2003).

Flow cytometry: This may be considered as the form of automated fluorescence microscopy in which instead of sample being fixed to a slide, it is injected into a fluid (dye), which passes through a sensing medium of flow cell. In flow cytometer the cells are carried by laminar flow of water through a focus of light the wavelength of which matches the absorption spectrum of the dye with which the cells have been stained. On passing through the focus each cell emits a pulse of fluorescence and the scattered light is collected by lenses and directed on to selective detectors (photomultiplier tubes). These detectors transform the light pulses into an equivalent electrical signal. The light scattering of the cells gives information on their size, shape and structure. This system is highly effective means for rapid analysis of individual cells at the rate of thousand cells per second.

Solid Phase Cytometry (SPC): SPC is a novel technique that allows rapid detection of bacteria at single cell level, without the need for growth phase (Haese and Nelis, 2002). The short lime detection inherent in this approach is of considerable advantage over conventional plating techniques especially for slow growing bacteria.

SPC combines aspects of flow cytometry and Epifluorescene microscopy. The microbes are isolated from their matrix from membrane filter fluoresecently labeled with argon laser excitable dye and automatically counted by laser scanning device. During 3 min scanning process the entire membrane filter surface is scanned yielding a theoretical detection limit of one cell per membrane filler. During scanning two photo multiplier tubes with wavelength 500 to 530 nm (green) and 540-585 nm (amber) detects the fluorescent light emitted by labeled cells. The signals are processed with software's which differentiate between viable signals (target cells) and background noises (electronic noise and fluorescent panicles). Scanned results are displayed as primary and secondary maps. Actual nature of each fluorescent spot can be further examined by epifluorescent microscope.

Electronic nose: This system comprises of sophisticated hardware with sensors, electronics, pumps, flow controllers, software's, data preprocessing and statistical analyzer. In microbiology the smell of the cultural bacteria often provides a clue to the identification of the organisms present which requires skill. Large amounts of different gaseous components are released from substrates contaminated with spoilage organisms. The traditional approach has been sample extraction followed by gas chromatography, which is tedious and requires some knowledge of the molecules involved. Electronic nose can he applied either in monitoring factors influencing spoilage or factors indicating spoilage. The samples from headspace are passed into the sensor which contains several odor sensors. A computer collects the sensory signals from the sensors where first pretreatment of data is done. The data are further analyzed by software and the results displayed. Several researches are underway to have a clear understanding of the principle of this technique in detection of spoilage organisms.

Immunological methods: Immunological methods rely on the specific binding of an antibody to an antigen. Immunoassay refers to the qualitative and quantitative determination of antigen and antibody in a specimen by immunological reaction. The increased use of immunoassay for rapid detection of microbes is due to:

- Development of new and highly sensitive essays
- Mechanical devices to automate tedious steps
- Techniques to construct predetermined antibodies of specificity (Hybridoma technology)

Polyclonal antibodies contain a collection of antibodies having different cellular origin and therefore somewhat different specificity. The development of Monoclonal antibodies greatly enhanced the field of immunology by providing a constant and reliable source of characterized antibodies. Immunoassays can be classified as:

- **Homogenous immunoassay (Marker free):** In this assay there is no need to separate the bound and unbound antibody; the antigen antibody complex formeda is directly visible or measurable. Incubation times are usually very short, e.g., agglutination reaction, immunodiffusion, turbidemetry
- **Heterogeneous assays:** In this the unbound antibodies must be separated from the bound antibody eg. ELISA. It is the earliest and probably the most prevalent format of antibody assay used for pathogen detection in food. Commercially available ELISA is usually designed as Sandwich assay. ELISA for pathogens have detection limits ranging from 10^{8}-$1O^{5}$ cfu mL^{-1} for whole bacterial cells and few ng mL^{-1} for toxins/protein. Therefore, direct detection of pathogens in food is not possible and enrichment is required for at least 16-24 h

Nucleic acid based assays: Advances in biotechnology have led to the development of a diverse array of assay for detection of food pathogens. Rapid analysis that used nucleic acid hybridization and nucleic acid amplification techniques offer more sensitivity and specificity than culture based methods as well as dramatic reduction in the time to get results. Many methods have also achieved the high level automation, facilitating their application as routine sample screening assays (Wang *et al*., 1997).

Although molecular techniques have improved food microbiology to a great extent, they are not wonder techniques. Certain techniques and methods look good and work well if used in research laboratories by skillful technician, but are not useful for routine testing of food pathogens (Rijpens and Herman, 2002). The essential principle of nucleic acid based assays is the specific formation of double stranded nucleic acid molecules from two complementary single stranded molecules under defined physical and chemical conditions. There are many nucleic acid based assays but only DNA probe and PCR has been developed commercially for detecting food pathogens (Wang, 2002). Recently a number of DNA based molecular typing methods, including Pulse Field Gel Electrophoresis (PFGE); Restriction Fragment Length Polymorphism (RFLP) and ribotyping have also been developed (Ritcher, 1993).

DNA HYBRIDIZATION

The identification of bacteria by DNA probe hybridization is based on the presence or absence of particular genes. A gene probe is composed of nucleic acid molecules; most often double stranded DNA. It consists of either an entire gene or a fragment of a gene with a known function. Alternatively, short pieces of single stranded DNA can be synthesized, based on the nucleotide sequence of the known gene (Laizrd *et al*., 1991). Double stranded DNA probes must be denatured before hybridization reaction, whereas, oligonucleotide and RNA probes, which are single stranded need not be denatured. Gene probes can be labeled with radioactive substances by two methods. Nick translation and random priming technique. Oligonucleotide probes are usually labeled at 5 with 32p, using bacteriophage T4 polynucleotide kinase and gamma AT 32p. Although, radioactive probes seem to have the greatest sensibility in hybridization process, they are potential hazards and disposal of radioactive wastes can be expensive. Currently, labeling of probes with non-radioactive substances such as alkaline phosphatase have been used without effecting the kinetics or specificity of the hybridization (Pitcher *et al*., 1989).

Target nucleic acids are denatured by high temperature (above 95°C) or high pH (above 12) and then the labeled gene probe is added. If the target nucleic acid in the sample contains the same nucleotide sequence as that of the gene probe, the probe will form hydrogen bond with the target. The unreacted, labeled probe is removed by washing the solid support and presence of probe target complexes is signaled by the bound label and detected by autoradiography (Laizrd *et al*., 1991).

POLYMERASE CHAIN REACTION

The PCR is an in-vitro method used to increase number of specific DNA sequence in a sample. PCR is used increasingly in research in food microbiology because of its high sensibility or specificity. By this method, a specific DNA fragment is amplified during a cyclic 3-step process (Olsen *et al*., 1995).

- The target DNA is denatured at high temperature
- Two synthetic oligonucleotides (primers) one annealed to opposite strands at a temperature that only allows hybridization to correct target

- Polymerization is performed with the oligonucleotide as primers for the enzymes and the target DNA as template

When this is performed over and over with namely synthesized DNA as template in addition to original target DNA, an exponential amplification of the DNA fragment between 2 primers is obtained (Biswas *et al*., 2008). Theoretically, PCR can amplify a single copy of DNA a million fold in less than 2 h, hence, it has the potential to disseminate or greatly reduce the dependence on cultural enrichment. In a PCR system, assuming a sensitivity of 1 cell/reaction tube, approximately 10^3 bacteria mL^{-1} sample required to ensure a reliable and repeatable amplification (Wang *et al*., 1997).

LIMITATIONS

Although PCR is a powerful technology, the reactions can be dramatically affected by the presence of inhibitory compounds in foods and selective microbiological media like bile salts and acriflavin. A problem to routine use of PCR in food testing lab is that the procedures are rather complicated and very clean environment is needed to perform the tests. Further, PCR can not distinguish between live and dead cells and hence providing more false negative results (Biswas *et al*., 2008).

DNA MICROARRAYS (GENE CHIP TECHNOLOGY)

This technology used photolithography, which was developed by computer chip makers. A gene chip can be made of glass or nylon membrane and there are two basic variants. In one format, target DNA is amplified by PCR and spread on to a membrane, which is then probed either singly or simultaneously by hundreds of labeled probes to determine specific hybridization. In other format, an array of oligonucleotides are synthesized directly on a glass chip and then exposed to labeled target DNA. DNA chip technology also makes it possible to detect diverse individual sequence in complex DNA samples (Pitcher *et al*., 1989). Development of this approach is continuing at a rapid pace and for microbiologists, this technology will be one of the major tools for the future.

REQUIREMENTS FOR ALTERNATIVE AND RAPID METHODS

There are several factors which must be considered before adapting a new alternative or rapid method:

- **Accuracy:** False-positive and false-negative results must be minimal or preferably zero. The Method must be as sensitive as possible and the detection limit as low as possible. In many cases, the demand is, less than one cell per 25 g of food, as small numbers of some pathogens may cause disease. Analytical tests for these agents need only be qualitative (presence / absence). For rapid screening methods, a higher false positive frequency may be acceptable, as positive screening tests are followed by confirmation tests
- **Validation:** The alternative test should be validated against standard tests and evaluated by collaborative studies. In these studies, preference should be given to naturally contaminated food specimens; the tests are then performed under conditions in which users will apply them. Results obtained with samples containing a low contamination level should be emphasized, since there is sufficient evidence that in most cases high numbers of target cells will lead to positive lest results

- **Speed:** Rapid tests for the detection of pathogens or toxins should give an accurate result within hours or at the utmost one day. However, many detection systems need an overnight enrichment for resuscitation and amplification of the target pathogens, as they rely on the presence of at least 10^4-10^5 organism mL^{-1} for results to be reliable
- **Automaton and computerization:** The ability to test many samples at the same time. Many systems utilizing the microtiter plate format can handle 96 samples at one time. However, for smaller laboratories, the availability of single unit tests is also very important
- **Sample matrix:** New systems should give a good performance of the matrices to be tested. Baseline extinction values may depend on the type of food being tested. Background flora, natural substances or debris can interfere with the lest method and invalidate the test result
- **Costs:** Purchasing reagents, supply, operational costs, up-keeping. The initial financial investment for rapid methods may be high, because, many systems require expensive instruments. Operating costs of many commercial rapid test kits are also high
- **Simplicity:** Methods should be user-friendly, which means easy to operate and manipulate
- Reagents and supply should be rapidly available
- Training, technical service and company support is essential
- **Space requirements:** Instruments are preferably compact and small.

A major disadvantage (Table 1) of alternative and rapid methods over cultural methods is that most methods need damaging of the cells and therefore, viable cells for confirmation and further characterization can only be obtained by repeat analysis using standard cultural procedures. Moreover, rapid methods usually detect only one specific pathogen, while cultural methods may simultaneously detect and isolate many pathogens by including several types of numerous microbiological examinations or samples, selective media in the analysis. The use of several rapid assays to do multi pathogen analyses on a food makes this analysis unacceptably expensive.

LIMITATIONS OF RAPID METHODS

AOAC international approved rapid methods are mostly designed for preliminary screening, negative results are regarded definitive, but positive results are considered presumptive and must be confirmed. Evaluation of rapid methods shows that same methods may perform better in some foods. This may be attributed mostly to interference by normal microbiota or inhibitors in food. In case of an illness investigation, the food implicated may be suspected to contain a particular pathogen based on clear symptoms, but the actual pathogen is unknown. In these situations, when multiple pathogen analysis may be needed it makes the procedure complex and costly.

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