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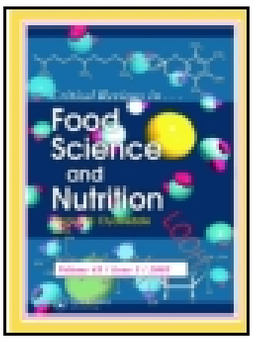

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Advanced Molecular Diagnostic Techniques for Detection of Food-borne Pathogens; Current Applications and Future Challenges

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ABSTRACT

The elimination of disease-causing microbes from the food supply is a primary goal and this review deals with the overall techniques availavle for detection of food-borne pathogens. Now-a-days conventional methods are replaced by advanced methods like Biosensors, Nucleic Acid-based Tests (NAT) and different PCR based techniques used in molecular biology to identify specific pathogens. *Bacillus cereus, Staphylococcus aureus, Proteus vulgaris, Escherichia coli, Campylobacter, Listeria monocytogenes, Salmonella* spp, *Aspergillus* spp. *Fusarium* spp. *Penicillium* spp., and pathogens are detected in contaminated food items which cause always diseases in human in any one or the other way. Identification of food-borne pathogens in a short period of time is still a challenge to the scientific field in general and food technology in particular. The low level of food contamination by major pathogens requires specific sensitive detection platforms and the present area of hot research looking forward to new nanomolecular

techniques for nanomaterials, make them suitable for the development of assays with high sensitivity, response time and portability. With the sound of these we attemet to highlight a comprehensive overview about food-borne pathogen detection by rapid, sensitive, accurate and cost affordable *in situ* analytical methods from conventional methods to recent molecular approaches for advanced food and microbiology research.

Keywords

Pathogens, Biosensor, Nanoparticle, 16S rRNA, PCR, NATs.

1. INTRODUCTION

Food-borne pathogens are of diverse in nature and keep causing major health problems in human worldwide. Many pathogens cause diseases in human which are transmitted through various food items. Microbiological safety of food has become an important concern of consumers, industry and regulatory agencies. Contamination may occur through pollution of air, water, soil and also at various points while raising food crop, harvesting stage, processing and during preparation. The presence of microbes (*Salmonella* spp, *Campylobacter*, *Listeria monocytogenes*, *E. coli.*, etc.) in food poses food poisoning problem. Assessment of quality and safety of food requires microbiological analysis. Growth of microbes can result in organoleptic changes in food rendering it unacceptable to the consumer. To address this problem, the industry is implementing quality assurance systems such as Hazard Analysis Critical Control Point (HACCP).

The rapid detection and identification of microorganisms is an imperative issue in scientific field. Good practices of hygiene would help health care systems significantly to reduce costs of health care, and also provide epidemiological data useful for monitoring infectious diseases (Yager et al., 2006). Microbiological testing is performed with two primary objectives: to establish the absence of pathogens or their toxins to ensure the safety of foods, and to enumerate total or indicator microbial load to monitor effectiveness of hygienic processing and verify product quality and shelf-life stability.

Traditional methods for the detection of microbes in foods rely on culturing of the microbes on to agar plates and these methods are time consuming. Currently available rapid methods are often unsuitable for use in industrial laboratories because lack of sensitivity, expensiveness,

complex to perform and require specialized expertise to operate. There is improvement in traditional methods (Kaltsas et al., 2005) and laboratories have begun to adopt Nucleic Acid-based Tests (NAT) to identify pathogens rapidly. The first NAT was used by the Food and Drug Administration (FDA) through the Gen-Probe PACE test (1988), that used to detect *Chlamydia* and *Gonococci*. The advantages of NAT include rapid results in a short period of time, even though low in number (theoretically a single cell) and detecting specific organism of interest. During the last decades, molecular methods developed extensively. Most important are the PCR-based techniques, which detect DNA of interest specifically. PCR is a very convenient technique for the detection of microbes and multiplex-PCR systems were applied for the detection of few target organisms by using target specific primers.

At present, there are many methods for detecting food-borne pathogens. In this review, we have discussed about using conventional methods, signal based methods and nucleic acid based methods to identify food-borne pathogens. The various rapid methods employed for identification of food-borne microorganisms and future aims are systemaized and discussed.

2. CURRENT CHALLENGES

Detecting the food-borne pathogens associated with many hindered factors in food draws attention for scientic research. There are many varieties of foods like solid, liquid, meat and ready to eat food complexity presents several obstacles in sampling, preparation and analysis. Even many inhibitors in food matrices show their potency in terms of obstacle in detection methods like DNA based assay, PCR technique and antigen-antibody specific assay (ELISA). One of the challenging task is in sample preparation, it is implemented before subjected to

detection. Further, many methodologies are used for the detection of pathogen DNA and toxin in food, but these assays have failed due to poor recovery rate which in turn due to reduced assay accuracy (Feng et al., 2007; Meng et al., 2007). Many amplification techniques would be useful that combine simple design, cost efficiency, roubustness for development of Point Of Care Testing (POCT) which is one of the big challenges in the research yet to achive.

3. CONVENTIONAL METHODS

3.1. Culture and colony based methods

Conventional microbiological method (cultureing of pathogens on media), considered as golden standard method, which is reliable and accurate, but time-consuming and labor intensive. These methods include, blending the food sample with a selective enrichment medium to increase the population of the target organisms and plating onto a suitable media to isolate pure cultures followed by examining the cultures by phenotypic analysis or morphological or metabolic fingerprinting (monitoring carbon or nitrogen utilization). This process takes 2--3 days to get results and next 7--10 days for confirmation (Adzitey et al., 2013). This is an obvious inconvenience in many industrial applications, particularly in food sector. Apart from the above disadvantages, conventional culture methods are still representing a field where progress is possible. These methods are often combined together with other detection methods like an automated or semi-automated DNA amplification, antibody or biochemical-based method to yield more robust results.

3.2. Immunology-based methods

Immunology has developed as a multipurpose technology with numerous applications in the field of agriculture, medicine and in many other areas of biological sciences. Immunological techniques have been demonstrated to be highly specific, sensitive, simple, rapid, cost-effective and it can be automated for large scale applications. Immunodiagnostics is used for the rapid detection of pathogens that cannot be easily identified by other conventional methods. Development of the Enzyme-Linked Immune Sorbent Assay (ELISA) is considered as an important milestone in the advancement of serological diagnosis of diseases caused by microbial pathogens especially by viruses (Clark and Adams, 1977). The basic principle of antibody-based detection (Immunoassay, Fig. 1) is the binding of antibodies to target antigens, followed by the detection of the antigen--antibody complex. ELISA is widely used to develop methods for detecting pathogenic bacteria and bacterial toxins in foods. There is less experimental time required compared to traditional culture based methods but antibody-based detection still lacks, the ability to detect microorganisms in "real-time." Only, if quantities of the pathogen are high enough, then immunoassays can be used to provide real-time information. But the problems that may arise are the low sensitivity of the assays, low affinity of the antibody to the pathogen or analyte being measured and interference from contaminants (Meng and Doyle, 2002).

Immunology-based methods coupled with other methods for pathogen detection like, immune magnetic separation on magnetic beads are coupled with Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF/MS) for detection of *Staphylococcal* enterotoxin B (Schlosser et al., 2007), and combination of immune magnetic separation with flow cytometry for detection of *Listeria monocytogenes* (Hibi et al., 2006; Jung

et al., 2003).The Table. 1. mentions the immunology based assay and its detection limit for food pathogens.

4. SIGNAL BASED METHODS: Biosensors in food-borne pathogen detection

4.1. Bioreceptor

A biosensor is an analytical device, which converts biological response/signals into an electrical signal. This has two main important components: one is bioreceptor to recognize and a transducer, to convert the recognition event to measurable sensitive electrical signal. A bioreceptor may be tissue, organelle, microorganism, enzyme, cell, antibody, biomimic, nucleic acid, etc. and the transduction may be optical, thermometric, electrochemical, magnetic and piezoelectric or combinations of one or more of the above techniques.

Antibodies are common bioreceptors it may be monoclonal, polyclonal or recombinant, based on respective properties and differ in the way they are synthesized. In any case, they are generally immobilized on a substrate near to the detector surface or a carrier (Lazcka et al., 2007). The first report of biosensor-based plant pathogen detection deals with epitope mapping of mAbs on *Tobacco Mosaic Virus* (*TMV*) and *Cowpea Mosaic Virus* (*CPMV)*. The authors tested one virus concentration, as such the assay was unfortunately not challenged in terms of sensitivity (Peter et al., 2008). Many immunological methods involve the use of labeled antibodies, enzymes, biotin, fluorophores and radioactive isotopes is to provide a detection signal in biological assays.

Use of enzymes as biorecognition elements is well developed and is a widely studied area. Enzymes are chosen based on their specific binding capability and their catalytic activity and with a suitable substrate which should provide sufficient electron transfer to the working

electrode (Vo-Dinh and Cullum, 2000). In the field of pathogen detection, the use of labeled enzymes gained more popularity in immunoassay detection than other labeled methods such as radioisotope and fluorescent tag. Enzymes offer the advantages of high sensitivity, the possibility of direct visualization and are stable for years. For example, a report by Chemburu et al., (2005) discussed the application of highly dispersed carbon particles which utilized sandwich immunoassay format used Horse Radish Peroxidase (HRP) enzyme to label the antibody for the detection of pathogenic bacterias such as *L. monocytogenes*, *Escherichia coli* and *Campylobacter jejuni*. Recent advances in nucleic acid recognition, especially the introduction of Peptide Nucleic Acid (PNA) has opened up exciting opportunities for DNA biosensors. PNA is a synthesized DNA in which the sugar--phosphate backbone is replaced with a pseudopeptide. PNA as a probe molecule has several advantages like superior hybridization characteristics, detection of single-base mismatches and improved chemical and enzymatic stability relative to nucleic acids. But the main drawback of PNA is that synthesis is highly expensive (Gall et al., 2003).

In cell based bioreceptors biorecognition is either based on whole cell/microorganism or a specific cellular component that is capable of specific binding to certain species. The ability of cells to recognize and respond to stimuli has made them attractive components for incorporation into biosensors (Sharma et al., 2013). In Cell-Based Biosensors (CBB), a whole cell serves as the molecular recognition element and requires two phases. First is the cells themselves are the primary transducers, converting the detected analyte into a cellular response. A second transducer is required to convert the cellular signal into an electronic signal that can be processed and analyzed. Zhao et al. (2006) developed an artificial cell-based biosensor that used a

liposome-doped silica nano composite, which mimics existing whole-cell assays for *Listeriolysin* O (LLO) a pore-forming hemolysin secreted by the food-borne pathogen *L. monocytogenes*. The immobilized liposomes act as a cellular compartment containing the fluorescent dyes. The dye release due to the pore formation by LLO indicates the presence of the toxin. In contrast to the mammalian cell-based biosensors, the synthetic cell based biosensors have very long self-life and preservation is easier, since these sensors are based on "non-living" system.

The proteins act as a cab used for transport of chemicals from one place to another, such as a carrier protein or gate protein involved in a cell surface. These proteins provide a means of molecular recognition through one or another type of mechanism specifically, active site or potential efficient sites. These types of proteins adhere to different types of transducers for bacterial pathogen detection. The lectin-based sensor array gets exposed to viable cells of Gram-negative and Gram-positive as well as yeast. With this type of biosensor, it was possible to distinguish between five microbial species including *Bacillus cereus, Staphylococcus aureus, Proteus vulgaris, E. coli* and *Enterobacter aerogenes* (Ertl and Mikkelsen, 2001).

A receptor that is designed to mimic like a bioreceptor (antibody, enzyme, cell or nucleic acids) is often termed as biosimilar or biomimetic receptor. The methods like genetically engineered molecules and molecular imprinting technique have emerged as attractive and highly accepted tools for the development of artificial mirror or recognition agents. The technique called molecular imprinting, produces artificial recognition sites by forming a polymer around a molecule which can be used as a template. Biochromic Conjugated Polymer (BCP) is a type of biometric based biosensor for detection of pathogens described by Song (2002). Cell membrane

components having biological activity are incorporated into conjugated polymers with desirable optical properties. Polydiacetylenic membrane-mimicking materials that mimic the cell membrane and conveniently report the presence of pathogens with a color change are used for the colorimetric detection of bacterial toxins and influenza virus.

Spike proteins present in the phages are used to recognize the bacterial receptors. In this case recognition event is highly specific and it can be used for typing of bacteria. Hence yhis has opened the path for the development of specific pathogen detection technologies. The use of phage as a biorecognition element for the detection of various pathogens such as *E. coli* (Singh et al., 2009), *S. aureus* (Balasubramanian et al., 2007) and *Bacillus anthracis* spores (Huang et al., 2008; Xie et al., 2009) by using different sensing platforms is reported.

4.2. Transducers

The transducer plays an important role in the detection process of biosensor. Biosensors can also be classified based upon the transduction methods they employ. Wide varieties of transduction methods have been developed in the past decade for the detection of food-borne pathogens. Although there are new types of transducers constantly being developed for use in biosensors, the transduction methods such as optical, electrochemical and mass based are given importance here since these are the most popular and common methods.

Optical businesses have received considerable interest for the bacterial pathogen detection due to their selectivity. Optical oriented detection offers a vast number of subclasses like Absorption, Refraction, Reflection, Infrared, Dispersion, Raman, Fluorescence, Chemiluminescence and Phosphorescence (Table. 2). However, all these subclasses require a suitable spectrometer to

record the spectrochemical properties of the analyte. The most commonly employed techniques of optical detection are Surface Plasmon Resonance (SPR) and Fluorescence Spectrometry due to their sensitivity. SPR is able to detect recording refractive index having even minor changes, which occur when cells bind to receptors immobilized on the transducer surface and it measures the change of the angle of the reflected light as a function of change of density of medium against time. Direct label-free detection of pathogens is also possible using this method. SPR based biosensors have been reported by many researchers for the detection of food-borne pathogens including *L. monocytogenes* (Bhunia et al., 2004; Koubova et al., 2001 and Taylor et al., 2006) and *Salmonella* (Bhunia et al., 2004).

Electrochemical based detection methods are another possible means of transduction, which have been used for food-borne pathogens identification and quantification. This biosensor can be classified into impedimetric, amperometric, conductometric and potentiometric based on the potential, current, conductance and impedance respectively and their target and assay time are depicted in Table. 3.

In amperometric based detection the sensor potential is set at a value where the analyst produces current. Thus, the applied potential serves as the driving force for the electron transfer and the measurement of current produced due electron transfer from the analyte. Singh et al., (2007) reported that the oxygen electrodes possibly represent the basis for simpler forms of amperometric biosensors, in which current is produced in proportion to the oxygen concentration. Many researchers have reported amperometric detection of food-borne pathogens

such as *E. coli O157:H7* (Varshney et al., 2005), *Salmonella* (Yang et al., 2001), *L. monocytogenes* and *C. jejuni* (Chemburu et al., 2005).

In potentiometric based detection, the bio-recognition process is converted into a potential signal. Usually a high impedance voltmeter is used to measure the electrical potential difference or Electro Motive Force (EMF) between two electrodes at near zero current. Not many potentiometric biosensors are available for the detection of pathogens. Light-Addressable Potentiometric Sensor (LAPS) for the detection of pathogens has been reported (Ercole et al., 2003). Bisha and Brehm, (2010) demonstrated the detection of *E. coli* cells in vegetable food using the Potentiometric Alternating Biosensing (PAB) system based on LAPS. Gehring et al. (1998) developed an Immuno Ligand Assay (ILA) in conjunction with LAPS for the rapid detection of *E. coli O157:H7* cells in buffered saline.

The integration of impedance with biological recognition technology for detection of pathogens has led to the development of impedance biosensors that are finding widespread use in recent years (Yang and Bashir, 2008). The Impedimetric transduction technique has been applied to detect or quantify a variety of food-borne pathogens. Electrochemical Impedance Spectroscopy (EIS) is playing an important role in the biosensor development. Yang et al. (2004) used inter digitized microelectrodes as impedance sensors for rapid detection of viable *Salmonella*.

Conductometric based biosensors bond the relationship between conductance and a bio-recognition technique. Normally, a conductometric biosensor consists of two metal electrodes separated by a certain distance and an AC voltage applied across the electrodes causes a current

flow. During a bio-recognition event the ionic composition changes and the change in conductance between the metal electrodes are measured.

The development of an 'electronic nose' for pathogen detection has received considerable attention in recent years. Balasubramanian et al. (2005) used a commercially available Cyranose-320™electronic nose system to identify *S. typhimurium* in inoculated beef samples. An electronic nose contains an array of 32 conducting polymer sensors to obtain the odour patterns of the headpiece of the meat samples was used. The volatile organic compounds emanating from vacuum packaged beef strip was analyzed. Their results proved that the electronic nose system was able to identify contamination of *S. typhimurium* in meat samples at a population concentration level ≥ 0.7 log10CFU/g. Recently, Arshak et al. (2007) reported the use of an array of conducting polymer composite sensors containing both carbon black and poly aniline to detect and identify the food-borne bacterial pathogens such as, *Salmonella* spp., *B. cereus* and *V. parahaemolyticus* through production of an individual response pattern for each bacterium. Their work demonstrated the potential application for the on-site identification of food-borne pathogens where these sensors could be interfaced with handheld devices to quantify emissions emanating from samples of contaminated foods. Apart from this, the advantages of biosensors are highly selective, fast, accurate and bind powerfully to the target under particular condition and have disadvantages like cost expensive, activity may lose at a particular time, response time is low in tissue material, recovery time is very high due to binding capacity and harsh condition is needed to reverse the reaction.

5. RECENT ADVANCES IN NUCLEIC ACID-BASED METHODS FOR DETECTING PATHOGENIC MICROORGANISMS

A researcher searching continuously for sensitive tools for detecting pathogenic microorganisms, which is currently the hot area for detecting pathogens is nucleic acid oriented techniques, and these are discussed below. In order to get the best performance and reproducibility of the results by various methods of identification, it is mandatory to follow the standard protocols with utmost care even for sample preparation, DNA isolation and preservation of the template DNA as these are going to decide the fate and quality of experiments (Weimer et al., 2001). The rapid NAT methods in molecular biology open up for food pathogen discrimination was explained in this study.

5.1. Cycling amplification technologies

5.1.1. Polymerase Chain Reaction

Since the mid-1980s the Polymerase Chain Reaction (PCR) technology has proved to be a valuable method for the detection of pathogens in food samples. Numerous reports have been published on detection by PCR pertaining to different food-borne pathogens including *E. coli* (Tsai et al., 1993 and Naravaneni and Jamil, 2005); *Salmonella* spp. (Rahn et al., 1992); *Shigella* (Frankel et al., 1990); *Yersinia* (Ibrahim et al., 1992); *Vibrio cholerae* (Shangkuan et al., 1995); *Vibrio parahaemolyticus* (Tada et al., 1992); *Vibrio vulnificus* (Brauns et al., 1991); *L. monocytogenes* (Simon et al., 1996), and *S. aureus.* (Wilson et al., 1991). Technique to detect a single bacterium was released almost 30 years ago with the invention of PCR. This method can detect a single copy of a target DNA sequence with respect to single pathogen in food. It is

permissible because it is amplifies the target organism sequence rather than the signal and by producing less false-positives. So, this amplify 1-million-fold of target DNA in less than an hour, with efficiency to the target pathogen (Batt, 2007). So PCR has become a very widely used detection method for the food-borne pathogens using nucleic acid as a target. PCR has distinct advantages over culture and other standard methods for the detection of microbial pathogens and offers the advantages of sensitivity, specificity, accuracy, rapidity and capacity to detect small amounts of target nucleic acid in a sample (Toze, 1999). Recently it was reported that PCR assay was found to be the most sensitive in the detection of *Salmonella* in seafood when compared to culture and ELISA methods. It is essential to include appropriate controls in the application of PCR for the detection of pathogens in food samples (Murphy et al. 2007).

This assay employs the utilization of DNA polymerase enzyme, which amplifies specific fragments of the target DNA molecule added to the reaction (Powledge, 2004). These nucleotides are named as primers and contain the sequences complementary to the target sequences of the target DNA molecule. First and most often used enzyme is *Taq* DNA polymerase (isolated from the bacterial species *Thermus aquaticus*) but the *Pfu* DNA polymerase isolated from *Pyrococcus furiosus* is also often used due to its high reliability of copying of the DNA sequence. Although these two enzymes are different they possess some mutual features that make them applicable in the PCR reaction: they can generate a new fingerprint of DNA, from the template DNA sequence (Valasek and Repa, 2005).

Thermal stability is necessary due to the fact that at the beginning of every PCR cycle double DNA helix is denatured to single strand form (‘'it is melted'’) by the application of heat at

high temperature (93-96°C) in the reaction tube. The temperature at which half of the DNA molecules become single-stranded is called melting temperature (Tm). The second phase of the PCR cycle is the primer annealing to the specific complementary sequences of the target single-stranded DNA molecule. Primers suppress the re-annealing of the single DNA strands and enable DNA polymerase to start the synthesis of a new strand. This is the primer annealing phase and it is performed at 65-75°C. Third phase is the elongation phase (at approximately 72°C) which involves binding of the nucleotides from the reaction mixture to the complementary ones of the target sequence. After that, primers get displaced resulting in the creation of two copies of a target DNA segment. The PCR primers used for amplifying the *ipaH* gene of *Shigella* (Hartman et al., 1990) were 59-GTTCCTTGACCGCCTTTCCGATACCGTC-39F and 59-GCCGGTCAGCCACCCTCTGAGAGTAC-39R; the primers used for amplifying the *invA* gene of *Salmonellae* (Eichelberg et al., 1994) were 59-ACCACGCTCTTTCGTCTGG-39F and 59-GAACTGACTACGTAGACGCTC-39R; and the *L. monocytogenes* having the gene hemolysin were amplified by the primer 59-CGGAGGTTCCGCAAAAGATG-39F and 59-CCTCCAGAGTGATCGATGTT-39R (Furrer et al., 1991). The expected size of the *Shigella ipaH* PCR product is 600 bp; the expected size of the *Salmonellae invA* PCR product is 941 bp; and a 234-bp product results from targeting the hemolysin gene of *L. monocytogenes*.

The homology between primers and the target DNA confers specificity to the amplification. The presence of given reaction amplified product reveals that the test sample having the target organism. The visualization of the amplified PCR product is done by ethidium bromide (EtBr) in agarose gel. Traditional method was recently replaced by the less toxic and sensitive SYBR GREEN, it is one of dye used to intercalate the double stranded DNA and detected by emitted

fluorescence. Real time PCR also uses the SYBR GREEN for quantification of the product on time.

Real time PCR or quantitative PCR (qPCR) is another adaptation of the PCR method to quantify the number of copies of nucleic acids during PCR. Thus, qPCR is used to quantify DNA and cDNA, determining gene or transcript numbers present within different samples (Lobert et al., 2010). qPCR offers advantages such as speed in getting the result, reduced risk of contamination and the ease in handling technology (Mackay et al., 2002; Maibach and Altwegg, 2003). The real-time PCR machine is a thermal cycler which is able to stimulate the fluorescent dye with a laser for quantifying amplification product every minute of cycle and show exponential and plateau phases. Real-time PCR has been used to quantify the amount of *Phytophthora* DNA with specific gene primer methods of detection and identification has proved to be inherently more specific and sensitive than traditional methods (Martin et al., 2009).

Most NATs for bacterial detection are DNA-based because the genomes of bacteria are DNA, rather than RNA as with some viruses. However, reverse-transcriptase PCR (RT-PCR)-based tests for bacteria have been published such as the one for the detection of hemolysin from *V. parahaemolyticus* described by Nakaguchi et al. (2004). RT-PCR is a method used for studying gene expression and uses RNA as its template to produce complementary DNA (cDNA). This PCR was designed to amplify RNA sequences (especially mRNA) through synthesis of cDNA by reverse transcriptase (RT). Subsequently, this cDNA is amplified using PCR. This type of PCR has been useful for diagnosis of RNA viruses, as well as for evaluation of antimicrobial therapy. It has also been used to study gene expression *in-vitro*, because the obtained cDNA

retains the original RNA sequence. The main challenge of using this technique is in mRNA of test samples especially handling of the low level mRNA of interest, coupled with low stability at room temperature and sensitivity to ribonucleases and change in pH.

Several alternative amplification methods have been already developed, such as Loop Mediated Isothermal Amplification (LAMP) (Tomita et al., 2008), Ligase Chain Reaction (LCR) (Wiedmann et al., 1994), Ligase Detection Reaction (LDR) (Cariani et al., 2012), Low-Stringency Single-Specific-Primer (LSSCP) (Mohamed, 2012) and Single Stand Conformation Polymorphism (SSCP) (Kanstantinos et al., 2008).

5.1.2. Loop Mediated Isothermal Amplification

Amplification of DNA is essential for the detection of target food pathogen. Loop Mediated Isothermal Amplification (LAMP) method does not require a cyclic process with a specific temperature profile, but amplifies DNA with greater specificity, efficiently and rapidly (Nagamine et al., 2002). Several software programs are presently available online for LAMP primers development and these include:

• LAMP designer (Premier Biosoft International, Palo Alto, Calif., USA).
http://www.premierbiosoft.com/tech_notes/Loop-Mediated-IsothermalAmplification.html

• Eiken Chemical Co., Ltd., Tokyo, Japan. PrimerExplorer V4 software.
http://primerexplorer.jp/e/v4_manual/index.html

To design the LAMP primers following things are nessory to remember: (1) both ends of the inner primers should not be AT-rich, (2) the Tm value for each domain should be ~55--65°C, (3) the distance from the 5'end of F2 to the 5'end of F1 and the 5'end of B2 to the 5'end of B1 sites

should be 40--60 bp, (4) the length of the amplified DNA region (from F2 to B2 sites inclusively) should not be >200 bp, and (5) HPLC-purified primers are recommended. LAMP has a simple visual amplicon detection system, having auto-cycling strand displacement DNA synthesis, which is carried out at 60-65°C for 45-60 min in the presence of *Bacillus stearothermophylus* (Bst) DNA polymerase, deoxyribonucleotide triphosphates (dNTPs), specific primers and the target DNA template. LAMP has a simpler sample preparation steps compared with conventional PCR and real-time PCR. With simple alteration the reaction product can be visualized without costly specialized equipment and LAMP reaction is taking 30-60 min (also resistance to various inhibitory compounds hidden in the samples compared to normal PCR). Hence there is no need of extensive DNA purification (Ha et al., 2007). During LAMP reaction large amount of amplification byproduct pyrophosphate ion is produced. Use of Mn++ in conjunction with calcein as fluorescence indicator of DNA amplification in LAMP reactions has been explained by Tomita et al. (2008). Calcein strongly fluoresces under U.V. forming chelation complexes with divalent metallic ions such as Ca++ and Mn++. When Mn++ ions complex with calcein fluorescence is quenched. Before the amplification reaction, calcein is combined with Mn ++ ions and makes UV fluorescence quenched and the reaction solution colour is orange. Newly generated pyrophosphate binds preferably with Mn++ and calcein combined with Mg ++ions, resulting in enhanced fluorescence. Combination with reverse transcription (RT), LAMP amplifies ribonucleic acid (RNA) sequences with more accuracy and detect target DNA at few copies in the reaction mixture. LAMP has the potential to help basic research on medicine, environmental hygiene, point-of-care testing and cost-effective diagnosis of infectious diseases

(Fakruddin, 2011). LAMP is as suitable for DNA sequencing as PCR, in terms of both Sanger sequencing and Pyrosequencing (Fakruddin and Chowdhury, 2012).

5.1.3. Ligase Chain Reaction PCR

LCR is a new technique used to detect nucleic acid sequence of microorganisms by DNA amplification. It is similar to PCR but, only probe molecules amplify through polymerization of nucleotides. Two probes for each DNA strand are ligated together to form a single probe. LCR uses thermostable DNA polymerase and a DNA ligase enzyme to drive the reaction. This technique has one disadvantage in the detection of food pathogen as, it can detect DNA from dead organisms. LCR employs specificity of ligase enzymes to achieve allelic discrimination. It is one of the newly developed amplification techniques for the detection of point mutation in microbial pathogens. The unique feature of LCR is the second primer set, mirror to the first pair, which is designed with the nucleotide at the 3' end of the upstream primer denoting the sequence difference. In the presence of target DNA, the adjacent probes are ligated by thermostable DNA ligase. If there is a mismatch at the primer junction, it will be discriminated against by thermostable ligase and the lack of PCR product. The absence of the ligated product indicates at least a single base-pair change in the target sequence. Subsequently, the ligated products can serve as templates and be amplified exponentially by thermal cycling (Wiedmann et al., 1994). One drawback is that, the target is amplified but contamination risk and variation in copy number of the plasmid containing the LCR target is also a source of error. LCR has a very well discriminating power at a target nucleotide site, such as single nucleotide base change.

5.1.4. Ligase Detection Reaction PCR

Detection of specific DNA molecules by enzymatic ligation was developed by Landegren and coworkers in 1988 to overcome the limitations of oligomeric probes in distinguishing single base mutations associated with genetic diseases (Landegren et al., 1988). The oligomeric ssDNA probes typically are specific at lengths of 20-25 nucleotides, their sensitivity is compromised due to their relatively low dissociation temperature (Td). Advantages of ligation based detection are both probe hybridization and catalyticall selectivity of DNA ligase to improve specificity and sensitivity of detection. Since ligases favor perfect complimentary of a double stranded DNA structure to successfully catalyze the sealing of a nick in the phosphodiester backbone, they can be used to link two adjacently hybridizing probes covalently together in the presence of a correct target molecule. The probes are target-specific which detect only if the probes become ligated. The "discriminating" probe is designed such that the 3'-end matches the target at a unique position which contains a nucleotide that distinguishes the target from other DNA strands. The first implementation of this technique employed the T4 ligase (Landegren et al., 1988), but further improvements have utilized thermostable ligases enabling thermal cycled reactions with higher ligation product yield and better sensitivity (Barany, 1991).

The presence of a point mutation is assessed by the ligation of the two adjacent oligonucleotides (Grossman et al., 1994). The LDR (Gerry et al., 1999), represents a reliable technique for identifying one or more sequences differing by change in single-base and due to chromosomal aberrations at target-nucleotide sequences. The two oligonucleotide are used as probes for target sequence, which are 5′-Fluorescently labeled and 3′-phosphorylated called 'Common Probe'. To the previously PCR amplified sample, the oligonucleotide probe pairs and a thermostable DNA ligase blended. The two probes hybridize consecutively along the template and the DNA ligase

joins their ends only in the case of a perfect match. The PCR--LDR approach usually, is associated with the hybridization onto are Universal Array (UA), where a set of artificial sequences, called Zip-codes are arranged (Gerry et al., 1999). This entire approach is rapid, sensitive and adaptable easily. Examples could be in environmental monitoring (Castiglioni et al., 2004; Rantala et al 2008), forensics and food industry (Bordoni et al., 2004).

5.1.5. Low-stringency Single-Specific-Primer PCR

Low-stringency Single-Specific-Primer PCR (LSSP-PCR) is an extremely simple PCR based technique, that allows the detection of single or multiple mutations in gene sized DNA fragments. In the two steps, the first step is specific PCR (sPCR) to obtain the DNA template to be used and the second one is LSSP-PCR, which uses low-stringency conditions and only one primer, usually used in the sPCR.

Briefly, template DNA fragments are subjected to PCR using high concentrations of a single specific oligonucleotide primer, with large amounts of Taq DNA polymerase and under low annealing temperature. Under these conditions, the primer hybridizes specifically to its complementary region and nonspecifically to multiple sites within the DNA fragments in a sequence-dependent manner, producing a heterogeneous set of reaction products that constitutes a unique “gene signature profile.” Thus the reaction yields a large number of products that can be resolved by electrophoresis to give rise to a multiband DNA signature that reflects the DNA template sequence. Small change in the single base mutation could drastically alter the signature band pattern, producing new signatures as a diagnostic tool (Vago et al., 1996; Pena et al., 1994). LSSP-PCR has been broadly used in the detection of mutations in human genetic diseases (Vago

et al., 1996), sequence variations in human mitochondrial DNA (Barreto et al., 1996) and for genetic typing of infectious agents such as *Human Papilloma Virus* (HPV) (Villa et al., 1995), *Trypanosoma cruzi* (Vago et al., 1996), *Trypanosoma rangeli* and *Leishmania infantum* (Alvarenga et al., 2012).

5.1.6. Single-Strand Conformation Polymorphism PCR

Single-Strand Conformation Polymorphism (SSCP) analysis is generally considered as the most suitable method for the detection of mutations in short stretches of DNA. The SSCP process involves PCR amplification of the target fragment, denaturation of PCR product with heat and formamide (or other denaturants like sodium hydroxide, urea and methyl mercury hydroxide) and electrophoresis on a non-denaturing polyacrylamide gel described by Chandrashekar et al. (2012). Single stranded DNA (ssDNA) fragments fall into unique conformations determined by their primary sequence whose structures are stabilized by intramolecular interactions (Kusakabe et al., 1993). As a consequence, even a single base alteration can result in a conformational change, which can be detected by the altered mobility of the ssDNA molecule in SSCP. Several parameters have been empirically found to affect the sensitivity of SSCP analysis (Hayashi and Yandell, 1993). Among them are included (i) type of mutation; (ii) size of DNA fragment; (iii) G and C content fragment; (iv) content of polyacrylamide or other gel matrix composition; (v) gel size and potential; (vi) gel temperature during electrophoresis; (vii) DNA concentration; (viii) run time of the electrophoresis; (ix) buffer composition, including ionic strength and pH and (x) buffer additives such as glycerol or sucrose.

The mobility of ssDNA depends on the secondary structure of amplified products. The different bands of ssDNA on the gel indicate different sequence SSCP fingerprints of particular pathogen. PCR--SSCP has also been used to locate mutated sequences in bacteria *Bacillus subtilis*, Hepatitis C virus (Lareu et al., 1997), *Human Papiloma Virus* type 16 (Van, 1995) and also searching for mitochondrial DNA point mutations (Jaksch, 1995). Moreover, the subtraction of mitochondrial cytochrome C oxidase subunit I (Hu et al., 2002) and identification of an *Ralstonia solanacearum* in tomato (Umesha et al., 2012), the diagnosis of *Leptospira* (Chandan and Umesha, 2013) were accomplished.

5.1.7. Restriction Fragment Length Polymorphism

Restriction Fragment Length Polymorphism (RFLP) is a very simple method that uses particular restriction enzyme digestion of the genomic DNA. It is used for the comparison of the number and size (mass) of the fragments produced by restriction endonucleases cutting at a specific recognition site of the target DNA (Ueda et al., 2005). The resulting DNA fragments are examined by electrophoretic separation. Presence, absence, or changes in the mass of the resulting DNA fragments are evidences of changing DNA sequences. This method requires pure culture for the discrimination of bacteria at the species level. RFLP in combination with PCR has been used for the accurate detection of *Staphylococcus* and *Listeria* spp. (Paillard et al., 2003).

5.1.8. Amplified Fragment Length Polymorphism

Amplified Fragment Length Polymorphism (AFLP) represents another genotyping technique based on selective amplification of restriction fragments of DNA molecule (Vos et al., 1995). The method involves restriction endonucleases digestion of total purified genomic DNA

followed by ligation of the resulting fragments by a double-stranded oligonucleotide adapter complementary to the base sequence of the restriction site. The adapters are designed such that the original restriction site is not restored after ligation, thus preventing further restriction digestion. Selective amplification of sets of these fragments in PCR is achieved with primers corresponding to the contiguous base sequences in the adapter, the restriction site plus one or more nucleotides in the original target DNA. The resulting PCR-amplified DNA fragments are then analyzed by gel electrophoresis. AFLP can be applied for the determination of sources of contamination, specially in cases such as live stocks (Siemer et al., 2005). AFLP combined with automated laser fluorescence analyzer been used for rapid and reliable identification at the strain level (Aarts et al., 1999).

Mango malformation disease occurring in tropical and subtropical areas is caused by the presumptive pathogens such as *Fuarium mangiferae, F. proliferatum, F. sacchari, F. sterilihyphosum* and *F. subglutinan*s. The isolates of *Fusarium* spp. were evaluated through analyses of AFLPs and DNA sequences of the genes encoding ß-tubulin (*tub2*) and translation elongation factor 1 (*tef1*) (Lima et al., 2009) were evaluated.

5.1.9. Random Amplified Polymorphic DNA Technique

The Random Amplified Polymorphic DNA (RAPD) technique is a PCR assay that uses arbitrary primers and it can be applied to differentiate races, strains and pathogenic or non-pathogenic isolates (Williams et al., 1990). The primers employed in this technique are very short pieces (ten or fewer bases) of DNA from a known source. It is highly probable that these primers may be able to find some complementary sequences in the target DNA, producing a mixture of DNA

fragments of various sizes. When the products of such a reaction are analyzed by gel electrophoresis, distinct banding patterns are produced and some of these patterns may prove to be specific to certain species or varieties or strains. The patterns themselves may be useful for detection and diagnosis of some pathogenic fungi, but some of the bands in certain cases, may be cut out of a gel and sequenced to produce specific primers for more precise PCR analysis or probes for dot hybridization and other detection procedures.

5.2. Isothermal amplification technologies

5.2.1. Nucleic Acid Sequence Based Amplification

Nucleic Acid Sequence Based Amplification (NASBA) is a technology developed in the early 90's to amplify nucleic acids without the use of a thermal cycler as like in PCR (Compton, 1991). It is isothermal, transcription-based amplification and is sensitive method adjusted for the detection of RNA molecules. Such reaction uses Avian Myeloblastosis Virus (AMV) reverse transcriptase, RNase-H and T7-RNA polymerase with a set of primer for the amplification of the target RNA molecule. In the reaction two oligonucleotide primers are complimentary to the target RNA region, dNTPs for the activity of AMV reverse transcriptase and ribonucleotide tri-phosphates for the activity of T7 RNA polymerase. The reaction is performed at 41°C for 1-2h. At this temperature the genomic DNA stays double stranded and therefore cannot replace the amplification substrate. One of the primers have the T7 promoter sequence amplifies cDNA strand using reverse transcriptase enzyme. The DNA/RNA hybrid formed is digested by RNase-H to produce an ssDNA. The second primer having complementary base pair of the cDNA binds it and polymerizes to form dsDNA by reverse transcriptase. The dsDNA then produces multiple

copies of single ssRNA with the help of T7 RNA polymerase. The resulted single-stranded product could be utilized for hybridization without prior denaturation step. Detection sensitivity of the NASBA assay was determined at 1 cfu/ml (Cook, 2003).

In the NSBA reaction 10-100 copies of target RNA molecules are generated in each of the cycle and after 4-5 cycles approximately 1 million copies of the target sequence are created (Compton, 1991). The number of cycles in the NASBA reaction is significantly less compared to conventional PCR methods where it is necessary to have approximately 20 cycles to obtain 1×10^9 molecules per reaction (Chan and Fox, 1999). NASBA technique is the promising diagnostic tool for the detection of viable microorganisms because it is based on detection and amplification of an RNA molecule. RNA and double strand cDNA accumulate exponentially and are detected by EtBr/agarose gel electrophoresis. Recently, fluorescently labeled probes and a fluorescence scanner are employed to follow the NASBA real-time amplification of a viral RNA genome (Leone et al., 1998). Since NASBA has the same speed and accuracy as PCR, and has added advantage of detection of live pathogens it is going to the represented as a promising laboratory method in food microbiology. The use of NASBA could contribute to the development of an affordable, portable and easy to use pathogen detection kit, suitable even for not so well equipped laboratories (Deborggraeve et al., 2006).

5.2.2. Strand Displacement Amplification

Strand Displacement Amplification (SDA), first described in 1992 (Walker, 1993), is also an isothermic amplification method, which utilizes four different primers containing a restriction site for HincII exonuclease, DNA as a template and an exonuclease-deficient fragment of *Eschericia coli* DNA polymerase 1 (exo-Klenow) for elongation of primers. In a single reaction,

10^9 copies of target DNA can be produced. SDA is the basis for some commercial detection tests such as BDProbeTec (Becton Dickinson, Franklin Lakes, NJ) and has been evaluated recently for the identification of *M. tuberculosis* directly from clinical specimens (McHugh et al., 2004).

5.2.3. Rolling circle amplification

In Rolling Circle Amplification (RCA), a single forward primer is extended by DNA polymerase along a circular template for many rounds, displacing upstream sequences and producing a long single-stranded DNA of multiple repeats. RCA technology enables the amplification of the probe DNA sequences more than 10-12 fold copies/h both in solution and on the solid phase at a single temperature. It has the ability to readily detect down to a few targets-specific circularized probes in a test sample. In RCA reaction, numerous rounds of isothermal enzymatic synthesis are involved. DNA polymerase extends a circle-hybridized primer by continuously around the circular DNA probe of several dozen nucleotides to replicate its sequence over and over again (Cho et al., 2005). A major advantage of RCA is that unlike PCR, this technology is resistant to contamination and requires little or no assay optimization. The capacity of RCA to yield the surface-bound amplification products, offers significant advantages to *in-situ* or microarray hybridization assays. RCA reactions exhibit an excellent sequence specificity that is favorable for genotyping or mutation detection.

5.3. Detection of pathogens by multiple targets

The disadvantage of specific assays is that they are limited in scope and useful only when a particular agent is suspected. For this reason, technologies such as mPCR, microarray, and

broad-range PCR assays have been developed for testing simultaneously more than one organism.

5.3.1. Multiplex PCR

The Multiplex PCR (mPCR) represents the method used for simultaneous identification of several gene sequences belonging to the same pathogen or originating from the mixture of different food-borne pathogens. The main advantage of the mPCR as compared to the conventional method is lower cost. The primary advantage is, less reagent and enzyme (Taq DNA polymerase) utilization. Another advantage is that like pathogens are analyzed individually in addition, short time is required for sample preparation and getting the results. The only limitation is that the amplified fragments of same length cannot be detected and lower quantity of amplified product may not be visible on agarose gel. This could be overcome by, designing the primers longer than those used in conventional PCR having a higher melting temperature (Tm). Magnesium concentration influences the reaction specificity which is one of the most significant factors in the PCR reaction (McPherson and Moller, 2000). Generally, $MgCl_2$ concentration in mPCR is higher than that used in the conventional PCR reaction. Anthony et al. (2000) used an universal primer for the detection of multiple pathogens simultaneously. In this method a single set of primer is used to amplify conserved stretches of DNA from 16S rDNA.

5.3.2. Microarray

Microarray refers to a small, two-dimensional high density matrix of DNA fragments which are printed or synthesized on a glass or silicon slide (chip) in a specific order. Hybridization of the DNA fragments to fluorescently labeled probes could be detected by advanced software and

instrumentation. Microbial Diagnostic Microarrays (MDMs) utilize three kinds of probes: (I) Short, (II) long oligonucleotides and (III) PCR amplicons. Short oligonucleotides have 1--2 mismatches and this display lowers binding capacity and thus there is necessity in utilizing PCR amplification. Long oligonucleotide probes and PCR products on the other hand, are characterized by lower discrimination potential (80--85% sequence homology) but higher binding capacity can also be combined with more generic amplification approaches (e.g., WGA-whole genome amplification). In addition to probe length, position of mismatch also plays a major role in hybridization specificity (Letowski et al., 2004). A planar glass slide with different surface modifications that allow covalent binding of probe molecules is the conventional microarray format widely accepted in research labs. Alternative formats have been developed in tubes or 96-well plates representing commercialized products in the routine market. Bead-coupled microarrays represent another alternative (e.g., xMAP) technology from Luminex (Dunbar, 2006).

The 16S rRNA gene serves as an evolutionary clock and taxonomic marker in bacterial systematic (Santos and Ochman, 2004). The reasons to choose 16S rRNA gene as a diagnostic marker is based on ubiquitous format, the presence of highly variable and conserved regions and the availability of an extensive and publicly accessible database. The alternative markers include universally conserved genes (e.g., *gyrB* or *rpoB*), group-specific functional genes (e.g., *pmoA* or *nirK*), virulence (e.g., set---*S. aureus* eneterotoxin genes) or other target-specific genes such as *iap* (*Listeria* spp. invasion-associated protein). [110] Utilization of more specific alternative marker genes (e.g., virulence or toxin genes) allows for additional typing of detected microorganisms. A clinical laboratory might also consider using a validated microarray for

detection of antimicrobial resistance genes. Recently, Yu et al. (2004) developed and validated a diagnostic microarray for the detection of fluoroquinolone-resistant *E. coli* clinical isolates. Some of the techniques in nucleic acid based detection of pathogens were compared with respect to assay time and detection limit (Table. 4).

5.4. Nucleic acid hybridization technologies in pathogen detection

The discovery of PCR has revolutionized molecular diagnostics by amplification of target molecules. However, non-amplification methods are also available, which can be easily performed in a clinical laboratory. Many of the commercially available non-amplification NATs rely on detection of a specific target by chemiluminescence, calorimetric, or fluorescent signals.

5.4.1. Fluorescent In-Situ Hybridization

Fluorescent In-Situ Hybridization (FISH) with oligonucleotide probes directed at rRNA is the most common method among molecular techniques not based on PCR. The probes used in FISH are 15--25 nucleotides in length, and are covalently labeled at their 5' end with fluorescent labels. It uses fluorescently labeled 16S rRNA probes and fluorescent microscopy to detect. After hybridization, the specifically stained cells are detected using epifluorescence microscopy (Wagner et al., 2003). FISH in combination with flow cytometry has been used for rapid culture-independent detection of *Salmonella* spp., (Bisha and Brehm, 2010). Family-genus, and species-specific FISH probes have been developed and published for the detection of *Chlamydia* spp.(Poppert et al., 2002), *Pseudomonas aeruginosa*, *Helicobacter* spp., (Jansen et al., 2000), *Streptococcus* spp., (Trebesius et al., 2001), *Staphylococcus* spp., (Table. 5) and others.

5.4.2. Line Probe Assay

The Line Probe Assay (LiPA) consists of a nitrocellulose strip with specific oligonucleotide probes attached as discreet parallel lines along the strip. Hybridization results in color change that can be detected visually or by an automated reader. Innogenetics (Gent, Belgium) produces several line probe NATs for bacterial detection like *Mycobacterium tuberculosis* complex and *Mycobacterium* spp., mutations in the rpoB gene showing rifampicin resistance and Treponema pallidum antibodies. Test results were 100% concordant with antibiogram results (Cirillo et al., 2004). The test was recently improved and evaluated with isolates of 642 Mycobacterial spp., and 27 non-mycobacterial isolates (Table. 5) and demonstrated 100% sensitivity and specificity and 99.2% accuracy (Mijs et al., 2002).

5.4.3. Hybridization Protection Assay

Hybridization Protection Assays (HPA) utilize a chemiluminescent acridinium ester detector molecule on a DNA probe that targets the specific bacterial rRNA. The RNA/DNA hybrid is detected in a luminometer. AccuProbe (Gen- Probe, San Diego, CA) HPA tests are available for the detection of *Mycobacterium avium* complex (Table. 5), *Mycobacterium intracellulare* and *Mycobacterium gordonae* (Lindholm and Sarkkinen, 2004).

5.4.4. Peptide Nucleic Acid-FISH

Nielson et al. (1991) discovered a DNA analogue called Peptide Nucleic Acid (PNA). Fluorescently labeled PNAs (Egholm et al., 1993) have been successfully used as hybridization probes in FISH. This probe has better advantages than DNA probes, including stability of

PNA/RNA hybrid due to the uncharged PNA. [121] Also, PNAs enter a bacterial cell easily because of their relative hydrophobicity. PNAs also have higher specificity than DNA oligomers due to the higher Tm of the PNA probe compared to its DNA. In addition to PNA-FISH, theoretically, PNAs could be substituted for DNA oligonucleotides to enhance an assay's performance. PNAs have been incorporated into Chemiluminescent In-Situ Hybridization assays (CISH) (Esiobu et al., 2004) and microarrays (Brandt et al., 2003).

6. Other Techniques in Molecular Biology for Pathogen Detection

6.1. Pulse Field Gel Electrophoresis

Pulse Field Gel Electrophoresis (PFGE) uses molecular scissors called restriction enzymes to cut bacterial DNA at possible restriction sites. These molecular scissors generate a fingerprint of DNA and get separated based on size. First the bacteria are loaded into an agarose suspension, similar to gelatin, then the bacterial cell is opened to release the DNA. PFGE has been used for characterizing *E. coli* (Bidet et al., 2000), *Salmonella*, *Listeria* (Table. 6) and other food-borne pathogens. Databases of these pathogens are stored in Pulse-Net and Food-Net, for access by the Centers for Disease Control and Prevention (CDCP), Food and Drug Administration (FDA) and United States Department of Agriculture (USDA).

6.2. Ribotyping

Ribotyping is a type of DNA based sub typing method in which bacterial DNA is firstly cuts into fragments with restriction enzymes. The restriction enzymes used is PFGE, which cut DNA in bigger fragments, but, in ribotyping reaction genomic DNA are cut into the high number of smaller fragments of 1 to 30 kb in size. The resulting fragments are separated according to their

size through electrophoresis. Further, in southern blotting, DNA probes are specifically bound to target DNA that contain genes coding rRNA synthesis, specially hybridized (to probe specific) for the 16S to 23S rRNA genes (Ryser et al., 1996; Wiedmann et al., 1997). Automated ribotyping has been used for the discrimination of *L. monocytogenes* (Gendel, 2004) and for the characterization of virulence gene polymorphism lineage (Wiedmann etal., 1997).

6.3. Nanoparticles in pathogen detection

Nanotechnology ("nanotech") is manipulation of matter on an atomic, molecular, and supramolecular scale. Nanotechnology involves characterization or manipulation of material structures, devices, materials that are between 1 and 100 nm in size. Advances in the manipulation of these nanomaterials permit binding of different biomolecules such as bacteria, toxins, proteins and nucleic acids. One of the major advantages of using nanomaterials for biosensing is due to large surface area, allowing a greater number of biomolecules to be immobilized and this consequently increases the number of reaction sites available for interaction with a target species. This property coupled with excellent electronic and optical properties facilitate the use of nanomaterials in 'label-free' detection and in the development of biosensors with enhanced sensitivities and improved response times (Gilmartin and O'Kennedy, 2012).

Metallic nanoparticles such as gold and silver (5-110nm in size) have been used in signal amplification of numerous bio-diagnostic devices. Gold nanoparticles (AuNPs) in particular have been used in a variable optical and electrical assays (Table. 7). AuNPs are chemically highly stable, change color on aggregation from blue to red, have ability to scatter the light and show

excellent conductivity. AuNPs are used for *Salmonella typhimurium* and *E. coli O157:*H7 organism's detection at 98.9 cfu/mL and 1--10 cfu/mL respectively. Magnetic nanoparticle elements like iron, nickel and cobalt (size range from 1-100 nm) show excellent conductivity. Quantum dots (2-10 nm) made up of semiconducting fluorescent nanoparticles consisting of a semiconductor material core (normally cadmium mixed with selenium or tellurium), which has been coated with an additional semiconductor shell (usually zinc sulphide) detected *E. coli 0157:H7* 10^3 cfu/ml (brain, heart infusion broth). Carbon nanotubes are allotrope of carbon consisting of grapheme sheets rolled up into cylinders; multi-walled nanotubes (MWNTs, 2--100 nm) are essentially a number of concentric single-walled nanotubes (SWNTs, 0.4--3nm) exhibit photoluminescence have excellent electrical properties; semi-conductors are used for the detection of *E. coli O157:H7* at1cell/mL limit (Yang and Li, 2006; Wang and Irudayaraj, 2008). Thiol modified oligonucleotide covalently bound with gold nanoparticles are being used as a probe in various rapid detection methods. The functioned chemistry is not so popular because of its cost. The approach was taken to use non functioned AuNPs for the detection of dsDNA and ssDNA. In this method citrate coated AuNPs have a characteristic red color in the colloidal state. The aggregation of AuNPs can be easily induced by the addition of salts resulting in purple color; the difference in color could be visualized with naked eye. The negatively charged AuNPs have electrostatic interaction with ssDNA which can uncoil in such a way so that its hydrophilic negatively charged phosphate backbone gets exposed to aqueous solutions and DNA bases interact with the AuNPs surface by VanderWaals forces giving negative charge for AuNPs enhancing their repulsion. These properties have been exploited to design a biosensor which can detect a PCR product directly in the same tubewithin a few minutes. The developed biosensor is

highly specific and sensitive and could detect low concentrations of DNA compared to existing methods (Rho et al., 2009).

6.4. Repetitive Extragenic Palindromic

The different typing methods used, in terms of time, accuracy and costs may allow mainly rep-PCR to obtain timely, accurate results with higher resolution among the different strains involved in hospital outbreak. Among the few publications regarding known methods for typing of *Ochrobactrum anthropi* the relevant papers are those of Bathe et al. (2006) describing the Repetitive Extragenic Palindromic PCR **(**REP-PCR) of *O. anthropi* (Table. 6). All patients developed infection during their stay in hospital Catanzaro, University Hospital (Italy) Oncology O.U Institution. No cases of infection due to *O. anthropi* had been diagnosed before. This was an accurate, efficient and more powerful technique, used for typing and monitoring the spread of bacteria and nosocomial infection control. Apart from the REP-PCR even PFGE and Matrix-Assisted Laser Desorption Ionization-Time-of-Flight (MALDI-TOF) were used for identification and typing of the *O. anthropi* strains recently in Italy (Quirino et al., 2014).

6.5. DNA sequencing techniques

The DNA sequencing technology has made it possible to sequence complete microbial genomes worldwide for scientist rapidly and efficiently. To access the entire DNA sequences of microbial genomes new strategies offers to analyze and understand microbes at the molecular level. Scientists are trying to detect pathogens in biological tissues and study variations in gene expression in response to the pathogenic invasion. These help in designing novel approaches for microbial pathogen detection and designing the drug and executing drug development. Every

single species of pathogen is having a unique genomic signature that differentiates from other organisms. One of the biggest challenges in the scientific field is to develop this nucleic acid signature for each microorganism of interest for rapid and specific detection. Recent evidence shows that variation in the mitochondrial DNA sequence obtained from the Whole-Exome Sequence (WES) data for off-target helps to identify the particular pathogen using point mutations. Currently WES would became a costly way of investigating a suspected mitochondrial disorder of a specific mtDNA disease. However, except cost, WES may become the first port of call for clinicians suspecting the mitochondrial disease in the future (Griffin etal., 2014).

6.6. Plasmid profile analysis

Plasmid DNA profile analysis has been used for typing of diverse species of Gram-negative and Gram-positive bacteria. It is applied and used as a marker for comparing strains in the field of health care and evaluating the potential spread of a resistance gene. Interestingly, plasmids have special properties like transferable to another strain by conjugation under selective pressure, but during the process can be gained or lost spontaneously. The gain or loss of plasmid generates a confusion of genetic relatedness to the isolate and it limits the short-term epidemiological studies. This is especially useful for organisms like *Staphylococcus* spp. (Table. 6) and enterobacteria (Ranjbar et al., 2014).

6.7. Multilocus sequence typing

The limited research publications dealing known Multi Locus Sequence Typing (MLST) and PFGE methods for typing of *O. anthropi* (Romano et al., 2009). Currently, different typing

methods are used for the same pathogen in different laboratories and even when the same method is used, the data are difficult to compare between laboratories and are often can not be used for evolutionary, phylogenetic or population genetic studies. Acceptance of MLST, as the "gold standard" for typing bacterial pathogens would resolve this highly unsatisfactory situation. Multi Locus Enzyme Electrophoresis (MLEE) commonly used for typing and population genetic analysis of pathogenic fungi and parasites, and MLST are useful for the determination of population structures of non bacterial haploid infectious agents and for portable molecular typing of those agents that are weakly or strongly clone (Table. 6). MLST is a nucleotide-based typing method that uses data from housekeeping genes in order to provide a sequence type. MLST provides molecular typing data that are highly discriminatory and electronically portable between laboratories, and is therefore suitable for investigating the genetic relationship of bacteria. The method has been validated with some important pathogens, including *Streptococcus pneumonia, Haemophilus influenzae* and *Neisseria meningitides* (Maiden et al., 1998; Jeffries et al., 2003).

6.8. DNase Treated DNA (DTD) PCR

The main objective of this work is to develop methods to overcome the problems associated with rapid detection of food borne pathogens using PCR based techniques. A multiplex PCR method was developed as a solution to the problem of testing single organism at a time. DNaseI enzyme treatment followed by PCR (that is DTD-PCR] was experimented to find a solution to the problem of false positive results obtained by amplification of DNA from dead cells. Four sets of primers were used for detection of *eaeA, hly, invA* and *gryB* genes of frequently occurring food borne pathogens like *Escherichia coli* O157:H7, *Listeria*

monocytogene, Salmonella enterica and *Vibrio parahaemolyticus* respectively (Table. 6). Experiments proved that DNaseI has the ability to remove DNA from dead cells without causing any damage to the DNA present inside live cells. DNaseI at a level of 10U/100 μL was found to remove DNA sourced from 5×107 dead cells in food systems within one hour of incubation. In the specificity test no interferences or non-specific amplification was observed when the multiplex protocol was tested with 89 strains of bacteria. The method developed was found to be sensitive to a minimum cell count of 102 cells in both pure cultures and in artificially spiked food systems. There were no interference or inhibitory actions when the protocol was applied to shrimps. Thus, this DTD multiplex PCR assay can be practically applied for simultaneous identification of viable cells of four important pathogens including *E. coli* O157:H7, *S. enterica, L. monocytogenes* and *V. parahaemolyticus* (Nadugala and Rakshit, 2007).

6.9. Lipidomics

It is an upcoming active field of biomedical and molecular area of research which includes complex lipidome analysis. Lipidome basically, a comprehensive and quantitative description of a total lipid moiety present in an organism. It involves identification and quantitation of thousands of networks in species and their interactions with other lipids, carbohydrates, proteins and other moieties in-vivo. Lipidomics is subdivided into membrane-lipidomics (description of membrane lipid constituents) and mediator-lipidomics (description of low abundant bioactive lipid constituents) in the species. Lipids act as membrane barriers, signal transducers and primarily sources of energy and by this way they influence outcomes in cardiovascular disease, Alzheimer's disease, bacterial as well as viral infection. When searching

for a new virulence factor like antigen among all lipids in an organism, it was not known at the outset (Layre and Moody, 2013) that *Tuberculosis* (TB) caused by the agent *Mycobacterium tuberculosis* had unique lipid profile among the bacteria since it has approximately 250 lipid enzymes in its genome (only 50 in *Escherichia coli*), and an unusually high number of distinct (Glyco) lipids in its cell membrane and wall. Only major components are known, but the description of the few meticulous chemical composition of many lipid species involved in their biosynthetic pathways and transport leaves great room for further investigations. Analysis of pathogen lipid profile is not totally new. Liquid chromatography and mass spectrometry are fueling the field of lipidomics to allow perception, characterization and quantification of many different classes of lipids. Challenging task to capture the full 'lipidome' of a cell or tissue in a single experiment. This work will open up to identify pathogen specific metabolic pathways. With the use of cell and molecular biology as a novel method will help to dissect the complicated lipid signaling during host--pathogen interactions for applications in drug and biomarker development (Wenk, 2006).

7. PRESENT AND FUTURE CHALLENGES

Contamination of food, water, medicine and ingestible household consumer products is a public health hazard that episodically causes thousands of deaths and each year sickens millions of population worldwide (Hoehl et al., 2012). In spite of the huge number of analytical developments and applications that have come up in food analysis, there are still a good number of issues that need to be improved in this hot area of research. For instance, still hundreds of foodborne infection cases occur throughout the world and population of one-third industrialized

nation's suffer from food-borne illness each year. As regards pathogen detection in foods, microbiologists have developed over the last decades reliable culture-based techniques. These are considered to be the "gold standard" methods but time-consuming. Microarray technologies represent an advance in nucleic acid testing methods. Nanomaterials have proven invaluable in the signal amplification of detection assays resulting in the detection of single cell in some foods. However, the matrix effects of some foods continue to limit the sensitivity of these assays. Functioned magnetic promising nanotechnologies coupled with advances in microfluidics and miniaturized devices could make portable, rapid, sensitive, easy-to-use diagnostic tools for food-borne detection a reality. As a consequence the "farm-to-fork" monitoring of food pathogens would become standard for food safety (Gilmartin and O'Kennedy, 2012).

Early detection methods for pathogens by NATs have drawback somewhere in identifying and quantification because of use of less sensitive methods for identifying pathogen even though they are accurate. A culture based method is time consuming, immunology based method needs specific antibody, that's costly, biosensor method needs highly pure sample preparation because, if any contamination is found in sample leads to false positive results. Without knowing experimental results error data will put to confusion. Even PCR based methods have some drawbacks like, identifying particular microbe needs specially designed probes, but labeling of isotope, fluorescence element or antibody of interest are of costly. To minimize these drawbacks it needs some new and cost adjustable approaches in the field of science to track contamination in food, agricultural commodities, export items from one place to another, packaged items and to identify disease and casual pathogens. Here our duty is to state future executable decisions, because nothing is impossible in science. Utilization of PCR, biosensor and nanotechnology are

the best hottest areas for the development of sensitive detection methods with cost effective, accurate, sensitivity with minimum time. A developing specific nanparticle for pathogen of interest, validation is highly interesting in the field of targeting biology.

There are still many problems waiting to be solved such as sample preparation, elimination of the effects caused by the unspecific binding and cross- hybridization and achieving highest sensitivity in the methods. However, the potential of molecular-biology techniques is almost revolutionary. Furthermore, biosensors are representing a new era in food-borne pathogen detection. It is believed that further development and advances in modern biotechnology, microbial biosensors will have a promising and bright future. More appropriate and presise analytical techniques are in need by the consumer protection and law enforcement for the detection of allergens in foods (Garcia etal., 2012; Xihong etal., 2014; Havelaar etal., 2010). Still many different approaches are emerging but more work is needed to show their application in food-borne pathogen detection line to get accepted by the scientific world. Due to the growing interest of Droplet PCR (dPCR) a "Minimum Information for Publication of Quantitative dPCR Experiments" guide has been recently published by Huggett et al., (2013). Apart from the above diffetent methods for detection of food-borne pathogens, commercially available assasy kits are also popularized because of work in point of care except the cost (Table. 8).

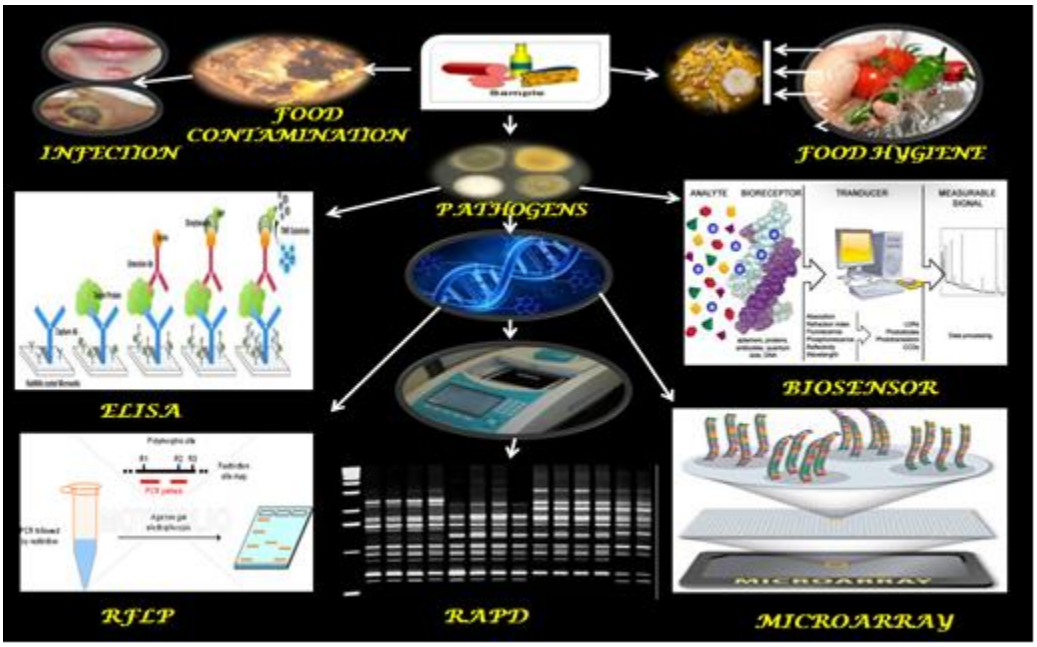
Molecular techniques applied in food microbiology are very promising alternatives to classical cultures, biochemical and serological identification and typification methods. In this regard, one analytical challenge that still remains in food safety is to present reliable results with respect to official guidelines as fast as possible without impairing method properties such as

recovery, accuracy, sensitivity, selectivity, and specificity. Among them, NATs based PCR have been successfully used, not only in control laboratories, but also in research to gain deeper knowledge in the biology and pathogenesis of bacterial species.

8. CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

TOC: Table of content



Highlights:

- Compare to the classical method, molecular based NATs is the best method.
- All NATs overview was represented to overcome the culture based methods.
- PCR based nucleic acid method is one of the suitable methods for pathogen detection.

- DTD-PCR explains the solution for problem associated with false positive results from dead cells.
- Next generation based nanotechnology and metabolite profile in pathogen detection was highlighted.

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Table. 1. Application of immunological-based methods for the detection of food-borne pathogens present in food samples.

	Detection method	**Foodborne pathogens**	**Detection limit**	**Food matrix**	**Assay time**	**References**
1	ELISA	*Escherichia coli O157:H7*	68 CFU/mL in PBS and	Artificially contaminated	3 h	Shen et al., 2014
			6.8×10^3 CFU/mL in	milk, vegetable and		
			food samples	ground beef		
2	Lateral Flow	*Salmonella typhi*	10^4--10^5 CFU/mL	Artificially contaminated	10 h	Kumar et al., 2008
	Immunoassay			food rinses (meat,		
				chicken and vegetables)		
				and milk		
		S. typhi	30 cells/25 g	Artificially contaminated	Not stated	Shukla et al., 2014

Table. 2. Different optical based biosensors for pathogen detection.

	Mode of detection	**Target pathogen**	**Limit of detection (CFU/mL)**	**Assay time**	**References**
1	Optic interferometer	*S. typhimurium*	10^5 CFU/mL	12 h	(Seo et al., 1999)
2	Resonant mirror detection	*Escherichia coli*			(Kiba et al., 2006)
3	Turbidimetry (O.D. measurements)	*E. coli 01 57:H7, Staphylococcus aureus and Yersinia enterocolitica*			(Barco et al., 2000)
4	Automated optical method	*Salmonella spp. and L.monocytogenes*	10--50 cells each in 25 g sample	24 h	(Peng and Shelef, 2001)
5	Optical method using laser beam	*E. coli*	45 cells		(Acharya et al., 2006)
6	Surface-enhanced infrared absorption spectroscopy	*Salmonella*			(Seelenbinder et al., 1999)

7	Bidiffractive grating biosensor (BDG)	*S. aureus enterotoxin B, Clostridium botulinum, ricin toxin and Francisella tularensis*			(O'Brien et al., 2000)
8	Near-infrared spectroscopy	*Septicemia/toxemia (septox) in chickens*			(Dey et al., 2003)
9	Imaging ellipsometry (IE)	*E. coli O157:H7, S. typhimurium, Y. enterocolitica, and Legionella pneumophila*	10^3 to 10^7 CFU/mL		(Choi and Oh, 2008)
10	Quantum dots	*E. coli O157:H7*	10^6 cells/mL		(Hahn et al., 2008)
11	Fluorescence microscopy	*E. coli O157:H7, S. typhimurium, Y. enterocolitica, and Legionella pneumophila*	10^2 CFU/mL		(Choi and Oh, 2008)

12	Chemiluminescence	*E. coli O157:H7*	10^2 to 10^5 CFU/mL	30 min	(Mathew et al., 2004)
13	Chemiluminescence enzyme immunoassay	*E. coli O157:H7*	10^1 to 10^2/g	24 h	(Kovacs and Rasky, 2001)
14	Chemiluminescent immunoassay	*E. O157:H7, Y. enterocolitica, S. typhimurium, and Listeria monocytogenes*	10^4 to 10^5 CFU/mL for all bacterial species		(Magliulo et al., 2007)
15	Bioluminescence	*E. coli*	10^3 cells/mL	2 h	(Frank et al., 2007)
16	Bacteriophage-based bioluminescene	*E. coli O157:H7*	10 CFU/mL	4 h	(Brigati et al., 2007)
		Salmonella	10^8 CFU/mL	1--3 h	(Chen and Griffiths, 1996)

Adapted from: Vijayalakshmi et al., 2010.

Table. 3. Different electrochemical based biosensors for pathogen detection.

	Mode of detection	**Target pathogen**	**Dynamic range/(LOD)**	**Assay time**	**References**
1	Amperometric	*E. coli O157:H7*	7.8×10^{1}	10 min	(Ruan et al., 2002)
		S. typhimurium	1.09×10^{3}	2.5 h	(Yang et al., 2001)
		Salmonella	1--5	6 h	(Brooks et al., 1992)
		E. coli	50 cells/mL	30 min	(Chemburu et al., 2005)
		L. monocytogenes, and C. jejuni	10 cells/mL		
			50 cells/mL		
		S. typhimurium	5×10^{3} cells/mL	2 h	(Che et al., 1999)
2	Potentiometric	*E. coli O157:H7*	7.1×10^{2} cells/mL	45 min	(Gehring et al., 1998)
3	Conductometric	*E. coli O157:H7 and Salmonella spp.*	7.9×10^{1}	10 min	(Muhammad and Alocilja, 2003)
		Bacillus cereus	35--88	6 min	(Pal et al., 2008)
4	Impedimetric	*S. typhimurium*	5.4×10^{5}	2.2 h	(Yang et al., 2004)
		L. monocytogenes			(Tully et al., 2008)
		Bacillus cereus			(Susmel et al., 2003)

		E. coli	10^1 to 10^7		(Munoz-Berbel et al., 2008)
5	Fluorescence	*S. cerevisiae*	0.01%		(Knight et al., 1999)
6	Luminescence	*E. coli*	0.15 ~ 5 mM		(Choi and Gu,2002)
					(Roda et al., 2013)
		M. gryphiswaldense	2 ~ 50%		
7	Voltametric	*E. coli*	2 ~ 400 μM		(Shitanda et al., 2009)

Table. 4. Different nucleic acid based methods for pathogen detection.

	Detection method	**Foodborne pathogens**	**Detection limit**	**Assay time**	**References**
1	Multiplex PCR	*Salmonella* spp.,	10^3 CFU/mL	24 h	Silva et al., 2011
		STEC O26, O103, O111, O145,	5×10^4 CFU/mL	24 h	Verstraete et al., 2012
		E. coli O157:H7,	10^3 CFU/mL	Not stated	Guan et al., 2013
		L. monocytogenes			
		Staphylococcus arueus,			
		Yersinia			
2	Real-time PCR	*L. monocytogenes, E. coli* O157,	<18 CFU/10 g	24 h	Suo et al., 2010
		Salmonella spp			
		L.	2×10^2 CFU/mL	24 h	Kawasaki

		monocytogenes, *E. coli* O157:H7,			et al., 2010
		Salmonella spp.			
		Salmonella spp., *L. monocytogenes*	5 CFU/25g	<30 h	Ruiz-Rueda et al., 2011
		S. aureus, *Salmonella*,	9.6 CFU/g,	<8 h	Ma et al., 2014
		Shigella	2.0 CFU/g and 6.8 CFU/g		
3	NASBA	*E. coli*	40 cells/mL	4 h	Min and Baeumner, 2002
		Salmonella Enteritidis	10^1 CFU/reaction	26 h	D'Souza and Jaykus, 2003.
		L.	400 CFU/mL	72 h	Nadal et

		monocytogenes			al., 2007
		Bacillus amyloliquefaciens, Bacillus cereus and	--		Gore et al., 2003
		Bacillus circulans			
		Salmonella Enteritidis and *Salmonella typhimurium*	<10 CFU/mL	<90 min	Mollasalehi and Yazdanparast, 2013
4	LAMP	*Salmonella* spp. and *Shigella* spp.	5 CFU/10 mL	<20 h	Shao et al., 2011
		Vibrio parahaemolyticus	10 CFU/reaction	16 h	Wang et al., 2013
		STEC O26, O45, O103,O111,	1--20 cells/reaction in pure culture and	Not stated	Wang et al., 2014

		O121,			
		O145, and O157	10^5--10^6 CFU/25 g in produce		
5	Oligonucleotide	*Escherichia coli O157:H7, Salmonella*	1×10^{-4} ng for each genomic DNA	Not stated	Suo et al., 2010
6	DNA microarray	*enterica, Listeria monocytogenes* and			
		Campylobacter jejuni			
		L. monocytogenes	8 logCFU/mL	Not stated	Bang et al., 2013
		Escherichia coli, Shigella spp.,	10 CFU/mL of pure culture	Not stated	Huang et al., 2014
		Salmonella spp., *Proteus* spp.,			

		Campylobacter jejuni, Listeria			
		faecalis, Yersinia enterocolitica,			
		Vibrio parahaemolyticus,			
		Vibrio fluvialis, β-*hemolytic Streptococcus, Staphylococcus aureus*			

NASBA: Nucleic acid sequence based amplification; LAMP: Loop Mediated Isothermal Amplification

Table. 5. Different nucleic acid hybridization technologies for food pathogen detection.

	Detection method	**Target pathogen**	**References**
1	FISH	*Salmonella* spp.,	Bisha and Brehm, 2010
		Chlamydia spp.,	Poppert et al., 2002
		Pseudomonas aeruginosa, *Helicobacter* spp.,	Jansen et al., 2000
		Streptococcus spp.,	Trebesius et al., 2001
2	LiPA	*Mycobacterium tuberculosis*	Mijs et al., 2002
3	HPA	*Mycobacterium avium*	Lindholm and Sarkkinen, 2004
		Mycobacterium intracellulare	
		Mycobacterium gordonae	

FISH: Fluorescent In-Situ Hybridization; LiPA: Line Probe Assay; HPA: Hybridization Protection Assay

Table. 6. Different other technologies for pathogen detection.

	Detection method	Target pathogen	References
1	PFGE	*E. coli*	
		Salmonella, Listeria spp.,	Bidet et al., 2000
2	Ribotyping	*L. monocytogenes*	Gendel, 2004
3	REP-PCR	*Ochrobactrum anthropi*	Bathe et al. (2006)
4	Plasmid DNA profileing	*Staphylococcus* spp.,	Ranjbar et al., 2014
5	MLST	*Streptococcus pneumonia*	Maiden et al., 1998;
		Haemophilus influenza	Jeffries et al., 2003
		Neisseria meningitides	
6	DTD-PCR	*E. coli* O157:H7	*Nadugala and Rakshit, 2007*
		S. enteric	
		L. monocytogenes	
		V. parahaemolyticus	
7	Lipidomics	*Mycobacterium tuberculosis*	Layre and Moody, 2013

PFGE: Pulse Field Gel Electrophoresis; REP-PCR: Repetitive Extragenic Palindromic-PCR; MLST: Multilocus sequence typing; DTD-PCR: DNase Treated DNA (DTD) PCR

Table. 7. Different natechnology based identification of pathogen detection.

	Nanomaterial	Target pathogen	Detection mode	Limit of detection (CFU/mL)	References
1	GNPOPs	*Salmonella and E. coli*	Inductively coupled plasma mass spectrometry (ICP-MS)		Lin and Hamme, 2015
2	MNPs	*L. monocytogenes*	ABeSPIONs; magnetic flux measurement by high-transition temperature SQUID	5.6- 10^6 cells/20 mL and 230 cells/1 nL	Jain et al., 2012
		E. coli	Incubation of target with fluorescein-labeled concanavalin A; epifluorescent microscopy	10^4 cells/mL	Chen et al., 2008
3	GNPs	*Vibrio parahaemolyticus*	AgaroseeGNPs on SPCE; amperometry	7.4 - 10^4 CFU/mL; 10^5e10^9 CFU/mL	Yakes et al., 2008

		E.coli O157:H7	eaeA gene-specific thiolated probe conjugated to piezoelectric biosensor	1.2 -10^2 CFU/mL; 10^2e10^6 CFU/mL	Rosi and Mirkin, 2005
		Salmonella	MABepolystyrene coupled with PABeGNPs; anodic stripping voltammetry	98.9 CFU/mL; 1.3-10^2e2.6 - 10^3 CFU/mL	Kalele et al., 2006
4	SNPs	*E. coli*	Rabbit IgG antibody conjugated with SNPs; SPR band shift using UV	5e10^9 cells/mL	Lin et al., 2008
5	PNPs	*E.coli O157:H7*	AB immobilized on PNPseSPCE; cyclic voltammetry		Mao et al., 2006
6	SiNPs	*E. coli O157:H7,*			
		S. typhimurium			
		and B. cereus	ABeSiNPs; plate-counting and	1-400 cells (plate-	Li and Church,

			fluorescence methods	counting method)	2014
				single cell (fluorescence method)	
7	GNRs	*Pseudomonas aeruginosa*	ABeGNRs by carbodiimide chemistry; NIR light-mediated staining of live/dead cells	75% decrease in cell viability	Grossman et al., 2004
8	CNTs	*Salmonella*	MABeCNTs conjugated to GCE; electrochemical impedance spectroscopy	1.6 _ 10^4 CFU/mL	Dungchai et al., 2008
9	SNC	*Salmonella*	Extent of SNC's bending proportional to bacterial count	25 cells/mL	Joo et al., 2012

MNPs: Magnetic Nanoparticles; GNPs: Gold Nanoparticles; SNPs: Silver Nanoparticles; PNPs: Peptide Nanotubes; SiNPs: Silica N Nanoparticles; GNRs: Gold Nanorods; CNTs: Carbon Nanotubes; SNC: Silicon-Nitride Cantilever; SPCE: Screen-Printed Carbon Electrode;

SPR: Surface Plasmon Resonance; SQUID: Superconducting Quantum Interference Device; AB:

Antibody; CFU: Colony Forming Units.

Table. 8. Different commercially available assay for detection of food-borne pathogens.

	Target pathogen	Method	Total time (h)	References
	Imunologically based			
1	*E. coli O157:H7*	Reveal®,	8.25-20.25	Neogen, 2011d
		Assurance®	10-20	BioControl, 2011a,
		EIA EHEC,	8.17- 18.17	SDIX, 2011a
		RapidChek	19.25-25.25	3M, 2011b
		3M™ Tecra™		
2	*Salmonella spp*	3M™ Tecra™	21-26.5	3M, 2011c,
		RapidChek®	24.17	SDIX, 2011b,
		TRANSIA™	24	BioContro l, 2011d,
3	*L. monocytogenes*	Reveal®	32-50	Neogen, 2011c,
4	*S. aureus*	TRANSIA™	2	BioControl, 2011b
		3M™ Tecra™	22-30	3M, 2011c,
	Nucleic acid based method			
1	*E. coli O157:H7*	BAX®,	11.5-27.5	DuPont, 2011
2	*Salmonella spp*	GeneQuence®	26-50	Neogen, 2011b
3	*L. monocytogenes*	TRANSIA™	46	BioControl, 2011c
4	*S.aureus*	GENE-TRAK ®	1	Neogen, 2011a

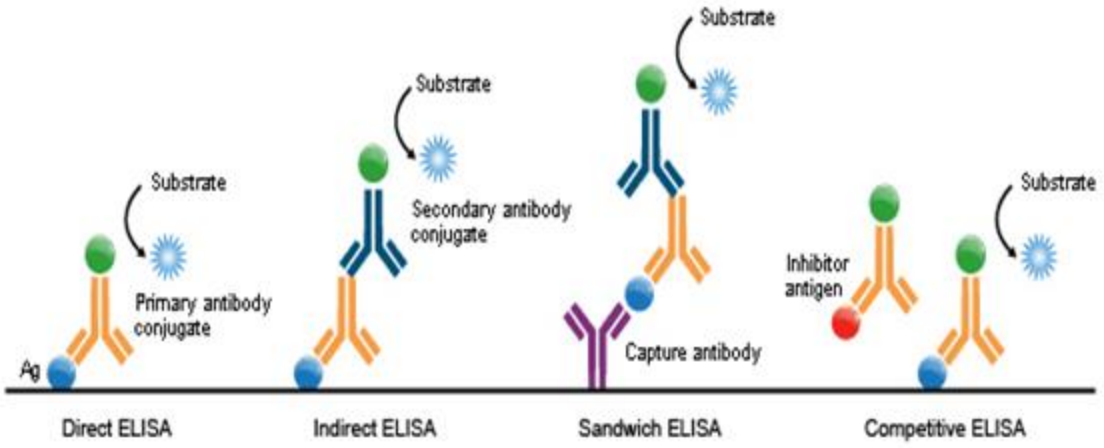

Figure.1: ELISA types and principle.

Detection of antigen can be performed using an enzyme-conjugated primary antibody (direct detection) or a set of unlabeled primary and conjugated secondary antibodies (indirect detection). But sandwich includes specific antigen and epitope interaction attached antibody to base and antigen pool added. The particular antigen of interest captured and it is assayed using primary and secondary antibody of interest. In competitive assay, both antigen and primary antibody of interest mixed and unbound antibody was washed. Then secondary antibody conjugated with enzyme specific to primary antibody added and substrate of interest added to know product by chromogenic or fluorescent signal.

Source: Adapteds from web.