RAPID DETECTION METHODS OF MICROBIAL PATHOGENS IN FOODS – A SHORT SURVEY

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Abstract: During harvesting, processing and handling operations foods may become contaminated with a wide range of microorganisms. This paper is presented as a short survey of recent used laboratory methods for foods microbial pathogen detection, briefly summarizing rapid, specific and sensitive methods useful for foods testing based on immunochemical and nucleic acid technologies. As the world becomes more concerned with safe foods, the demand for rapid detecting will only increase.

Key words: foods pathogen detection, immunological methods, molecular genetic methods

1. Introduction

Microorganisms, such as bacteria and viruses, are found widely in the environment, foods, marine and estuarine waters, soil, and the intestinal tracts of humans and animals. Many of these organisms have an essential function in nature, but certain potentially harmful microorganisms can have profound negative effects on both animals and humans, costing the food industry (and indirectly, the consumer) many millions of dollars each year. It is estimated that infectious diseases cause about 40% of the approximately 50 million total annual deaths worldwide (KRAMER *et al.,* 2005; INVITSKI *et al.,* 1999). The recent well published million-dollar food recalls due to food poisoning bacteria such as *Listeria monocytogenes* (LIN *et al.,* 2005; DONNELLY, 2001; SCHLECH, 2000) has increased the need for more rapid, sensitive and specific methods of detecting these microbial contaminants.

The aim of this overview is to summarize the rapid and sensitive detection methods of pathogens in foods. Here reviewed methods are focused particularly on detection of antigens (immunological methods), and on detection of species-specific DNA sequences (molecular genetic methods). The highlights of these methods lie on their high specificity, rapidity and sensitivity purposes.

2. Conventional detection methods

Traditionally, the detection and identification of bacteria mainly rely on specific microbiological and biochemical identification. Although these methods can be sensitive, inexpensive and give both qualitative and quantitative information on the number and the nature of the microorganisms tested, they are greatly restricted by

assay time, with initial enrichment needed to detect pathogens, which typically occur in low numbers in food and water (LEONARD *et al.,* 2003).

Culture is the term used to describe the biological amplification of cultivatable bacteria using artificial growth media. These media may be liquid (broth), solid (due to the addition of agar, and used for their growth-supporting surface), enriched (with growth-promoting substrates) and selective (with inhibitors to reduce growth of unwanted species) or contain specific indicators to reveal growth of species of particular interest. Bacterial growth also requires the right temperature and other physiochemical and chemical conditions. The bacteria have first to be recovered in pure culture. This requires growth of each, individual species in single colonies. The first identification tests performed include observation of bacterial colony morphology, alteration of the surrounding agar medium, the Gram stain, termed benchtop tests such as coagulase, catalase and oxidase reactions and specific antibody agglutination reactions (CHOW *et al.,* 2006).

3. Immunological methods

Detection with antibodies is perhaps the only technique that has been successfully employed for the detection of cells, spores, viruses and toxins alike (IQBAL *et al.,* 2000). Polyclonal antibodies can be raised quickly and cheaply and do not require the time or expertise associated with the production of monoclonal antibodies (CAHILL *et al.,* 1995). However, polyclonal antibodies are limited both in terms of their specificity and abundance. Since the development of hybridoma techniques (KÖHLER and MILSTEIN, 1975) and the emergence of recombinant antibody phage display technology, developed during the past decade (PETTY *et al.,* 2007), immunological detection of microbial contamination has become more sensitive, specific, reproducible and reliable with many commercial immunoassays available for the detection of a wide variety of microbes and their products (LEONARD *et al.,* 2003). Some of these methods are briefly listed below.

3.1 Serotyping

Serotyping is most widely applied to Gram-negative enteric bacterial pathogens such as *Salmonella* and *Escherichia*. Among Gram-positives, serotyping is important for the genus *Listeria*. The gist of a typical serotyping scheme is the use of specific antibodies (antiserum) to identify homologous antigens (Fig. 1). In many foodborne pathogens, the antigens are particulate, and agglutination methods are employed (JO *et al.,* 2004; LUKINMAA *et al.,* 2004; BAILEY *et al.,* 2002; GORMAN and ADLEY, 2004).

3.2 Fluorescent Antibody (FA)

This technique has had extensive use in both clinical and food microbiology since its development in 1942. An antibody to a given antigen is made fluorescent by coupling it to a fluorescent compound and when the antibody reacts with its antigen,

the antigen-antibody complex emits fluorescence and can be detected by the use of fluorescent microscope (Fig. 2).
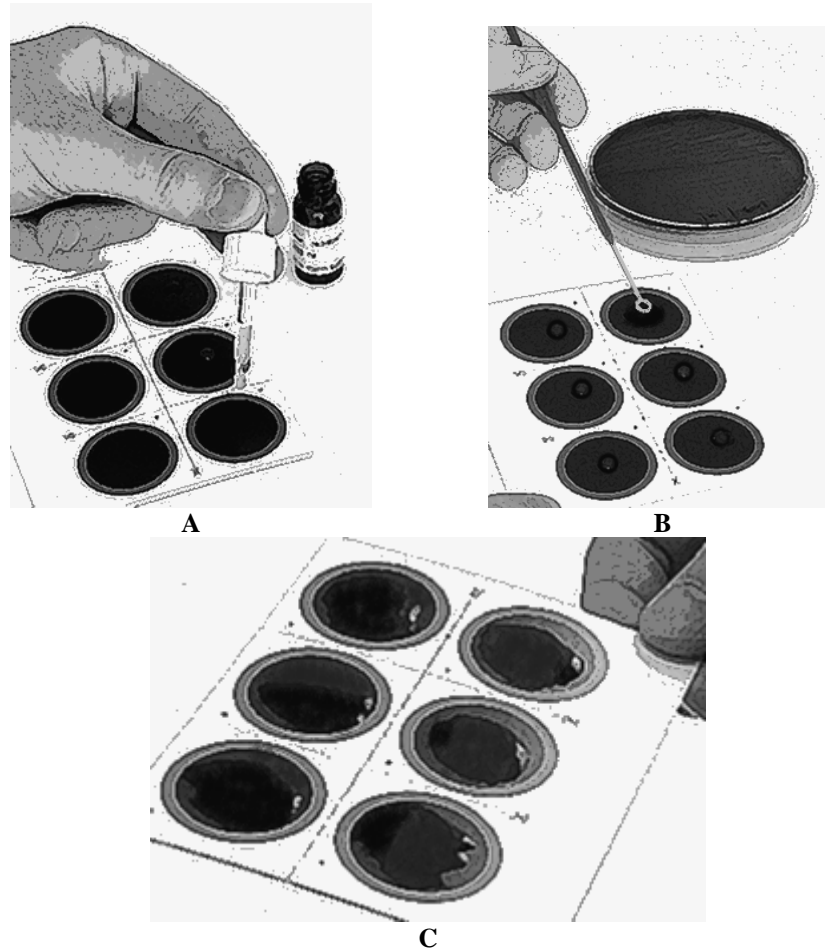

Fig. 1. Group B Streptococcus [GBS] Serotyping Test fy Essum AB. (A) Placing of reagent onto the agglutination site of the test plate. (B) Picking some fresh colonies from the plate where the bacteria have been isolated and their transfer adjacent to the test plate. (C) Gentle agitating the test plate and reading the agglutination pattern while agitating (processed according to PERSSON *et al*., 2004)

The fluorescent markers used are rhodamine B, fluorescein isocyanate, and fluorescein isothiocyanate, with the last being the most widely used. The FA technique obviates the necessity of pure culture isolations of *Salmonellae* OH globulin labeled with fluorescein isothiocyanate with somatic groups A to Z represented (HILKER and SOLBERG, 1973; THOMASON, 1971; INSALATA *et al.,* 1973). Because of the cross-reactivity of salmonellae antisera with other closely related organisms (e.g., *Arizona*, *Citrobacter*, *E. coli*), false positive results are to be expected when naturally

contaminated foods are examined (CHERRY and MOODY, 1965). An indirect fluorescent antibody technique based on highly specific monoclonal antibody for *Streptococcus inae* was developed and was found to be suitable for the detection and identification of *S. inae* from experimentally and naturally infected tilapia (KLESIUS *et al.* 2006).
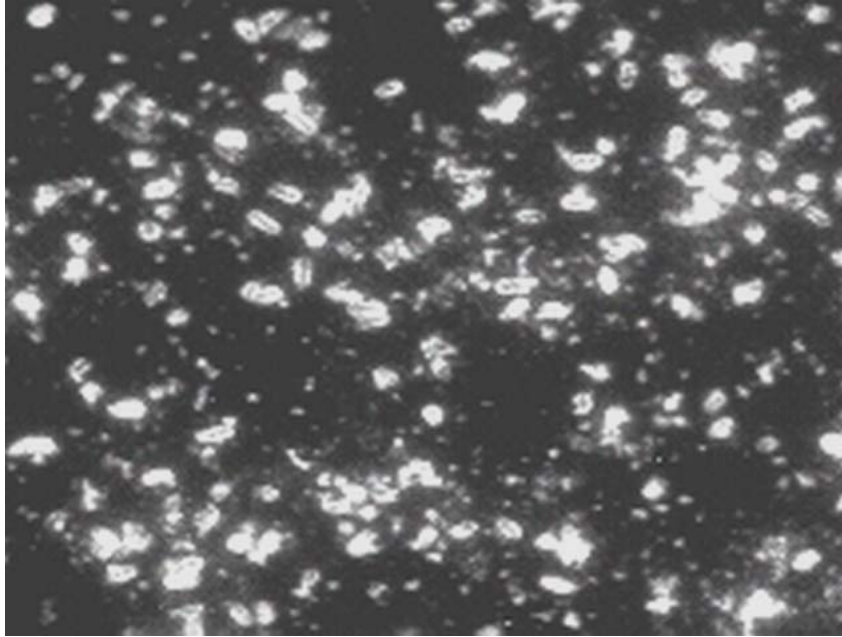

Fig. 2. Fluorescent antibody identification of *Yersinia pestis*. (photo: Abdul Ghaffar)

3.3 Radioimmunoassay (RIA)

This technique consist of adding a radioactive label to an antigen, allowing the labeled antigen to react with its specific antibody, and measuring the amount of antigen that combined with the antibody by a counter to measure radioactivity. The label used by many workers is ^{125}I.

The RIA technique lends itself to the examination of foods for other biological hazards such as endotoxins, paralytic shellfish toxins, etc. The detection and identification of bacterial cells within 8-10 minutes have been achieved (STRANGE *et al.* 1971) by the use of ^{125}I-labeled homologous antibody filtered and washed on a Millipore membrane. Because of its requirement for an isotope and its lack of portability, the RIA method is rarely used now for food microbiology.

3.4 Enzyme-linked immunosorbent assay (ELISA)

The ELISA (enzyme immunoassay or EIA) is an immunological method similar to RIA but employing an enzyme coupled to antigen or an antibody rather than a

radioactive isotope. A typical ELISA is performed with a solid-phase (polystyrene) coated with antigen and incubated with antiserum. Following incubation and washing, an enzyme-labeled reparation of anti-immunoglobulin is added (Fig. 3A). After gentle washing, the enzyme remaining in the tube or microtiter well is assayed to determine the amount of specific antibodies in the initial serum. The amount of enzyme present is ascertained by the colorimetric determination of enzyme substrate. Variation of this basic ELISA consists of a "sandwich" ELISA that allows detection and quantification of pathogen related antigen (Fig. 3B) (VALDIVIESO-GARCIA *et al.,* 2001; GILROYA and SMITH, 2003). In this case, the antibody specific for the antigen is coated to the surface of microtiter wells to which the antigen containing test sample is added. After unbound antigens are washed away, the antibody antigen complexes are than detected by an enzyme-linked antibody, which is specific for a different epitope on the antigen. Next, unbound reporter antibodies are washed away, the substrate is added and the coloured reaction product is measured. The "double sandwich" ELISA is a variation of the latter method, and it employs a third antibody. The ELISA technique is used widely to detect and quantitate organisms and/or their products in foods (BENNETT, 2005).
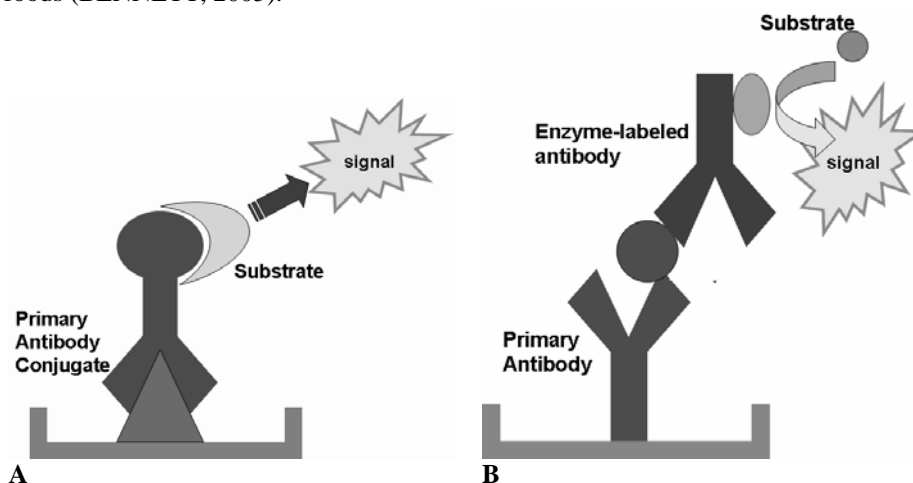

Fig. 3 ELISA assay (**A**) Typical ELISA assay (**B**) Sandwich ELISA.

3.5 Immunomagnetic separation

This method employs paramagnetic beads (about 2-3 μm in size, about 106-108/ml) (Fig. 4) that are surface activated and can be coated with antibody by incubating in the refrigerator for varying periods of time up to 24 hours. The unabsorbed antibody is removed by washing. When properly treated, the coated beads are added to food slurry that contains the homologous antigen (toxin or whole cells in Gram-negative bacteria), thoroughly mixed, and allowed to incubate from a few minutes to several hours to allow for reaction of antigen with antibody-coated beads. The concentrated antigen is assayed by other methods. In one study, immunomagnetic

separation was combined with flow cytometry for the detection of *E. coli* O157:H7. The antigens were labeled with fluorescent antibody, which was measured by flow cytometry, and the combined method could detect <103 cfu/g of pure culture or 103-104 cfu/g in ground beef (FU *et al.,* 2005; DRYSDALE *et al.,* 2004; TSAI *et al.,* 2006). This method was also used for measuring the number of *L. monocytogenes* cells (HIBI *et al.,* 2006).

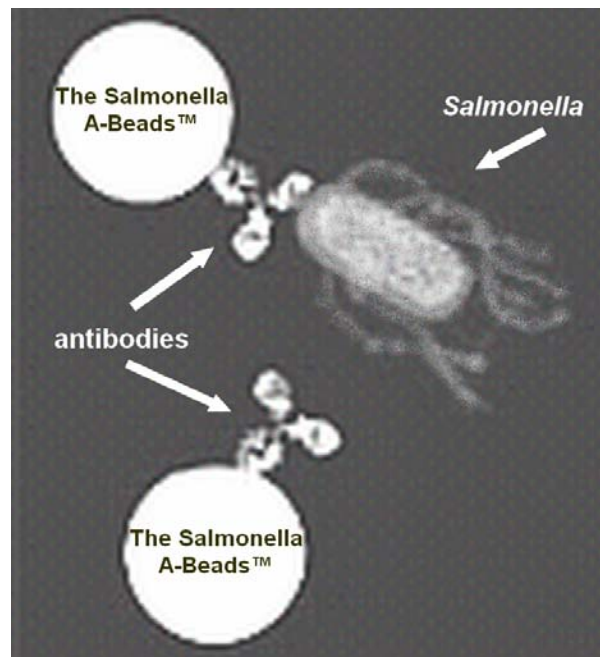

Fig. 4 The Aureon Biosystems Salmonella A-Beads™: polydisperse 1.5 um cluster type paramagnetic particles with affinity-purified antibodies against Salmonella spp. covalently bound to the surface. The Salmonella A-Beads™ will bind specifically to Salmonella in a mixed flora sample and is designed for the rapid and specific isolation of Salmonella from food and environmental samples (edited according photo by Charles J. DiComo).

4. Molecular genetic methods

The progress made in nucleic acids techniques for diagnostics is enormous in relation to the period during which it has been applied to the development of assay systems as compared to that during which phenotypic *in vitro* analysis has developed. Indeed, nucleic acid technology has now assumed an essential role in various areas of *in vitro* diagnosis. Basically, this approach employs genetic materials (DNA and RNA) as diagnostic targets for identifying suspected pathogens. The rapid advancement of nucleic acid diagnostic is a consequent of two essential advantages: (1) nucleic acids can be rapidly and sensitively measured, and (2) the sequence of nucleotides in a given gene is highly specific and it can be used to distinguish closely related serotypes

(KWANG, 2006). The 16S rRNA species-specific sequences are reliable tool for bacterial diagnosis. The importance of 16S rRNA was shown at proposing the establishment of three kingdoms of life forms, namely Eukaryotes, Archaebacteria, and Prokaryotes. The 70S bacterial ribosome contains three definable rRNA fractions, 5S, 16S and 23S. The 5S contains ca. 120 nucleotides while 16S and 23S contain ca. 1.500 and ca. 3.000, respectively. The 16S rRNA has been shown to be an excellent chronometer of life forms, especially bacteria. It was by the use of 16S rRNA sequence and hybridization data that the class *Proteobacteria* was established. By use of these sequences, new bacterial genera can be defined on a genotypic rather than a phenotypic basis. From this and other molecular genetic information, the genus *Pseudomonas* has been reduced by the transfer of over 50 species to 10 new genera. The existence of many of gene banks for many other bacterial taxa of importance in foods suggests that generic and species realignments will continue (JAY *et al.* 2005).

Besides, nucleic acids detection offers many advantages over immunological assays. Nucleic acids are more stable than proteins to high temperatures, high pH, organic solvents, and other chemicals; hence samples can be treated in a relatively harsh manner without destroying the nucleic acid for detection. Tools used for application of nucleic acid detection like DNA primers and probes are more defined entities than antibodies and antigens, and their composition can be accurately checked by sequence analysis, and produced in DNA synthesizers whenever necessary. The more commonly applied nucleic acid assay technologies in diagnostic include nucleic acid probes and polymerase chain reaction (KWANG, 2006).

4.1 Nucleic acid probes

The fundament of the specific association of nucleic acid sequences by complementary base pairing through hydrogen bonding, which centralizes based on adenine complementarities to thymine and uridine, while guanine complementary to cytosine, has allowed the development of several nucleic acid diagnostic methods for detection. Nucleic acid probes are short fragments of nucleic acid sequences that detect specific sequences associated with pathogen by nucleic acid hybridization with highly conserved sequences. If pathogen contains sequences complementary to the probe, the two sequences can hybridize to form a double-stranded molecule. The probe can be chemically synthesized according to the target gene sequence, which can be derived if a short amino acid sequence of the protein encoded by the target gene is known. During synthesis of the probe, labeled nucleotides are incorporated, to enable detection of the probe after hybridization. The types of labeled probes include radioactive (which gives a radioactive signal), biotin (which generate a color) and chemiluminescent (which is enzyme-linked).

Nucleic acid hybridization is often performed in the form of Southern blotting that detects for DNA and Northern blotting that detects for RNA. In both Southern and Western blotting, denatured DNA and RNA samples are separated by electrophoresis and immobilized onto a solid support system, usually nitrocellulose or nylon membrane, respectively, before detection with radioisotope-labeled or enzyme-labeled sequence-specific probe(s). Hybridization is allowed to occur at an optimized

temperature in which considerable sequence homology between the target sequence and the probe is necessary to form a stable duplex. Following washing to remove un-hybridized probe, the hybridization is detect according to how the probe was labeled (DOOLEY, 1994). Nucleic acid probes can be designed for identifying all serotypes or some interesting serotypes of a pathogen. However, when used alone probes may not be very sensitive. Although, nucleic acid hybridization procedures can be combined with PCR due to provide a powerful tool, which exhibits remarkable specificity and sensitivity. It is especially useful when the target pathogen cannot be cultivated, or is difficult to cultivate. On the other hand, nucleic acid probes in diagnostics is designed for use in the food industry, such as for the detection of *Salmonella* and *Staphylococcus* employ probe dipsticks, which remove hybridized DNA from liquid solution. For this method, a two-component probe is used, in which one serves to bind to the specific sequence of the pathogen, and the other serves to bind to the dipstick. Following hybridization of the two-component probe to target sequence, the dipstick is inserted into the hybridization solution to remove the hybridized DNA for measurement (JAY *et al.,* 2005).

4.2 Polymerase chain reaction (PCR)

This method is fast becoming the most widely used of all molecular genetic methods for detecting and identifying bacteria in foods. Its increasing use is due to its high sensitivity, specificity, its availability in many formats, and the commercial availability of PCR-based methods in kit-like formats. The polymerase chain reaction diagnostic methods detect for specific nucleic acid sequence found in the genome of pathogens (CHOTÁR *et al.,* 2006, Fig. 5).
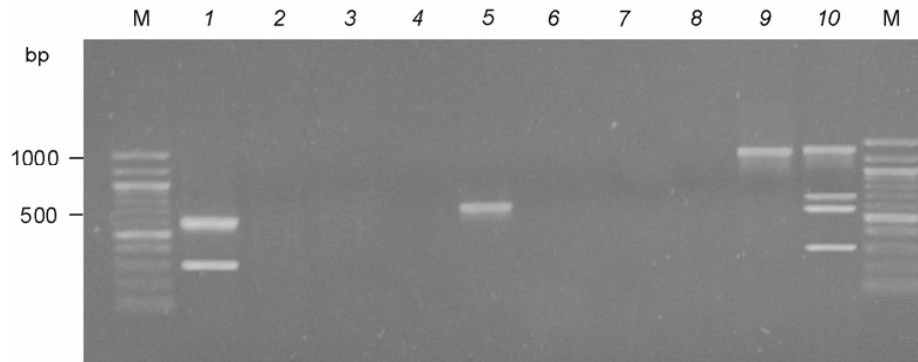

Fig. 5. Determinations of specificity of primers in PCR assay; amplification products of the different primer combinations were analyzed by electrophoresis in 0.9 % agarose gel. Lanes: 1–3 – DNA *S. agalactiae* with primers SAGA1 and SAGA2 and SIP3(F) and SIP4(R) (1), Ecoli1 and Ecoli2 (2), SAU1 and SAU2 (3); 4–6 – DNA *E. coli* with primers SAGA1 and SAGA2 (4), Ecoli1 and Ecoli2 (5), SAU1 and SAU2 (6); 7–9 – DNA *S. aureus* with primers SAGA1 and SAGA2 (7), Ecoli1 and Ecoli2 (8), SAU1 and SAU2 (9); 10 – multiplex PCR with all 8 primers; M – 100-bp DNA ladder (BioLabs) (CHOTÁR *et al.,* 2006, photo: Barbora Vidová).
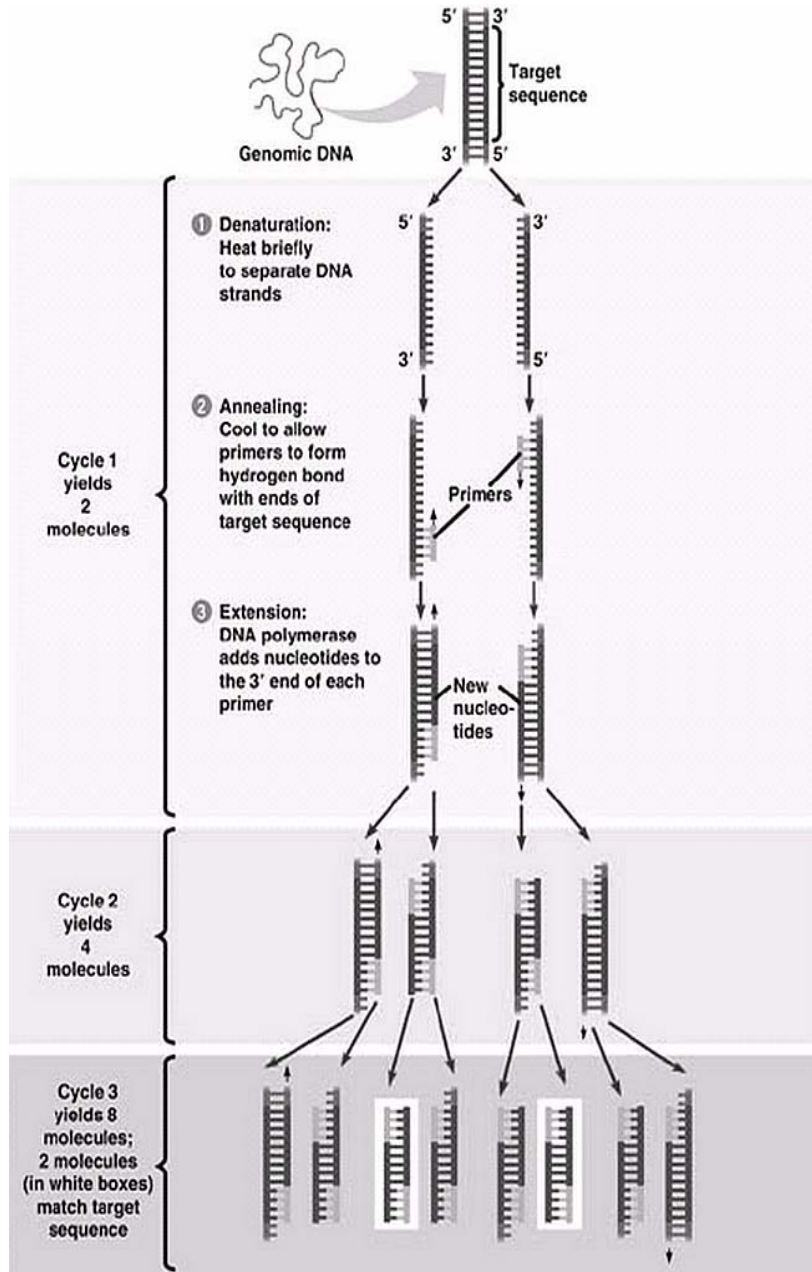
Fig. 6. Polymerase chain reaction procedure (drawn by Michael Gaines).

It is based on DNA replication *in vitro* to produce large quantities of a target sequence of pathogen isolated from a complex mixture of heterogeneous DNA

molecules (e.g. host genome, nucleic acid from other microbes present). The reaction is set up using the following reagents - reaction buffer, DNA template, deoxynucleotides (dNTPs), primers and thermo stable DNA polymerase. A pair of primers is used for the reaction. The primers are single-stranded DNA molecules of about 20-30 nucleotides long (oligonucleotides) designed from the sequence flanking the two ends of the target genome. These primers are specific to the pathogen to maximize the specificity of the assay. The amplification of DNA by PCR is accomplished through cycling a succession of incubation steps of different temperatures optimized for DNA replication (Fig. 6).

Briefly, a sample containing mixture of DNA molecules is heat-denatured to separate complementary strands of DNA into single-stranded molecules for reaction. Next, the reaction is brought down to a lower temperature to allow pathogen-specific primers to search and anneal to the complementary sequence on opposite strands of the target DNA. This step is followed by DNA extension at a higher temperature, with DNA polymerase adding on dNTPs using the annealed DNA sequence as a template to produce copies of the targeted sequence. The three steps make up one cycling reaction, with each cycle producing duplicates of the DNA targets. The cycle of steps is then repeated, in which the newly synthesized DNA strands can also serve as templates for primer extension. These steps are repeated 20-40 times, yielding exponential amplification of the target DNA sequences. The amplified products can then be visualized on agarose gel, or further assayed by nucleic acid probing for increased sensitivity and specificity (SNUSTAD and SIMMONS, 2000).

PCR offers a major advantage of detecting pathogens in a single cell particle. This saves the hassle of isolating the pathogens in culture. PCR has distinct advantages over culture and other standard methods for the detection of microbial pathogens and offers the advantages of specificity, sensitivity, rapidity, accuracy and capacity to detect small amounts of target nucleic acid in a sample (TOZE, 1999). PCR have been used extensively for several years for identification and characterization of bacteria in food samples, including meat and dairy products (HILL, 1996; WANG *et al.,* 1997; ASLAM *et al.,* 2003; ERCOLINI *et al.,* 2004; ALARCÓN *et al.,* 2006; JENSEN *et al.,* 1993; FACH and POPOFF, 1997; TSEN *et al.,* 1998), viruses (SCHWAB *et al.,* 1996; DUBOIS *et al.,* 1997; TRAORE *et al.,* 1998), protozoa (ROCHELLE *et al.,* 1997; STINEAR *et al.,* 1996) and helminths (GEARY, 1996; MATHIS *et al.,* 1996) and multiple primers can be used to detect different pathogens in one multiplex reaction. Alternatively, diagnostic methods like nucleic acid probes, restriction fragment length polymorphism, or sequence analysis can also be used to confirm the identity of the PCR product and to further characterize the genome (VOGEL *et al.,* 2004).

4.3 Real-time polymerase chain reaction

Real-time PCR, also known as kinetic PCR or quantitative real time PCR, has been increasingly employed in diagnostic microbiology, virology and parasitology. In the biotechnology, real-time PCR has been used to genotype identification of genetically modified organisms, monitor gene expression in bioprocesses and for evaluating the

safety of biologicals. The importance and wide use of real-time PCR as a quantitative tool in the life sciences is due to the inherent wide dynamic range of quantification (107-109 fold), high assay sensitivity (detection of less than 10 copies), and high precision with no post-PCR manipulations, thus minimizing the risk of cross-contaminations. Thus, in view of the high accuracy of measurement and reliability of real-time PCR, this technology will continue to be an essential tool for the quantification of gene expressions and genotyping.

TaqMan real-time PCR pathogen detection assays amplify target nucleic acid sequences from select microbes present in samples collected from complex biological environments. Specific amplification of target sequences is directed by custom designed primers and probes. The first degree of specificity is achieved by the combination of amplification primer sequences. An additional degree of specificity results from a probe that hybridizes to a region of nucleic acid sequence that identifies the microbe of interest. As PCR amplification proceeds, fluorescence is excited by laser or halogen light and is detected by a charge-coupled device (CCD) camera. Because the probe does not inhibit the PCR reaction, these systems give a linear response to template concentration over at least five orders of magnitude. Target sequences that confer specificity to pathogenic microorganisms are found within the portion of the microbe's genome that encodes their virulent agents. The genes or a portion of the genes that contribute to the disease phenotype of the pathogen distinguish a target microbe from its nearest neighbors (JOHNSON *et al.,* 2005; OBERST *et al.,* 1998).
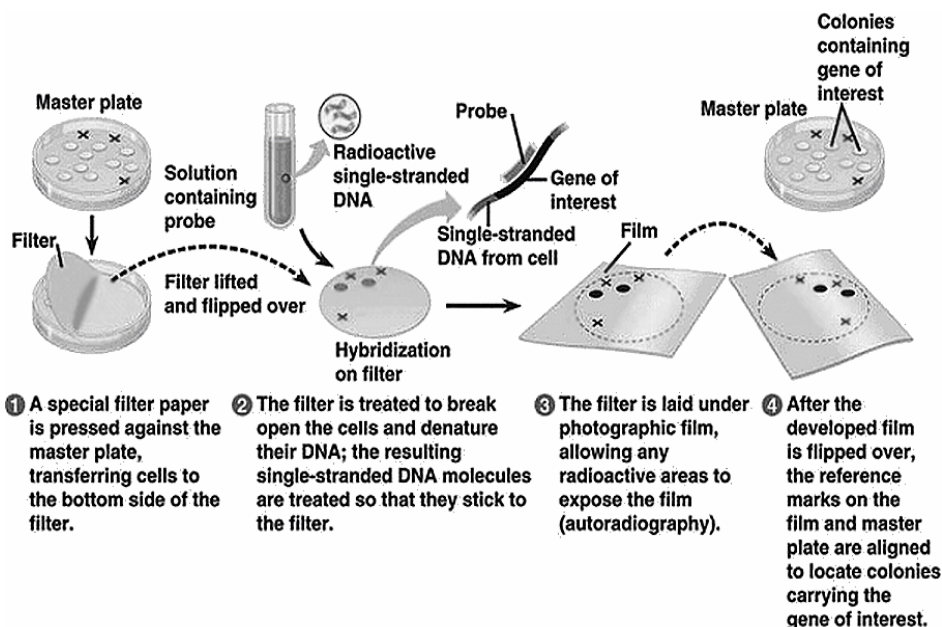

Fig. 7. Ribotyping (drawn by Michael Gaines)

4.4 Ribotyping

DNA is extracted from cells and digested with a restriction endonuclease such as EcoRI, and the fragments are separated by agarose gel electrophoresis. Separated fragments are transferred to a nylon membrane and hybridized with an appropriately labeled copy DNA (cDNA) probe derived from ribosomal RNA (rRNA) by reverse transcriptase. The pattern radioactive / chemiluminescent) that is created is recorded. The automated device creates riboprints that are matched or compared to those of known strains stored on computer software (JAY *et al.,* 2005; GRIF *et al.,* 2006) (Fig. 7). In a study of *Salmonella* serotype Enteritidis, ribotyping was the most discriminating and accurate of the genetic methods used to distinguish among food, water, and pathogenic strains (LANDERS *et al.,* 1998).
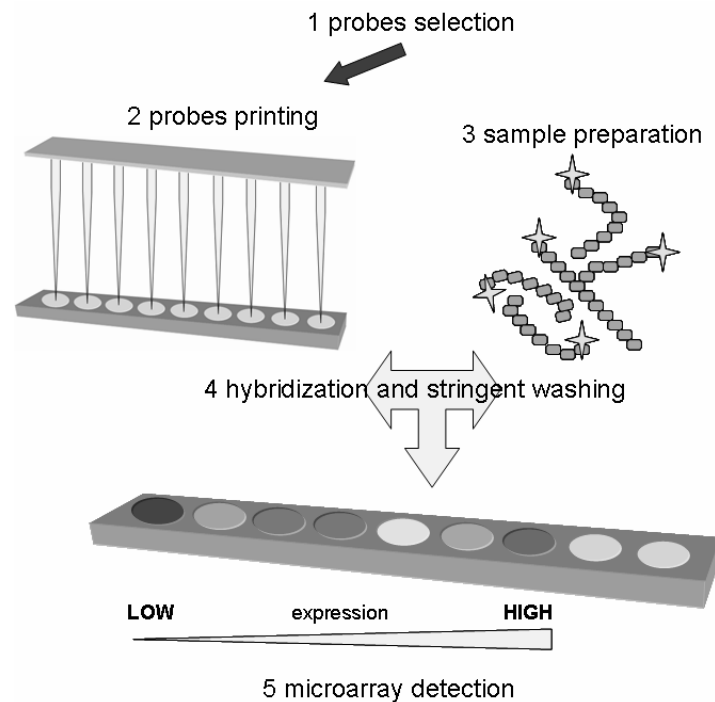
Fig. 8. Microarray assay. Microarray is produced by selecting (1) and printing (2) probes in an array on s glass plate (microscope slide). The sample (target) is prepared, labelled (3) and then let to react with the microarray in a process called hybridization (4). After hybridization, the slides are washe with a weak salt solution (4) that removes unspecific binding of the target molecules (stringent washing). The target molecules that have bound to the probes on the array are detected (5) using fluorescent scanning or other scanning techniques. Finally, quantified values of the "spots" are analysed to give a genotype.

4.5 Microarrays

A very simple microarray may consist of a solid surface (such as a nylon membrane, glass slide, or silicon chips) onto that are attached small quantities of a

single-stranded DNA (ssDNA) from different known bacterial species (Fig. 8). When ssDNA from as many unknown species is exposed to these array (DNA chip), complementary strains will bind to their respective sites on the chip. If a reporter molecule is used, the identity of the unknown species can be confirmed. A DNA microarray is, in essence, a dot blot set-up with the capacity to obtain and process large amounts of data. A DNA microarray for microorganisms begins with the construction of oligonucleotides (primers), probes, hybridization, and data analysis. The overall process has been presented and reviewed by YE *et al.* (2001).

As currently used, several hundred to several thousand specimens or probes may be applied to a solid surface. In a study of *Xanthomonas* pathovars, a 47-probe microarray was employed to fingerprint 14 closely related strains, and the fingerprints showed clear differences between the test strains (KINGSLEY *et al.,* 2002). A DNA microarray has been developed for the detection of bacteria in the microbial consortium of ready-to-eat vegetable salads, with as few as 100 cfu being detectable in < 1 hour (RUDI *et al.,* 2002).

5. Conclusion

In the field of food protection, it is extremely important to be able to identify and to type pathogenic and spoilage microorganisms at an early stage. Here reviewed methods based on immunochemical and nucleic acid technologies provide a very useful alternative against conventional microbiological laboratory methods, because conventional methods involve enriching the food sample and performing various media-based metabolic tests (agar plates or slants), which typically require 3–7 days to obtain a result. On the other hand rapid-screening tests based on immunological or nucleic acid technologies developed for food testing can provide results within hours.

An important area for further investigation will lie in the expansion of whole genome sequence comparisons to related pathogens to identify those genes that confer detrimental properties, and to other microorganisms including closely related nonpathogenic species to ascertain evolutionary relationships and to identify novel virulence factors. The use of DNA microarrays will facilitate whole genome comparisons among diverse strains and the identification of strain- and lineage-specific sequences.

Specific (serotype) markers could be used to develop accurate identification and subtyping methods for use in both health institutions and the food industry. The combination of such pathogen specific markers with detection on DNA microarrays might provide a more sensitive, rapid and informative detection of pathogens in food and food production sites than do classical culture methods. More rigorous and systematic assessment and development procedures are needed to realize the full potential of microarrays for use in industrial and clinical settings, however, because currently this technique is prohibitively expensive and requires highly specialized knowledge. Efficient cooperation between academia and industry is required so that DNA microarray technology and other novel detection methods can be tailored to the practical needs of the food industry and health institutions. There is also a need to understand better the ways in which pathogens can protect themselves against the

conditions that are used in food preservation, because this might lead to the optimization of current foodprocessing strategies. Complete genome sequences have already provided us with an unprecedented insight into the evolution and lifestyles of food-borne pathogens, including species that previously have not been studied so well. The availability of several microbial genome sequences has catalyzed a burst of genomics-driven fundamental research in the ecology, physiology and virulence of foodborne pathogens. The coming years will bring the first practical benefits to the field of microbial food safety, including strategies and tools for the detection, identification and control of these pathogens.

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