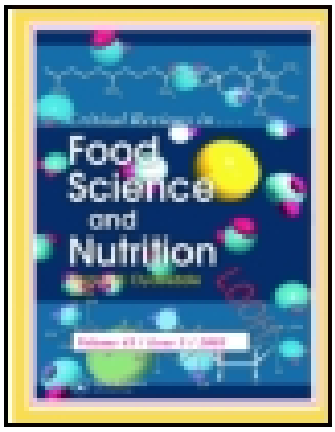


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# Molecular Methods for Microbiological Quality Control of Meat and Meat Products: A Review

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# Molecular Methods for Microbiological Quality Control of Meat and Meat Products: A Review

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## ABSTRACT

Achieving food safety is a global health goal and the food-borne diseases take a major check on global health. Therefore, detection of microbial pathogens in food is the solution to the prevention and recognition of problems related to health and safety. Conventional and standard bacterial detection methods such as culture and colony counting methods and immunology-based methods may take up to several hours or even a few days to yield a result. Obviously this is inadequate, and recently many researchers are focusing towards the progress of rapid diagnostic methods. The advent of molecular techniques has led to the development of a diverse array of assay for quality control of meat and meat products. Rapid analysis using DNA hybridization and amplification techniques offer more sensitivity and specificity to get results than culture based methods as well as dramatic reduction in the time to get results. Many methods have also achieved the high level automation, facilitating their application as routine sample screening assays. This review is intended to provide an overview of the molecular methods for microbiological quality control of meat and meat products.

**Keywords** meat, meat products, PCR, DNA analysis, molecular methods, quality control

## *INTRODUCTION*

Meat-borne illness is a major problem in terms of social and economic loss. However, because the illness typically resolves on its own, it is often not regarded as serious; this may often result in under-reporting of outbreaks. Conventional methods for the detection and identification of microbial pathogenic agents mainly rely up on specific microbiological and biochemical identification where the culture and colony counting methods involve counting of bacteria and immunology-based methods involve antigen-antibody interactions. While these methods can be sensitive, inexpensive and give both qualitative and quantitative information of the tested microorganisms, they are greatly restricted by assay time; also initial pre and/or enrichment is needed in order to detect pathogens which typically occur in low numbers in meat and meat products.

## *MOLECULAR METHODS*

Advances in biotechnology have allowed the development of powerful tests by which particular bacterial strains can be rapidly identified without the need for isolating pure cultures (Rasmussen *et al.,* 1994). The polymerase chain reaction (PCR) is a technique for *in vitro* amplification of specific segments of DNA, using a pair of primers (Nguyen *et al.,* 1994). A million-fold amplification of a particular region can often be realized, allowing, among a myriad of other uses, the sensitive detection of specific genes in samples. PCR can be used to amplify genes specific to taxonomic groups of bacteria and also to detect genes involved in the virulence of food-borne bacteria (Finlay and Falkow, 1988; Bej *et al.*, 1994). Modifications of PCR methods like multiplex PCR and real time PCR methods could be used for more specific

identification of pathogens. Hybridization using DNA probes has been employed with Microarray methods for detection of many antigens in one test format.

***DNA HYBRIDIZATION***

DNA has a unique property to stick to the complementary base pair of itself and this property can be utilized to identify the specific sequence within the genome. Duplex DNA is denatured into two intact strands followed by immobilisation of the two on a membrane or support as a target for the probe. Probes are labelled single stranded complementary DNA that is added in solution. Random collisions of the probe with the target will base pair along complementary regions, the stringency of the conditions affects how closely sequences must match for hybridization to occur. Excess unbound probe is washed off and the labelled double stranded DNA is detected. Radio labelled (usually $P^{32}$ or $S^{35}$) or non-radioactively labelled digoxigenin and biotin probes are detected visually. It can detect as low as 0.1 picograms of homologous DNA.

Wang *et al.* (2011) developed a colony hybridization method for the detection and isolation of diarrhoeagenic *Escherichia coli* (DEC) from those samples containing numerous coliform bacteria. Digoxigenin-labelled DNA probes were designed to detect seven pathotypes of DEC based on type-specific genes. A total of 615 meat, food and faeces samples identified as DEC positive by multiple real-time PCR for the virulence genes were analyzed by a colony hybridization method.

***POLYMERASE CHAIN REACTION (PCR)***

The PCR is an *in vitro* enzymatic method which allows several million fold amplification of a specific DNA sequence within few hours. This technique was invented by Mullis *et al.* (1986) and is not only useful for molecular biologist and geneticist but also useful for food microbiologists. Now a day's PCR assay is gaining immense popularity in detection of meat-borne pathogens. This is a sensitive, cost effective, precise, authentic and potentially applicable technique for microbiological quality control of meat and meat products due to its lesser complexity and fast reliable nature.



The ingredients required for successful PCR amplification are template DNA, pair of forward and reverse oligonucleotide primers, all four deoxynucleotide triphosphates, a thermostable DNA polymerase enzyme and reaction buffer. The PCR technique involves denaturation, primer annealing and elongation steps for a set number of times depending on the degree of amplification required. This generates billions of copies of desired DNA segment from picograms quantities of starting DNA in matter of few hours (Chikuni *et al.*, 1994). However, the other parameters are also important for successful amplification of desired PCR products, such as DNA quality, primer concentration, different thermo cyclers, brand of DNA polymerase, $Mg^{++}$ concentration, annealing temperature and final extension periods (Meunier and Grimont, 1993; Macpherson *et al.*, 1993). Theoretically, PCR can amplify a single copy of DNA a million fold in less than 2 hr, hence, it has the potential to disseminate or greatly reduce the dependence on cultural enrichment. In a PCR system, assuming a sensitivity of 1 cell/reaction tube, approximately 103 bacteria/ml sample required to ensure a reliable and repeatable amplification (Wang *et al.*, 1997). Although PCR is a powerful technology, the reactions can be dramatically affected by the presence of inhibitory compounds in foods and selective microbiological media

like bile salts and acriflavin. A problem to routine use of PCR in food testing laboratory is that the procedures are rather complicated and very clean environment is needed to perform the tests. Further, PCR can not distinguish between live and dead cells and hence providing more false negative results (Biswas *et al.,* 2008).

Naravaneni and Jamil (2005) developed PCR method for the rapid identification of the food borne pathogens *Salmonella* and *Escherichia coli*. Suitable primers were designed based on the specific gene *fimA* of *Salmonella* and gene *afa* of pathogenic *E.coli* for amplification. Agarose gel electrophoresis and subsequent staining with ethidium bromide were used for the identification of PCR products. Donhauser *et al.* (2011) identified and quantified *E.coli* O157:H7, *Salmonella Enterica* and *Campylobacter jejuni* by PCR assay coupled with chemiluminescence flow through DNA microarray analysis. The DNA microarray assay results were comparable with Real-time PCR.



***MULTIPLEX PCR***

Multiplex PCR is a technique in which more than one pair of primer is used to amplify the different fragment of target gene simultaneously to save time and minimize the expense on detection of food borne pathogens (Slavik *et al.,* 2003; Bottero *et al.,* 2004). However, great care must be taken to ensure that primers have same melting temperature and they must not interact with each other. Kawasaski *et al.* (2009) used a multiplex PCR system for simultaneous detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 in foods. The multiplex PCR assay was able to detect all three pathogens when found in food at less than or equal to 5 bacteria per 25 gm of food. Fukushima *et al.* (2010) reported simultaneous

screening of 24 target genes of food borne pathogens in 35 food borne outbreaks using multiplex PCR assays. Zhou *et al.* (2011) used multiplex PCR method for identification of *Campylobacter* isolates in retail broiler meat and typed using pulsed-field gel electrophoresis (PFGE).

***REAL TIME PCR***

Real-time PCR has become a very attractive method for the detection of meat borne pathogens since it offers a continuous monitoring technique for PCR product formation throughout the reaction, which eliminates post-PCR analysis process, shortens the detection time compared to standard PCR, and reduces the risk of amplicons contamination by laboratory environments (Heid *et al.*, 1996; Klein and Juneja, 1997). In addition, Real-time PCR is a quantitative method and is often used to determine the number of pathogens in various samples (Heid *et al.*, 1996; Long *et al.*, 2008). Real time PCR method allows built in product detection (both quantitative and qualitative) during the entire reaction period. RT-PCR is so named as one can continuously monitor the development of amplicons in a fluorimeter. SYBR-Green (a cyanine dye, binding preferably to dsDNA) or other fluorescent labelled probes that emit lights during amplification are widely used in RT-PCR. The emitted light signals corresponding to DNA amplification recorded at frequent intervals generating a curve showing product generation. The more targets DNA amplifies in the sample, the earlier amplicons can be detected and the peak curve is generated.

Baggi *et al.* (2005) have used RT-PCR for rapid detection of diarrheagenic *E. coli* using SYBR Green dye and best sensitivity and specificity was observed. For the detection of food samples 5’ nuclease multiplex PCR can also be employed. The method uses the 5’ nuclease

activity of T*aq* polymerase (Holland *et al.*, 1991) and FRET (Fluorescence Resonance Energy Transfer) technology in same experimental setup with multiple primers and probes. The method can be optimized by the amount of each primer pair to achieve the maximum amplification in separate reaction condition and the target loci must be checked separately for amplification with same reaction conditions and same PCR program. Real-time PCR using RNA as template is more authentic since the RNA is present only in viable microbes. RNA is first reversely transcribed to cDNA and in second step used for amplification. Merck KGaA developed a Real-time PCR assay kit for detection of food borne pathogens, beer spoilage and genetically modified organisms. The kits are developed for the molecular detection of pathogenic bacteria in foods and environmental samples, such as *Salmonella, Listeria monocytogenes, E. coli* O157:H7, *Campylobacter* and *Cronoobacter sakazukii*. Huang *et al.* (2007) identified 8 food borne pathogens by multicolour combinational probe coding technology (MCPC) in a single real time PCR.



***RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS (RAPD)***

RAPD, a PCR based technique, generates pattern of DNA bands on gel electrophoresis using amplification of random DNA segments with primers of arbitrary nucleotide sequence. Short length primers bind randomly along the prokaryotic genome resulting number of different DNA fragments. The array of fragments is examined for similarity of genotypes based upon the number and size of the amplicons. Wong *et al.* (1999) used RAPD method for molecular typing of *Vibrio parahaemolyticus* isolates obtained from patients involved in food poisoning outbreaks in Taiwan. RAPD has also been used in detection of *Listeria* species in poultry processing

environment and vegetable processing plants to identify the source of contamination and dissemination routes (Gilmour and Lawrence, 1995; Garcia-jailon *et al.,* 2004).

***LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)***

Loop mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that relies on an auto-cycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (Notomi *et al.,* 2000; Nagamine *et al.,* 2001). *Bst* DNA polymerase is the largest fragment of *Bacillus stearothermophilus* DNA polymerase protein that contains the 5'- 3' polymerase activity, but lacks the 5'- 3' exonuclease domain which performs 5'- 3'exonuclease activity. *Bst* DNA polymerase has strand displacement activity which synthesizes DNA with loop forming primers to yield long stem loop products under isothermal condition. LAMP is different from PCR in that four to six primers perform the amplification of the targeted gene, the amplification uses a single temperature step at 60-65$^0$C for about 60 min, and the amplification products have many types of structures in large amounts. Thus, LAMP is faster and easier to perform than PCR, as well as being more specific. Furthermore, gel electrophoresis is not required, because the LAMP products can be detected indirectly by the turbidity that arises due to a large amount of by-products, pyrophosphate ion being produces, yielding an insoluble white precipitate of magnesium pyrophosphate in the reaction mixture. Haro-Kudo *et al.* (2005) used LAMP assay for the rapid detection of within 60 min. The 220 strains of 39 serotypes of *Salmonella Enterica* subsp. *Arizonae* were amplified, but not 62 strains of 23 bacterial species other than *Salmonella*. Tang *et al.* (2011) used LAMP for rapid and sensitive detection os *Listeria monocytogenes* in food.

***DNA MICROARRAY***

DNA microarray represents the latest advance in molecular biology (Yoo and Lee, 2008), in which many microscopic spots of DNA oligonucleotides are arrayed series. Each spot contains picomoles of a specific DNA sequence which may be a short section of a gene or other DNA elements that are used as probes to hybridize a cDNA or cRNA sample under high stringency conditions. The relative abundance of nucleic acid sequences in the target can be detected and quantified by fluorophore-labeled probes. An array usually contains thousands of probes, and a micro array experiment is able to achieve goals from many pathogen detection tests in parallel. Therefore, DNA microarrays have tremendously promoted many types of investigations and offered a useful tool for microbiological quality control of meat and meat products.

Identification of various food pathogens simultaneously was carried out using the microarray technique by Jiang *et al.* (2004). Oligonucleotide array is gaining popularity in investigating the clinical samples as it does not require costly reagents and machines. Zhang *et al.* (2006) used an electronic microarray technique for detection and differentiation of viable *Campylobacter* species, *C. jejuni, C. coli* and *C. lari.* This is achieved by using mRNA of the 60 kDa heat shock protein as the viability marker. This technique was able to detect as few as two viable Campylobacter cells. Liu *et al.* (2006) same microarray method for detection of viable pathogenic *Escherichia coli, Vibrio cholera* and *Salmonella typhi*. Four unique genes, the *E. coli* O157:H7 LPS gene (*rfbE*) and H7 flagelling gene (*fliC*), the *V. cholera* O1 LPS gene (*rfbE*), and the *S. typhi* LPS gene (*tyv*) were chosen as the targets for detection. The technique was able to detect as few as two to 150 cells of *E. coli* O157:H7. Bugarel e*t al.* (2011) developed microarray

 

system based on multiplex Real-time PCR assay for detection and characterization of *Salmonella Enterica* subsp. *enterica* implicated in food-borne diseases.

***CONCLUSIONS***

Adoption of tests in combination with routine culture is unlikely to be cost effective; however as the costs of rapid technologies decrease, total replacements with of rapid technologies like molecular techniques may be feasible. Further research is needed on the effectiveness and cost-effectiveness of emerging rapid molecular diagnostics in meat borne infection, particularly those being developed to test for more than one organism at a time, such as multiplex PCR and DNA microarray technologies. Multiple pathogens will be detected in a significant number of samples if more sensitive methods are employed. Therefore, tests to determine the microbial agents responsible for the symptoms are to be developed.

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