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Molecular methods for the detection and characterization of foodborne pathogens*

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Abstract: The surveillance of foodborne pathogens in food industries has shown the urgent need for rapid and dependable methods to detect and characterize the organisms in food and environments of clinical and epidemiologic importance. Recent studies on rapid methods in microbiology have been focused on biochemical characterization, immunoassays, and molecular methods. Many molecular methods have been developed and applied to the detection and characterization of foodborne pathogens in laboratories and food industries. They can be mainly divided into DNA banding pattern-based tests and DNA sequence-based tests. The former includes nucleic acid hybridization, polymerase chain reaction (PCR), amplified restriction length polymorphism, and randomly amplified polymorphic DNA, etc. Most of these methods in commercial applications are based on PCR or hybridization techniques. The principle, characteristics, and application of molecular methods for the detection and characterization of foodborne pathogens were reviewed in this article.

Keywords: detection; foodborne pathogens; genotyping; molecular methods; polymerase chain reaction.

INTRODUCTION

Foodborne pathogens have long been recognized as one of the most important sources causing food poisoning worldwide and in some occasions even deaths. Conventional microbiological quantification techniques such as the plate count methods and the most-probable-number (MPN) methods are time-consuming and require 5 to 6 days to finish the whole process. Rapid methods for the detection and characterization of foodborne pathogens are vital not only to food industries for hygienic purposes but also to governments for increasing protection level of public health and for ensuring the safety of consumers. In recent years, various rapid methods have been established to detect foodborne pathogens [1–5], among which nucleic acid-based molecular methods are commonly used instead of protein-based immunological techniques because of simplicity in operation, stable detection results, and savings in time [6,7]. The purpose of this paper is to review some recently developed methods based on nucleic acid approaches and to discuss some newer and more novel molecular methods under laboratory investigations for the rapid and dependable detection and characterization of foodborne pathogens.

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DETECTION METHODS USING PCR

Polymerase chain reaction (PCR) has become the most frequently used method for amplifying nucleic acids since it was developed by Mullis [8,9]. The reaction system includes a heat-stable DNA polymerase, a template DNA from the pathogens being detected, and two complementary oligonucleotide primers that are designed to flank the sequence on the template DNA. A typical amplification needs 20 to 40 cycles, which amplifies specific pieces of template DNA at more than a billion-fold. Each amplification cycle of a PCR consists of a heat denaturation phase when single strands are generated from a double-stranded DNA, an annealing phase when the primers bind to the single-stranded target sequences, and an extension phase when the DNA polymerase makes a strand that is complementary to the template. This approach has been frequently used by a growing number of studies to detect and characterize foodborne pathogens.

Multiplex PCR

The cost and limited volume of test samples are the key points for the pathogen detection in food industries. A process termed multiplex PCR, in which multiple sets of primers were included in a single reaction tube [10,11], has advantages for these purposes. In this procedure, more than one target sequence are amplified in one reaction system, including more than one pair of primers. Multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics [12–14]. A key point in the development of a multiplex PCR assay is the design of the primers. All of the primers must be designed with very close annealing temperature, and the amplification products need to be at markedly different sizes so as to be easily determined by agarose gel electrophoresis. In addition, the multiplex primers might cause interference in the amplification process, which often makes it difficult for optimization of the reaction, especially when the number of primer pairs in the reaction system increases [15]. Rachlin et al. [16] developed a Web-enabled system called MuPlex that is specific for the design of multiplex PCR primers. MuPlex is used to specify a set of DNA sequences along with primer selection criteria, interaction parameters, and the target multiplexing level, and to provide multiple solution alternatives that reveal tradeoffs among competing objectives. An additional linker is also suggested to solve the competition between multiplex sets of primers [17].

Nested PCR

The sensitivity and specificity are the most important parameters of a detection method, and nested PCR has been developed for this purpose [18,19], in which two sets of PCR primers were sequentially used. The first primer set is used to amplify a target sequence, which then serves as the template for a second amplification. The second primer set lies internal of the first amplicon. This secondary amplification would not occur if the primary amplification was nonspecific. A lot of foodborne pathogens have been detected and characterized by this method [20–22]. A major shortcoming of nested PCR is that the reaction vessel needs to be opened in order to let the second primer set join in, which increases the contamination probability from the laboratory environments [23].

Reverse-transcription PCR

Reverse-transcription PCR is a modified PCR method in which RNA is used rather than DNA as the initial template. In contrast to the detection of DNA from nonviable organisms using standard PCR, the detection of cDNA from messenger RNA encoded by a pathogen using reverse-transcription PCR could be evidence of active cells [24]. In reverse-transcription PCR, the RNA target was first converted into a complementary DNA copy (cDNA) by the reverse transcriptase. This cDNA is used as template and amplified by standard PCR methods. Reverse-transcription PCR is used not only to detect genes of

foodborne viral pathogens via RNA [25–28], but also to detect the specific expression of certain genes during the course of growth or infection since they are amplified at the much higher number of messenger or ribosomal RNA than the number of DNA copies present in foodborne pathogens. However, RNA is unstable, and reverse-transcription PCR is therefore more skillful at handling when quantification is required for foodborne pathogen detection.

Real-time PCR

Real-time PCR becomes a very attractive method for the detection of foodborne pathogens since it offers a continuously monitoring technique for PCR product formation throughout the reaction, which eliminates post-PCR analysis process, shortens detection time compared to standard PCR, and reduces the risk of amplicon contamination by laboratory environments [3,29]. In addition, real-time PCR is a quantitative method and is often used to determine the number of pathogens in various samples [29,30]. Four types of indicators have been used most frequently in real-time PCR methods for pathogen detection: TaqMan probes [31,32], molecular beacons [33,34], fluorescence resonance energy transfer (FRET) hybridization probes [35,36] and SYBR Green dyes [37–39]. In addition to the specific primer set, one or two probes are required in the former three real-time PCR methods to be coupled with fluorescent dye to improve the detection signals, which are used for the increase of detection specificity and for the design of multiplex detection methods [40–42].

OTHER DETECTION METHODS

DNA microarray

DNA microarray represents the latest advance in molecular technology [43], 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 microarray 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 the detection of foodborne microorganisms [44–46].

Recently, serious deviations in reproducibility and accuracy of microarray technology were noted for results obtained from different laboratories or using different experimental platforms as this technology is more and more frequently adopted to diagnostic and clinical applications [47]. This led the U.S. Food and Drug Administration (FDA) to launch the MicroArray Quality Control (MAQC) project to assay the reliability of microarray technology [48,49]. This project involved researchers from government departments, academic institutions, and industries to establish a strictly controlled "gold standard", and more than 1300 microarray-based hybridizations were compared during the entire project. The MAQC project demonstrated that the key factors influencing variations were the biological samples and man-based factors, rather than technical diversity [49], and implementation of standardized methods and tools therefore needs to be improved or perfected. This study proved that the microarray technique was mature enough for applications in detection and regulatory settings of foodborne pathogens [50],

Loop-mediated isothermal amplification (LAMP)

LAMP is a novel nucleic acid amplification method based on the principle of autocycling strand displacement DNA synthesis performed by the Bst DNA polymerase [51], which offers a number of ad-

vantages compared to PCR. First, all reactions can be carried out under isothermal conditions ranging from 60 to 65 °C. Second, six primers recognizing eight distinct regions on the target nucleotides are used to acquire an extremely high specificity [52]. Third, detection is simplified by visual judgment with the unaided eye without post-amplification electrophoresis [53,54]. Thus, a LAMP assay is independent of expensive equipment [55], and it is specific, fast, and easy to perform [53]. These features represent a simple, rapid, and cost-effective analysis that is desired in modern detection methods for foodborne pathogens [53,56]. Furthermore, the increase in the turbidity of the reaction mixture in accordance with the production of precipitate correlates with the amount of DNA synthesized and is suitable for quantitative [55] and real-time assays.

Recently, derivative LAMP assays, such as reverse-transcription LAMP assay [57], multiplex LAMP assay [58], in situ LAMP assay [59], and real-time reverse-transcription LAMP assay [60] have been developed and employed for the detection of various foodborne pathogens, including porcine parvovirus [61], *Campylobacter jejuni* and *Campylobacter coli* [62], *Vibrio parahaemolyticus* [63], *Aeromonas caviae* [64], *Escherichia coli* [55], *Staphylococcus aureus* [65], etc.

Nucleic acid sequence-based amplification (NASBA)

Compared with traditional PCR methods, NASBA relies on the isothermal amplification of RNA for detection of target organisms, and thermal cycling equipment is not required. Three steps are included in this method. Firstly, a primer binds to the target RNA sequence and a cDNA strand is produced with a reverse transcriptase. Secondly, RNase H digests the template RNA and the cDNA is bound with a second primer, with which the reverse transcriptase is used to produce double-stranded cDNA. Last, T7 RNA polymerase is used to produce RNA transcripts via an amplification process. This method particularly suited to the detection of RNA viruses because an RNA polymerase is used to amplify RNA without conversion to cDNA. This assay has been evaluated with several foodborne pathogens [66–68], and it was indicated that NASBA was a sensitive, specific, and rapid analysis method.

Ligase amplification reaction (LAR)

The LAR is also termed the ligase chain reaction (LCR), which is another technique for detecting or amplifying a target nucleic acid sequence compared to the above-mentioned amplification-based methods [69]. Like PCR, this amplification reaction is cyclic with denaturation, annealing, and ligation. A thermostable enzyme is used to join two oligonucleotides that are immediately adjacent to each other. The ligated oligonucleotide pairs along with the original sequence then become templates for the next cycle. This reaction yields a 10^6-fold increase in copies of the original target sequences from 20 to 30 cycles. This method is able to detect many target foodborne pathogens [70]. Gilpin et al. described the use of LAR to detect *Mycobacterium tuberculosis* in a clinical laboratory [71].

GENOTYPING METHODS

Many genotyping techniques have been recently developed for the identification and classification of bacteria. The DNA fingerprinting is a typical kind of these techniques which have become the most powerful typing methods and have been extensively applied for genotyping of foodborne pathogens isolated from food products as well as from the human gastrointestinal tract.

Pulse field gel electrophoresis (PFGE)

PFGE is an agarose-gel electrophoresis method by separating the directly digested fragments of the intact chromosome. The chromosome is the most fundamental component of identity of a pathogen and therefore contains characteristic information for assessing strain interrelatedness. When the chromo-

some DNA is digested with restriction enzymes, a series of fragments at different sizes are generated which are referred to as restriction fragment length polymorphisms (RFLPs). The generated DNA fingerprints depend on the specificity of the restriction enzyme and the sequence of the bacterial genome. The different patterns are produced by PFGEs, which are therefore characteristic of a particular species or strain of bacteria. The PFGE method represents the characteristics of a complete genome and thus is specially used to detect and compare specific changes of genes (DNA deletion, insertions, or rearrangements) within different strains. Its high discriminatory power has been reported for the differentiation of foodborne pathogens such as *E. coli* and *Salmonella spp.* [73,74].

Ribotyping

Ribotyping is a reliable and rapid subtyping technique that is effective for the classification of foodborne pathogens. In this method, the genomic DNA is digested by selected restriction endonuclease to generate small DNA fragments, which are separated by gel electrophoresis and identified by Southern hybridization. Specifically labeled oligonucleotide probes are used to hybridize with target sequences within the fragments, and the detection of these fragments is achieved. The probes used in ribotyping were designed based on partial sequences of the rDNA genes or the intergenic spacer regions of the whole rDNA operon. Ribotyping has been successfully used for the identification of foodborne pathogens [75–80]. However, it should be indicated that it provides high discriminatory power at the species and subspecies level rather than at the strain level.

Randomly amplified polymorphic DNA (RAPD)

RAPD is a PCR-based technique in which arbitrary primers are used, and it has been widely used as a rapid, sensitive, and inexpensive method for subtyping of different strains [20,81–84]. The primers are able to bind under low stringency to many partially or perfectly complementary sequences located in the genome of pathogens. If binding sites locate in a region that allows amplification of DNA fragments, fingerprint patterns are generated that are specific to each strain. It is reported that the reproducibility and discriminatory power of the RAPD is influenced by several factors such as annealing temperature, DNA template purity and concentration, and primer combinations.

Amplified restriction-length polymorphism (AFLP)

AFLP combines the advantages of RFLP with PCR-based methods by ligating primer-recognition sequences (adaptors) to the digested DNA. In the AFLP method, two restriction enzymes are used to digest total genome DNA, one with an average cutting frequency and another with a higher cutting frequency. The DNA fragments are ligated by the double-stranded nucleotide adapters which serve as primer binding sites for PCR amplification. After that, the PCR primers bind to the adapter and produce strain-specific amplification patterns by DNA polymerase. At present, AFLP has been used not only in clinical studies, but also in strain genotyping of foodborne pathogens [85].

Denaturing gradient gel electrophoresis (DGGE)

DGGE is a comprehensive tool for the characterization of bacteria by the separation of PCR-amplified segments of 16S rRNA genes. In this method, double-stranded DNA is denatured due to a linearly increasing denaturing gradient of urea and formamide at increased temperatures. After staining, a mixture of PCR products from 16S rRNA genes forms a binding pattern that displays the different melting behaviors of the various fragments. The DGGE-generated patterns make it possible to monitor specific changes in the gene structure of bacteria. Therefore, DGGE offers a useful tool for rapid detection and

identification of pathogens in food products as well as those derived from the gastrointestinal tract [86–88].

KEY ISSUES ON FUTURE DEVELOPMENT OF MOLECULAR METHODS FOR THE DETECTION AND CHARACTERIZATION OF FOODBORNE PATHOGENS

Novel molecular methods need new targets

Detection targets, i.e., specific DNA sequences from either genomes or plasmids of organisms of interest, are crucial to the accuracy of molecular diagnosis. Most of the frequently used detection targets were identified a decade ago along with gene function studies and immunological analyses. Those methods for target mining were time- and labor-consuming since there was limited information available on whole genomic and plasmid sequences at that time. Therefore, verification of the accuracy of those targets has been recommended and is carried out in our laboratory in recent years.

Genome sequences, whether complete or partial, of more than 1000 microorganisms are publicly available at the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>), which include most of the known foodborne pathogens. Based on such abundant sequence information and bioinformatics technology, the target mining for the detection of foodborne pathogens has become an active research area. In general, genus-, species-, serogroup- and even serovar-specific DNA sequences can be generated as candidate detection targets by aligning the genome sequences of target and non-target pathogens and verified by further screening. Searching and screening conditions need to be adapted to meet the specificity requirement of PCR, hybridization, and array-based assays. For example, Kim et al. [89] successfully used comparative genomics to find out 70-mer oligonucleotide probes specific for the microarray detection of 11 major foodborne pathogens.

Several supporting software packages such as nWayComp [90] and GenomeBlast [91] have been developed based upon rapid and automatic sequence alignment with the purpose of mining specific DNA sequences for the detection of foodborne pathogens. Furthermore, some new molecular targets have recently been yielded for the detection of *Salmonella* spp. [92] and *S. aureus* [93] by the application of a novel, specific DNA mining platform in combination with downloaded genome DNA sequences.

Internal amplification control is effective to assure right results of PCR assays

When a PCR method is designed for the detection of pathogenic bacteria in food and environmental samples, it is highly recommended to include internal amplification controls (IACs) in this assay, as the inhibition of complex food materials on the amplification system is likely to compromise its accuracy by introducing false-negative results. An IAC is a heterogenic DNA fragment flanked by either the same primer pair as the target and termed competitive IAC, or a completely different primer pair and termed noncompetitive IAC [94]. In conventional PCR assays, IAC and target are differentiated by the length of the products, whereas in real-time PCR they are differentiated by the wavelength of probe signals.

There are several methods available for IAC construction in which synthesized single-stranded DNA [95,96], double-stranded DNA [97], plasmid [41], genomic DNA from other strains [98], different strains transformed with a plasmid [99], etc. Long et al. [30] introduced a novel computational DNA random shuffling method for IAC design, which shared the same length and G+C content with target sequence. A mutant strain was generated after this IAC sequence was inserted into the genome of *L. monocytogenes*, which was employed as an IAC to develop a real-time PCR assay for novel indication of false-positive detection results during accurate qualification assay.

Both detection and characterization of foodborne pathogens are desired

Molecular technology has been employed for the detection of almost all common foodborne pathogens. However, information regarding identification and/or typing of these organisms is limited, which includes serotype, toxin secretion type, virulence factors, drug resistance factors, and so on. These characterization jobs are very important for the requirements of food safety and quality control in food industries and clinical diagnostics.

Future result data from investigations will be derived from the combination of detection with characterization assays. Multiplex PCR technology is competent for this purpose, and a lot of such methods have been developed, such as a multiplex PCR for the detection of enterotoxigenic *S. aureus* [100], multiplex PCR and real-time TaqMan PCR assays for the detection of *Salmonella enterica* and the highly virulent serovars Choleraesuis and Paratyphi C [40], real-time multiplex PCR for the detection of different *Salmonella* serotypes including Enteritidis, Gallinarum, Typhimurium, Kentucky, and Dublin [42], melting-curve analysis and real-time multiplex PCR for the detection of diarrheagenic *E. coli* [101], and Cytolethal distending toxin (cdt) gene-based species-specific multiplex PCR assay for the detection and identification of *C. jejuni*, *C. coli*, and *Campylobacter fetus* [102].

Microarray technology holds great potentials for high-throughout detection of foodborne pathogens. Based on this technology, novel assays have been developed for the analysis of drug resistance by *Plasmodium falciparum* [103], detection and simultaneous subtyping for *Newcastle disease* and avian influenza [104] and for *Plum pox virus* [105], serogroups and virulence gene patterns detection for *E. coli* [106]. However, some obstacles still hinder the application of microarrays and other molecular technologies for the detection and characterization of foodborne pathogens. For example, sufficient fluorescently labeled DNA, which is sometime difficult to be achieved, is required before successful visualization of microarray hybridization [107,108]. Moreover, suitable molecular detection targets are still not abundant enough for the development of such a high-throughout method.

The risk of foodborne diseases caused by pathogens still remains, thus, molecular technologies need to be continuously improved to meet the challenges for the control of this threat.

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