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Molecular Tools To Study Preharvest Food Safety Challenges

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ABSTRACT Preharvest food safety research and activities have advanced over time with the recognition of the importance and complicated nature of the preharvest phase of food production. In developed nations, implementation of preharvest food safety procedures along with strict monitoring and containment at various postharvest stages such as slaughter, processing, storage, and distribution have remarkably reduced the burden of foodborne pathogens in humans. Early detection and adequate surveillance of pathogens at the preharvest stage is of the utmost importance to ensure a safe meat supply. There is an urgent need to develop rapid, cost-effective, and point-of-care diagnostics which could be used at the preharvest stage and would complement postmortem and other quality checks performed at the postharvest stage. With newer methods and technologies, more efforts need to be directed toward developing rapid, sensitive, and specific methods for detection or screening of foodborne pathogens at the preharvest stage. In this review, we will discuss the molecular methods available for detection and molecular typing of bacterial foodborne pathogens at the farm. Such methods include conventional techniques such as endpoint PCR, real-time PCR, DNA microarray, and more advanced techniques such as matrix-assisted layer desorption ionization–time of flight mass spectrometry and whole-genome sequencing.

INTRODUCTION

There has been a marked shift in consumer preferences about food choices. Today, consumers not only demand safe, inexpensive, tasty, and healthy food but also expect animal welfare and environmental safety. Consumers have become more curious about the source of meat—whether it comes from a conventional or an antibiotic-free farm (ABF) and if biosecurity requirements were met. To ensure the supply of safe meat to consumers, preventive measures need to be taken at the farm when animals are alive. This is where the concept of preharvest food safety comes from. Preharvest food safety is the combination of measures and interventions adopted at the farm to detect harmful pathogens and to reduce pathogen load in the food chain. Preharvest food safety is not only about the food animals and the microbes they carry but also includes the surrounding environment and human activities. The farm is a dynamic environment where pathogens, food animals, human interventions, environmental factors, and other animal species interact closely.

Safe meat production has to be a holistic approach instead of a single-point inspection at slaughter or during processing. Earlier, all food safety interventions were largely focused on postmortem inspection of the carcass and other quality checks administered before the finished product could reach the market. These quality

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checks would categorize meat into categories such as acceptable, unacceptable, and unsafe for human consumption. It was believed that because the postharvest stages (slaughter and processing) in meat production are closest to the final product, by controlling the incidence of pathogens in the finished product, the risk of foodborne diseases in humans could be reduced. Nevertheless, postharvest food safety measures such as meat inspection and detection of meat that is unfit for human consumption do contribute to consumer protection but do not completely prevent major food safety issues in the final product; i.e., these are only quality checkpoints at the end of an on-farm production phase.

Another reason is that the controlled environment of a slaughterhouse or processing unit is unlike an open and complex farm environment, which comprises different pathogens moving freely and looking for an opportunity to colonize food animals. Classical zoonotic diseases such as tuberculosis and brucellosis could be recognized at the farm stage, and their lesions could be detected during postmortem inspection. However, foodborne pathogens such as multidrug-resistant *Salmonella* and *Campylobacter*, enterohemorrhagic *Escherichia coli* (*E. coli* O157:H7), *Toxoplasma*, and *Yersinia* are only detectable through targeted monitoring systems, because they cause neither clinical symptoms nor lesions to be detected during postmortem inspection of the carcass (1). The traditional mandatory meat inspection procedures are essential, but they are unable to prevent new/emerging foodborne pathogens posing risks to human health (2, 3).

Moreover, issues such as the emergence of antimicrobial-resistant (AMR) bacterial pathogens (*Salmonella* and *Campylobacter*) and the presence of antimicrobial and chemical residues in meat have forced researchers and the meat industry to focus on the preharvest stages of meat production. In particular, the use of antimicrobials and the emergence of multidrug resistance bacteria are the most important preharvest food safety issues affecting public health. The modern food animal production system depends a lot on the use of antimicrobials for prophylaxis and growth promotion in addition to the treatment of animals. Domestic sales and distribution of antimicrobials approved for use in food animals in the United States in 2015 was approximately 15.6 million kg (4). Subtherapeutic doses of antimicrobials are used in food animal production for rapid growth. Such doses, however, are not able to kill microbes and lead to the emergence of antimicrobial resistance in microbes. AMR bacteria can pass through the food chain to the consumers. Extensive usage of antimicrobials in production animals creates selective pressure on bacteria that eventually develop resistance to survive (5, 6). These resistant bacteria originating from production systems could travel to the slaughter and processing units and transfer antimicrobial-resistant attributes to the native microbial population.

Preharvest food safety research and activities have advanced over time with the recognition of the importance and complicated nature of the preharvest phase of food production. In developed nations, implementation of preharvest food safety procedures along with strict monitoring and containment at various postharvest stages such as slaughter, processing, storage, and distribution have remarkably reduced the burden of foodborne pathogens in humans (1). Early detection and adequate surveillance of pathogens at the preharvest stage is of the utmost importance to ensure a safe meat supply. There is an urgent need to develop rapid, cost-effective, and point-of-care diagnostics which could be used at the preharvest stage and which complement postmortem and other quality checks performed at the postharvest stage. With newer methods and technologies, more effort needs to be directed toward developing rapid, sensitive, and specific methods for detection or screening of foodborne pathogens at the preharvest stage. In this review, we will discuss the molecular methods available for detection and molecular typing of bacterial foodborne pathogens at the farm. Such methods include conventional techniques such as endpoint PCR, real-time PCR, DNA microarray, restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), and more advanced techniques such as matrix-assisted layer desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), whole-genome sequencing (WGS), and multilocus sequence typing (MLST).

MOLECULAR DETECTION TECHNIQUES

The development and application of new tools for the rapid identification of microorganisms is of paramount importance. Culture and isolation of bacterial pathogens is time-consuming and typically takes 36 to 48 h from sample processing to complete results (7). This time is considerably increased if the growth of the pathogen is slow or delayed (8). The use of molecular methods is a key approach for the rapid and accurate identification of foodborne pathogens. One of the major reasons for the wide acceptance of molecular techniques in pathogen detection is their ability to generate results within a short time compared to culture-based methods. In this section,

we discuss molecular methods available for pathogen detection.

Endpoint PCRs

The rapid and accurate detection of foodborne pathogens became possible with the arrival of PCR. PCR involves the amplification of a specific segment of DNA of any pathogen under restricted conditions of reagents and time-temperature combinations. PCR is divided into two categories, endpoint PCR and real-time PCR, on the basis of the time to result interpretation and the detection of the amount of amplified DNA. Endpoint PCR is further divided into simplex and multiplex PCR, the details of which are given below.

Simplex PCR

The easiest molecular approach to detecting pathogens is the amplification of genomic DNA using PCR with oligonucleotide primers that are specific for the genus or species of the bacteria. Simplex PCR can identify only one gene/target in a single PCR reaction and requires designing highly specific primers against the gene (9). PCR amplification is followed by endpoint visualization of the amplification product(s) in an agarose gel (10, 11). Simplex PCRs can identify the known microorganisms at the genus or species level in a very short time. Early detection of foodborne pathogens in farm settings is particularly important because it provides sufficient time to set up measures to prevent further spread and to strengthen biosecurity. However, it has limited utility in outbreak investigations involving new pathogens with no sequence information available in GenBank/NCBI. In such cases, PCR needs to be coupled with gene sequencing or restriction digestion of the amplified product for identification and differentiation of the new pathogens involved (9). Simplex PCR has been extensively utilized in prevalence studies for detection of an array of pathogens at the production (preharvest), slaughter, and processing stages (12–14).

Multiplex PCR

The inability of simplex PCR to amplify multiple targets/genes led to the development of multiplex PCR. Multiplex PCR can be used for simultaneous detection of multiple pathogens in a single PCR reaction. However, designing primers for a multiplex reaction is a challenge. Primers should be designed such that each primer sequence is able to produce a unique amplicon for the target DNA. Moreover, identification of a common annealing temperature is a prerequisite for successful design of a multiplex PCR (15).

Multiplex PCR has been highly commercialized, and several multiplex PCR detection kits are available. To date, several multiplex PCRs able to detect different genera or species of foodborne pathogens have been developed (16–21). For example, multiplex PCR was developed for concurrent detection of five major foodborne pathogens, namely *Staphylococcus aureus*, *Listeria monocytogenes*, *E. coli* O157:H7, *Salmonella enterica* serovar Enteritidis, and *Shigella flexneri*, targeting 16S rDNA, listeriolysin O (*hlyA*), the intimin (*eaeA*) gene, *invA*, and the invasion plasmid antigen H (*ipaH*) genes, respectively (22). This multiplex PCR assay was able to simultaneously detect all five of these organisms in artificially contaminated pork samples. Multiplex PCR has been successfully applied to the differentiation of species, serotypes, subtypes, toxin genes, and antimicrobial resistance genes of bacteria. Recently, Rawool and coworkers reported a multiplex PCR for simultaneous detection of the *Listeria* genus, *L. monocytogenes*, and three important lineages (LI, LII, and LIII) of *L. monocytogenes* (23). A multiplex PCR was reported for rapid identification of multiple serovars of *Salmonella*, namely Typhimurium, Choleraesuis, Infantis, Hadar, Enteritidis, Dublin, and Gallinarum (24). Application of such multiplex PCRs could be very useful in disease investigation because sometimes multiple serovars of *Salmonella* are found in food or food animals. This will not only help to significantly reduce the cost of diagnosis but will also reduce the time required for serotyping the isolates. Such PCRs could be very useful in detecting multiple pathogens from samples and in outbreak investigations involving several etiological agents. These can be successfully used in low-resource countries for rapid identification of pathogens.

Real-Time PCR

The most remarkable development in PCR technology has been the introduction of real-time monitoring of DNA amplification during a PCR reaction (25, 26). Also known as quantitative PCR, real-time PCR is a well-recognized method for the detection and quantification of foodborne pathogens. Real-time PCR involves target amplification and detection in a single step (27). Post-PCR procedures such as gel electrophoresis and gel imaging are not required in real-time PCR. Real-time PCR merges PCR with the use of fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction. Foodborne pathogens such as *Salmonella*, *Campylobacter*, *E. coli*, and *Listeria* are the leading causes of foodborne infections and outbreaks worldwide. Rapid assays are needed

to identify these pathogens quickly from fecal and environmental samples at food production sites to minimize the infection across the food chain.

Real-time PCR amplification can be mainly categorized into a non-amplicon sequence-specific method and sequence-specific methods which are based on fluorescent labeled probes (28). The nonspecific method is based on the use of double-stranded intercalating DNA binding dyes, such as SYBR green I and EvaGreen. Amplicon sequence-specific detection methods such as TaqMan, molecular beacons, and Scorpions are based on the use of oligonucleotide probes labeled with a donor fluorophore and an acceptor dye known as quencher (29). Both of these approaches use some kind of fluorescent marker which binds to the DNA, and as the number of DNA copies increases during the PCR reaction, the fluorescence signal also increases.

SYBR green is a double-stranded DNA binding fluorescent dye (30). This nonspecific intercalating dye emits slight fluorescence, and the fluorescence signal is enhanced when bound to the minor groove of the DNA double helix (31, 32). Comparison with reference samples of known concentration allows the quantification of the initial concentration of the target DNA. In initial cycles the fluorescence is too low to be distinguishable from the background. However, the point at which the fluorescence intensity increases above the detectable level corresponds proportionally to the initial number of template DNA molecules in the sample. The SYBR green real-time PCR cannot differentiate between specific target and nonspecific amplifications or primer dimers (33). The advantage of using SYBR green is that a DNA melting curve can be generated after PCR along with the calculation of the T_m value of the amplified products. SYBR green lacks specificity and binds to all double-stranded DNA; thus, it can be used to detect any PCR product (34).

TaqMan probes are short oligonucleotides (normally 10 bp long—10-mer) specific to the target sequence between the two primers used in the PCR. TaqMan probes carry a fluorescent reporter molecule at one end and a quencher molecule capable of quenching the fluorescence of the reporter molecule at the other end. However, during the PCR the TaqMan probe binds to the target sequence and is cleaved by the polymerase. Therefore, the reporter and quencher molecules are physically separated, and the fluorescence increases. The fluorescence, measured after each cycle in a real-time PCR, is proportional to the amount of the specific target amplification product and does not include PCR artifacts (35). TaqMan probes and molecular beacons are sequence-specific probes and only bind to the target sequence (36).

The major advantages of real-time PCR include (i) fast and high-throughput detection and quantification of target DNA sequences, (ii) simultaneous amplification and visualization of newly synthesized DNA amplicons, (iii) no post-PCR processing or gel imaging, (iv) very low chances of cross-contamination after PCR amplification, and (v) the ability to multiplex several targets in a single reaction (37). Real-time PCR has become an indispensable tool in the detection of viral pathogens because isolating viruses in the laboratory is difficult and time-consuming. Moreover, methods like enzyme-linked immunosorbent assay have low sensitivity, specificity, and reproducibility. For RNA viruses, real-time PCR is used in conjunction with reverse transcription to synthesize cDNA, which works as a template for PCR. Several real-time reverse transcriptase PCRs have been reported for the detection of RNA viruses (38, 39). However, a major limitation of real-time PCR is its inability to differentiate between live and dead cells. This may lead to false-positive results when estimating the presence of viable pathogens in a sample. Detection of dead cells may overestimate the risk associated with the foodborne pathogens. Also, real-time PCR has limited utility in the detection of new or unreported pathogens (40).

In a farm setting, food animals can be infected by several pathogens at a time. Therefore, it is desirable to develop molecular assays that can detect multiple pathogens concurrently. Multiplex PCR has been widely used for amplifying multiple targets by using multiple primer pairs in a single reaction. However, developing multiplex PCR is difficult and requires expertise during standardization. Moreover, including multiple targets in a PCR reaction significantly decreases sensitivity, and hence, the number of targets is kept to a minimum (28). Compared to conventional PCR, real-time PCR offers better options for multiplexing several targets in a single reaction. Recently, there has been an increase in the development of real-time multiplex PCRs for the detection of multiple pathogens including foodborne pathogens (41–46). In one such study, Hu et al. developed a molecular beacon-based multiplex real-time PCR for the detection of various foodborne pathogens, namely *S. enterica* subsp. *enterica*, *L. monocytogenes*, *E. coli* O157, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Campylobacter jejuni*, *Enterobacter sakazakii*, and *Shigella* spp. (46). Specific genes were targeted for simultaneous detection of eight foodborne pathogens. Fukushima et al. used a SYBR green-based multiplex real-time PCR for simultaneous identification of 24 genes of several foodborne patho-

gens (42). This assay was validated for the detection of foodborne pathogens in 33 out of 35 cases of foodborne outbreaks (42). Another study reported a rapid real-time multiplex PCR assay using four primer sets and four TaqMan probes for the detection of multiple *Salmonella* serotypes. The assay was found to be sensitive and correctly identified different *Salmonella* serotypes in artificially contaminated chicken skin and chicken meat samples (41). Fratamico et al. detected Shiga toxin-producing *E. coli* serogroups O26, O45, O103, O111, O121, and O145 with a TaqMan-based real-time multiplex PCR with primers targeting the *stx1*, *stx2*, *eae*, and *wzx* genes (43). Apart from pathogen detection, real-time PCR has been commonly utilized for the detection of antimicrobial resistance genes in foodborne pathogens (47–49). For instance, Roschanski et al. reported a multiplex real-time PCR for the detection of class A beta-lactamase genes *bla*CTX-M, *bla*SHV, and *bla*TEM and CIT-type AmpCs in *Enterobacteriaceae* (50). The authors correctly identified resistance genes in previously identified resistance gene subtypes and a few animal and environmental isolates (50).

DNA Microarray

DNA microarray is considered a rapid and sensitive pathogen detection technique that overcomes the limitations of PCR-based methods (9, 51). Microarrays allow a large number of specific DNA sequences to be detected simultaneously. A microarray consists of a microscopic glass slide (also known as a chip) containing thousands of chemically synthesized short sequences/probes of 25 to 80 bases adhered to the surface of the slide (35, 52). These probes have nucleotide sequences that are complementary to one or more target genes/organisms. Nucleic acids are extracted from the sample and labeled with a fluorescent or radioactive dye. DNA is denatured into single-stranded DNA fragments and incubated over the surface of the glass slide for several hours for hybridization to take place. DNA fragments hybridize to their complementary or near complementary DNA probes on the glass slide. The array is then washed to get rid of the unbound additional DNA and scanned to capture and quantify the fluorescence signal probe-sample complex. The fluorescence intensity is directly proportional to the concentration of fluorescent dye-loaded nucleic acid (53).

Several microarrays have been developed for the detection of foodborne pathogens (51, 54–62). For instance, Guo et al. developed a microarray system for simultaneous identification of 46 *Salmonella* O serogroups targeting O antigen-specific genes. The assay accurately identified 40 serogroups. The remaining six serogroups were identified in the combination of three pairs and needed further differentiation using PCR or conventional serotyping. The assay correctly identified 98% of the *Salmonella* strains tested, with a low detection sensitivity of 50 ng genomic DNA. This was the first time a comprehensive microarray system was developed for simultaneous identification of as many as 46 *Salmonella* O serogroups (51). In a similar study, Braun et al. developed a microarray assay to identify multiple *Salmonella* serovars prevalent in Europe and North America (59). This assay differentiated O antigens and 86 H antigens of *Salmonella*. The authors successfully evaluated their microarray system in 117 of 132 reference strains of *Salmonella*. The other 15 serovars gave a similar band pattern shared by multiple serovars, which could be due to high homogeneity between serogroups A and D1. Further, this assay was also used to identify a panel of *Salmonella* field isolates; all were correctly identified at the genus level, and the results of 88.6% of the isolates correctly matched the conventional serotyping data (59).

In addition to *Salmonella*, the microarray method has also been used for the identification and differentiation of other foodborne pathogens. For example, Wang et al. reported an oligonucleotide microarray for the detection of 22 foodborne bacterial pathogens. Their assay identified most of the major foodborne pathogens, namely *Salmonella* spp., *C. jejuni*, *Clostridium perfringens*, *S. aureus*, *L. monocytogenes*, *Bacillus cereus*, *Proteus vulgaris*, *V. parahaemolyticus*, and *Clostridium botulinum*, to name a few (54). This assay possessed good specificity and a low sensitivity of 10^2 CFU/ml of bacterium. Moreover, the assay was also validated using field samples (54). Chiang et al. developed a nylon chip-based microarray system targeting 16S rRNA for simultaneous identification of *Bacillus* spp., *E. coli*, *Salmonella* spp., *Staphylococcus* spp., and *Vibrio* spp. (63). Microarrays have also been used for the detection of resistance genes in bacterial pathogens. In one such study, Perreten et al. reported a microarray system for simultaneous detection of 90 antibiotic resistance genes in Gram-positive bacteria (64). The chip contained 137 oligonucleotide probes and was hybridized with bacterial strains carrying specific antibiotic resistance genes. Microarray results were in accordance with the phenotypic resistance (64).

Microarrays allow simultaneous detection of multiple targets in a single assay. Several identification assays can be run in parallel, with each probe representing a specific small section of a genome or a sequence common

to multiple genomes (65). A major limitation of this method is the complexity and the amount of time invested to design genetic regions which are diverse enough to allow discrimination of several species (66).

Loop-Mediated Isothermal Amplification (LAMP) Assay

LAMP is an innovative nucleic acid amplification technique used for rapid, sensitive, and specific detection of foodborne pathogens (53, 67). LAMP is considered an ideal technique for use in field conditions because it does not require costly laboratory equipment (thermal cycler) for DNA amplification. LAMP is an isothermal nucleic acid amplification technique in which amplification is carried out at a single constant temperature of 60° to 65°C. For amplification, LAMP uses two inner and two outer primers to target six distinct regions within the target DNA. A pair of "loop primers" is also used to accelerate the amplification process. A polymerase enzyme with high displacement activity (usually *Bst* DNA polymerase) facilitates DNA amplification at a constant temperature, unlike conventional PCR, where amplification is carried out in an alternating time-temperature combination. Amplified DNA can be visually detected as increased turbidity resulting from the accumulation of magnesium pyrophosphate (an amplification byproduct) (68), by measuring the turbidity over time, or by measuring fluorescence using intercalating dyes such as SYBR green I (69). Several variants of the LAMP assay are in vogue, namely multiplex LAMP (70), reverse-transcription LAMP (71), real-time LAMP (72), RFLP LAMP (73), multiple inner primers LAMP (74), and *in situ* LAMP (75).

The LAMP assay has several advantages over conventional molecular detection methods. Due to the high specificity of the primers, the amount of DNA produced in the LAMP assay is significantly higher and the detection limit is lower than conventional PCRs (53, 76). Moreover, a LAMP assay takes only 30 to 60 min for amplification (77, 78), which is considerably less than the time taken by conventional PCRs (4 to 6 hours). One of the major limitations of LAMP is its poor ability to multiplex (70). Multiplexing in LAMP increases the probability of false-positive amplification because of the presence of several pairs of primers targeting different regions.

LAMP has been applied for the detection of various foodborne pathogens due to its rapidity, high sensitivity, and ease of producing results. Dong et al. developed a LAMP assay targeting the *hip*O gene to detect *C. jejuni* in samples from cattle on farms (79). The assay was found to be specific (100% inclusivity and exclusivity), sensitive (100 fg/μl detection limit), and quantifiable. The assay took less than 30 min to produce amplification for all *C. jejuni* isolates (79). Yamazaki et al. compared the LAMP assay with conventional isolation methods for the detection of *C. jejuni* and *C. coli* in naturally contaminated chicken meat samples (80). The LAMP assay showed high sensitivity and specificity of 98.5% and 97.4%, respectively. Moreover, LAMP required only 23.5 to 25.5 hours for the complete detection of the bacteria starting from the enrichment culture compared to 3 to 4 days for conventional culture methods (80). A six-primer LAMP system targeting eight regions of the *hlyA* gene has been reported for the detection of *L. monocytogenes* (81). This LAMP assay was rapid and produced results within 40 min. Sensitivity of the LAMP method for the detection of *L. monocytogenes* in pure cultures was 2.0 CFU per reaction, and the sensitivity was 100-fold higher compared to conventional PCR (81).

The LAMP assay has also been used for the simultaneous detection of antimicrobial resistance genes and the genus or species of the bacteria. In one such instance, the LAMP assay simultaneously detected staphylococcal *mecA* (methicillin resistance) and *spa* (*S. aureus*) genes (82). The LAMP assay amplified both the genes within 60 min with high specificity and sensitivity. In a recent study, Chen et al. developed a LAMP system for identification of methicillin-resistant *S. aureus* by simultaneously amplifying the *mecA* (methicillin resistance specific), *nuc* (*S. aureus* specific), and *femB* (virulence marker) genes (83). The LAMP assay identified all three genes in less than 60 min. There was uniformity in the results of LAMP and PCR assays (83).

MALDI-TOF MS

MALDI-TOF MS is a rapid, sensitive, and cost-effective technique currently used for pathogen detection. It generates unique mass-spectral fingerprint signatures for individual microorganisms, which helps in genus and species identification (84). In this technique, a sample is mixed with an organic compound known as a matrix. Matrix components vary according to the type of sample analyzed and the type of laser used (84, 85). The most commonly used matrix components are α-cyano-4-hydroxycinnamic acid, 2,5-dihydroxy benzoic acid, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), ferulic acid, and 2,4-hydroxy-phenyl benzoic acid. These matrix compounds have strong optical absorption capabilities within the range of laser wavelength used. The samples are mixed with the matrix and deposited on a

metal plate. This mixture is then left to dry, which results in crystallization of the matrix along with the sample. The sample embedded in the matrix is then bombarded with a pulsed laser beam, which results in desorption of the analytes in the matrix. The analytes are vaporized and ionized into the gaseous phase. Desorption and ionization generates singly charged ions from the analytes by either gain or loss of more than one proton. The soft ionization approach used in MALDI is beneficial because it does not lead to a loss of sample integrity. The ionized analyte molecules are accelerated through an electrostatic field at a fixed potential and made to travel through a metal tube toward a detector. The detector determines the TOF taken by gaseous analyte molecules to travel through the flight tube and reach the detector (86). Detection of each bioanalyte molecule depends on the molecular mass (*m*), the charge (*z*), the ratio mass/charge (*m/z*), and the relative intensity of the signal (87). Small bioanalyte molecules travel faster than larger molecules (higher molecular weight) and are detected early. Different biomolecules in a sample with different mass (*m*) to charge (*z*) ratios form a mass spectrum on the basis of the TOF. Currently, various applications of MALDI-TOF MS are being utilized for AMR detection in bacterial pathogens. These include detection of enzymes/antibiotics and/or their degraded products, direct MALDI-TOF MS analysis of bacterial extracts, and mini-sequencing-primer extension followed by MALDI-TOF assay.

During the past few years, a surge has been observed in publications using the MALDI approach for pathogen detection, including foodborne pathogens. MALDI-TOF MS has been developed and validated for several bacterial foodborne pathogens, namely *Salmonella* (88, 89), *Campylobacter* (90), *S. aureus* (91, 92), and *E. coli* (93). Dieckmann and Malorny successfully used MALDI-TOF MS for identification of the five most frequently isolated *S. enterica* serovars, namely Enteritidis, Typhimurium, Virchow, Infantis, and Hadar (89). They also reported 100% inclusivity and exclusivity of serovar-identifying biomarker ions for these *Salmonella* serovars (89). A study validated MALDI-TOF MS as a powerful tool for the identification of clinical isolates of coagulase-negative staphylococci (94). Mandrell et al. reported the identification and differentiation of multiple strains of *Campylobacter coli*, *C. jejuni*, *Campylobacter helveticus*, *Campylobacter lari*, *Campylobacter sputorum*, and *Campylobacter upsaliensis* isolated from animal, clinical, or food samples using a MALDI-TOF MS system (90).

MALDI-TOF MS is a rapid, cost-effective, sensitive, and specific technique (7, 95). The expected result turnaround time for MALDI-TOF MS is less than 5 min, which is significantly faster than other molecular techniques used for pathogen detection (77). Another advantage is its ability to detect unknown organisms, which is not limited to the prespecified targets (7). Mass spectra obtained from unknown organisms are compared with the already available MS database to identify the organism. Once identified, the mass spectra of unidentified species can be added to the MS database after identification by sequencing for future reference (96). Disadvantages of MALDI-TOF MS include its initial high setup cost, which limits its use as a routine diagnostic method in resource-limited countries. However, in comparison to conventional methods, MALDI is much less labor intensive, with a low overall cost. Very few reagents and laboratory supplies are needed for a MALDI experiment; these include a metal slide, matrix solution, pipette tips, and loops for sample application. A research group reported that use of MALDI-TOF MS resulted in net savings of 87.8% in reagent costs annually compared to conventional methods (97). The authors further stated that the routine use of MALDI in pathogen detection could offset the initial high cost of MALDI equipment in approximately 3 years (97). Because of its ease of operation, rapid result turnaround time, and very low overall cost per sample, the MALDI technique is revolutionizing the field of pathogen detection.

MOLECULAR TYPING TECHNIQUES

Several PCR-based molecular typing methods have been developed and applied to study the population structure and molecular epidemiology of foodborne pathogens at the preharvest and postharvest stages. These methods are based on PCR amplification and subsequent analysis of the banding pattern in gel electrophoresis. One of the main advantages of typing techniques is their utility in the investigation of foodborne outbreaks and the complete understanding of the epidemiology of foodborne infections (98). Rapid typing methods can significantly reduce costs associated with detection, containment, and decontamination. Working principles, advantages, disadvantages, and the application of such methods in food safety research are discussed in the following subsections.

PCR-RFLP

RFLP is a molecular typing technique which involves amplification of genomic DNA and digestion with suitable restriction enzymes. Restriction fragments are then separated using gel electrophoresis. Restriction enzymes,

also known as restriction endonucleases, cut the bacterial DNA at specific sites and produce restriction products of specific sizes. The presence or absence of these restriction fragments of different sizes constitutes a specific banding pattern for each target that can be visualized over gel electrophoresis. RFLP is a simple method to detect known mutations that alter recognition sites of restriction endonucleases (99). However, with the advent of pulsed-field gel electrophoresis (PFGE) and other inexpensive techniques for molecular typing, PCR-RFLP has become obsolete (100, 101). The advantages of using PCR-RFLP include its low cost and lack of requirement of expensive and advanced instruments. It can be performed in a laboratory with basic PCR facilities, and no specialized personnel training is required. PCR-RFLP is a very sensitive technique for strain identification and differentiation of microbes. The disadvantages include the requirement for specific restriction enzymes and difficulties in identifying the perfect combination of enzymes targeting different sites in the target sequence. Moreover, the process is time-consuming and involves several steps, such as PCR amplification, gel extraction, restriction digestion using endonucleases, and final gel electrophoresis for result interpretation. PCR-RFLP is not a suitable technique for preharvest food safety research because simultaneous detection of multiple pathogens is difficult due to the requirement of specific primers and restriction enzymes for each target. This technique can be used for strain differentiation when time is not a constraint and there is no urgency to get rapid results.

RAPD

RAPD is a molecular subtyping technique that is widely used for typing for various bacterial pathogens (102). It involves PCR amplification of random segments of genomic DNA with a single primer of arbitrary nucleotide sequence (9 to 10 bp). Unlike conventional PCR, the size of the target PCR products is unknown in RAPD. The arbitrary primer may or may not amplify the segment of the target genome, depending on the positions that are complementary to the primer sequence. Moreover, the arbitrary primer can simultaneously anneal to multiple sites in the whole genome and produce amplification products of varied sizes; this difference in the banding pattern is the basis of strain differentiation. PCR products can be visualized over gel electrophoresis, and banding patterns of different strains/isolates can be compared.

RAPD has been extensively used for molecular subtyping of various foodborne pathogens. For instance, Lin et al. developed and optimized a RAPD protocol for molecular subtyping of *S.* Enteritidis strains. They used a panel of primers to characterize isolates of *S.* Enteritidis, which had been previously characterized by phage typing, ribotyping, and PFGE. RAPD distinguished isolates into different RAPD subtypes. Moreover, RAPD successfully differentiated eight *S.* Enteritidis isolates which were previously untyped by the above three methods. It was also reported that RAPD had more discriminatory power than any of the other three subtyping methods applied individually (103). Two years later, a RAPD method was developed to differentiate strains of *Salmonella* Typhi and other *Salmonella* spp. (104). Five-primer RAPD successfully differentiated several strains of *Salmonella* Typhi into 21 distinct RAPD profiles. Moreover, RAPD was also able to differentiate 65 other *Salmonella* isolates representing 42 serotypes. Yoshida et al. used four-primer RAPD to characterize 20 unrelated *L. monocytogenes* strains isolated from different animals and locations at different points in time (105). All strains were classified into 18 unique subtypes. The authors further characterized seven epidemiologically related *L. monocytogenes* strains isolated from raw milk and a bulk tank collected from a dairy farm and found that all *L. monocytogenes* strains had the same RAPD profile (105). Hernandez et al. studied a set of *C. jejuni* and *C. coli* isolates recovered from human feces, seawater, and poultry products and reported a total of 118 different RAPD profiles, with each profile having 4 to 11 bands (106). These authors found the RAPD method to be highly discriminatory for *Campylobacter* subtyping. The RAPD protocol reported by Hernandez et al. (106) was later validated by Açik and coworkers (107) in *C. jejuni* and *C. coli* isolated from healthy cattle and sheep. The authors found a high degree of heterogeneity among *Campylobacter* isolates using RAPD (107). Another study successfully used RAPD to distinguish 20 reference strains of *Clostridium difficile* (108).

One of the major drawbacks of RAPD is low reproducibility. Highly standardized experimental conditions with consistent reagents and cycling conditions are required to get consistent results. The quality and concentration of DNA and PCR cycling and reaction conditions can affect the reproducibility of the RAPD-PCR (102). Another limitation is the requirement of very-high-quality DNA for the reaction. Precautions need to be taken to avoid contamination of DNA because the short random primers (9 to 10 bp) used for target amplification can cross-amplify similar sequences of other pathogens.

Amplified Fragment Length Polymorphism (AFLP-PCR)

AFLP-PCR combines the advantages of RFLP and PCR assays. This technique involves the digestion of entire bacterial DNA by one or more restriction enzymes, commonly MseI and EcoRI (109). The genomic DNA is incubated with restriction enzymes for complete digestion of the bacterial DNA into restriction fragments. Digestion is followed by the ligation of the resulting fragments to 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 these fragments is done by using primers targeting specific sequences ligated to either end of restriction endonuclease-digested genomic DNA rather than genomic DNA itself; this generates a high number of fragments for analysis. The resulting patterns of PCR-amplified DNA fragments are then analyzed by gel electrophoresis to assign subtypes (110, 111). AFLP-PCR is considered reliable and robust because it uses stringent reaction conditions for primer annealing (112). The high discriminatory ability of this assay is due to the generation of a higher number of fragments for analysis (113). Prior knowledge of the bacterial DNA sequence is not required for analysis. Moreover, highly restrictive amplification conditions due to high specificity of primers toward adaptors minimizes nontarget amplification. The discriminatory power of AFLP is considered to be equal to PFGE, the current gold standard in molecular typing.

This whole-genome fingerprinting technique has been used for high-resolution typing of several bacteria in epidemiological studies (113). Guerra et al. developed a single-enzyme (EcoR1) AFLP assay for molecular typing of *L. monocytogenes* (111). They evaluated this method with 84 *L. monocytogenes* cultures and compared AFLP-PCR results with serotyping, phage-typing, and cadmium and arsenic resistance typing. The technique was found to be reproducible, and a range of different banding patterns was obtained. AFLP produced indistinguishable banding patterns for *L. monocytogenes* isolates related by origin and serovar. AFLP, phage typing, and cadmium/arsenic typing produced indistinguishable patterns for the majority of epidemiologically related groups of *L. monocytogenes* cultures (111).

Since its inception, a few variants of AFLP technique have been reported. An example of these is fluorescent AFLP (FAFLP). FAFLP uses fluorescent oligonucleotide primers, which enables accurate and high-resolution detection of the amplified digested fragments. Several studies have used FAFLP for high-resolution genotyping of the foodborne pathogens (109, 114, 115). Recently, Roussel and coworkers compared FAFLP and PFGE for molecular subtyping of *L. monocytogenes* isolated from humans, food, food processing environments, and animals (109). Both the techniques were able to produce unique types for epidemiologically associated strains. The discriminatory power of FAFLP was similar to that of PFGE. The authors concluded that as a less labor-intensive assay, FAFLP could be used for outbreak investigations and source tracking of the pathogens (109). However, they also highlighted the necessity of complete standardization of FAFLP protocols and reproducibility assessment through trials before using FAFLP for routine typing (109). In a similar study, FAFLP and PFGE were compared for molecular typing of *Salmonella* Typhimurium DT126 to differentiate between outbreak-associated and epidemiologically unrelated DT126 isolates in Australia. In this study, the discriminatory power of AFLP was found to be greater than that of PFGE. Both techniques differentiated between isolates from separate outbreaks. However, neither assay could separate epidemiologically unrelated isolates from the outbreak isolates (116).

MLST

MLST was first proposed in 1998 as a typing approach that enables the unambiguous characterization of bacterial isolates in a standardized, reproducible, and portable way (117). Since its discovery in 1998, MLST has been widely applied to a large number of pathogens of public health and food safety importance. MLST involves the extraction of genomic DNA and PCR amplification of highly conserved housekeeping genes (preferably seven). After amplification, amplicons are sequenced, aligned, and investigated using bioinformatics software. Housekeeping genes are distributed in different loci around the chromosome of microorganisms (118, 119). For each housekeeping gene, different sequences are assigned as alleles, and the alleles at the multiple loci constitute an allelic profile, which unambiguously defines the sequence type of each isolate. Reasons for targeting housekeeping genes in MLST include their uniform presence in all organisms, their conserved nature, and their ability to provide adequate discrimination for molecular typing. Moreover, sequence data submitted by researchers located anywhere in the world are freely available online via the internet. Research laboratories can easily compare their sequences with other laboratories. Online collection of

sequence data helps with the rapid comparison of sequences of bacteria obtained from any part of the world (120).

MLST data available online have been applied in several food animal farm-based epidemiological and evolutionary studies (121–127). For example, a study used MLST to compare *C. coli* isolates selected from fecal, environmental, and carcass samples of antibiotic-free and conventional swine production systems (128). The authors reported a genotypically diverse *C. coli* population with the presence of *C. coli* isolates sharing a common ancestry in both production systems. They concluded that a common population structure between *C. coli* from conventional and antibiotic-free farm systems may be the reason for persistence of antimicrobial-resistant *C. coli* in antibiotic-free farms (128).

MLST has several advantages over other genotyping techniques. First, it is a DNA-based technique and does not require processing of live cultures. Second, MLST detects changes at the genotypic level (DNA), which may not be apparent by phenotypic typing methods such as serotyping. MLST targets coding regions and uses variation that accumulates very slowly in the population, in contrast to PFGE. Moreover, the online availability of the sequence data from various parts of the world makes it easy to compare sequences from different places. These qualities make MLST an excellent molecular tool for long-term epidemiological investigations (117). Although MLST is easy, rapid, and less expensive than WGS, it is costlier than other genotyping methods such as PFGE and PCR-RFLP.

PFGE

PFGE is the most widely used molecular typing system for bacterial pathogens. It is considered one of the most discriminatory subtyping methods and thus is regarded as a gold standard in molecular epidemiological studies (129, 130). In 1996, the Centers for Disease Control and Prevention (CDC) established a nationwide molecular subtyping network called PulseNet for molecular surveillance of foodborne diseases to facilitate early outbreak detection and capacity building in disease investigation. PulseNet uses DNA fingerprints of the foodborne bacteria to detect an outbreak. Once a PFGE pattern is generated, the laboratory analyzes the pattern using bioinformatics software (BioNumerics). Then the DNA fingerprint is uploaded to the PulseNet database, where it is investigated and analyzed to determine if it is causing an outbreak. The PulseNet PFGE database helps with the quick identification of outbreaks that are geographically separated and caused by a similar pathogen or strain across states. PulseNet facilitates rapid web-based sharing of PFGE fingerprints of the bacterial pathogens. Online sharing of DNA fingerprints helps with detection of temporally and spatially related foodborne disease outbreaks. PFGE is based on the digestion of bacterial genomic DNA with restriction enzymes, which allows analysis of fragments up to 10,000 kb (131). Large fragments of DNA molecules are separated by applying an electric field that changes direction periodically in a gel matrix. The comparison of banding patterns of genomic DNA fragments after digestion with a restriction enzyme is the basis of PFGE. Briefly, bacterial cells are incubated overnight, immobilized in agarose, and digested with a rare restriction enzyme. The enzyme-treated plugs are then loaded onto an agarose gel, and the DNA fragments are separated based on size using an alternating electric field. This results in unique DNA fingerprints/PFGE patterns of the bacterial pathogens. The PFGE electrophoretic banding patterns are exceptionally reproducible and specific for various bacterial organisms (132). The major limitations of PFGE are that it is labor intensive and requires 3 to 4 days to generate complete results. Moreover, some strains of bacteria cannot be typed using PFGE.

However, PFGE has been reported as an efficient, time-saving, and cost-effective method for serogrouping of *Salmonella* isolates (133). In a study from Taiwan, 45 *S.* Typhimurium isolates which caused food-poisoning diarrhea during 1991 to 1994 were subjected to restriction digestion with XbaI, AvrII, and SpeI, followed by PFGE, resulting in 26 PFGE patterns. Since isolates of the same patterns were recovered from unrelated food-poisoning cases, it was suggested that these isolates could be prevalent and circulating in Taiwan (134). McCullagh et al. used PFGE for molecular typing of 109 *S. aureus* isolates in Northern Ireland, which included 47 isolates from broilers with clinical conditions and 62 strains from hatcheries (135). PFGE analysis showed a similarity between 85% of the strains from clinical sources and 71% of the hatchery isolates. On the basis of the PFGE data, the authors concluded that the hatchery was a potential source of infection for clinical broiler disease (135). Another study used PFGE for identification and differentiation of various *Salmonella* serotypes, namely Heidelberg, Javiana, Typhimurium, Newport, Enteritidis, Dublin, Pullorum, and Choleraesuis (136). Molla et al. used PFGE to investigate the occurrence and genotypic relatedness of *S. enterica* isolated from feed and fecal samples in commercial swine farms (137). They reported that more than 50% of the *Salmonella* isolates recovered from feed shared geno-

typic clonality with those detected in swine fecal samples, suggesting the dissemination of *Salmonella* via feed (137). The presence of highly clonal *Salmonella* isolates indicated an epidemiological link between *Salmonella* in feed and in feces (137). PFGE can be used for short-term as well as for long-term epidemiological surveillance programs. Refsum et al. performed PFGE to analyze 142 isolates of *S.* Typhimurium obtained from passerines (n = 46), gulls (n = 26), domestic animals (n = 50), and the environment (n = 13) collected over a period of 30 years and revealed that passerines are the major source of human infection in Norway (138).

WGS

WGS of bacterial pathogens using next-generation techniques (NGS) can provide comprehensive genetic information, including genus and species identification and sequence information on drug resistance and virulence determinants (139). The term "next-generation sequencing" specifically refers to the strategies that have succeeded the old sequencing method, i.e., Sanger sequencing. Popular platforms available for NGS include pyrosequencing (454/Roche), Illumina (Hiseq, Miseq), ion-torrent sequencing, SOLiD, PacBio RS, and Oxford Nanopore (140, 141). Each NGS platform, however, follows the same workflow sequence, which involves nucleic acid extraction, library preparation, sequencing, and data analysis using bioinformatics software. Illumina sequencing involves DNA synthesis by a DNA polymerase incorporating deoxynucleoside triphosphates labeled with fluorescent terminators which are detected in real time (142). Illumina offers a range of sequencing instruments which includes low-throughput benchtop machines as well as ultra-high-throughput machines. The most extensively used Illumina platforms are MiSeq and HiSeq (143). MiSeq has been shown to be the best regarding both throughput per run and error rates compared to pyrosequencing and ion-torrent sequencing platforms (77). Ion-torrent sequencing utilizes the "sequencing-by-synthesis" approach. It is performed on a semiconductor chip and is based on the detection of hydrogen ions that are released during DNA synthesis (9, 142).

The huge amount and comprehensiveness of the sequence data and the low cost per base makes NGS an interesting alternative to current PCR and culture-based detection methods. Moreover, apart from molecular typing, NGS also has utility in virtual screening/detection of antimicrobial resistance and virulence determinants in the whole genome of the microorganism. Once a microorganism is sequenced, the whole genome of the bacteria is screened for the presence of antimicrobial resistance and virulence determinants/markers using gene prediction programs (ResFinder). This approach helps in predicting future resistance. However, the reliability of WGS as a tool for predicting antimicrobial resistance is critically dependent on the availability of a regularly updated resistance database and prior knowledge of the target sequence. WGS data in the absence of any phenotypic support from traditional tests might lead to false-positive or -negative results. For example, the absence of a known gene sequence in the whole genome does not necessarily mean that the isolate is susceptible. There might be other novel or unreported molecular mechanisms that confer the same resistance, which is not characterized or sequenced yet. Similarly, NGS may not be able to identify and interpret the presence of an unreported/unknown sequence. NGS analysis depends a lot on the annotation interpretation of the genome sequence using prior knowledge of such sequences (144). In our opinion, until a technological advancement is made which can accurately predict antimicrobial resistance, at least some support from the phenotypic tests will be required. Another major limitation of NGS is the requirement of a highly trained professional for comprehensive analysis and interpretation of the WGS data. Relevant information needs to be skillfully extracted from the vast NGS data sets using bioinformatics software packages. Also, the high initial setup cost and the scarcity of user-friendly bioinformatics tools curtails its use in resource-limited countries.

However, these limitations could be overcome by the fact that NGS methods provide high-resolution sequence data in a short time frame of only 2 to 5 days. Moreover, the cost of performing WGS is expected to go down in the near future due to improvement in the methodology and increasing competition among the commercial houses to provide affordable and cost-effective genome sequencing options. Keeping all these facts in mind, it can be safely assumed that WGS will become an indispensable tool in molecular diagnostics, pathogen typing, and detection of antimicrobial resistance determinants in the near future.

CONCLUSION

Current methods for pathogen detection and identification are mainly culture based. Although they are accurate and complete, culture methods are time-consuming and laborious, which seriously hampers the in-time implementation of biosecurity measures on farms. To overcome the limitations of bacterial isolation, rapid

molecular detection methods have been developed. In particular, DNA base detection methods such as PCR, real-time PCR, multiplex PCR, and microarray have been extensively used for pathogen detection at the pre- and postharvest stages. However, these methods still require a prior culture step and a trained individual to perform the tests. LAMP is a unique nucleic acid amplification technique used for rapid, sensitive, and specific detection of foodborne pathogens in a single step. LAMP is called "equipment-free technology" because it does not require an expensive thermal cycler for DNA amplification, and visualization with the naked eye is sufficient for result interpretation. No post-PCR gel electrophoresis is required. Moreover, result turnaround time for a LAMP protocol is only 30 min to 1 h. These features make LAMP a method of choice for use in field conditions. However, the only limitation of LAMP is its inability to multiplex various targets. The introduction of techniques like MALDI-TOF MS has completely revolutionized the field of pathogen detection. MALDI is based on the production of mass-spectral fingerprints for each microorganism, which helps in precise genus and species identification. This technique is very fast and sensitive and provides results within 5 min from a single isolated bacterial colony. Another advantage of MALDI is its ability to detect unknown organisms. The initial equipment cost of MALDI-TOF MS is very high, but research suggests that routine use of this technique could save 87.8% in reagent costs annually compared to conventional methods. Once the equipment is installed, the cost of processing samples becomes very low.

For molecular typing of microorganisms, several methods are in vogue. However, PFGE is considered the gold standard for molecular typing and is used extensively worldwide for epidemiological typing of foodborne pathogens. NGS, with its high multiplexing capability, is rapidly becoming the method of choice for pathogen and AMR detection. In addition to pathogen detection, WGS can accurately predict the presence of antimicrobial resistance and virulence determinants within the whole-genome sequence. MLST, although it is a good typing technique, is less likely to be the method of choice because of the high cost of sequencing multiple housekeeping genes for a single isolate. Moreover, MLST is not considered an ideal technique for serovar differentiation. MLST is more suited for long-term epidemiological investigations. Although a lot of progress has been made in developing rapid and sensitive methods for pathogen detection; culture methods still remain essential. Culture methods provide useful information about phenotypic characteristics such as antimicrobial resistance, which is crucial in deciding on preventive measures. We strongly believe that recent molecular-based diagnostic methods will not be able to completely replace conventional microbiological and biochemical methods for the detection of genus/species and antimicrobial resistance. We foresee in-tandem use of traditional phenotypic methods and the latest molecular methods, at least for some time to come.

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