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Review

Molecular detection of foodborne pathogens with emphasis on multiplex-allele-specific PCR, computational primer design, and gene amplification approaches

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ABSTRACT

Foodborne diseases continue to be major public health challenges around the world, with contaminated food linked to an estimated 420,000 deaths annually, many of which are children under five. Traditional testing methods for foodborne pathogens (i.e., microbial culturing and biochemical assays), while generally reliable, are slow and labor-intensive and significantly limit their ability to protect the public from exposure to foodborne pathogens. Reflecting on these limitations, a growing number of methods using rapid detection approaches, which are more efficient for detecting foodborne pathogens, have been developed, including nucleic acid-based methods, immunological methods, and biosensor-based methods. In particular, multiplex polymerase chain reaction (mPCR) offers a good method for the rapid detection of multiple pathogens in food samples. This review highlighted both the progress and prospects of multiplex-allele-specific PCR (MAS-PCR) technology and its use to detect and genotype foodborne pathogens in food samples. Also, the MAS-PCR used with probe-based methods has the possibility of being more specific and sensitive than the single PCR assay alone. The subsequent new approaches are designed to minimize the time it takes to identify pathogens to improve food safety and avoid disease outbreaks. Ultimately, advanced technologies would demonstrate increased speed and accuracy of pathogen detection, creating a greater contribution towards global food safety and public health.

1. Introduction

Foodborne diseases are a significant public health concern. The WHO estimates that 420,000 deaths occur annually due to consumption of contaminated food. Children under 5 years bear a substantial burden, accounting for 125,000 deaths and carrying a 40% burden of foodborne diseases. The incidence of foodborne diseases has surged globally, particularly in India (Villanueva-Segura et al., 2020; Pires et al., 2021; Almaary, 2023). Food-associated microbes (FAMs) could be defined as the microbes/microorganisms that live in food; contribute to food fermentation and production; or contaminate the food (Lee et al., 2022; López et al., 2025). Bacteria, viruses, fungi, algae, protozoa, and archaea are the different classes of FAMs. FAMs are used by food industries for the production of wine, beer, dairy products, and bakery products (Narayana Saibaba, 2022). Conversely, FAMs growth is also concerned about the food spoilage and contamination; sometimes it would be pathogenic microbial growth in the foodstuff. The most prevalent pathogens causing foodborne diseases are viruses, bacteria, and parasites (Adley and Ryan, 2025); foodborne diseases linked to fungus are also established (Long et al., 2023). See Table 1 listing the examples for pathogenic microbes from food sources. Consumption of contaminated food containing these pathogens results in foodborne diseases. Globally;

Abbreviation: AGPase, ADP- Glucose Pyrophosorylase; Bt1, Brittle-1 (ADP-glucose transporter); CRISPRCas 9, Clustered Regularly Interspaced Short Palindromic Repeats Cas9 Protein; dCas, Deactivated CRISPR- associtated protein; FAO, Food and Agriculture Organizations; FOS, Fructooligosaccharides; GBSS, Granule Bound Starch Synthase; GMO, Genetically Modified Organism; IPK1, Inositol Pentakisphoshate 2-Kinase; MIPS, Myo- Inositol-1- Phosphate Synthase; NFHS, National Family Health Survey; QMO, Quality Protein Maize; QTL, Quantitative Trait Loci; RDA, Recommended Dietary Allowance; RFOs, Raffinose Family Oligosaccharides; RNAi, RNA Interference; SBE, Starch Branching Enzyme; SDGs, Sustainable Development Goals; SPS, Sucrose Phosphate Synthase; T6P, Trehalose-6- Phophate; TALENs, Transcription Activator -Like Effector Nuclease; TCA, Tricarboxylic Acid; VBNC, viable but non-culturable.

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Table 1
Foodborne pathogens, their species, and associated food sources.

Classification	Species name	Food source	Reference
Viruses	*Norovirus*	leafy greens (such as lettuce), fresh fruits, and shellfish (such as oysters)	CDC (Centers for Disease Control and Prevention); 2021; WHO (World Health Organization); 2015
	Hepatitis A virus	fruits, vegetables, shellfish, ice, and water consumption of uncooked/	Patel et al.; 2016; CDC (Centers for Disease Control and Prevention); 2021; WHO (World Health Organization); 2015
	Hepatitis E virus (genotype 3)	undercooked pork or deer	Patel et al.; 2016
	Hepatitis E virus (genotype 4)	Meat	Patel et al.; 2016; WHO (World Health Organization); 2015; FAO (Food and Agriculture Organization); 2019
	Aichivirus (AiV)	Consumption of shellfish By ingestion of HAV-contaminated food or water	Scallan et al.; 2011
	Astrovirus (AstV)	oysters and shellfish	Scallan et al.; 2011
	Coronaviruses		Scallan et al.; 2011
	Rotavirus (RV)	contaminated water and food, especially shellfish	CDC (Centers for Disease Control and Prevention); 2021; WHO (World Health Organization); 2015
	Sapovirus (SaV)	contaminated water and food, especially shellfish	CDC (Centers for Disease Control and Prevention); 2021; WHO (World Health Organization); 2015
Parasites			
Helminths	*Trichinella*	undercooked meat and Pork	FAO (Food and Agriculture Organization); 2019; WHO (World Health Organization); 2015
	Taeniasaginata	Cow	FAO (Food and Agriculture Organization); 2019; WHO (World Health Organization); 2015
	T. solium/T. asiatica	Pork	FAO (Food and Agriculture Organization); 2019; WHO (World Health Organization); 2015
	Anisakis simplex	raw or undercooked fish	FAO (Food and Agriculture Organization); 2019; WHO (World Health Organization); 2015
	Pseudoterranova	raw or undercooked fish	FAO (Food and Agriculture Organization); 2019; WHO (World Health Organization); 2015
	Diphyllobothriumlatum	raw or undercooked fish	FAO (Food and Agriculture Organization); 2019; WHO (World Health Organization); 2015
	Clonorchissinensis, Paragonimuswestermani, capillariaphilippinensis,	fish or marine source	FAO (Food and Agriculture Organization); 2019; WHO (World Health Organization); 2015; Tatonova et al.; 2018
	Gnathostoma roundworms, Heterophylesheterophyles, Metagonimusyokagawai, Echinostomum, Nanophyetussalmincola, Eustrongylides, Phylometra, and Nybeliniasurmenicola		
Bacteria (Non spore forming)			
Brucella spp.	*B. abortus, B. melitensis, B. suis, B. ovis, B. canis, and B. neotomae*	raw or undercooked meat, unpasteurized milk, and dairy products and raw fish	CDC, 2021; WHO (World Health Organization); 2015
Campylobacter spp.	*Campylobacter avium, C. canadensis, C. coli, C. concisus, C. corcagiensis, C. cuniculorum, C. curvus, C. fetus, C. gracilis,*	king prawns and chilli sauce	CDC, 2021; WHO (World Health Organization); 2015
	C. helveticus, C. hominis, C. hyointestinalis, C. insulaenigrae, C. iquaniorum,	raw chicken liver and cooked	CDC, 2021; WHO (World Health Organization); 2015

(continued on next page)

Table 1 (*continued*)

Classification	Species name	Food source	Reference
	C. jejuni, C. lanienae, C. lari, C. mucosalis, C. peloridis, C. rectus, C. showae, C. sputorum, C. subantarcticus, C. troglodytis, C. upsaliensis, C. ureolyticus, and C. volucris	Food poor food handling hygiene and deficient kitchen facilities	CDC (Centers for Disease Control and Prevention); 2021; WHO (World Health Organization); 2015
Salmonella spp.	*S. enterica are: S. enterica subsp. enterica, S. enterica* subsp. *salamae, S. enterica* subsp. *arizonae, S. enterica subsp. diarizonae, S. enterica* subsp. *houtenae, and S. enterica* subsp. *Indica*	meat products and ready-to-eat food	Scallan et al.; 2011; CDC (Centers for Disease Control and Prevention); 2021; WHO (World Health Organization); 2015
Yersinia spp.	*Y. pestis, Y. pseudotuberculosis, Y. enterocolitica, Y. aldovae, Y. bercovieri, Y. entomophaga, Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. mollaretii, Y. rohdei, Y. ruckeri, Y. aleksiciae, Y. massiliensis, and Y. simili*		Hurst et al.; 2018 Le Guern and Pizarro-Cerdá; 2022
Listeria spp.	*L. monocytogenes, L. ivanovii, L. aquatica, L. booriae, L. cornellensis, L. fleischmannii, L. floridensis, L. grandensis, L. grayi, L. innocua, L. marthii, L. newyorkensis, L. riparia, L. rocourtiae, L. seeligeri, L. weihenstephanensis, and* L. *welshimeri.*		Carlin et al.; 2021
Escherichia coli spp.	*Escherichia coli, E. blattae, E. hermannii, E. vulneris, E. fergusonii, and E. albertii*	raw milk and dairy products	Scallan et al.; 2011; WHO (World Health Organization); 2015
Bacteria (Spore forming)			
Brucella spp.	*B. cereus,B. subilis, B. licheniformis, and B. pumilus*	meat, vegetables, puddings, and milk products	CDC (Centers for Disease Control and Prevention); 2021; WHO (World Health Organization); 2015
Clostridium spp.	*C. botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, C. butyricum, and Clostridium sordellii.*	cow and pig feces;spores in foods, milking machinery and sources of milk contamination	

this subject is considered to be a major threat for human health (Awad et al., 2024). Henceforth, it becomes crucial to test the quality of the food for safety purposes.

2. Pathogens and risks

Shigella spp. is an important group of pathogens responsible for human intestinal infectious diseases that disrupt intestinal homeostasis, separated into four subgroups based on their surface antigens: *S. dysenteriae, S. flexneri, S. boydii, and S. sonnei* (Pakbin et al., 2023; Park et al., 2023a, 2023b). The *S. flexneri* strain is the leading pathogen in developing countries. *Staphylococcus aureus* is an important pathogen of food poisoning and suppurative diseases in humans (Chen et al., 2021) and the leading pathogen of nosocomial infections and is a common environmental contaminant representing the most common gram-positive bacteria. The rate of clinical isolation of multidrug-resistant *S. aureus* has increased in recent years; and it is thought that this resistance is more likely to be related to sources associated with pigs rather than pets. *Yersinia enterocolitica* is capable of inducing diarrhea (Talim et al., 2024) and can be associated with atypical complications, including erythema nodosum and reactive arthritis. *Listeria monocytogenes* is an important zoonotic pathogen that induces listeriosis in humans and animals, often resulting in symptoms of sepsis, meningitis, and mononucleosis. Its existence in food is regarded as a great risk to human health, in addition to being a serious concern for the cold storage safety of refrigerated foods.

2.1. Methods for the detection of foodborne pathogens

In general, the evaluation of food quality has a number of facets, including the study of the FAMs found in foods and using several methodologies, for example, microbial culturing (plating methods), biochemical investigations, immunoassays (the most common is an ELISA), nucleic acid-based testing (polymerase chain reaction (PCR)), and lastly, using biosensor-based methods (López et al., 2025; Oslan et al., 2024). Therefore, there is a continual demand to develop rapid laboratory research methods for control of risks from food-borne pathogens to societies worldwide, but especially in countries that are developing, such as India. Traditional methods to detect foodborne pathogens are tedious and require excessive time, hence the need for rapid and effective alternatives (Quintela et al., 2022). Traditional detection of foodborne bacterial pathogens requires culturing of the microorganism on an agar plate followed by traditional biochemical identifications (Lee et al., 2022; Zhou et al., 2020; Zhao and Wu, 2020). Although these methods are inexpensive and simple to do, they take long laboratory hours, from up to 2 to 3 days for first identification of foodborne pathogens to over a week to confirm the species. Additionally, this detection method has low sensitivity and can generate false

negative results due to viable but non-culturable (VBNC) pathogens. For these reasons, rapid foodborne pathogen detection methods have developed and can be grouped into nucleic acid-based methods, biosensor-based methods, and immunological-based methods (Harrison et al., 2022; Oslan et al., 2024; Watier-Grillot et al., 2025). These rapid methods are important to the food industry, with many more advantages, such as the immediate detection of pathogens in raw and processed food with sensitivities, time, and less human error (Gonçalves et al., 2023; Harrison et al., 2022; Lewis et al., 2020). Despite advantages, challenges such as the high cost of sophisticated instruments, chemical reagents, experienced personnel, long sample preparation, and long generation time delay effective treatment. Researchers are constantly developing new methods for detection and continuing to improve in regard to rapidity, sensitivity, specificity, suitability for in situ analysis and the distinction of viable cells (Panwar et al., 2023; Zhou et al., 2020). Each rapid method possesses its unique advantages and limitations, emphasizing the ongoing need for innovation in this critical area of food safety.

The development of detection methods and methodologies is of utmost importance, particularly within the realm of molecular biology, where the MAS-PCR is a valuable tool for the detection of multiple pathogenic microorganisms at the same time (Watier-Grillot et al., 2025). This methodology offers numerous benefits; including speed; efficiency; ease of use; and most importantly; cost-effectiveness. PCR has proven instrumental in detecting various foodborne pathogens such as *Listeria monocytogenes*; *Escherichia coli* O157:H7; *Streptococcus agalactiae*; *S. aureus; Campylobacter jejuni*; *Salmonella* spp.; and *Shigella* spp. (Watier-Grillot et al., 2025; Hu et al., 2023a; Zhou et al., 2020; Zhao and Wu, 2020).

Multiplex polymerase chain reaction (mPCR) has, in recent times, proliferated to be one of the leading derivative techniques of polymerase chain reaction (PCR) due to its abundance of efficiency and versatility and has thus been followed widely and used internationally. Multiplex PCR is a method developed off of traditional PCR technology that amplifies multiple DNA fragments in one reaction system. The multiplex PCR assay offers diagnostics and identification of multiple genomes in a matter of hours. For example, multiplex PCR can detect highly virulent *Listeria monocytogenes* at a very low minimum concentration of 10^4 CFU/mL within 48 h. Hernández et al. (2022) shed additional light on this use of multiplex PCR to identify as well as classify food into pathogenic categories and found that 1 to 10 CFU of *Shigella* and *Salmonella* were detected in foods in a matter of 24 h. Similarly; Li et al. (2020) used multiplex PCR to detect the genomic DNA of four pathogenic bacteria with a sensitivity of 100 pg/μL. Additionally; Li et al. (2022a) utilized multiplex PCR to detect pathogenic bacteria in milk at a concentration of 10^4 CFU/mL. Therefore, multiplex PCR is a more comprehensive and cost-effective method for identification than single PCR. The purpose of this review, however, is not only to review the recent developments in rapid diagnostic approaches, especially MAS-PCR along with probe-based approaches, but also to consider delivery technologies that may support prompt identification of the genotypes of the foodborne pathogens in food products to decrease and hopefully prevent foodborne pathogens from contaminating products and contributing to foodborne disease outbreaks to play a role in food safety. This review considers the limitations as well as capabilities of these approaches in food safety and public health and function as a basis for the additional research in the area and an advisement for practice.

3. Global significance of foodborne diseases

Foodborne illnesses are a significant global health challenge that results in millions of illnesses, hospitalizations, and deaths each year (Keddy et al., 2025; Hu et al., 2023b). At-risk groups, such as children, the elderly and immune-compromised individuals, are particularly susceptible to severe outcomes from foodborne illnesses. Quality of life and medicinal treatment, along with lost productivity, result in a tremendous economic burden. Foodborne diseases also have an impact on finished product quality and food security. As a result, foodborne diseases have become a significant public health priority for global health and food safety agencies. Foodborne disease emergence across borders due to increased international travel and food trade is increasing the need for global coordination and surveillance (WHO (World Health Organization), 2022). Overall; advancements in diagnostics and surveillance technologies have allowed for more robust detection and monitoring of foodborne pathogens; faster response times; and better outbreak management. Coordination at the global level by organizations such as the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) is also critical to ensuring a safe food supply and protecting the health of the public. The global incidence of foodborne diseases is higher than ever; presenting a serious public health issue in some countries; including India (Singha et al., 2023; Villanueva-Segura et al., 2020). According to the WHO, food that is contaminated causes an estimated 420,000 deaths globally every year, 40% of which occur in children younger than five years old, thus emphasizing the critical need for improved detection methods (WHO (World Health Organization), 2022; WHO (World Health Organization), 2024).

3.1. Epidemiology of foodborne diseases from 2010 to 2024

The epidemiology of foodborne diseases between 2010 and 2024 was highly variable by geographic region, sources of the food supply, and regulatory frameworks (He et al., 2023; WHO (World Health Organization), 2024; Zhang et al., 2023). In the years 2010–2014; more surveillance and reporting of foodborne diseases occurred; largely in developed countries with tighter regulatory controls. The years included global reports of outbreaks of foodborne disease; specifically *listeriosis; salmonellosis;* and *E. coli* (Chiang et al., 2012; Salazar et al., 2015). Global initiatives to develop a better understanding of the burden of foodborne disease, like the World Health Organization Foodborne Disease Burden Epidemiology Reference Group (FERG), developed during this period of time. From 2015 to 2019, foodborne diseases known as burdens remained a concern, while also focusing on improving food safety measures and prevention procedures globally. Significant foodborne disease outbreaks occurred in all areas of the world, including outbreaks related to contaminated produce or processed food.

The adoption of rapid detection and genomic sequencing methods improved capability for rapid detection and trace back of foodborne pathogens during outbreaks. Between 2020 and 2024, COVID-19 pandemic disruption to food supply chains may have contributed to a lapse in direct food safety attention to foodborne illness prevention. The need for rapid detection methods, particularly molecular and biosensor methods was underscored for improved detection of pathogens and implementing mitigation measures intended to reduce the possibility of outbreaks. In addition, improving international collaborations and data sharing around foodborne illness spurred renewed focus to improve food safety efforts both domestically and globally. There were outbreaks in 2020–2024 during the time period from *Salmonella*, *Listeria*, and *E. coli* infections associated with different food products, underscoring the importance of prevention and ongoing attention in areas related to the food safety continuum (Chiang et al., 2012; Igo et al., 2022; López et al., 2025; Sun et al., 2025). Overall, there was sustained purposeful attention to understanding foodborne illnesses and how to lessen their consequences through detection, surveillance, and prevention efforts from 2010 to 2024.

4. Classification of foodborne diseases

Foodborne diseases represent a major public health burden on a global scale, with millions of instances documented annually. These diseases are characterized by diverse disease-causing organisms of etiologies including bacterial, viral, and parasitic infections, chemical poisoning, and allergic reactions (Gaballa et al., 2021; Park et al., 2023a,

2023b). It is valuable to know the classification and mechanisms of pathogenesis of foodborne diseases to enable scientific evidence-based intervention methods for the protection of public health. Fig. 1 shows in a convenient form with variety the causative agents and the mechanisms of transmission for different foodborne diseases.

4.1. Bacterial infections

Bacterial pathogens, including *Salmonella, Escherichia coli* (*E. coli*), *Campylobacter*, and *Listeria monocytogenes*, are frequent sources of foodborne illness (Chiang et al., 2012; Watier-Grillot et al., 2025). These microorganisms can infect food during the production and processing of food products, resulting in human consumption and illness. Foodborne illnesses vary from mild gastroenteritis to severe systemic illness depending on the virulence of the strain and host susceptibility.

4.2. Viral infections

Foodborne illnesses are largely caused by viruses such as norovirus and hepatitis A virus. These pathogens infect food and water through fecal contamination. After a person consumes infected food or water, viral particles are capable of replicating in the gastrointestinal tract, leading to the development of gastroenteritis symptoms and other systemic illnesses.

4.3. Parasitic infections

Parasitic foodborne pathogens include protozoa (e.g., *Toxoplasma gondii, Giardia*) and helminths (e.g., *Taenia* spp.). These microorganisms can originate from environmental sources or from infected human or animal hosts and can contaminate food products. If a person eats contaminated food, they may become infected with the pathogen, which can ultimately lead to illness. Symptoms may be localized to gastrointestinal diseases or may become systemic.

4.4. Chemical poisoning

Chemically induced contamination of food, such as heavy metals, pesticides, or mold toxins, can result in either acute or chronic poisoning in humans. Contaminants may enter the food chain from agricultural sources, food processing, or the environment. After ingestion, they exert toxic effects on various organ systems, culminating in symptoms from nausea or vomiting to multi-organ failure.

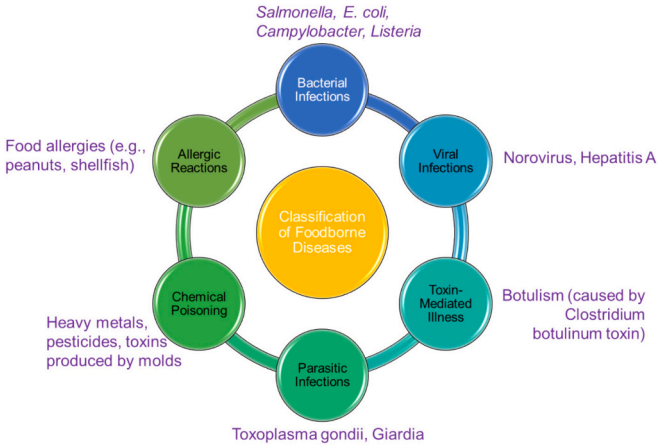

Fig. 1. Schematic diagram illustrating the overview of classification of foodborne diseases.

4.5. Allergic reactions

Food allergies are hypersensitivity reactions that are immune-mediated to specific food antigens (allergens). The following foods are common allergens: peanuts, tree nuts, shellfish, dairy products, etc. In predisposed individuals, eating allergenic foods can induce an allergic response, which can vary according to the individual. Allergic responses can be as mild as a skin rash or as severe as anaphylaxis, which could lead to death.

4.6. Toxin-mediated illness

Toxins produced by certain bacteria (e.g., *Clostridium botulinum*) may contaminate food and result in toxin-mediated illness. For example, botulism is caused by the ingestion of botulinum toxin made by *C. botulinum* in inadequately processed and/or preserved food. The toxin affects the neuromuscular junction, causes paralysis, and can result in respiratory failure and death.

5. Microbial culturing and biochemical studies

Identifying foodborne bacteria generally uses traditional methods where bacteria are grown on agar. The advantages of these methods include that they are generally inexpensive and uncomplicated; bacteria can grow on different types of culture media (i.e., selective plating, pre-enrichment, and enrichment media). However, they are labor-intensive. First, traditional methods take a long time-two to three days for preliminary identification and an additional week or more to confirm a species. Second, traditional methods are not very sensitive; false-negative results are possible, especially for viruses. This is important, since motile but VBNC can compromise food safety assessments. Consequently, there is a greater risk for foodborne illness resulting from pathogens that are not identified quickly. These limitations illustrate the need for a more sensitive and efficient visual identification system to quickly and reliably detect foodborne pathogens and reduce the potential for illness from ingesting contaminated food.

5.1. Immunoassays (ELISA)

Immunoassay, particularly as demonstrated in the Enzyme-Linked Immunosorbent Assay (ELISA), is based on antigen-antibody reactions (Sakamoto et al., 2018). The immunoassay procedure has multiple benefits. Firstly, the steps involved are relatively straightforward in the sense that they can be accomplished with little training or resources in a number of different locations. Second, because of the specificity of antigen-antibody interactions, immunoassays possess significant sensitivity and specificity, thereby adding accuracy and reproducibility to the analyte target measurements. Immunoassays have high efficiency, as they can be performed without pre-treatment steps, and multiple sample batches can be worked on at once. Nonetheless, immunoassays have some limitations. One of the significant limitations is the antibody preparation step, which is labor-intensive and costly. Most antibody development requires significant quantities of time, costs, and expertise, which adds to the timeliness, costs, and quality efforts of an immunoassay testing procedure.

Also, even when reactivity is surprisingly high, technically, immunoassays can lead to false-positive or false-negative results. Cross-reactivity or other substances in the sample can result in inaccurate results. Additionally, antibodies, as utilized in immunoassays, can be unstable, potentially affecting result reliability and reproducibility over time. In spite of this, immunoassays (including ELISA) are still a valuable method utilized in areas such as clinical diagnostics, food safety testing, and environmental monitoring. To overcome some of the limitations indicated, future improvements in antibody engineering, assay design, and quality control methods are expected to enhance the ease of use and reliability of immunoassay techniques.

6. Challenges with conventional detection methods

Foodborne bacterial pathogens were traditionally identified through culturing microorganisms on agar plates and using standard biochemical identifications (Zhou et al., 2020; Zhou et al., 2020). Although these traditional approaches are culturally cost-effective, they are resource-intensive, requiring 2 to 3 days for preliminary identification and approximately 7 days for species confirmation. In addition, traditional approaches are limited based on their sensitivity, the presence or absence of viable cells (false negatives), and the limitation of the VBNC state of the pathogens; practicing these poses a considerable limitation.

7. Advancements in detection technologies and the rise of rapid detection methods

Due to the limitations of traditional methods, there has been an emergence of a range of rapid detection approaches, such as nucleic acid-based, biosensor-based, and immunological-based methods. These approaches enable rapid pathogen detection and offer advantages in sensitivity and specificity, or reduced time for analysis, and are of critical importance in the food industry for immediate detection of pathogens in raw or processed foods. The advent of novel molecular tools has resulted in a revolutionary transition in monitoring the microbial quality of food and water (Paruch, 2022; Villanueva-Segura et al., 2020). Technologies such as high-density microarrays, quantitative real-time PCR (qPCR), and sequencing are 'breakthrough' technologies that allow the rapid, high-throughput, sensitive, and specific capability to detect a broad cross-section of microbial pathogens. These technologies, as well as technologies such as MAS-PCR, are soon to be the majority detection and discovery of pathogen testing, ultimately changing the paradigm from traditional culture- and time-based techniques.

7.1. Nucleic acid-based methods

Nucleic acid-based methods, especially polymerase chain reaction (PCR), have emerged as a powerful approach to detect foodborne pathogens (Aladhadh, 2023; Pang et al., 2024; Zhou et al., 2020; Zhao and Wu, 2020). PCR allows for the rapid amplification of sequences of DNA for efficient testing and has high sensitivity. A limitation of conventional PCR is its use in detecting multiple pathogens at the same time. Nucleic acid-based methods are dependent on detecting a particular DNA or RNA sequence for the target pathogen. This involves using a synthetic oligonucleotide (either probes or primers) that is complementary to the target oligonucleotide and hybridizes with the target nucleic acid sequence (Pang et al., 2024). Nucleic acid-based methods also prevent ambiguous/misinterpreted information by using specific genes of the target pathogens. Techniques range from different forms of PCR methods to expanded next-generation sequencing methods.

7.2. Computational approaches for identification and primer design

Rapid and precise identification of pathogens is essential during food-borne disease outbreaks, and this review outlines the available computational methods to identify food pathogens, food pathogen-specific genes, and primer sequences to amplify foodborne pathogen-specific genes. Based on our analysis of literature, we promote the use of computational methods to enhance the molecular diagnostics of foodborne pathogens (Aziz et al., 2008; Overbeek et al., 2014).

7.3. Significance of identifying organism-specific genes

In order to improve targeted methods of detection, it is essential to understand the genetic signature of foodborne pathogens. Certain genes serve as the basis for use as biomarkers identifying pathogenic organisms. Then primers are designed based on the specific identification of the genes to amplify in molecular assays that are robust and selective.

7.4. Computational approaches in gene identification

a. Genome mining strategies:

Genome mining computational tools, including genomics data algorithms, are valuable for identifying the genes responsible for virulence, pathogenicity, or certain metabolic pathways and are often outlined in principal genome mining techniques.

b. Comparative Genomics advancements:

Relevant are recently published reports that used comparative genomics to locate conserved regions between strains of a pathogen. This approach help us locate the genes specific to our pathogen of interest.

c. Bioinformatics tools to inform our actions:

Published articles that described the use of bioinformatics tools, such as BLAST, to cross-reference genes that we identified to available databases, increase the validity of our gene identification.

7.5. Primer design strategies

a. Existing literature on primer specificity:

Examining existing literature emphasizes the relevance of primer specificity. Modeling software helps design primers with high specificity, which can limit the potential for cross-reactivity.

b. Recommended primer length and melting temperature:

Recent literature emphasizes the significance of recommended primer length and melting temperature to improve PCR efficiency. Several studies review algorithms that predict these criteria, which demonstrate advances in the primer design strategies.

c. In silico *validation procedures*:

A review of published articles that discusses the utility of in silico validation, computational modeling of primer binding efficiency, and other secondary structures. In silico validation is useful for refining designs prior to experimental validation.

7.5.1. Examples and applications in practice

a. Example 1: Genes of *Listeria monocytogenes*:

Some examples provided in recent reputable publications were examined, and they included studies focused on the identification of genes of *Listeria monocytogenes*. We also highlighted the computational tools and approaches applied in each study.

b. Example 2: Primer Design for Escherichia coli O157:H7:

A review of a more recent publication that identifies several case studies on primer design for *Escherichia coli* O157:H7 allows us to show how computational tools assist in designing primers that provide more specificity and/or sensitivity.

7.6. Multiplex allele-specific PCR (MAS-PCR)

To address the need for simultaneous detection of multiple pathogens, MAS-PCR has emerged as a groundbreaking technology (Table 2) (Fan et al., 2022; Zhao and Wu, 2020; Liu et al., 2019). MAS-PCR streamlines the detection process by allowing the simultaneous identification of several pathogens, which is crucial given the diverse range of foodborne pathogens that can contribute to health risks in any water sample. Fig. 2 provides a schematic overview of the MAS-PCR procedure.

7.6.1. Applications of MAS-PCR

The application of MAS-PCR encompasses the diagnosis of infectious diseases; genetic screening; agriculture crop; food safety; forensics; and cancer, among others, due to rapid turnaround time, allele specificity, cost, and platform adaptability; however, careful primer design and validation are important considerations in order to limit cross-reactivity/bias (Table 3). Table 4 contains a comparison of various types of MAS-PCR methodologies, probe enhancements, and their applications/uses from key publications.

Table 2
Summarizes the bacterial species, target genes, and corresponding multiplex PCR methods used for the detection of foodborne pathogens, along with amplicon sizes (in base pairs). The species listed include *Salmonella enterica* (various serotypes), *Escherichia coli* (including enterohemorrhagic strains), *Staphylococcus aureus, Listeria monocytogenes, Shigella*spp.,*Campylobacter* spp., *Clostridium botulinum*, *Vibrio cholerae*, and *Bacillus amyloliquefaciens*. Each entry provides the specific genes targeted (e.g., *mcr-9, flaA, ctxA*), the amplicon size range, and references to studies employing multiplex PCR methods for pathogen detection in food safety and microbiological research.

Bacterial Species	Target Gene	Multiplex PCR Method	References
Salmonella enterica Various serotypes (e.g., *Salmonella Typhimurium, Salmonella Enteritidis*	***mcr*-9, Extended-spectrum β-lactamases (ESBLs) and PQMR (*IncN/IncFIB/IncP/IncA/C/IncHI2*)**	**80 to 280 kb**	Zhang et al.; 2016; Upadhyaya et al.; 2015; Rahman et al., 2022
***Escherichia coli* (*E. coli*):** Enterohemorrhagic*E. coli (EHEC)* - e.g., *E. coli* O157:H7	***ileS, thrB,* and*polB***	**401, 337, and 232 kb**	Rahman et al., 2022
S.aureus, L. monocytogenes, S. flexneri, Y. enterocolitica and C. difficile	***Nuc, hlyA ((haemolysin A), ipaH, lysP, tpi***	**484, 345, 204, 156, 88 kb**	Sharma et al.; 2021 Chen et al.; 2021; Chen et al.; 2022
Campylobacter *Campylobacter jejuni, Campylobacter coli*	***flaA, flaB, cadF, ciaB***		Koolman et al.; 2015
Clostridium botulinum	***botA, botB, botC***		Zhang et al.; 2013
Staphylococcus aureus	**enterotoxingenes (e.g., *sea, seb, sec, sed)***		Schwendimann et al.; 2021
Vibrio cholera	***ctxA, ctxB, tcpA***		Zareitaher et al.; 2022;
Shigella	***ipaH, ial, virA***		Zhu et al.; 2021
Bacillus amyloliquefaciens	***hblA, hblB, hblC, nheA, nheB,* and*nheC***		Jung et al.; 2021

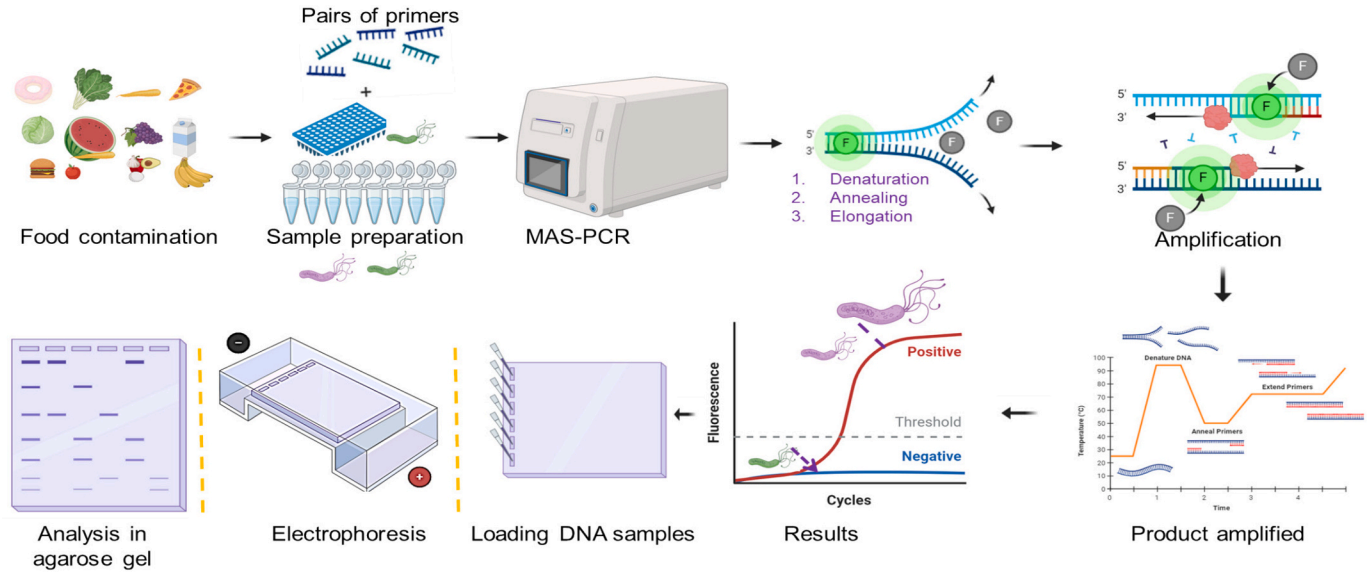

Fig. 2. Schematic representation and representative results of Multiplex Allele-Specific Polymerase Chain Reaction (MAS-PCR) coupled with probe-based analysis. Initially, genomic DNA is extracted from the sample of interest. Subsequently, allele-specific primers are designed to amplify target alleles selectively. The multiplex PCR setup combines the DNA template, allele-specific primers, and PCR reagents. The PCR amplification involves multiple cycles of denaturation, annealing, and extension, resulting in the amplification of the targeted DNA sequences. Gel electrophoresis is performed after amplification to separate the PCR products based on size. Visualization of the gel under UV light after staining with a DNA-specific dye allows for the interpretation of results. Additionally, it depicts representative MAS-PCR results, where each lane represents a specific sample or control. Amplification bands in lanes 2–4 correspond to different alleles, while the absence of bands in lanes 5–6 indicates the absence of target alleles. These results exemplify the specificity and sensitivity of MAS-PCR in simultaneously detecting multiple alleles in a single reaction.

7.6.2. Infectious disease and AMR surveillance

MAS-PCR is widely used for drug resistance profiling in tuberculosis. It targets *rpoB* codons 516, 526, and 531 for rifampicin resistance; katG315 and *inhA* minus 15 for isoniazid resistance; *gyrA* codons 90 and 94 for fluoroquinolone resistance; and rrs 1401 for kanamycin resistance. The assays detected phenotypic resistant (80–98%) to antibiotics within 4 h instead of the standard ~21 days for culture-based antibacterial susceptibility testing. The assays exhibited 95.2% sensitivity and a 30% decrease in treatment failures for treated patients whose treatment was guided by an empirical culture result after 21 days and compared to those whose treatment was guided by the results of a panel of 50 sputum specimens analyzed for phenotypic resistance (Chia et al., 2012; Maruri et al., 2012; Kim et al., 2025; Xiao et al., 2024). Battery-operated thermocyclers in field-deployable 8-plex format have been developed to screen for second-line agents (capreomycin/amikacin) in low-resource settings (Limberis and Metcalfe, 2023). This multiplex LAMP-based screening provides single-base specificity for blaCTX-M alleles in AMR surveillance (Higgins et al., 2023). PCR-reverse line blot hybridization is used for the detection of multiple pathogens to allow epidemiological analysis (Kong and Gilbert, 2006) while the assays have also been used to identify virulence markers of *Vibrio vulnificus* (Bier et al., 2015) and *Helicobacter pylori vacA* types from biopsies (Chisholm et al., 2002). During COVID-19 (2021−2023); MAS-PCR assays were used to monitor SARS-CoV-2 variant (Alpha/Delta/Omicron) levels in wastewater at a limit of detection (LOD) of 10^3 copies/mL (Limberis and Metcalfe, 2023). Within veterinary medicine; the PRRS virus (PRRSv)

Table 3
List of bacterial pathogens, their gene targets, primer sequences, and corresponding amplicon sizes used for molecular detection. The table includes the left and right hybridization sequences (forward and reverse primers), along with the expected amplicon size in base pairs (bps) and references for primer source.

S. No	Pathogen	Gene target	Left hybridization sequence	Right hybridization sequence	Amplicon size (bps)	References
1	*Bacillus cereus*	*groEL*	CCTTCACGAATCATAGCTTGTGCTAATACAGTTGC	AGTTGTTGTTCCGTCACCAGCTACGTCATTTG	109	Chang et al., 2003
2	*Bacillus cereus*	*csr*	TGGTGAAATTGAAGCGGCAATTGCTGCATTAGAA	GATGTAGTACAAACAATTGTTACAACAATGACGG	110	Chung et al., 2012
3	*Clostridium perfringens*	*plc*	TGGCAAAGAGGAAACTATAAACAAGCTACATTC	TATCTTGGAGAGGCTATGCACTATTTTGGAG	106	Chung et al., 2012
4	*Campylobacter coli*	*glyA*	GAGAGATTGCGGATGAAGTTGGAGCTTATCTT	TTTGCAGACATTGCACACATTGCTGGAC	102	Fukushima et al., 2010
5	*Campylobacter jejuni*	*hip*	TAAATCTTTATTTTCAACCTGCTGAAGAGGG	TTTGGGTGGTGCTAAGGCAATGAT	97	Onori et al., 2014
6	*Cronobactersakazakii*	*MMS*	CTCGTCTGTACTAATTCCTCAGGGGATATTGTCC	CCTGAAACAGACAGAGTAGTTGTAGAGGCC	115	Fukushimaet al., 2010
7	*E. coli O157:H7*	*tir/eaeA*	TATATCCATAATCATTTTATTTAGAGGGAGGGAGGG	GGGAAGTCTAACTAAGGTCAATTTTTCAGG		Liu et al.; 2019; Zeinhom et al., 2012
8	*Enterococcus* spp.	*tuf*	CGATTTCCCAGGCGATGATGTTCCAGTTAT	CGCAGGTTCTGCTTTGAAAGCTTTAG		Zeinhom et al., 2012
9	*Listeria monocytogenes*	*hly*	TGTGAATGCAATTTCGAGCCTAACCTA	TCCAGGTGCTCTCGTAAAAGCGAA	93	Liu et al.; 2019; Zeinhom et al., 2012
10	*Salmonella* spp.	inv	CGATAGGGATAATATGGGGCGGAATAT	CATGACGCAGCTGTTGAACAACCCAT	85	Liu et al.; 2019; Kim et al., 2016
11	*Shigella* spp.	*ipaH*	CTCCACTGCCGTGAAGGAAATGCGTTT	CTATGGCGTGTCGGGAGTGACAGCAAAT	97	Fukushima et al.; 2010
12	*Shigellaflexneri*	*ipaH*	GTTCCTTGACCGCCTTTCCGATA	CCGTCTCTGCAGGCAATACCTC	92	Chung et al., 2012
13	*Staphylococcus aureus*	*nuc*	TGAAACAAAGCATCCTAAAAAGGTGTAGAGAAA	TATGGTCCTGAAGCAAGTGCATTTACG	103	Chung et al., 2012
14	*Staphylococcus aureus*	*nuc*	CCTGCGACATTAATTAAAGCGATTGATG	GTGATACGGTTAAATTAATGTACAAAGGTCAA	102	Fukushimaet al., 2010
15	*Salmonella* spp.	inv	CGATACGGATAATATGGGGCGGAATAT	CATGACGCAGCTGTTGAACAACCCAT	95	Fukushima et al.; 2010
16	*Salmonella enteritidis*	*sefA*	TGGCTTTGTTGGTAACAAAGCAGAGG	TTCAGGCAGCGGTTACTATTGCAG	92	Chung et al., 2012
17	*Vibrio vulnificus*	*vvh*	CTCAGTTTATGGTTCTGCGGGCTCAT	CAACCAACAGCAGTACCGTGAAACAAC	95	Fukushima Kim et al.2010
18	*Vibrio parahaemolyticus*	*tdhS*	TGAGCTTCCATCTGTCCCTTTTCC	TGCCCCCGGTTCTGATGAGATA	88	Liu et al.; 2019; Mustapha et al., 2006
19	*Salmonella enterica*	*hilA*	CAACCTACGACTCATACA	GCGTAATTGATCCATGAG	192	Liu et al.; 2019
				TCAAGAATATCCTTAACACTGCGGC		
20	*Streptococcus pyogenes*	*scpA*	CAGACATTAAAGCAAATACTG	TCTGCTATTGTTTCTTCTG	154	Liu et al.; 2019
21	*Yersinia enterocolitica*	*foxA*	CGGTGATGTGAACAATAC	GCCATATAACGCAGAAGA	136	Liu et al.; 2019
22	*Enterococcus faecalis*	*ddl*	CCCATAGTAAAGGATACATAC	CGCTGTGATTTCTTCTTA	100	Liu et al.; 2019
23	*Proteus mirabilis*	*ureR*	CCATCAGATTATGTCATTCAA	GAGGAAAATGCAATTTATCTTTA	88	Liu et al.; 2019
24	*Vibrio fluvialis*	*toxR*	TTCGCAGTCTAAATTTCG	TCCACCATATTTTCTTACG	124	Liu et al.; 2019

has been genotyped in swine to identify the potential for vaccine escape (Richardson et al., 2009; Xiao et al., 2023) and *E. coli* pathotype has been genotyped (O/H antigens [62-plex]) to assist in outbreak tracing (Huang et al., 2022).

7.6.3. Genetic screening, agriculture, and food safety

Using triplex MAS-PCR for genetic screening of HFE C282Y/H63D mutations from whole blood tissue is successful with 100% accuracy without performing any extractions (Limberis and Metcalfe, 2023). Forensic geneticists have confirmed that they can obtain profiles from fecal/aged bone using the 40-plex SNP CODIS+/Ancestral panels for investigative ancestry research (Xie et al.; 2022). 20-Plex Y-Chromosome Microdeletion Assays for Population Genetics using AZFa / AZFb / AZFc showed that karyotyping methods are unable to detect 15% of AZFc cases (Huang et al., 2022). Continue; evidence for the use of KASP as molecular markers assisting in the selection of plant material that exhibits resistance to Fusarium tonation in wheat Fhb7 / Pm21 has been demonstrated using ~10 k samples (Wang et al., 2025). KASP molecular markers have also been used to genotype apple MdACS1 / MdACO1 SNPs and to create homogenous line F_2 apple trees at 95% confidence (Xiao et al., 2024). Twelve-plex *Salmonella* spp. assays have been effectively used to detect >99% specific *Salmonella* spp. at levels of 1 CFU in <25 g samples of poultry (Mamishi et al., 2019).

7.6.4. Oncology and advanced genomics

Liquid biopsy for circulating cell-free DNA (cfDNA) targeted non-small cell lung cancer (NSCLC) with MAS-PCR, detecting EGFR exon 19 deletions, L858R, and T790M mutations at 0.5% variant allele frequency (VAF) using plasma (cfDNA). In comparison with next-generation sequencing (NGS), the method demonstrated 92% concordance in a cohort of 200 patients and was associated with poorer progression-free survival, with a hazard ratio of 2.8 and p less than 0.05. Additionally, both turnaround time (2 h) and test cost ($5) for MAS-PCR were significantly lower than for NGS.

The use of stool samples obtained from KRAS / NRAS / BRAF to facilitate early diagnosis of colorectal cancer via DNA analysis resulted in a total detection rate of 88%). Other recent technological developments in the field of genomics include multiplex nested RT-PCR for the analysis of α/β T-cell receptor (TCR α /β) genes; and multiplex ligation dependent probe amplification (MLPA) for quantifying deletion and duplication of LAMA2 gene (Dash et al., 2011; Oliveira et al., 2014) both of which are achieved using metagenomic sequencing (MGV Seq) techniques to explore the varying degree of biological variability among individual strain of microbes within microbial communities (Li et al., 2025) and measuring the amount of human leukocyte antigen class I open reading frames by evaluating specific complementary DNA amplicons made from total RNA (Lank et al., 2010). The availability of next generation sequencing (NGS) to evaluate BRCA1/2 is often limited by the fact that formalin-fixed; paraffin-embedded (FFPE); tissues have low coverage; however; there are now optimized protocols available to allow for the use of NGS when assessing this region (Zakrzewski et al., 2019).

In summary, MAS-PCR applications include pathogen detection, virulence genotyping, and antimicrobial resistance surveillance, mutation detection for cancers, characterizing resistance genes in plants, immunogenetic profiling, and microbial species-level monitoring. The primary advantages of the MAS-PCR technique are rapid turnaround times, the ability to discriminate alleles, cost-effectiveness, and the ability to adapt to multiple platforms; however, appropriate design of primers and validation of assays to eliminate cross-reactivity and amplification bias must still be considered.

7.6.5. Methodologies of MAS-PCR

MAS-PCR is a PCR method that selectively amplifies the target alleles (SNPs/mutations) located at multiple sites simultaneously by adding a specific “mismatch” or “discriminatory/stabilizing” mismatch to the

Table 4
Comparison of MAS-PCR methodologies, probe enhancements, and applications across key studies.

Study	Application	Methodology Highlights	Probe Enhancement	Sensitivity/Specificity (%)	LOD (Copies/ Reaction)
Chia et al. (2012)	MDR-TB genotyping (*rpoB*, *katG*)	Touchdown PCR, 8-plex, sputum lysate	None (gel electrophoresis)	98.4/100	10
Li et al. (2017)	EGFR mutations in NSCLC plasma	4-plex AS primers, SYBR Green qMAS-PCR	PCR-SERS (Cy3/R6G-AgNPs)	92/95	0.1% VAF
Limberis and Metcalfe (2023)	MTB drug resistance (*rrs/inhA*)	primerJinn design, 12-plex, high-fidelity Q5	HRM (EvaGreen)	95.2/99	5
Xie et al. (2022)	Wheat SNP genotyping (*Fhb7/ Pm21*)	Simulated annealing, 20-plex KASP	FRET cassettes (FAM/ HEX)	100/100	1 ng DNA
Pu and Wu (2025).	cfDNA EGFR/T790M	Nested MAS-PCR, 384-well	TaqMan (FAM/BHQ1)	96/98	0.01% VAF
Yang et al. (2023)	XDR-TB *se*cond-line drugs	Direct lysis, 10-plex	Molecular beacons	94/97	10
Huang et al. (2022)	Forensic 62-plex CODIS	5′-flap endonuclease Taq	MeltArray MBs (3 dyes)	98.5/99	1
Kim et al. (2021)	Respiratory viruses (6-plex)	Paper-based microfluidics	SERS-paper	99/98	10
Wang et al. (2025)	Apple crispness SNPs	Multi-KASP duplex	Universal FRET	100/100	N/A
Mamishi et al. (2019)	*Salmonella serovars* (12-plex)	Crude food lysate	SYBR melt curve	99/99	1 CFU/25 g

Note: MAS-PCR = multiplex allele-specific PCR; LOD = limit of detection; VAF = variant allele frequency; TB = tuberculosis; NSCLC = non-small cell lung cancer. Data summarized from PubMed-sourced reviews and primary studies.

primer sequence. Thus, the researcher can carry out multiplex assays on a population of SNPs/mutations with one PCR tube and precisely identify the target alleles among a pool of individuals from different sources. The design and layout of the competitive multiplex assay uses allele-specific forward primers (ASFs) along with a common reverse primer but has been designed such that the antepenultimate/penultimate base of each ASF has created a required mismatch to enhance target vs. non-target SNP/mutation differentiation (Limberis and Metcalfe, 2023). The annealing temperatures of ASFs are usually adjusted to be 2–5 °C greater than the estimated T*m* of the corresponding reverse primer in order to ensure that only perfect match extensions occur; therefore the touchdown protocol begins at 65 °C and ends at 55 °C Over 10–15 cycles to minimize non-specific product generation (Kaushik et al., 2025).

As a result of the recent advancements being made in the field of MAS-PCR, there is an increased requirement for high fidelity DNA polymerases (i.e., Q5 or Phusion), in order to reduce the potential for error incorporation into multiplex-MAS-PCR (i.e., >10-Plex) assays thus ensuring they meet the accuracy requirements (>99%) and sample input amounts (<1–10 ng) of genomic or total nucleic acid DNA extracted from clinical specimens (Limberis and Metcalfe, 2023). One additional consideration that must be taken into version when optimizing buffer conditions (5–10% DMSO/betaine and 1.5–2.5 mM $MgCl_2$) for GC-rich sequence amplification is that Bovine Serum Albumin (BSA; 0.1–0.2 μg/μL) must be used to reduce inhibition effects that will occur due to the presence of inhibitors found in raw samples (sputum or serum) (Xiao et al., 2023). A MAS-PCR procedure consists of a pre-defined thermocycling protocol and typically requires 35–40 thermocycles (95 °C × 15 s/denature, 58 °C–62 °C × 30 s/anneal, 72 °C × 30 s/extend) with at least 2–3% agarose gel electrophoresis or capillary electrophoresis being used to visualize the MAS-PCR products post amplification. Consequently, the genotype confirmed by the observation of specific product size between 100 and 300 bp associated with the presence of the desired allele for each individual analyzed by MAS-PCR (Pu & Wu., 2025).

There are tools available to use the SYBR Green I quantitative assay to observe the status of a samples in real-time and perform melting curve analysis on an amplicon in the range of 65 °C - 95 °C where the melting temperature (T*m*) of an individual amplicon can be used to distinguish the amplicon based on Tm resulting from a single nucleotide polymorphism (Ahmed & Gulhan., 2024). Dimer formation risk may be evaluated using a computational assay, such as Primer3Plus or OligoAnalyzer, to design primer sets with at least 20 unique sets of non-cross-hybridizing primers as reported by Brodin et al. (2013); as demonstrated through pairwise scoring using annealing algorithms, including the primerJinn Tool. Using automated methods to assess primer quality and suitability for sequencing data minimizes off-target primer use and allows detection of contamination at very low densities relative to high-fidelity screening methods (Xie et al., 2022; Limberis & Metcalfe., 2023). Validating assay methodology can establish 100% concordance between a Sanger sequencing assay result of an individual target sequence from 50 or greater independent samples taken from the same source for quantification and determination of pathogen limit of detection (LOD) using pathogenicity for genotyping methodology of <10 genome copies (Chakraborty et al., 2023; Xiao et al., 2023).

Real-world applications of these optimizations also include the use of MAS-PCR, which allows for the lysis of crude lysates without the need for extracting DNA during pathogen diagnostics, thus decreasing the test turnaround time to less than two hours (Limberis & Metcalfe., 2023). In the field of oncology, plasma cfDNA (circulating free DNA) samples are diluted then subjected to nested MAS-PCR, with a dilution factor of 1:10 using TE buffer to lessen the amount of inhibitors and increase the sensitivity of the assay to provide <0.1% variant allele frequency (Chakraborty et al., 2023; Pu and Wu, 2025). These methods can be performed in 384-well plates, allowing for >1000 samples to be processed per day in a laboratory setting (Ahmed and Gulhan, 2024).

7.6.6. *Probe-based enhancements*

Working in probe integration with MAS-PCR has made it significantly more sensitive, enabling it to be run in closed-tube qulantitative formats that reduce the risk of contamination after PCR. TaqMan allele-specific probes (labeled with 5'-FAM/BHQ1) hybridize internally to the amplicons and produce real-time signals (Ct difference > 5 for discrimination) by cleaving generated amplicons with 5′ nuclease; multiplexing 4 EGFR loci at 0.01% LOD in cfDNA was achieved through optimizations (Pu and Wu, 2025). Molecular beacons l (MBs)-hairpin-shaped probes containing 5′-fluor/quencher-developed for use with Taq's 5′-flap endonuclease in MeltArray enable detection of up to 62 different targets by using Taq's unique Tm clusters (FAM/HEX/TAMRA) and were validated on a panel of 100 clinical samples with an accuracy of 98.5% (Xiao et al., 2023; Wang et al., 2025). Universal probes have provided a further means to detect point mutations through single-base extension, thereby reducing the background (Pu and Wu, 2025).

Plasma detection of multiplexed EGFR exons 19 and 21 using PCR-SERS (Surface Enhanced Raman Scattering) with Rhodamine 6G/ Cyanine 3 (Rh6G/Cy3) or Cyanine 3/Rhodamine 6G (Cy3/Rh6G) Raman dye-labeled antisense primers (AS) on silver-coated nanospheres (AgNPs) yielded 0.1% variant allele frequencies (VAF) with significant differences across clusters by Analysis of Variance, using of 0.92 versus high resolution melt (HRM) (Pu and Wu, 2025; Chakraborty et al., 2023; Kim et al., 2021). Paper-based SERS-microfluidics multiplexed 6 respiratory viruses (e.g., Respiratory Syncytial Virus (RSV), influenza, and SARS-CoV-2) using multiplex amplification of specific targets (MAS)-

PCR with a limit of detection (LOD) of 10 copies per μL, allowing for field use (Kim et al., 2021).

KASP assay methods can vary substantially from others by allowing the use of biallelic or universal FRET cassette (FAM/HEX) primers/scoring for clusters through analysis of SDS analysis software. A unique feature of multi-KASP is the ability to perform a duplex analysis on non-homologous loci (Fhb7/Pm21) to easily resolve all 500 wheat samples at 100%. InDel genotype resolution by PACE (PCR Allele Competitive Extension) uses locked nucleic acids (LNA) to increase specificity 20 times greater (Kalendar et al., 2022). After MAS-PCR, high resolution melt analysis (HRM) using EVA Green intercalating dyes on probes differentiated single nucleotide polymorphisms (SNPs) with ΔTm between 0.5 °C and 2 °C; Future HRM-MAS real-time analysis is capable of detecting Mycobacterium tuberculosis rpoB526 with 99% specificity. Digital PCR (dPCR)-MAS utilizes 20,000 droplet partitions of reaction for absolute quantification and unidentified mutation detection (0.001% VAF) in tuberculosis (TB) patients. CRISPR-Cas12a probes collateralize MAS amplicons, cleaving FQ reporters for ultra-sensitive readout (fg/μL LOD), multiplexed via LwaCas12a for 5 TB drugs. Nanopore-direct MAS products enable long-read discrimination without probes. Table 5 showed the comparison of MAS-PCR with other techniques.

7.6.7. MAS PCR primer design

Designing several sets of highly specific, non-cross-reacting primers for MAS-PCR is critical in order to simultaneously genotype pathogenic organisms. However, the nature of multiplexing generates a number of unique challenges, including amplified interactions between primers, dimer formation, and amplification bias; as well as the fact that these issues grow in magnitude (quadratically) with progressive plex levels (i.e., there are 4950 primer pairs possible for each 50-plex panel), making it unlikely that traditional algorithms address these issues effectively (Zhang et al., 2022; Goyani et al., 2025). There are a variety of strategies that can address these issues when performing foodborne pathogen assays. These strategies include: targeting conserved regions of the pan-genome, using simulated annealing for pairwise ΔG threshold scoring to limit (−9 kcal/mol) asymmetric 3′-mismatches and off-targets (i.e., FBPP and primerJinn enforce strict Tm/GC requirements to reduce 3′-mismatches), and enabling reliable detection of Salmonella at 12-plex and 1 CFU/25 g. According to Yang et al. (2023) and Dey et al. (2012), the MAS-PCR technique has the following advantages over qPCR: MAS-PCR is more capable of performing multiplex PCRs than qPCR; it is less expensive and faster than MassARRAY; it provides 82–98% accurate detection of drug resistance mutations found in the most frequently identified mutations, while the results for detecting drug resistance mutations located in rare variants are poorer than qPCR. The major challenges in developing multiplex MAS PCR primers are identified in Table 6.

7.6.8. Primer design complexity in MAS-PCR

The use of specific primer pairs for each allele in MAS-PCR creates a 3′ terminal mismatch to ensure the primers are allele specific for each SNP. This requirement, when extending this approach to multiple sets (e.g., many SNPs), imposes requirements for potential cross reactivity between (2n) primer pairs, such that many sets have similar melting temperatures (60–68 °C), amplicon sizes (100–500 bp), and low self- and hetero-dimer formation, all of which exist in a non-convex optimization space with respect to defining a collection of 2n interactions across potentially many $p = 5–10^*$ sets, which grows quadratically (e.g., 4950) as the number of genotyping primers increases ($n = 2$) for any single p = 5–10* set of (e.g., $n = 100$) primers / 50-plex for SNP genotyping (Goyani et al., 2025). Designing penultimate and antepenultimate mismatches artificially increases the number of 3′-specific design choices for each SNP to nine per SNP; however, random designs currently yield an estimated ~30% specificity and do especially poorly

Table 5
Comparison of MAS-PCR with other techniques.

S·No	Method	Principle	Typical LOD (Food Samples)	Speed	Key Advantages / Limitations
1	MAS-PCR	Allele-specific multiplex amplification	10^1–10^2 CFU/mL	3–4 h	Simultaneous detection & genotyping; complex primer design (Tao et al.; 2020)
2	Conventional PCR	Single-target amplification	10^2–10^3 CFU/mL	4–6 h	Simple & low cost; post-PCR processing required (Chin et al.; 2022)
3	Multiplex PCR	Multiple primer sets in one reaction	10^1–10^2 CFU/mL	3–5 h	Multi-pathogen detection; optimization challenges (Ngamwongsatit et al.; 2023)
4	Real-Time PCR (qPCR)	Fluorescent monitoring of amplification	10–10^2 CFU/mL	2–3 h	Quantitative & sensitive; costly equipment (Hodzic et al., 2023; Postollec et al., 2011)
5	TaqMan Probe qPCR	Sequence-specific fluorescent probe detection	1–10 CFU/mL	2–3 h	Very high specificity; probe expense (Xue et al.; 2025)
6	SYBR Green qPCR	DNA-binding dye fluorescence	10–10^2 CFU/mL	2–3 h	Cost-effective; risk of non-specific signals (Kim et al.; 2025)
7	Digital PCR (dPCR)	Partitioned absolute quantification	1–10 CFU/mL	3–4 h	Ultra-sensitive; high cost (Sun et al.; 2025)
8	LAMP	Isothermal amplification	10–100 CFU/mL	<1 h	Rapid; no thermocycler; primer complexity (Li et al., 2025)
9	Multiplex LAMP	Multi-target isothermal amplification	10–100 CFU/mL	<1 h	Fast screening; interpretation difficulty (Xie et al., 2022)
10	RPA	Recombinase-based amplification	1–100 CFU/mL	20–40 min	Very rapid; primer specificity issues (Wang et al.; 2024)
11	CRISPR-Cas Detection	Cas enzyme-mediated target recognition	1–10 CFU/mL	~1 h	Extremely specific; emerging method (Rathore et al.; 2024)
12	Nested PCR	Two-step amplification	1–10 CFU/mL	5–6 h	High sensitivity; contamination risk (Zhang et al.; 2024)
13	HRM Analysis	Melting curve-based genotyping	10–100 CFU/mL	2–3 h	Probe-free genotyping; limited multiplexing (Słomka et al.; 2017)
14	DNA Microarray	Hybridization to specific probes	10^2 CFU/mL	6–8 h	High-throughput; expensive (Igo et al.; 2022)
15	Next-Generation Sequencing	Massive parallel sequencing	1–10 CFU/mL	24–48 h	Comprehensive profiling; costly & complex (D.-G. Park et al., 2023)
16	Whole Genome Sequencing	Complete genome analysis	1–10 CFU/mL	24–72 h	Strain-level resolution; not rapid routine test (Brown et al., 2019; Gomes et al., 2025)
17	Immuno-PCR	Antibody-DNA coupled amplification	1–10 CFU/mL	4–5 h	Combines antigen specificity & PCR sensitivity; complex setup (Radomirović et al.; 2025)
18	Biosensor-coupled PCR	PCR with sensor-based readout	10–100 CFU/mL	2–4 h	Portable options; device-dependent (Biswas et al.; 2024)
19	RT-PCR	Reverse transcription PCR (RNA detection)	10–100 copies	3–4 h	Suitable for RNA pathogens; additional step (Botello-Morte et al.; 2022)
20	Probe-enhanced LAMP	Probe-based isothermal amplification	1–10 CFU/mL	<1 h	Improved specificity; higher reagent cost (Atceken et al.; 2023)

Table 6
Key Challenges in Multiplex MAS-PCR Primer Design.

Challenges	Description	Impact on MAS-PCR Specificity	Example Mitigation
Dimer Formation	Quadratic increase in homo/heterodimers with primer number	Cross-reactivity, reagent depletion (Xie et al., 2022)	Thermolabile OXP primers
3′ Mismatch Optimization	Selecting artificial mismatches for allele discrimination	Low ~30% success rate randomly	Penultimate mismatches (Liu et al.; 2012)
Amplification Bias	Preferential shorter/GC-low targets; length/loop effects	Uneven allele detection (Liu et al.; 2012)	PCR suppression, one primer/target (Broude et al.; 2001)
Cross-Hybridization	Primers binding off-target alleles/ sequences	False positives in multi-SNP panels (Kaderali et al.; 2003)	High Tm (65–68 °C), long primers (30–40 nt) (Broude et al.; 2001)
Computational Intractability	Non-convex landscape for multi-primer optimization	Trial-and-error heavy(Xie et al., 2022)	Simulated annealing algorithms (Xie et al., 2022)

on GC-rich genomes like that of *Mycobacterium tuberculosis* due to GC bias, uneven melting (e.g., 55–60 °C), and off-target hybridization from shared flanking sequences (Limberis et al., 2023).

7.6.9. Core challenges and mechanisms

The prevalence of primer-dimer production/mispriming at allele-specific 3′-ends results in false positive risks due to weak non-specific binding occurring in unified conditions. When comparing sets, cross-reactivity in homologous/repetitive areas can lead to false positive results. This can result from the sequestering of polymerases/dNTPs as well with the existence of spurious bands on gels or absent targets in the case of false positive results (Goyani et al., 2025). Furthermore; the amplification of bias further modifies the output due to the influence of the GC content; length of amplicon (>500 bp poorly amplified); secondary structure of the template; accessibility to the template; and the inherent low efficiency/misalignment of the primers used for amplification due in part to the presence of PS loops [approximately 200 nt optimal] and the competition between the template and the product of the PCR amplification; serves as a limiting factor for concurrent multiplex amplification of 5–10 targets (Polz and Cavanaugh, 1998; Zhang et al., 2022). Inherited parameters include 3′-mismatch stability ($\Delta G^{\circ} \approx -11.5$ kcal/mol online); however, they are still under an active search for optimally functioning values. SNPs (<1%) also represent an area where subsequent single-plex amplification procedures must be followed for validation of the SNP sequence due to limited access to template (Dey et al., 2012).

7.6.10. Optimization strategies and trade-offs

Using simulated annealing algorithms (i.e., PrimerJinn) as mitigation methods, along with *insilico* validation, should provide for successful 14-plex amplification while equalizing yield across all target amplicon lengths, which would yield more successful results than unmodified triplex products (Goyani et al., 2025). However, full optimization require empirical balancing of the concentration of Mg^{2+} (2–4 mM), primer concentrations (100–300 nM), and annealing temperature, whereby although higher concentrations of Mg^{2+} enhance yield, they also decrease specificity; combinations of larger sets frequently do not yield success in the laboratory (Limberis et al., 2023).

7.6.11. Strategies to overcome challenges

Chemically modified primer technologies such as OXP phosphotriester serve as hot-start primers and greatly reduce dimer formation by 80–100% in 9-plex reactions due to their ability to prevent low-temperature extension. PCR suppression as described by Broude et al. (2001) halved primer requirements when using n + 1 tag for n-plex reactions; provided hairpin structure increased specificity and allowed for the use of four individual PCR reactions in single-base resolution as verified in CFTR ΔF508 assays. Additionally; computational aids for simulated annealing design sets to avoid dimer formation and a mix of polymerases (e.g.; Taq + AmpliTaq) resulted in a greater consistency of yields. Nested or two-step PCR techniques increased the signal from weak alleles; while uniform-sized amplicons less than 400 bp helped minimize bias (Liu et al., 2012, Xie et al., 2022).

7.6.12. Implications for applications

In genotyping, pathogen detection, or cancer miRNA profiling-relevant to microbial tech-these challenges limit MAS-PCR to low-plex without mods, but solutions like Xie et al. (2022) extend to 20+ targets cost-effectively. Future directions include AI-optimized designs and universal adapters for ultra-high plexing.

8. Research and development on a global scale: MAS-PCR in pathogen detection

Among the rapid detection methods, MAS-PCR has emerged as a pioneering method, providing sensitive and specific identification of multiple pathogens simultaneously (Fan et al., 2022; Zhao et al.; 2020). The comprehensive analysis function allows for the surety of food samples; in turn; allowing for food safety determinations to be performed faster and more efficiently. Variously around the world; different research groups are working towards developing methods for pathogen detection in food; which generally use probes that successfully target species-specific genes (Chiang et al., 2012; Villanueva-Segura et al., 2020; Zhou et al., 2020; Zhao et al., 2020). DNA-based molecular methods, including MAS-PCRs, allow for rapid and direct detection of foodborne pathogens, overcoming the challenge of cultivation-based methods. There are an increasing number of foodborne disease cases across the world, and hence there is an urgent need for rapid laboratory research methods, particularly in developing countries, such as India. Rapid methods allow for fast detection and minimize human error, in addition to minimizing the health risks associated with the consumption of contaminated food.

Hosseinali et al. (2023) studied multi-drug resistance (MDR) associated with tuberculosis detection using MAS-PCR. They detected 67 MDR-TB and 31 drug-sensitive isolates tested using drug susceptibility testing. MAS-PCR targeted mutation hotspots associated with isoniazid and rifampin resistance; which was confirmed via whole-genome sequencing. The sensitivity of MAS-PCR for detection of isoniazid resistance and resistance to rifampin was 82.8% and 98.4%; respectively. The results demonstrated the potential of using MAS-PCR to detect drug resistance in tuberculosis with important implications for effective patient treatments and control measures. Sun et al. (2023) developed a single-tube MAS-PCR assay to detect mutations. They examined eight patterns of resistance: resistance patterns I through VIII defined by specific combinations of resistance to *BosVHR, FluoMR, PenHR, CarR, BosMR, FluoSS, PenS, FluoSS, PenLR, BosLR*, and *CarS*. They successfully developed nine AS-PCR assays to detect mutation types and determined sensitivity and specificity. Most assays had a detection limit of 100 pg/μl, and there was high sensitivity at 10 pg/μl for B-H278R and D-G109V mutations. Validation using extracted DNA with known mutations demonstrated a strong agreement between AS-PCR and sequencing. They also developed a single-tube multiplex MAS-PCR (MAS-PCR) assay for rapid detection of mutations in the *CcSdhD* gene.

Rega et al. (2022) conducted an investigation that examined chromosomal gene mutations *gyrA* Ser83 (gyrA83), *gyrA* Asp87 (gyrA87), *parC* Ser80 (parC80), and *parC* Glu84 (parC84) related to ciprofloxacin- and nalidixic acid-resistant *E. coli* using MAS-PCR. They assessed meat

samples obtained from farmed versus wild boar pigs to identify antimicrobial-resistant foodborne microorganisms, confirming that pathogens may be transmitted from animals to consumers through the consumption of animal products. The authors concluded that antimicrobial-resistant microorganisms in pork and wild boar pose a significant potential threat to consumers, especially if these organisms are biofilm-producing and establish colonization on food processing equipment and surfaces.

Goodarzi and Doosti (2015) reported they detected twenty-two mutations located at the second base of codon 83 and twenty mutations located at the first base of codon 87 using MAS-touchdown PCR. Although they observed the number of mutations was still relatively low; they reiterated the need for control and eradication. Liu et al. (2019) reported they developed a PCR assay that is rapid, low-cost, and high-throughput for detecting 12 common foodborne pathogens, including *Escherichia coli O157:H7, Listeria monocytogenes/ivanovii, Salmonella enterica, Vibrio parahaemolyticus, β-streptococcus hemolyticus, Yersinia enterocolitica, Enterococcus faecalis, Shigella* spp., *Proteus mirabilis, Vibrio fluvialis, Staphylococcus aureus,* and *Campylobacter jejuni*. They developed specific primers and probes for virulence genes that displayed specificity without cross-reactivity. Diagnostic analytical sensitivity was down to 1 copy/μL for certain pathogens and caused them to detect contamination in meat samples at low concentrations. The authors state their findings demonstrate the feasibility of routine food safety monitoring for timely and accurate pathogen detection to alleviate the risk of public health hazards.

9. Conclusion

The MAS-PCR assay, in conjunction with probe-based methodologies, represents a novel approach for the rapid and sensitive detection of foodborne pathogens present in food samples. While food security continues to be examined, foodborne illness remains a major public health problem globally, killing 2.2 million people each year. The increasing prominence of the detection of foodborne pathogens is attributable to improved surveillance methods and increasing visibility of reported outbreaks. Traditional microbial detection is limited by both rapidity and specificity, whereas newer technologies, e.g., qPCR and MAS-PCR, offer improvements in both the rapidity to obtain results and specificity to detect pathogenic strains. Innovative detection of foodborne pathogens using real-time rapid diagnostic methods will be critical for the early detection of foodborne illness and food safety surveillance, to improve public health.

CRediT authorship contribution statement

Rajkumar Prabhakaran: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Meenakshi Sundari Rajendran:** Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Conceptualization. **Ramkumar Muthu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Data curation, Conceptualization.

Consent for publication

All authors have reviewed and approved the final version of the manuscript and consent to its submission and potential publication in this Journal.

Ethics approval and consent to participate

Not relevant.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Data availability

Data will be made available on request.

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