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Integral detection of bacterial macromolecules: applications of nucleic acid-based approaches

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ABSTRACT

Bacteria are the relevant causes of a vast range of diseases influencing humans, plants, and animals. Rapid detection of pathogenic macromolecules including their virulence factors, spores, and toxins has become ever more significant in food safety, biological weapons defense, and public health. Consequently, over the past decade, numerous developments have been observed in both conventional and recent approaches for bacterial detection from different sources. Many researchers have recently organized their efforts toward the development of rapid and accurate methods. This review offers an overview of trends in bacterial detection from different sources and describes different nucleic acid-based methods. Next-generation sequencing technology opened a new horizon to the world where knowledge knows no boundaries. Nevertheless, much investigation and effort are needed before they become a reliable method.

Keywords: *Bacterial detection, Molecular techniques, Next-generation sequencing, PCR-based methods, Microarray, Medicine, Food-borne.*

1. INTRODUCTION

Micro-organisms are ubiquitous from earth (e.g., waters, soil, air, plants) to the human body. Several of these micro-organisms play a vital role in nature; however, certain potentially dangerous ones have negative impacts on humans and animals and cost the consumers and the food industries billions of dollars every year. In 1990, approximately 16 million people died from infections while this number had fallen to 15 million in 2010. It is estimated that infectious diseases cause about 13 million deaths worldwide in 2050 [1]. Identification of bacteria in the disease cycle, food industry, and the environment are important to control the role of a specific bacterium in the incidence of a definite disease, infection, or illness. Furthermore, outbreaks of pathogenic bacteria are repeatedly in the news, either naturally occurring ones or due to contamination in the environment. Thus, the integral detection and identification of bacteria are essential in our world today.

The culturing of bacteria, the oldest detection method, remains the standard technique. Since culturing methods are extremely time-consuming, other approaches are much needed in bacterial monitoring. Therefore, several analytical methods have been designated to detect and identify bacteria to overcome the analysis time and detection limitations [2-4]. Molecular-based approaches have been the most important tools for the analysis of bacteria from different environments and it is possible to be more rapid and sensitive for detecting a broad range of bacterial molecules in a single test [5]. Generally, molecular approaches can detect or analyse definite nucleic acid or gene sequences, proteins, cell surface antigens, and cellular compounds or structures [6]. The most general method is the polymerase chain reaction (PCR) that, due to its high sensitivity, has yielded the most favorable results. Since the development of PCR, numerous variations of this original technique have been established to achieve the quantification, discriminating the viable bacteria, and simultaneous detection of several bacteria from food, soil, water, air, and the human body (Table 1). DNA microarray or biochips method has applied for rapid analysis of thousands of nucleic acid sequences and can be considered as an accurate technology for testing micro-organisms. Moreover, the advent of new technologies, namely next-generation sequencing (NGS), has brought promising approaches.

The purpose of this paper is to provide a review of the available molecular approaches for bacterial detection in different environments and to give informative information on the power, and effectiveness of each technique. As well, relevant applications and disadvantages of the methods are discussed. Moreover, monitoring of bacteria in different environments of interest (mainly water, food, and medicine) is covered in this review.

2. The importance of rapid detection of bacterial macromolecules

2.1. In medicine

The triumph of modern medicine depends on the detection of bacteria in water, food, plants, soil, and humans. Infectious diseases are the most leading reasons for human mortality [7]. Therefore, in order to reduce patients' mortality and the spread of the diseases as well as facilitating appropriate patient management

and improve public health, development of rapid and precise diagnostic tools is crucial for detection and identification of pathogenic microorganisms. Most importantly, the treatment of the disease in the early stages demands rapid diagnosis of pathogens, especially where the correct drug choice would be serious.

It is estimated that each year 78 to 330 million new cases of genitourinary tract infections are diagnosed worldwide mainly due to *Mycoplasma genitalium*, *Chlamydia trachomatis*, *Ureaplasma urealyiticum*, and *Neisseria gonorrhoeae* [8]. *Escherichia coli (E. coli)* O157:H7 and Shiga toxin-producing (STEC) strains are significant pathogens that are related to a spectrum of human infections (e.g., diarrhea, hemolytic-uremic syndrome, and hemorrhagic colitis). Now, these pathogens are considered the main public health problems because of the rate of morbidity and mortality associated with their outbreaks [9]. Additionally, case studies of microorganisms in aseptic cleanroom operations, rapid detection of bacteria in blood [10], cerebrospinal fluid (CSF) [11], oral cavity [12], and urine [13] are essential in the clinic.

It is estimated that the risk of bacterial contamination in platelet concentrates transfusion is higher (50 to 250 times) than the pooled risk of hepatitis B and C virus, HIV, and human T-cell leukemia virus infections [14, 15]. Unlike the transmission of virus elements, bacterial contaminations are connected with rapid onset of sepsis, acute reactions, and high rate of mortality nearly following transfusion [15]. Increasing evidence suggests that the wounds microbial composition has an essential role in wound healing [16], suggesting the necessity of studying their composition. At last but not least, microbiota, a community of micro-organisms in the human body, is an essential key player in human health and disease [17] and the study of human microbiota opens an interesting area of research in medicine [18-20] and forensic fields [21]. Moreover, bidirectional relations exist between gastrointestinal microbiota and the toxicity of environmental pollutants [22]. These reports altogether demonstrate the importance of bacterial detection in medicine.

2.2. In the food industry

The food industry is the foremost organization worried about the existence of pathogenic bacteria. More than 250 foodborne diseases are existed [23] from diarrhea to cancers, most of which caused by bacteria. Based on the World Health Organization (WHO), foodborne diseases resulted in 600 million people falling ill and 420,000 deaths every year worldwide [24], mostly in children (< 5 years of age). The most commonly recognized foodborne infections are those caused by Enterohaemorrhagic *E. coli*, *Salmonella* spp., and *Campylobacter*, *Vibrio cholera*, and *Listeria*. These pathogens can be found in several foods including raw and ready-to-eat products (e.g., dairy products, fruits, vegetables) [4, 25]. So, the examination of food for the presence of bacteria is a standard application for confirming food quality and safety.

2.3. In the environment

Water and safe drinking water is essential to all life since waterborne diseases possibly represent the most significant risk to human and public health by affecting more than half of the population in the developing world. Therefore, an appropriate evaluation of microbial water quality is vital for public health. Due to the recurrent contagion of drinking water by pathogens especially *E. coli* 0157:H7, *Giardia*, and *Cryptosporidium*, alarms with water quality have increased [26]. Besides, waterborne pathogens like *Shigella*, *Salmonella*, *Vibrio prahaemolyticus*, *Eterohaemorrhagic Escherichia*, etc. continue to infect water supplies and lead to the outbreaks of waterborne diseases in spite of the present guidelines to control or inhibit their spread [27]. In particular, waterborne infections like typhoid fever, cholera, dysentery, and diarrhea [27], as well as the presence of intestinal pathogens create a major public health hazard, especially in the developing countries [28]. About 2.2 million people die every year because of basic hygiene-related infections, where the great majorities are kids [29]. On the other hand, sewage contaminations in drinking and recreational waters, during which time swimmers are at risk, threaten the humans [30]. Furthermore, biological wastewater treatment plants can be harmfully affected by toxicity [31]. Consequently, monitoring of pathogenic bacteria in aquatic environments such as drinking water and its delivery systems, surface water, wastewater, and groundwater is required as a measure to prevent the spread of waterborne diseases.

Many problematic airborne bacterial pathogens like *Mycobacterium tuberculosis*, *Bacillus anthracis*, and *Streptococcus pneumoniae* affect human health and agricultural applications since they are transmitted rapidly in the form of aerosols, spores, dust particles, or a combination of them [32]. Bacterial agents and cell components spread as bioaerosols and can produce infections and asthmatic troubles. Tuberculosis (TB) is a major cause of morbidity and mortality and about one-third of the world's population could be infected with *Mycobacterium tuberculosis*. Therefore, the rapid study of airborne pathogens has an essential role in early warning of a disease spreading and prevention.

As a result, in areas such as environmental monitoring, various industry organizations such as food, pharmaceuticals, cosmetics and water, and assessment of public health hazards and disease diagnosis it is vital to screen and identify microbial contaminations or community. For this purpose, several methods have been investigated. In the following section, we have an overview of the nucleic acid-based molecular methods for detection of bacterial macromolecules with an emphasis on DNA and RNA.

Table 1. Advantages and disadvantages of PCR-based bacterial detection methods.

Method	Nucleic acid method	Definition	Advantage and applications	Disadvantage	Ref.
Nested PCR	DNA amplification	Employs the initial PCR product for a 2nd round of amplification.	-High sensitivity and amplification efficiency since non-specific primer annealing is avoided	-	
Multiplex PCR	DNA amplification	Simultaneous detection of specific genes using different primers	-High sensitivity and specificity -A practical protocol for detection/identification of pathogenic bacteria from food, clinic, or environmental samples. -Separation of multiple bacterial species within single general -Separation of mixed pathogens associated with an infectious disease	-Primer design is crucial. -The number of primer sets usually does not exceed six. - Difficult to discriminate viable and non-viable cells. -Reaction can be Affected by PCR inhibitors	[86]
RT-PCR	RNA amplification	Reverse transcription of mRNAs results in a single-strand cDNAs that utilized as template sequence for following PCR reaction	- Detecting viable cells -Easy to accomplish and leads to sensitive and specific results.	-The isolation and processing of samples needs time and costly equipment	[86]
qPCR	RNA amplification	The specificity of conventional PCR combines with the quantitative measurement for evaluation of gene expression levels in samples	-High sensitivity and specificity -Rapid cycling -Real-time monitoring of PCR amplification products without post-amplification products processing - Differentiate viable and nonviable cells - Simultaneous detection of multiple pathogens	- High cost - Occurrence of cross contamination - Needs trained personnel - Influences by inhibitors	[86]
ddPCR	DNA amplification	Partitions a single PCR reaction into 20,000 droplets and increases the sensitivity for detection of rare targets and absolute quantitation by analysis of the frequency of positive droplets.	- Measures the absolute copy number of nucleic acid targets without the need of external standards -Increases the tolerance of PCR to inhibitors, further improving assay sensitivity for samples such as stool - Detection of bacteria from inhibition-prone samples (environmental water and stool)	-	[29, 87]
NASBA	isothermal based RNA amplification	Is an isothermal based method of RNA amplification using an enzyme mixture (RNase H, AMV RT, and T7 RNA polymerase) at a fixed temperature (41° C)	- Sensitive and specific - Low cost - Detects viable microorganisms - It does not require a thermal cycler, so; improves portability - May be more sensitive than PCR for detection of bacteria based on rRNA-based amplification	- Difficulties in RNA handling - amplification inhibitors and RNA integrity are the central causes of concern for NASBA - Needs viable microorganisms - The enzymes used are not thermostable. - The amplified RNA target length should be in the range of 120 to 250 nucleotides	[86, 88]
LAMP		Is based on auto-cycling strand displacement DNA synthesis under isothermal conditions (59 - 65°C for 60 min). 4 primers comprising 2 inner and 2 outer primers are used to target six specific regions of target DNA.	- Low cost - Easy to perform - High sensitivity and specificity - It does not require a thermal cycling system	- Insufficient to detect unknown or unsequenced targets - Primer design is complicated	[86]
RFLP	DNA/ typing	After amplification step, genomic DNA is digested by RE for subtyping gene variants.	-The analysis of single cultures of bacteria, or samples with a low biodiversity (clinical samples or food) -High discriminatory power -Reproducible	-It can only be performed on single species of microorganism. -Needs pure culture for the discrimination of bacteria at the species level. -Needs large amount of high-quality DNA -Laborious and time consuming -Wants expensive computer software and experienced personnel	[5, 41]
AFLP	DNA/ typing	After digestion of total purified genomic DNA, complementary aptamers ligated to the fragments. Then selective amplification of sets of these fragments is achieved.	Can be applied for the determination of sources of contamination -A reproducible approach -Can be automated	-Time-consuming -Expensive	[5, 38]
RAPD	DNA/ typing	a single short length primer with a low annealing temperature bind to many genomic sites and produces a mixture of DNA fragments with several sizes.	-Simple, inexpensive, rapid and easy in use -It can be applied to any species without any information about the nucleotide sequence. -Can be applied to distinguish strains, races, and pathogenic or non-pathogenic bacteria	-It is sensitive to subtle differences in reagents, protocols, and machines - Low reproducibility because of very low annealing temperatures	[5, 38]

Method	Nucleic acid method	Definition	Advantage and applications	Disadvantage	Ref.
			-Can analyze phylogenetic relations among closely related species -Can differentiate strains within a species		

PCR: Polymerase Chain Reaction,qPCR: quantitative PCR, ddPCR: Droplet Digital PCR, DIG- dUTP: digoxigenin- UTP, NASBA: Nucleic acid sequence based amplification, LAMP: Loop-mediated isothermal amplification, AMV RT: avian myeloblastosis virus reverse transcriptase, RFLP: Restriction Fragment Length Polymorphisms, RAPD: Random amplified polymorphic DNA, AFLP: Amplified fragment length polymorphism, LAMP: Loop-Mediated Isothermal Amplification.

Table 2. Different bacterial macromolecules detection in medicine.

Pathogen	Target gene/protein	Detection method	Clinical sample	Medical application	Ref.
Mycoplasma pneumoniae	*p1 adhesin*	PCR	Throat swabs	Patients with respiratory tract infections	[89]
Haemophilusinfluenzae, S. pneumonia, *Mycoplasma pneumonia* *Chlamydophila pneumonia* *Legionella pneumophila*	- - *p1 adhesin* *16S rRNA, 5S rRNA*	Multiplex PCR	Sputum	Patients with Community-Acquired Acute Bronchitis	[90]
Ehrlichia spp., *Anaplasmaphagocytophilum*	*groEL*	qPCR	Blood	Human Infection with *Ehrlichiamuris*–like Pathogen	[91]
P. aeruginosa, Staphylococcus aureus, Klebsiellapneumoniae, S. mitis	Not stated	qPCR	Cough specimens and sputum	Adults with cystic fibrosis	[92]
S. pneumoniae	*comX*	qPCR	Serum	Patients with bacteraemic pneumococcal infections.	[2]
L. gasseri, L. crispatus, L. iners, S. salivarius, S. mutans, Enterococcusspp, Staphylococcus aureus.	Not stated	qPCR	Vaginal fluids	Forensic	[93]
Enterobacteriacea, Staphylococcus spp., *Streptococcus* spp., *E. coli, S. agalactiae, S. aureus; Lactobacillus spp.*	Not stated	qPCR	Vaginal samples	Healthy women	[94]
Enterobacteriaceae	*mcr-1*	qPCR	Stool	Not stated	[95]
EAEC, ETEC, EIEC, EPEC	*agg*Rand*aat*A, *elt* and *est, ipa*H, *eae*A	qPCR	Stool	Detection of diarrheagenic*E. coli*	[96]
C. difficile	*Tox*A/B	qPCR	Stool	Patients with cancer	[97]
19 diarrhea-related pathogens	*stx1, stx2, est-h, gyrB, invA, lysP, aggR, astA, aggR, eae, ...*	qPCR panel assay	Stool	Screening of suspected enteric pathogens, applied for surveillance of acute diarrhea	[97]
Carbapenemase-producing strains	*IMP, NDM, VIM, KPC and OXA-48*	qPCR	Isolates and stool samples	Clinical samples	[98]
E. coli, Klebsiella spp. Enterobacter spp. Citrobacter spp., Proteus mirabilis, Enterococcus faecalis, P.aeruginosa	*16S rRNA*	multiplex qPCR	Urine	Patients with urinary tract infections	[99]
Enterococcus species	*vanA, vanB, vanC1,2, vanD4, vanM, vanN, ddl, 16S rRNA*	multiplex qPCR	Clinical samples	Discrimination of vancomycin-resistant enterococcal species	[100]
S. pneumoniae, Haemophilusinfluenzae, S. aureus, Moraxella catarrhalis, E. coli, Klebsiellapneumoniae, P. aeruginosa, Acinetobacterbaumannii	*lytA, fucK, nuc, copB, yccT, gltA, gyrB, blaOXA-51-like*	multiplex qPCR	Sputum	Respiratory tract infections	[101]
S. pneumoniae, Haemophilusinfluenzae, Neisseria meningitidis, S. agalactiae, and Listeria monocytogenes	*lytA,bexA,ctrA, cfb, hlyA*	multiplex qPCR	Cerebrospinal fluid	Patients with Meningitis and Culture-Negative Cerebrospinal Fluid Specimens	[102]
E. coli, Enterococcus faecium, Enterococcus faecalis, Acinetobacterbaumannii, and Staphylococcus aureus	*gadA*and*gadB*, Hypothetical ORF, ncRNA Ref12A, 23S rDNA, *tuf*	multiplex qPCR	Whole blood samples	Critically ill patients with sepsis	[103]

Pathogen	Target gene/protein	Detection method	Clinical sample	Medical application	Ref.
C. difficile, STEC, EPEC, ETEC, EIEC, Shigella, EAEC	*tcdA and tcdB, stx1 or stx2, eae, lt or st, ipah, aatA*	Film Array rapid multiplex qPCR	Stool	Peediatricdiarrhoea	[104]
Klebsiellapneumoniae	*IMP, VIM, KPC, NDM-1,* and *OXA-48*	rep-PCR	Various clinical samples	Intensive care unit patients	[105]
Helicobacter pylori	*Urease A*	Nested PCR	Gastric biopsies	Oral Cavity	[106]
Salmonella typhi	*tviA*and*tviB*	Nested PCR	Blood	Suspected typhoid patients	[107]
S. pneumonia, S. pyogenes, Moraxella catarrhalis Haemophilus influenza,Staphylococcus aureus	*lytA, ntpC, ompJ, hdp nuc*	Species-specific qPCRs	Nasopharyngeal swab	Pre-school children	[108]
Capnocytophagacanimorsus	*16S rRNA*	PCR	CSF	Patient with meningitis	[109]
E. coli, S. aureus, E. faecalis, E. faecium, Klebsiella pneumonia, S. agalactiae, S. epidermidis, S. pyogenes, Proteus mirabilis, P. aeruginosa, Enterococcus casseliflavus, Enterococcus gallinarum, C. difficile	*pflB, mecA, BckdE1, Fms14, Khe, CspA2, gehD, MstA, DiaA, ArcC, DVanC, VanC1, TdcA*	LAMP	Blood, urine, wound, sputum and stool	Hospital patients with suspected clinical infection	[110]
Treponemapallidum	*bmp*	LAMP	Peripheral Blood	Secondary Syphilis Patients	[111]
Leptospira	*LigB*	LAMP	Urine	Clinical samples	[112]
S. aureus,S. epidermidis,Enterococcus faecium,Enterococcus faecalis,E. coli,Klebsiellapneumoniae,P. aeruginosaEnterobacter cloacae,Acinetobacterspp	*16S rDNA*	NASBA-MB	Blood	Bloodstream infections patients	[110]
Mycobacteria strains	*hsp65*	PCR-RFLP	Sputum, synovial fluid, urine, tissue biopsy and bronchial fluid	Suspected tuberculosis patients	[113]
Pseudomonas aeruginosa	-	RAPD-PCR	Nose, throat, and lesion swab, liquor, urine, sputum, tracheal aspirate, blood culture and…	Epidemiological analyses	[114]
L. rhamnosus, L. paracaseisubsp. paracasei	*fsw, cas*, Cro/CI family	ddPCR/qPCR	Piglet faeces	Preclinical and clinical trial for quantifying survival of ingested probiotics.	[115]
Campylobacter jejuni, avian influenza, *infectious laryngotracheitis virus*	specific target	ddPCR using color-coded Luminex beads	-	Multiple pathogen biomarker detection	[116]
S. pneumoniae	*cps* loci	PCR	Biorepository	Serotyping of *S. pneumonia* in molecular laboratory	[117]
Campylobacter spp., Salmonella spp, Y. enterocolitica, EIEC/Shigellaspp, E. coli 0157, C. difficile, Aeromonasspp	*stx1/stx2*	multiplex PCR	Stool	Diagnosis of bacterial gastrointestinal infections	[118]
Ureaplasmaurealyticum	*16S rRNA*	qPCR/pyrosequencing	Amniotic fluid	Diagnosis of amniotic fluid infection	[119]

C.: Clostridium, L: Lactobacillus, S: Streptococcus, E. coli: Escherichia coli, P. Pseudomonas, EPEC/EHEC: enteropathogenicenterohaemorrhagic Escherichia coli, PCR: polymerase chain reaction, EPEC: *Enteropathogenic E. coli*, STEC: *Shigatoxigenic E. coli*, EIEC: *Enteroinvasive E. coli*,

EAEC: *Enteroaggregative E. coli*, ETEC: *Enterotoxigenic E. coli*, qPCR: Real-time PCR, CSF: Cerebrospinal fluid, PCR: Polymerase Chain Reaction, qPCR: quantitative PCR, NASBA: Nucleic acid sequence based amplification, RFLP: Restriction Fragment Length Polymorphisms, RAPD: Random amplified polymorphic DNA

Table 3. Food-borne bacterial detection methods.

Detection Method	Food-borne pathogens	Detection limit	Food matrix	Assay time	Ref
PCR	*Campylobacter jejuni, Salmonella Enteritidis, Listeria monocytogenes, Escherichia coli*	Not stated	Poultry meat	-	[120]
	Salmonella enterica	3 CFU/g	Ground beef	4.5 h	[121]
Multiplex PCR	*Staphylococcus aureus, Shigellaflexneri, Listeria monocytogenes, Escherichia coli O157:H7, Salmonella Enteritidis*	10-17 CFU/g^{-1}	Artificially contaminated pork		[122]
	Escherichia coli O157:H7, Salmonella, Listeria monocytogenes Staphylococcus aureus, Yersinia enterocolitica	10^3 CFU/mL	Artificially contaminated pork	Not stated	[86]
Real-Time PCR	*Enterohemorrhagic, Escherichia coli (EHEC, 1), Salmonella spp., Vibrio parahaemolyticus, Campylobacter jejuni, Campylobacter coli, Staphylococcus aureus, Bacillus cereus, Clostridium perfringens*	1.0×10^1 to 1.0×10^3 CFU/ml	Human Feces	-	[123]
	Salmonella typhimurium *Salmonella anatum* *Salmonella montevideo*	1.8×10^4 6.4×10^3 4.3×10^3	Cattle lymph nodes	-	[124]
	Listeria monocytogenes, Escherichia coli, Salmonella spp.	6.4×10^6 CFU/g	Natural Cheeses		[125]
	Aspergillusniger, Aspergilluswelwitschiae	-	Coffee	-	[126]
MCMRT-PCR	*Escherichia coli* *Salmonella* *Shigella* *Staphylococcus aureus* *Vibrio parahaemolyticus* *Listeria monocytogenes*	1.2×10^2 CFU/mL 3.0×10^2 CFU/mL 2.5×10^2 CFU/mL 3.9×10^2 CFU/mL 2.1×10^2 CFU/mL 4.4×10^2 CFU/mL	Artificially contaminated milk		[127]
LAMP	*Vibrio vulnificus*	2.5×10^3 CFU/g	Artificially contaminated raw oysters	8 h	[128]
	Vibrio parahaemolyticus	10 CFU/reaction	Naturally contaminated seafoodsamples: fish, shrimp and mussel	16 h	[129]
	Shiga toxin-producing Escherichia coli	1–20 cells/reaction in pure culture and 10^5–10^6CFU/25 g in produce	Artificially contaminated lettuce, spinach and sprouts	Not stated	[130]
NASBA	*Salmonella Enteritidis*	10^1 CFU/reaction	Artificially contaminated fresh meats,poultry, fish, ready-to-eat salads andbakery products	26 h	[131]
	Listeria monocytogenes	400 CFU/mL	Artificially contaminated cooked ham	72 h	[132]

MCMRT-PCR: Melting curve-based multiplex real time PCR, NASBA: Nucleic acid sequence based amplification, LAMP: Loop-Mediated Isothermal Amplification.

3. Nucleic acid-based techniques for detection of bacteria

Molecular-based methods are reliable tools for the bacterial detection from different sources and offer ways to study a wide range of them in a single test. Based on their reproducibility, simplicity, and the discriminatory power variety of molecular-based techniques have been developed to reduce the analysis period and increase the types and number of bacteria [30]. Methods based on nucleic acid amplification and typing for rapid detection and identification of bacteria in different samples are discussed in the following parts.

3.1. Nucleic acid amplification methods

PCR is broadly used in bacterial detection. The PCR amplifies a particular sequence of the genome including a specific gene, arbitrary sequences or repetitive areas to the detected level, so, the targeted sequence of DNA should be known for the synthesis of the oligonucleotides. Generally targeted DNA areas for detection/identification of bacteria are bacterial virulence factors, antigens, receptors, toxins, cellular metabolites, and ribosomal RNA (rRNA). 16S rRNA gene is a favorable PCR amplification target for identification and phylogenic purposes, and detection and monitoring of particular populations in the environment [33].

Limitations of PCR applications lay in sample preparation for analysis, the cells should be lysed and purified in the case of contaminated environmental samples. Moreover, it is not easy to control all factors that affect PCR result, these factors include DNA template quality, environmental factors (e.g., temperature, humidity, and microbiological cleanliness), tools, reaction materials and conditions, and personal practice [34]. Due to the presence of inhibitory factors like humic acids and metal ions that may influence the result, the use of PCR is insufficient for sediment and soil samples. Furthermore, food may have substances that stop enzyme activity and degrade the target DNA in the PCR reaction [35]. In addition, PCR assays alone cannot confirm the existence of toxins in the food and determine live/dead differentiation [36]. So, PCR systems may also lead to false positive result because they may amplify the DNA of dead and/or noninfective organisms [37]. Inability to distinguish between culturable and nonculturable micro-organisms is the other limitation of this technique. Although the technique of PCR is simple and quick, it still needs improvements for bacterial investigations in different fields.

The approval and application of nucleic-acid amplification approaches in regular bacterial detection have been limited due to the susceptibility of PCR to inhibitors, contamination, and experimental conditions, standardization, and validation of PCR protocols that can hamper the reaction.

3.2. Molecular typing of pathogens

For investigation of infectious diseases in modern medicine, pathogenic typing, and identification of different strains of organisms within a species is vital. Bacterial typing methods are important epidemiological tools that can offer prevention strategies and inhibit future outbreaks of an infection [38, 39]. Differences in the sequences of nucleic acid in the plasmid or the whole genome DNA can be analyzed using restriction enzymes without having any knowledge of the actual DNA sequence of the micro-organism. Here, we will review the existing molecular typing approaches for outbreak detection and epidemiological surveillance of pathogenic bacteria, aiming to give an overview of their specific advantages and disadvantages (Table 2).

3.2.1. Ribotyping. Ribotyping is a method used to identify and characterize micro-organisms. In this method DNA sequences generated from digestion of 16S rRNA genes by restriction enzymes are analyzed. In ribotyping, genes within the rRNA operon are amplified by PCR, digested with restriction enzymes, separated on a gel and finally probed. The generated pattern of separated DNA fragments by electrophoresis is digitized and computer-based programs are utilized to compare the patterns of these fragments with the one from *E. coli*, as a reference, in a database. The bacteria origin is determined using the bacterial specific pattern of DNA sequences (riboprints). Ribotyping is a rapid, simple, and specific technique to identify bacteria [5]. Ribotyping has been becoming a well-known technique for ecological, epidemiological, systematics, and population studies of bacteria. There have been numerous modifications in this method, one of which is the automated ribotyping that has high-throughput applications in food and pharmaceutical industries [40].

3.2.2. Pulsed-field gel electrophoresis (PFGE). Pulsed-field gel electrophoresis (PFGE), similar to ribotyping, utilizes digested DNA fragments to identify bacteria. However, unlike ribotyping in which rRNA is analyzed, in PFGE whole DNA genome is digested by rare cutting enzymes. Separation of digested DNA fragments by conventional gel electrophoresis is not achievable. Gel matrix separates the different lengths of DNA fragments under the unidirectional electric field. In PFGE DNA fragments are subjected to successive alternating electric fields. In this condition, DNA molecules migrate continuously in different directions. Therefore, the basis of sieving in PFGE differs from conventional agarose gel electrophoresis in which separation occurs based on the size of the DNA molecules. In PFGE, uncoiled DNA fragments move parallel to the electric field and enter a pore opening in the agarose. By stopping the electric field and applying a new electric field perpendicular to the uncoiled DNA, reorientation happens and DNA fragments enter a new opening. The speed of change in the field direction is a key factor as slow switching prohibits reorientation of DNA fragment and the result will be similar to the conventional electrophoresis. Size of DNA fragments will be analyzed by a size marker and retrieved pattern will be compared to the pattern of other bacterial fingerprints.

Among molecular typing methods, PFGE technique is considered as the ‘gold standard’ for numerous bacteria in the clinic[38]. Despite being widely used, PFGE is technically demanding and time-consuming. Moreover, it may not be able to discriminate between bands which are close or identical in size [38].

3.2.3. Repetitive-element PCR. In repetitive-element PCR (rep-PCR) method, noncoding intergenic repetitive sequences (RSs) are amplified and based on the distribution of these elements through the genome several amplicons with different sizes are produced.

After electrophoresis, the pattern of the bands is compared to define the genetic similarity between the isolated bacteria. It is applicable in the differentiation of different mycobacteria strains [41]. rep-PCR is a very rapid and cheap approach and can result in real-time strain-typing using Web-based software. The main drawback of the method is the lack of adequate reproducibility, resulted from differences in reagents and gel electrophoresis systems [38, 41].

3.2.4. Whole genome sequencing (WGS). WGS provides extensive detailed data about genome of bacteria and facilitates detection and characterization of bacteria in clinical samples, food and environment. WGS compares whole genome of pathogens base by base and is useful in unraveling the evolution of bacterial strains. Also, it is considered as the frontline for epidemiological surveillance typing and outbreak investigations of clinically important pathogens such as *Campylobacter jejuni*[42]. WGS based approaches have been used as a high resolution typing tool for pathogenic bacteria such as *L. monocytogenes*[43], *Salmonellaspp*.[44] and *Staphylococcus aureus*[45] as the major sources of foodborne diseases. However, high quality reference sequences are required to validate the results, especially in phylogenetic studies. More efficient analysis techniques are required to use the acquired data for routine clinical applications [46].

3.3. Microarrays.

Microarray-based approaches can be applied to positively identify and differentiate strains of bacteria from the relatively innocuous to the highly pathogenic ones. Microarray technique utilizes immobilized probes on usually glass slides to detect the target bacteria in hybridization assay [47]. To amplify one or more genes, PCR is used and then the products are hybridized to the array to detect species-specific polymorphism within the genes of interest [47]. This technology rapidly identifies pathogenic strains of different bacteria and also multidrug resistant isolates in clinical samples (e.g. *Mycobacterium tuberculosis*) [48]. A microarray approach used gyrB gene fragment and detected mycobacterial species in clinical samples with 99.8% sensitivity and 100% specificity [49]. Moreover, it is useful to detect foodborne pathogenic bacteria and finding health risks in environmental resources (e.g. aquatic system) [50]. *Listeria monocytogenes* and *Salmonella spp*. were successfully identified in milk using microarray approach [51, 52]. Moreover, the microbiota of body organs such as oral and vagina samples have been successfully characterized [53, 54]. A number of disadvantages of microarray-based technique cause inaccurate analysis of samples. The signal intensity after probe hybridization is not quantitatively proportional to DNA concentration. Moreover, due to the presence of homology among some genes, nucleic acids in the sample will mismatch to the probes and will lead to decreased specificity. Furthermore, if no complimentary probe is designed for a gene on the microarray chip, the related fragment will not be detected [47]. However, still modified versions of microarray technique are developing and are in use for various purposes.

3.4. Next generation sequencing (NGS).

NGS techniques are developed and high-throughput technology to sequence and analyze the massive amount of nucleic acids simultaneously. It has facilitated WGS and whole exome sequencing and has been translated into clinical and research centers. Targeted amplicon sequencing that amplifies conserved regions (e.g., 16S rRNA gene sequencing) of genome is performed by this technique. RNA sequencing (e.g., mRNA rRNA, tRNA, and non-coding RNAs) unravel transcription profiling and expression pattern of even unknown genes in bacterial species in various conditions and environments [55]. Moreover, epigenetic (e.g whole genome methylation sequencing and chromatin immunoprecipitation followed by sequencing (ChIP-Seq) studies can be performed by NGS methods. Next generation sequencing (NGS) technologies have tremendously facilitated these procedures and provide a deep perspective of bacterial genomics. NGS strategies overcome the limitations of culture- and cloning dependent methods and provide cost- and time effective techniques. Sanger's dideoxy chain termination technique was first introduced in the late 1970s [56] and was used in first generation sequencers. Since then, a variety of cost-effective NGS technologies became available including second generation sequencing techniques (454 (Roche), SOLiD (ABI), Solexa (Illumina)) and third generation sequencing systems or single molecule long read sequencing (Nanopore (Oxford Nanopore Technologies), single-molecule-real-time technology (Pacific Bioscience), and Heliscope (Helicos Ion Torrent (Life Technologies)) [57, 58]. Each sequencing strategy uses a unique detection platform deeply reviewed by Goodwin et al. [59]. Analysis of generated data requires sophisticated computational bioinformatic methods. Efforts are in progress to facilitate data processing step for easy usage of techniques in diagnostic laboratories [60]. In each technique the attempt is to improve the particular features of sequencing techniques in terms of improving the length of amplicons, a number of reads, error rate, and cost [58].

NGS has been implemented in some medical microbiology laboratories to detect pathogenic bacteria, either one or several species in each clinical sample varying from body fluids such as saliva [61], abscess samples [62] to organ tissues [63]. In clinic and public health, NGS is used to develop new therapeutics for infectious bacteria by profiling virulence and drug resistant genes such as in tetracycline-resistant *Streptococcus pneumoniae*[64, 65], and understanding the molecular responses in exposure to different therapies [66]. Moreover, NGS technology monitors the generation of drug resistant bacteria [67], predicts the treatment responses [68] and trace pathogen dissemination and outbreaks [69]. NGS makes the characterization of bacteria feasible and provides detailed information about the taxonomy and phylogeny of bacteria to discriminate them from other closely related strains [70] and also characterizes the zoonotic bacteria with potency to infect humans[71]. Microbiome composition of different organs in healthy and diseased condition has been identified [72-74]. Moreover, NGS furthers our understanding of interactions between different bacterial populations in each microbiome [75] which is pivotal to understand food-related microbiomes [57, 76]. In the last decade, this was routinely performed by target amplicon sequencing in food microbiology laboratories [76]. NGS provides information about the bacteria involved in food processing steps such as fermentation, characterization of spoilage microorganisms and contaminating bacteria to monitor and improve food quality, safety and preservation [57]. *E. coli O157:H7, Shigella spp., Salmonella spp., Listeria monocytogenes,* and *Campylobacter spp.*

are among frequently identified food-borne pathogens [77]. The information obtained by NGS, in combination with other–omics data, are promising in identification and prevention of food-borne diseases, tracing the source of contamination and toxins in food-borne outbreaks and in risk assessment [78]. To control food-borne diseases, NGS effectively track emergence, virulence and pathogenicity of new pathogenic strains such as Shiga toxin producing *E. coli* O104:H4[79]. NGS techniques are used to analyze biodiversity of various ecosystems such as soil [80], freshwater [81], and benthic samples [82], to assess water quality and track the source of water pollution [83], to determine the effect of contaminations on bacteria population in an ecosystem [84], and for diet analysis of animals [85].

4. CONCLUSIONS

Bacterial infection is a major public concern that occasionally leads to thousands of deaths worldwide. Consequently, monitoring and detection of bacteria in different environmental samples has essential impacts on human health. Over the past decade, numerous developments have been observed and many researchers have organized their efforts toward the development of rapid and accurate ones. Next-generation sequencing technology opened a new horizon to the world where knowledge knows no boundaries. Despite the vast number of developments in bacterial detection methods in the analysis of different samples, there are still some unsolved issues that need to be addressed in this hot area of research. Much investigation and effort are needed before they become a reliable method.

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