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Review Article

Advanced Molecular Techniques in the Diagnosis of Pathogenic Bacteria: An Applied Study on PCR and Gene Sequencing Techniques

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Abstract: Diseases caused by waterborne and foodborne pathogenic bacteria present a significant threat to global health and economic stability. Although traditional culture-based methods have long been used, they suffer from time-consuming procedures, underestimation of viable counts, and the inability to detect viable but non-culturable (VBNC) cells. Therefore, this research provides a comprehensive applied study of advanced molecular techniques as precise and rapid diagnostic tools. The study highlights the effectiveness of Polymerase Chain Reaction (PCR) variants and Gene Sequencing techniques in detecting and characterizing enteric and non-enteric pathogenic bacteria (such as E. coli O157:H7, Campylobacter, Salmonella, Shigella, and Legionella) in wastewater environments and wastewater-based epidemiology (WBE). The findings show that molecular methods offer high sensitivity and specificity, effectively overcoming matrix inhibitors and serving as a crucial foundation for early warning and outbreak control.

Keywords: Advanced Molecular Methods, Polymerase Chain Reaction (PCR), Gene Sequencing, Pathogenic Bacteria, Wastewater-Based Epidemiology.

INTRODUCTION

Diseases brought on by germs that come from humans pose a significant danger to health around the globe, the World Health Organization (WHO) estimates that 600 million individuals, which is nearly one in ten, become ill because of eating food that is unsafe to consume. Sadly, out of these, 420,000 people lose their lives each year, resulting in a loss equivalent to 33 million years of healthy living [1]. Eating unsafe food leads to an annual economic loss of USD 110 billion in healthcare expenses and lost productivity in lower and middle-income countries. Furthermore, the growing issue of diseases spread through water adds to this global challenge, resulting in nearly USD 12 billion in financial losses and more than 2.2 million deaths yearly. Diseases caused by contaminated food and water put a strain on healthcare systems and impact economies worldwide, which slows down social and economic growth [2].

Among the many germs that spread through food and water, disease-causing bacteria are the largest and most frequent category. Bacteria that cause illnesses through food and water, such as Escherichia coli, Campylobacter species (including C. jejuni and C. coli), Legionella species, Salmonella species, Shigella species, are associated with the majority of infection cases, with some resulting in severe or deadly outcomes [3].

In recent times, while there has been a drop in outbreaks linked to water and food, thanks to better public health initiatives, the challenge posed by infectious diseases related to water and food remains a serious worldwide concern. Additionally, the rise of antimicrobial resistance among germs hampers effective ways to prevent and treat a growing variety of infections. The old way of finding harmful bacteria mostly depends on growing them in a lab. These culture methods are inexpensive, straightforward to use, and very consistent, making them popular for monitoring bad bacteria, like measuring fecal indicator bacteria in swimming areas [4].

However, these methods have significant drawbacks, such as being unable to distinguish between the bacteria of interest and other similar microorganisms

present in the same samples, as well as yielding false positive or negative results. They also require a lot of time and effort and cannot detect nonculturable yet viable cells, Furthermore, when using culture methods for counting purposes, the actual number of bacteria is often underestimated [5].

This affects how accurately we can measure the amount of harmful bacteria and leads to underestimating how common these pathogens are in the community. Additionally, nonculturable cells can still become active and lead to illness, increasing risks to public health. As a result, molecular methods have emerged as a fast and precise tool for detecting harmful bacteria, becoming the leading technique in this area [6].

Based on the biological markers used, molecular techniques can be classified into two main types: those that focus on nucleic acids and those that target proteins or antigens. The nucleic acid targeting techniques include methods that enhance visibility, such as the polymerase chain reaction (PCR), real-time PCR (qPCR), digital PCR (dPCR), DNA microarrays, fluorescence in situ hybridization (FISH), and molecular beacons, as well as sequencing methods like pyrosequencing, Illumina sequencing, and nanopore sequencing [7]. The methods that target proteins and antigens involve traditional approaches that use the interaction between antibodies and antigens, resembling immunological techniques like lateral flow tests (LFTs). Additionally, by merging basic molecular detection techniques with a metal and paper platform, biosensor and paper-based devices have emerged as rapid, low-cost, and portable options for detecting harmful bacteria on-site [8].

Wastewater-based epidemiology, or WBE, is a technique that gathers both quality and quantity information regarding how chemicals are used or exposed to and the number of infections among people living in a specific wastewater area by examining chemical substances, germs, and specific markers found in untreated sewage [1]. Sewage from a wastewater treatment facility or drainage systems acts like a combined sample of urine and feces from a community and can help assess the overall health of that community [9].

Research on wastewater has demonstrated that it can indicate not just illegal drug usage, eating habits, and lifestyles, but also the presence of disease outbreaks in a population [3]. Moreover, wastewater testing can identify germs released by people who do not show any symptoms yet making it a valuable resource for early detection and quick response to infectious diseases [10].

Traditional techniques generally include filtering, staining, and examining samples under a microscope. More sensitive and specific methods such as PCR-based techniques and DNA sequencing have also been used in the analysis of human pathogens in wastewater. However, the concentration of pathogenic bacteria is usually lower than that of indicator microorganisms and thus requires a highly sensitive detection method. The complex wastewater matrix often leads to false-negative results due to the presence of various inhibitors. Additionally, sample processing methods vary for different downstream analytical approaches [11].

Thus, it remains challenging to employ molecular detection methods for accurate and quantitative identification of pathogenic bacteria in raw wastewater. This review centers on molecular analytical techniques for pathogenic bacteria in wastewater and outlines recent advancements of these methods in wastewater-based epidemiology. The potential of these approaches in wastewater analysis is examined to offer insights for further application [12].

Chapter One: Pathogenic Bacteria in the Environment and Wastewater

General Classification of Microbial

Infectious agents that can lead to severe illnesses or even death pose a significant risk to public health worldwide. Different types of harmful agents in humans can be categorized as [13]:

1. Bacteria (for instance, Enterohemorrhagic E. coli (EHEC), Campylobacter species, and Salmonella species, among others).
2. Viruses (like influenza viruses, hepatitis virus, rotaviruses, and Norwalk viruses, to name a few).
3. Protozoa (such as Giardia lamblia and Cryptosporidium parvum).
4. Parasites (including ascaris and Ancylostoma).

At present, there are roughly 538 kinds of harmful bacteria that can infect humans. This count is significantly greater than the total for other types of pathogens, which include about 208 types of viruses, roughly 57 species of parasitic protozoa, as well as certain fungi and worms [14].

Escherichia Coli O157:H7 (Virulence Factors and Toxins)

The type E. coli that leads to diarrhea is known as diarrheagenic E. coli. Among the various types of diarrheagenic E. coli, enterohemorrhagic E. coli (EHEC) is notable for its ability to produce Shiga toxin [15].

Both E. coli O157:H7 and other non-O157 strains that produce Shiga toxin, such as O26:H11, O111:H8, and O118:H16, can generate these toxins, but only the O157:H7 type can cause illness in humans. The other strains usually reside in cattle without causing any health issues. E. coli O157:H7 is the most common and dangerous strain within the harmful group of E. coli. It can lead to not only diarrhea, severe intestinal inflammation but also serious condition called hemolytic

uremic syndrome, which primarily affects children and can result in kidney failure and even death. The harmful traits of E. coli O157:H7 include the type III secretion system, Shiga-like toxins 1 and 2, a system that helps it tolerate acid, hemolysin, and an enzyme known as extracellular serine protease [16].

Campylobacter spp: (Epidemiological Significance)

Is one of the main reasons for diarrhea, and it is also seen as the leading reason for human stomach infections around the world. There are 13 harmful Campylobacter types known to cause infections in humans, including C. jejuni, C. coli, C. lari, C. concisus, C. rectus, C. hyointestinalis, C. insulaenigrae, C. sputorum, C. helveticus, C. fetus, C. mucosalis, C. upsaliensis, and C. ureolyticus. Of the 17 types and six subtypes of Campylobacter, C. jejuni and C. coli are most closely tied to infections, accounting for 80–85% and 10–15% of all infections, respectively [17].

C. jejuni and C. coli are also the main types commonly found and collected from wastewater. Harmful Campylobacter causes 400–500 million infections each year. In Europe, nearly 230,000 cases have been reported annually since 2015. It is thought that the amount needed to cause campylobacteriosis is very small, with just 360 colony-forming units (CFU) being sufficient to lead to illness [18].

Salmonella spp: (Serotypes, Antigens, and Target Genes Such as invA)

Salmonellosis is a significant disease that can spread from animals to humans, primarily caused by Salmonella bacteria. People typically get infected by consuming raw food items. Some types, including S. typhimurium and S. enteritis, mostly infect humans through poultry. Eggs and chicken are the two types of food most often linked to Salmonella infections. According to the Centers for Disease Control and Prevention, between 2006 and 2017, about 53.4% of food-related illness outbreaks in the United States were due to Salmonella, and around 32.7% of these cases were connected to eating fruits and vegetables. Furthermore, S. Typhi and Paratyphi are responsible for typhoid fever and paratyphoid fever, respectively. These bacteria are specific to humans, are Gram-negative, and only affect people. They can be transmitted from one person to another through contaminated food or water, or by interacting with someone who is acutely or chronically infected [19, 20].

Although some methods have been created to grow and separate these two types of Salmonella from various samples, it remains challenging to cultivate them because they are very particular about their growing conditions. The molecular method is viewed as a more effective way to find and measure Salmonella bacteria compared to traditional culture methods [21].

Various techniques have been developed to focus on different genes or gene areas that are unique to Salmonella. The most common genes targeted are invA and flagellin genes (fliC-d for Salmonella Typhi, fliC-a for Salmonella Paratyphi A). Some tests combine multiple approaches to enhance sensitivity and accuracy. Additionally, protein markers like the membrane vesicle protein PagC are being used to detect harmful Salmonella as a new type of biomarker [22].

Shigella spp: (Diagnostic Characteristics)

Salmonellosis is a major disease that people can get from a germ called Salmonella spp., which spreads to humans through uncooked food. Some types of this germ, like S. typhimurium and S. enteritis, mainly come from chickens and can infect people. The two types of food most often linked to Salmonella infections are eggs and chicken meat. The Centers for Disease Control and Prevention report that between 2006 and 2017, about 53.4% of foodborne illness outbreaks in the USA were due to Salmonella, with roughly 32.7% of those linked to consuming fruits and vegetables. Furthermore, Typhi and S. Paratyphi are the primary bacteria that cause typhoid fever and paratyphoid fever, respectively [23].

Both are types of germs that only affect humans and are Gram-negative. These germs can spread from one person to another through contaminated food or water or by coming into contact with someone who is infected [24].

Shigella is a type of bacteria that is Gram-negative and belongs to the Enterobacteriaceae family, capable of living with or without oxygen. It is believed to cause an illness known as shigellosis or bacillary dysentery. There are four main types of Shigella: S. dysenteriae, S. flexneri, S. boydii, and S. sonnei. Shigellosis is an infection of the colon primarily caused by S. sonnei and S. flexneri. This bacterium spreads through the fecal-oral route, and it only takes about 10 bacterial cells to trigger an infection [25]. Since the bacteria do not survive well outside the human body during certain stages, they continuously spread from person to person. Every year, there are around 165 million cases of Shigella infection globally, leading to about 1 million deaths, especially in poorer nations. It has been found that the traits of Shigella species are very similar This close genetic similarity means that harmful genes are common in both Shigella and E. coli, complicating the process of identifying which types are harmful [26].

Clostridioides difficile, often called Clostridium difficile or C. difficile for short, is a kind of bacteria that can make spores and is classified as Gram-positive. Certain harmful types of C. difficile can lead to severe diarrhea and a risky condition known as pseudomembranous colitis, which usually requires antibiotics for treatment. These dangerous types are mainly identified by their ability to generate harmful

substances called enterotoxin A (TcdA) and cytotoxin B (TcdB). The diarrhea associated with C. difficile, referred to as CDAD, is a widespread infection seen in hospitals, linked to serious health issues and high death rates, imposing a heavy financial burden on healthcare systems [27]. In the last twenty years, toxin-producing C. difficile has become a leading cause of infections in hospitals, iarrhea and can cause lifethreatening pseudomembranous colitis. It has been observed that C. difficile infections contracted outside of healthcare settings are increasing among people who don't seem to have interacted with healthcare facilities and do not have known risk factors for these infections (CDI) [28].

Pneumonia that comes from Legionella spp. is a serious lung infection mainly caused by Legionella pneumophila. Furthermore, an additional 19 types have been identified as harmful to humans through tests done on clinical samples [29].

Legionella spp. do not respond to beta-lactam antibiotics because they need to live inside cells. Therefore, treating these infections calls for a substantial dose of either quinolones or macrolides. Quick detection of LD is essential for tracking outbreaks and providing care in hospitals. Legionella has been found in wastewater, with amounts reaching as high as 10^8 CFU/L,The Mycobacterium family consists of over 170 species, with at least two, Mycobacterium tuberculosis and Mycobacterium leprae, considered important harmful germs for humans. Most of the others are germs that can cause illness in both people and animals when conditions are right. Typically, Mycobacterium is divided into two main categories: the closely related M [30].

Chapter Two: Advanced Molecular Techniques for Detection of Pathogenic Bacteria

Biomarkers such as nucleic acids, proteins, antigens, adenosine triphosphate (ATP), and metabolic byproducts are used to study microorganisms. To distinguish different microorganisms in a single sample, nucleic acids (DNA/RNA), proteins, and antigens are often selected as biomarkers because of their unique physical and chemical traits present in various pathogens [31]. Finding DNA/RNA relies on the precise hybridization and multiplication of target materials, which allows for high levels of specificity and accuracy the key biomarker is the DNA or RNA remnants from these pathogens. The biomarkers can include genes specific to certain genera or species, functional genes, and genes related to resistance to drugs. Also, when analyzing resistance to antibiotics, gene transfer is another important aspect. Different mobile genetic components like plasmids, transposons, bacteriophages, integrons, and combinations of these serve as important nucleic acid targets for studying how resistance genes spread and are found in bacteria [32].

ATP tests, enzyme activity measurements, and analysis of metabolic products are primarily used to evaluate the behavior of living cells. Since there is a direct relationship between the total amount of ATP and the total colony-forming units, the cells that are metabolically active can be measured by the ATP level. These byproducts can be detected using electrochemical techniques [33].

Table 1: Molecular detection methods and examples of their applications in the analyzed pathogenic bacteria

Molecular Method	Target Bacteria/Genes	Sample Type	Limit of Detection | Reference
Polymerase chain reaction (PCR)-based technique	Multiplex-PCR (mPCR)	Enteropathogens	Wastewater
Single qPCR	Salmonella	Salmonella Isolates	-
Microfluidic quantitative PCR	Antibiotic resistance and heavy metal	Wastewater resistance genes	Droplet digital

Second: Gene Sequencing Techniques

Sequencing is the technique used to determine the order of nucleotides in A segment of DNA. It encompasses any method used to determine the sequence of the four bases: adenine, guanine, cytosine, and thymine (or uracil in the case of RNA). Frederick Sanger was the first to develop DNA sequencing technology, based on the chain-termination method, also known as Sanger sequencing [34]. Initially, sequencing was utilized for small genomes like those of viruses and organelles. It was not feasible to fully sequence a bacterium's genome due to financial and technical constraints. Ultimately, the introduction of the shotgun method developed by Sanger and others made it possible to sequence entire bacterial genomes. The shotgun method is regarded as the standard approach, and over the years, whole genome sequencing of various bacteria has been performed using this technique [35].

Next-generation sequencing (NGS), commonly referred to as high-throughput sequencing, is a broad term that covers various contemporary sequencing methods. These technologies enable the sequencing of DNA and RNA much faster and cheaper compared to the older Sanger sequencing method, which was once the standard, thus changing the landscape of genomics and molecular biology research the efficiency and cost-effectiveness of NGS come from its ability to analyze a large number of sequences simultaneously, allowing for 300 Gb of DNA to be processed in a single run using one chip [36].

Immunological methods rely on the specific interaction between antibodies and antigens. These techniques include enzyme-linked immunosorbent assays (ELISA), immunofluorescence assays (IFA), and serum neutralization tests (SNTs) [6]. In these approaches, specific fluorochrome-labeled antibodies are used to capture target antigens, enabling the counting of fluorescently labeled cells by detecting the fluorescence signal via microscopy or flow cytometry [37].

Table 2: Applications of molecular techniques in studying harmful bacteria in wastewater

Detection	Method	Growing	DNA/RNA Extraction	Target Pathogen	Biomarker	Sample Type
PCR	Yes	Yes	Campylobacter sp., C.jejuni, C.coli	Limit of Detection (LoD)	16S rDNA, mapA, ceuE	Waste-water
Yes	Yes	E. coli O157:H7	stx2	Wastewater	2 CFU/10 mL	-
Yes	Yes	S.typhimurium	stx1	Wastewater	qPCR	-
No	Yes	E. coli	uidA gene	Wastewater	10 pgs/fraction	standard curve
No	Yes	Shigella dysenteriae	16S rRNA gene	Wastewater	5 gc/fraction	standard curve
No	Yes	S. enterica serovar Typhi	invA	Wastewater	-	-
No	Yes	S. enterica serovar Paratyphi A	rfbA	Wastewater	0.05-0.005	of seeded waste-water

Sequencing, as a strong analytical tool, has been broadly used for analyzing bacterial diversity and possible pathogens in wastewater. The DNA sequencing method can carry out extensive parallel analysis of PCR products and environmental nucleic acids. This provides a new approach for the analysis of harmful bacteria in wastewater [38].

The method of using NGS technologies in wastewater research can be split into four parts: whole genome sequencing (WGS), metagenomic sequencing, metatranscriptomic sequencing, and sequencing of a targeted amplified gene (e. g., 16S rRNA and 18S rRNA genes). WGS is a strong method for identifying microorganisms in wastewater, as it depends on isolating and growing bacteria, extracting long DNA, and creating long read sequencing platforms. Future improvements in these areas could enhance and make its use in wastewater analysis easier [39].

Table 3: Comparison of Old Cultural Techniques and Modern Molecular Methods

Parameter	Old Cultural Techniques	Modern Molecular Methods (PCR and Sequencing)
Time to Results	24 to 72 hours (or longer)	2 to 6 hours (Rapid diagnosis)
Sensitivity & Specificity	Moderate can miss low bacterial loads	High; targets specific DNA/RNA sequences
Detection of VBNC Cells	Unable to detect (requires culturability)	Can easily detect Viable but Non-Culturable cells
Throughput & Automation	Low; labor-intensive and manual	High; capable of high-throughput analysis

This table provides a comprehensive comparison between conventional methods and modern molecular approaches used in microbiology diagnostics:

Time to Results:

Traditional culture methods typically require 24 to 72 hours (or even longer) because they rely on microbial growth on agar plates. In contrast, advanced molecular techniques (such as PCR and sequencing) deliver results rapidly within 2 to 6 hours.

Sensitivity & Specificity:

Traditional methods have moderate sensitivity and can sometimes miss low bacterial loads or slow-growing organisms. Molecular techniques offer exceptional sensitivity and specificity by targeting precise and unique DNA or RNA sequences.

Finding VBNC Cells:

Traditional techniques are unable to identify "Viable but Non-Culturable" (VBNC) cells since these methods depend on the bacteria growing and creating visible colonies. On the other hand, molecular techniques can effortlessly discover these cells by monitoring their DNA.

Throughput & Automation:

Culture-based methods are generally manual, labor-intensive, and have low sample throughput. Advanced molecular techniques allow for high-throughput analysis, automation, and handling large volumes of samples simultaneously.

Table 4: Key Polymerase Chain Reaction (PCR) Variants and Their Applications in Pathogenic Bacteria Detection

PCR Technique	Target Pathogens / Biomarkers	Main Purpose / Application
Conventional PCR	Escherichia coli, Salmonella spp	Basic identification and presence/absence screening
Multiplex PCR	Salmonella enterica, Shigella flexneri, Listeria monocytogenes	Detecting various germs at the same time in one test
Quantitative Real-Time PCR (qPCR)	Campylobacter jejuni, Legionella spp	Quantification of bacterial load and gene expression
Digital PCR (dPCR)	Antibiotic Resistance Genes (ARGs) in wastewater	Absolute quantification without relying on standard curves

Their Applications in Pathogenic Bacteria Detection

This table categorizes the various types of PCR technologies based on their specific targets and primary applications in pathogen detection:

- **Conventional PCR:** Used chiefly for basic identification, screening, and determining the presence or absence of target pathogenic bacteria such as Escherichia coli and Salmonella spp.
- **Multiplex PCR:** Designed to detect multiple pathogens at the same time within a single reaction tube (e.g., Salmonella enterica, Shigella flexneri, and Listeria monocytogenes), saving time and resources.
- **Quantitative Real-Time PCR (qPCR):** Utilized for precise quantification of bacterial load and monitoring gene expression, commonly applied to pathogens like Campylobacter jejuni and Legionella spp.
- **Digital PCR (dPCR):** A highly advanced variation used for absolute quantification (especially for tracking Antibiotic Resistance Genes - ARGs in wastewater) without the need for a standard calibration curve.

Table 5: Overview of Gene Sequencing Techniques Used in Wastewater-Based Epidemiology (WBE)

Sequencing Technology	Primary Function / Output	Application in Pathogen Diagnostics
Sanger Sequencing	Single gene or short fragment sequencing	Confirmation of specific PCR amplicons and validation
Next-Generation Sequencing (NGS)	High-throughput, massive parallel	sequencing | Comprehensive screening and surveillance of complex samples
Whole Genome Sequencing (WGS)	Complete mapping of an organism's genome	Tracking outbreak sources, virulence factors, and strain typing
Metagenomic Sequencing	Total DNA/RNA sequencing from environmental matrices	Profiling overall microbial diversity and detecting unknown pathogens

This table outlines the prominent gene sequencing platforms and their critical roles in environmental surveillance and pathogen tracking:

- **Sanger Sequencing:** Focuses on single genes or short DNA fragments; it is Primarily utilized for verifying particular PCR amplicons and confirming genetic objectives.
- **Next-Generation Sequencing (NGS):** Offers extensive, simultaneous sequencing abilities, which makes it perfect for thorough assessments and monitoring at the community level of complicated environmental samples.
- **Whole Genome Sequencing (WGS):** Provides complete genetic mapping of an organism's entire genome, allowing researchers to track outbreak sources, identify virulence factors, and perform accurate strain typing.
- **Metagenomic Sequencing:** Involves sequencing total DNA and RNA extracted directly from environmental samples without prior cultivation, enabling the profiling of overall microbial communities and the discovery of unknown or emerging pathogens [40].

CONCLUSIONS

As worries grow about diseases from harmful bacteria and their resistance to treatments, many see that good monitoring is vital for quickly addressing and managing outbreaks. Wastewater-based epidemiology has gained popularity because it is an effective method for tracking health issues across large populations and serves as a warning system for potential disease outbreaks. With improved techniques for identifying harmful bacteria and their related markers, the use of WBE is broadening to include a wide variety of pathogens through different advanced molecular approaches suited for analyzing wastewater.

In brief, this study highlights a significant change from older, lengthy culture-based techniques to modern molecular methods that allow for the quick and precise identification of harmful bacteria in water and food settings. Utilizing different types of Polymerase Chain Reaction (PCR), such as multiplex PCR and quantitative real-time PCR (qPCR), along with advanced Gene Sequencing and Next-Generation Sequencing (NGS), has revamped the way we monitor the

environment and execute Wastewater-Based Epidemiology.

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