DEVELOPMENT OF MULTIPLEX PCR FOR THE SIMULTANEOUS DETECTION OF *Pseudomonas aeruginosa*(toxA) AND *Escherichia 1coli* (eae) in CLINICAL SAMPLE

DISSERTATION SUBMITTED TO ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM
IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF

DEGREE OF MASTER OF SCIENCE IN ZOOLOGY

SUBMITTED BY,

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CERTIFICATE

This is to certify that the dissertation entitled "Development of multiplex PCR for the simultaneous detection of *Pseudomonas aeruginosa*(toxA) AND *Escherichia coli*(eae) in clinical sample "is an authentic record of original project work carried out by Ms. Maria Edward (Reg.No:SM23ZOO005) during the academic year 2023-2025, under my guidance in requirement of the Degree of Master of Science in Zoology from St. Teresa's College (Autonomous), Ernakulam.

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BONAFIDE CERTIFICATE

Certified that this thesis titled, **"Development of Multiplex PCR Panel for the Simultaneous detection of *Pseudomonas aeruginosa* (toxA gene) and *Escherichia coli* (eae gene) in Clinical Samples"** is a bonafide work of **MARIA EDWARD, Reg no: SM23ZOO005** who carried out the Research under my supervision. Certified further, that to the best of my knowledge, the work reported herein does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an occasion on this or any other candidate.

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I hereby declare that the dissertation entitled "Development of multiplex PCR for the simultaneous detection of *Pseudomonas aeruginosa*(toxA) AND *Escherichia coli*(eae) in clinical sample" submitted to St. Teresa's college (Autonomous), Ernakulam in partial fulfilment of the requirement for the award of the Degree of Master of Science in Zoology is a record of original research work done by me under the supervision and guidance of Dr. Reema Kuriakose Associate professor, Department of Zoology, St. Teresa's college (Autonomous), Ernakulam and to the best of my knowledge and belief, this project contains no material previously published or written by another person, except where due reference is made.

MARIA EDWARD

ACKNWOLEDGEMENT

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MARIA EDWARD

LIST OF ABBREVIATIONS

1	%	Percentage
2	°C	Degree Celsius
3	e.g.	Example
4	Et Al.	And other
5	Etc.	And other similar things
6	DNA	Deoxyribose Nucleic Acid
7	µL	Microliter
8	UTI	Urinary Tract Infection
9	Tm	Melting temperature
10	Ta	Annealing temperature
11	Stx	Shiga toxin
12	STEC	Shiga toxin-producing Escherichia coli.
13	HUS	Haemolytic Uremic Syndrome.
14	BD	Bloody Diarrhoea
15	EHEC	Enterohemorrhagic Escherichia coli
16	EPEC	Enteropathogenic Escherichia coli
17	FAS	Fluorescence Actin Staining
18	VFGs	Virulent Factor Genes
19	DFA	Direct immunofluorescence Assay
20	STH	Somatotropic Hormone
21	FECT	Formalin–Ethyl acetate Concentration Technique
22	CAUTI	Catheter-Associated Urinary Tract Infection
23	PAUTI	*Pseudomonas aeruginosa* Urinary Tract Infection
24	PSR	Polymerase Spiral Reaction

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1.ABSTRACT

The accurate and fast detection of pathogens in scientific samples is important for powerful disease control in patients, especially in the case of bacterial infections like urinary tract infections. *Escherichia coli* and *Pseudomonas aeruginosa* species, are the main pathogens associated with an extensive variety of infections, including urinary tract infections, pneumonia, wound infections etc. Traditional diagnostics such as bacterial subculture, biochemical checking out, and individual polymerase chain reaction (PCR) assays are broadly used, as they are time consuming, labour-intensive, and might lack sensitivity, especially in the case of polymicrobial infections or when the pathogens are found in low quantities. Therefore, the improvement of a multiplex PCR assay for the simultaneous detection of *E. Coli* and *P. aeruginosa* in clinical samples represents an important advancement in diagnostic microbiology.

This project aims to the development of multiplex PCR panel for detecting pathogens. A major challenge in this attempt is, ensuring the uniqueness of the PCR assay while avoiding cross-reactivity between two species, especially given genetic diversity and stress variation within each genus. To address this, specific primers were designed targeting highly conserved genomic regions. For *E. coli*, the primers targeting the eae gene, which encodes for intimin protein and for *P. aeruginosa* the primers targeting gene is toxA which encodes Exotoxin A. The primers were carefully adapted to their efficiency in a multiplex PCR format, taking into account the different melting temperature and reducing the primer-dimer formation. The primer obtained is then used in the PCR reaction along with specific DNAs isolated from each sample. Gel electrophoresis is done to check whether the genes are amplified or not. Each showed band near to their product length and successfully amplified. After single plex PCR reaction multiplex were done which amplified both the *E. Coli* and *P. aeruginosa* at the same time and established high sensitivity and specificity for both pathogens. And the panel developed is validated by testing with a urine sample which showed the presence of *P. aeruginosa*

In conclusion development of multiplex PCR panel for the detection of pathogens has more advantages over the conventional method. This assay provides rapid, efficient, accurate results which helps for the early diagnosis of the disease and in the improvement of personalized medicine.

2. INTRODUCTION

The organisms that cause disease are called pathogens. Those microorganisms that cause disease are classified into five groups: viruses, bacteria, fungi, protozoa, and helminths. Parasites and helminths come under the group parasites and the study of parasites are known as parasitology. Study of viruses, bacteria, fungi are called microbiology. They are microscopic, present everywhere on earth- air, water, soil, human body, even in high temperature etc. Some bacteria play a crucial role in our daily lives, keeping us healthy.

Viruses are small piece of genetic information (DNA or RNA) and can infect host like animals, humans etc. common cold, influenza COVID 19 are the disease caused by virus. Secondly the bacteria, which are small microscopic organisms which are present everywhere even inside our body, some bacteria cause disease like urinary tract infection, food poisoning etc. While some helps for digestion like lactobacillus which produce lactic acid that helps for releasing digestive enzymes. Thirdly the fungi, fungi are the eukaryotic organism, that are incredibly important to the ecosystem and human health, being among the most extensively spread creatures on the planet. Whereas some fungi live freely in soil or water and others associate with plants or animals in parasitic or symbiotic ways. Disease caused by fungus are aspergillosis, candidiasis etc. Fourthly Protozoa, they are single celled free-living organism, depending on the parasite's species, strain, and host resistance, they may cause disease like giardia, malaria etc. and infections can range from asymptomatic to fatal. Helminthes are worm like parasite, which cause disease like ascariasis. describe

Our body's first line of defence against intruders like bacteria is our immune system. It keeps us healthy and speeds up the action of recovery from illnesses or injuries. Immune system is classified into two: Innate immunity and Adaptive immunity. Innate immunity is the immunity that are present by birth itself and adaptive immunity are the immunity that is acquired during lifetime like vaccination etc. Mobilization of antimicrobial defence system cause inflammations like redness, heat, swelling and pain. Every pathogen fights back against, and tries to overcome, our defences using “virulence factors”. These factors are encoded by specific genes located on the chromosome. In order to help our natural defence systems in their fight against pathogens, we use a variety of drugs such as antibiotics. They are becoming more and more problematic to deal with, as many microorganisms have developed resistance to the drugs that is used to treat infections.

Infectious diseases are a main reason of death and debility. They pose a significant threat to public health, with the possibility to cause extensive epidemics and pandemics that can have devastating consequences on individuals, communities, and ecosystems. Therefore, it is crucial to implement effective infectious disease stoppage and control measures to minimize the spread of these diseases and protect public health. These diseases occur when pathogens attack the body, immune system, and disrupt normal physical functions. In many infectious pathogens, two types of bacteria that often cause diseases in humans are *Escherichia coli* (E. coli) and *Pseudomonas aeruginosa* (*P. aeruginosa*). Both these bacteria have different mechanisms of pathogenesis and affect the body in different ways. The study of infectious

illnesses' emergence, transmission, and containment at the population level, along with the methods for doing so, is known as infectious disease epidemiology.

The subject of the present study involves infectious disease-causing agents like. *E. coli* and *P. aeruginosa. E. coli*, is often found in the digestive tracts of warm-blooded animals and humans. They are rod-shaped and gram-negative bacteria belongs to the Enterobacteriaceae family. *E coli* was first isolated from infant stool. There are hundreds of different strains of *E. coli* bacteria. Different strains have unique properties. *E. coli* O157:H7 is a strain of *E. coli* that causes serious intestinal infections in people. It is the most frequent strain that causes sickness in humans. It differs from other *E. coli* in that it produces a strong toxin that destroys the intestinal wall lining, resulting in bloody diarrhoea. It is also known as enterohemorrhagic *E. coli* infection.

Each *E. coli* has specific characteristics of pathogenicity and virulence factors which is coded by particular genes. Mainly *E. coli* possesses genes coding for Shiga toxins(stx)and for intimin (eae). The majority of *E. Coli* strains are safe. Though, some strains like Shiga Toxin (STEC), can result in serious foodborne infections. The eae gene of Enteropathogenic *E. coli* (EPEC) is required for intimate attachment to epithelial cells in vitro. Enterohemorrhagic *E. coli* (EHEC) strains also have an eae gene and can form intimate attachments and efface microvillus in vitro and in animal models. This cause bacterial adherence to the urinary tract cell which cause urinary tract infection

Pseudomonas aeruginosa is a gram negative, aerobic, non-spore forming bacteria which cause infectious disease. It is a multidrug resistant pathogen and also, they possess strong resistance to a number of antibiotics by selectively preventing them from passing through its outer membrane. Individuals with immunocompromised conditions such as cystic fibrosis, bronchiectasis, neutropenia, burns, cancer, AIDS, organ transplants, uncontrolled diabetes mellitus, and ICU hospitalization are more prevalent to *P. aeruginosa* disease. toxA gene of pseudomonas is virulent gene which cause pathogenicity, which encodes exotoxin A, which act as a cytotoxin, this toxin causes the destruction of the cell and a wide variety of disease including urinary tract infection.

Mechanism of action of exotoxin A: its structure has two functional domains; domain 1 and domain 2. Domain1 is enzymatic domain which has enzymatic activity and domain2 is binding domain which helps to bind with the receptor of the host organism. After binding with the receptor, it enters into the host organism by endocytosis. This then activates domain 2 which cause the inhibition of protein synthesis and thus cause cell death. It causes several diseases including lung disorders, urinary tract infections. This gene has the ability to enter into the endothelial cells of urinary tract causing destruction and urinary tract infection. *Escherichia coli* and *Pseudomonas aeruginosa* are the two major culprits of urinary tract infection. *E coli*.is less affecting compared to *P. aeruginosa* it causes less complicated infections. But becomes serious issue if left untreated. While pseudomonas is the one which cause severe infections.

Present study involves the use of multiplex PCR. Polymerase chain reaction is a technique which is used to amplify DNA and produce a greater number of copies of a specific segment

of DNA. The three main steps in PCR includes denaturation, primer annealing, and extension. Firstly, Denaturation is the process of separation of double stranded DNA into single stranded. By providing high temperature causes the breakage of hydrogen bond and double strand get breaks. Second step is the primer annealing in which the primer gets attached at the 3-prime end. Thirdly, the extension step or the elongation step, in which Taq polymerase, that is isolated from the Thermus aquaticus is a temperature resistant enzyme that is used in this invitro method of amplifying the specific region of DNA adds nucleotides to the annealed primer.

In multiplex PCR, the reaction contains two or more primer sets intended for amplification of distinct targets. This method allows for the amplification of many target sequences in a single tube. In addition to the practical applications of PCR, this method can reduce the effort and time. When using multiplex reactions, primers need to be carefully chosen such that their annealing temperatures should be similar and they don't complement one another. Two or more pathogens can be detected simultaneously which reduce cost and time, this the way it differs from single plex PCR. Here through multiplex PCR, we are creating a diagnostic panel for the fast detection of pathogens such as, *E. coli and P. aeruginosa.* So, we could detect disease in the fastest way.

3. AIM AND OBJECTIVE

AIM:

The aim of this project is to **develop a multiplex PCR panel** for the **simultaneous detection** of *Escherichia coli* and *Pseudomonas aeruginosa* in clinical urine samples.

OBJECTIVES:

- to design and synthesize specific primers for the virulence gene eae for *E. coli* and toxA *for P. aeruginosa*.
- to develop multiplex PCR panel for the simultaneous detection of pathogens.
- Validation of multiplex PCR panel using clinical sample.

RELEVANCE

Development of multiplex PCR panel for the simultaneous detection of pathogens is relevant in modern biology as it has many advantages over the conventional methods. Usually, detection of pathogens from samples are done via microbiological methods which is time consuming and require a lot of steps. As urinary tract infection is very common disease, it should be diagnosed early and treated properly, this experiment aims for the fast detection of pathogens. Some of the advantages of multiplex PCR are:

- Rapid detection and accurate diagnosis
- Highly specific and sensitive
- Cost effective
- Improved patient management

4.REVIW OF LITERATURE

Detection of pathogens are very important, especially *E. coli and P. aeruginosa*, they are pathogens which cause serious infections like Urinary tract infections which should be detected and diagnosed at the earliest for improving patient management. Many methods are done to detect these pathogens here we are using multiplex PCR panel. It's a technique that helps in simultaneous amplification of pathogens in a single reaction. Unlike the traditional methods here mPCR uses multiple set of primers to amplify the target gene.

Several studies were conducted on the detection of *E. coli* and *P. aeruginosa* with respect to their virulent gene, detections using multiplex PCR, urinary tract infections and pathogens causing it.

4.1 PRIMER DESIGNING

Primer designing is an important step which is used in techniques like PCR, DNA sequencing etc. This involves creating a short single stranded sequence that binds with the target sequence that is to be amplified. Karry Mullis in 1983 found that there should be a complementary sequence that should bind to the target sequence of DNA to get amplified later many computational techniques have been developed. To be a good primer that amplifies the target sequence correctly should have some factors. The melting temperatures (Tm) of the primers used should not be more than 5°C, equation to calculate Tm is: Tm = 4(G + C) + 2(A + T) °C. Also, some conditions like annealing temperature (Ta) should be 3-5°C lower than the Tm. The efficiency of PCR can be increased only if the GC content of each primer is in the range of 40-60%. Distribution of G and C nucleotides should be in a uniform manner or else may cause non-specific priming. (P. Borah 2011). Here the present study was also followed many conditions to increase the specificity, efficiency and to be a primer with all the requirements to conduct a PCR reaction.

4.2 *Escherichia coli* AND eae Gene

E. coli is a common bacterium which cause most infections and the virulent gene for the present study was identified as eae gene which encodes intimin protein that cause adherence and colonization to the urinary tract causing urinary tract infection. (Wang et al.,2023) studied and examined the prevalence and polymorphism of eae gene in clinical strains. Shiga toxin (Stx)-producing *Escherichia coli* (STEC) infections cause occurrences of severe disease in children ranging from bloody diarrhoea to haemolytic uremic syndrome (HUS). They took the samples of children under 17-year-old with and without HUS, and assessed the pathogenic risk of different *eae* subtypes. They studied 240 STEC strains isolated from paediatric patients in Finland with whole genome sequencing. The gene *eae* was present in 209 (87.1%) strains, among which 49 (23.4%) were from patients with HUS, and 160 (76.6%) were from patients without HUS. Twenty-three different eae genotypes were found and are classified into five subgroups- γ1, β3, ε1, θ and ζ3. The subtype *eae*-γ1 was pointedly overrepresented in strains from patients aged 5-17 years, while β3 and ε1 were more commonly found in strains from patients under 5 years. In conclusion this study demonstrated a high occurrence and genetic variety of *eae* in clinical STEC and that *eae* is not essential for STEC-associated HUS.

Similarly, characteristics of eae gene were studied and the prevalence and genetic diversity of *eae* among clinical Shiga toxin producing Escherichia coli (STEC) and isolated from the patients with diarrhoea, bloody diarrhoea (BD), haemolytic uremic syndrome (HUS) as well as from asymptomatic STEC-positive individuals in Sweden with whole-genome sequencing. They found that 173 out of 239 (72.4%) of clinical STEC strains were *eae* positive. In summary, this study demonstrated a high prevalence of *eae* in clinical STEC strains and considerable genetic diversity of *eae* in STEC strains in Sweden from 1994 through 2018, and revealed association between *eae* subtypes and disease severity. (Ying et al., 2020)

Previously eae gene from enteropathogenic *Escherichia coli* (EPEC) was shown to be essential for production of the 'attaching and effacing' histopathology characteristic of EPEC infections cloned the *eae* gene from enterohaemorrhagic *E. coli* (EHEC) which, in addition to producing Shiga-like cytotoxins, also produces the attaching and effacing effect (Yu et.al.,1992). The sequence homology between the EPEC and EHEC sequences was 86% and 83% at the nucleotide and amino acid levels.

Similarly, another experiment was explained by Donnenberg et al (1993) on the role of the eae Gene of Enterohemorrhagic Escherichia coli in Intimate Attachment of In Vitro and in a Porcine Model. They constructed an eae deletion/insertion mutation in wild-type EHEC 0157:H7 strain 86-24 by using linear electroporation of a recombinant allele. The mutant obtained was deficient in inducing f-actin accumulation in HEp-2 cells and was incapable of attaching intimately to colonic epithelial cells in a newborn piglet model of infection. Intimate attachment in vivo was restored when the EHEC eae gene or the eaeA gene of EPEC was introduced into the mutant on a plasmid. These results indicate that the eae gene is necessary for intimate attachment of EHEC in vivo.

Likely the presence of eae gene in domestic animals which cause disease and transmit it to humans were conducted (Krause et al., 2005). They took seven different species (cattle, sheep, dogs, cats, pigs, chicken and goats) and investigated them as natural reservoirs for attaching and effacing *Escherichia coli* (AEEC). In this study 2165 *E. coli* strains from faeces of 803 animals were examined for the presence of the intimin -(*eae*) gene as a characteristic of AEEC strains. Ten percent of the animals were found to excrete AEEC, most frequently found in sheep and pigs followed by cattle, dogs, cats and poultry. In conclusion they found that EPEC strains are frequently occurring in domestic animals and transmit it to humans.

Schmidt et al. (1994) studied about the Differentiation in virulence patterns of *Escherichia coli* possessing *eae* genes. They screened 98 *Escherichia coli* strains which belonged to enteropathogenic (EPEC) serotypes and 82 enterohemorrhagic *E. coli* (EHEC) strains using PCR. They were also examined by the fluorescence actin staining (FAS) test to check the enteropathogenic adherence factor. The result obtained was 26 of class I EPEC with localized adherence to HEp-2 cells carried EAF and *eae* genes. While 72 EPEC strains with no or diffuse adherence have only 1 strain with EAF positive and 6 strains had *eae*. Out of 82 EHEC 72 carried eae gene.

Hinata et al. (2004) detected *E. coli* using Realtime PCR and compared with traditional culture method. The method used here was they collected 200 urine samples targeting the uspA gene of *E. coli* and amplified using Realtime PCR. This method was then compared with the standard urine culture. And came to the conclusion that the result of the quantification of *E. coli* using real-time PCR strongly correlated with the result of urine culture.

4.2 *Pseudomonas aeruginosa* and toxA Gene

Like *E. coli, P. aeruginosa* is a bacterium which cause several infectious diseases. Virulent gene of *P. aeruginosa* is toxA gene which encodes Exotoxin A (ETA), that cause cell death.

Dong et al. (2015) in his study conducted the rapid detection of *P. aeruginosa* targeting toxA gene. They established a polymerase spiral reaction (PSR) method for rapid detection of *P. aeruginosa* by targeting the *toxA* gene, which regulates exotoxin A synthesis. To assess this reaction chromogenic visualization with hydroxy naphthol blue were used. All 17 non- *P. aeruginosa* strains tested negative, indicating the high specificity of the PSR primers, and developed a *P. aeruginosa* PSR assay, which could be a useful tool for clinical screening, especially in case of poor resources, or for point-of-care testing.

Similarly, a study was conducted on the detection of *P. aeruginosa* with respect to toxA gene and its resistance to antibiotics. 100 samples were collected from different patients and isolated and subjected to PCR for amplification. These isolates were tested against the antibiotic resistance showed the highest percentage of the resistance level, especially those strain associated with detection of *toxA* gene-positive PCR product. (Alabdali.,2021).

4.3 URINARY TRACT INFECTION AND ASSOSCIATED PATHOGENS (*E. coli* and *P. aeruginosa)*

Previous study conducted (Rahul et al.,2009) on the presence of *P. aeruginosa* in patients with urinary tract infection. Urinary tract is the second most common type of infection in the body. Catheterization of the urinary tract is the most common factor, which predisposes the host to these infections. Catheter-associated UTI (CAUTI) is responsible for 40% of nosocomial infections, making it the most common cause of nosocomial infection. This study summarizes to increase the advancement in the detection of pathogens and its diagnosis.

Similarly, another study analysed the mortality among hospitalized patients with *Pseudomonas aeruginosa* urinary tract infection (PAUTI) and the impact of antibiotic treatment on survival. And concluded that PAUTI are associated with high mortality in hospitalized patients, which increases significantly in those with severe comorbidity such as chronic renal failure, advanced liver disease or diabetes mellitus. Inadequate antibiotic treatment is associated with poor outcome, which remarks the importance of adjusting empirical antibiotic treatment based on the microbiological susceptibility results.

Ejrnæs et al. (2011) done an experiment to look for bacterial characteristics of importance for recurrence of UTI caused by E. coli. The studies were done on 236 Swedish women with community-acquired symptomatic lower UTI from a large study of 1162 patients treated with one of the three dosing placebos. And concluded that the uropathogenic E. coli were present in the bladder also VFGs associated act as a potential target for the prevention and treatment.

Another study (Storby;2004) investigated the distribution and antimicrobial resistance in urinary tract pathogens, primarily Escherichia coli, in two age groups, children of 2 y and adults 18-50 y, for a period of 12 years. Urinary tract culture is done by microbiological methods and antibiotic susceptibility is noted. From their findings they concluded that *E. coli* were the common pathogen present and were resistant to antibiotics in the case of children later increased in adult.

4.4 MULTIPLEX PCR

In the present study the development of Multiplex PCR panel which is a modern technique that helps in amplifying multiple targets in a single reaction. Many studies have conducted in showing the development of Multiplex PCR panel for the detection of many pathogens.

Similar study was conducted by Thong et al. (2011) investigated the Simultaneous Detection of *Methicillin-Resistant Staphylococcus aureus, Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* by Multiplex PCR The methodology of the study were: six pairs of selected primers targeting specific genes of the bacteriocyte conditions were optimized for the multiplex PCR to ensure specific amplification of the selected targets. Sensitivity and specificity tests were also carried out using a blind test approach on 50 bacterial cultures and resulted in 100 for both positive and negative predictive values.

Similarly (Zhihao et al., 2019) developed a multiplex PCR assay for the simultaneous and rapid detection of six pathogenic bacteria in poultry. Here, a multiplex polymerase chain reaction (m-PCR) assay is reported to rapidly identify targets genes (phoA, KMT1, ureR, toxA, invA, and nuc) of these six pathogens in clinical samples. Six pairs of specific primers were designed. The optimal reaction conditions, specificity, and sensitivity of the m-PCR assay were investigated. The results showed that betaine remarkably improved amplification of the target genes. Specific test results showed that all six pathogens were detected by the proposed m-PCR protocol without cross-amplification with viruses or parasites.

Another study related to the present study were conducted by (Padmavati et al.,2012) on the detection of major uropathogens in a single-pot multiplex PCR assay by conducted an experiment by taking 50 urine samples and established a complete diagnostic system. They have developed a single-tube multiplex PCR assay (mPCR) for the detection of the four major uropathogens such as *Proteus mirabilis, Pseudomonas aeruginosa*, Klebsiella pneumoniae and Escherichia coli. They also used mPCR on spiked urine samples using 40 clinical isolates to demonstrate its application under different strain. In conclusion the mPCR reported were rapid and simple screening tool that compete with conventional biochemical-based screening assay.

François et al. (2006) conducted study that shows the competency of multiplex PCR reaction with the conventional methods for the diagnosis of respiratory disease. Methodology of the study was comparing multiplex PCR with direct immunofluorescence assay (DFA) and HuH7 cell culture for the detection of viruses in 263 children admitted to hospital with an acute respiratory illness. PCR detected 124 positive response which were not identified by the conventional methods and then they came to the conclusion that multiplex PCR is an advanced technology which is rapid and highly sensitive foe the detection of pathogens.

Likewise, the efficiency of multiplex PCR was detected by the study conducted by (Sanprasert et al., 2019), here they developed multiplex PCR panel for the simultaneous detection of soil transmitted helminths (STHs) and established a conventional multiplex PCR for simultaneous rapid detection of *Ascaris lumbricoides, Necator americanus,* and *Strongyloides stercoralis* in stool samples. And the results show that the multiplex PCR could detect the DNA of STHs at a very low target gene concentrations with no cross-amplification. Came to the conclusion that the Multiplex PCR had five times higher sensitivity than the formalin–ethyl acetate concentration technique (FECT) in the detection of multiple infections.

Giorgio et al. (2009) done an experimental study to compare the multiplex PCR with conventional method in species identification. They carried out the study on colonies and on oral rinse solutions from 95 subjects with suspected oral candidiasis and results were compared with those from seven commonly used phenotypic identification systems. From the result four to nine species were detected by the phenotypic method while the PCR detected pathogens in 74% of the sample and concluded that Multiplex PCR was rapid and effective in the identification of Candida species and allowed the detection of more than one species in the same sample.

Caliendo (2011) studied the detection of respiratory pathogens using multiplex PCR and concluded that it's difficult to identify pathogens by preparing cultures, and developed PCR panel which shows fast detection and accuracy.

Safdar et al. (2023) explained that multiplex PCR is a powerful technique which amplifies multiple genes in a single reaction. This study was done in foodborne pathogens form raw and processed samples. Also, this study shows several challenges encountered during the PCR reaction. In related to present study these challenges were noted and tackled.

5. METHODOLOGY

Urinary tract infections are very common disease which should be detected and treated properly. For this multiplex PCR panel were developed for the simultaneous detection of pathogens like *E. coli* and *P. aeruginosa*. The methodology section details the step-by-step process followed to design, optimize, and validate the multiplex PCR assay for the detection of *E. coli* and *P. aeruginosa* from urine samples. This approach includes sample preparation, primer design, PCR optimization, validation, and performance testing.

5.1 SAMPLE COLLECTION

For the present study three samples which was already maintained at Veta Genomics lab were used, that include *E. coli*, *P. aeruginosa* and urine

5.2 PRIMER DESIGNING

Primer designing is the first and important step as it synthesizes primer which is required for Polymerase Chain Reaction for the amplification of DNA. At first virulent gene which is specific to each pathogen were identified using NCBI. Then the FASTA sequence of the specific gene is used to obtain the primer product using PRIMER BLAST. After that using PRIMER STAT, efficiency of the primer were studied. The primer pairs that satisfies the required conditions were selected for synthesis.

5.3 PRIMER SYNTHESIS

The primer obtained is then synthesized by outsourcing to Sigma Aldrich company.

5.4 DNA ISOLATION

DNA isolation has mainly four steps Lysis, Precipitation, Purification, Elution. The kit used for DNA isolation was XPLOREGEN. The first step of lysis was done by taking 1000 µL of Xploregen Buffer A 1(XBA1) to the beaded vial. The culture (*P. aeruginosa* and *E. coli*) is taken using a swab and mixed with the XBA1 and then mixed horizontally in the vortex for 10 min. Then 300 µL of XBA2 was added to the vial and again horizontally vortexed for 7 min. Then the tube was centrifuged for 2min at 10,000rpm and the 950 µL supernatant was transferred to 2ml vial. Second step precipitation was done by adding 200 µL of XBA3 solution and vortexed for mixing and centrifuged at 10,000 rpm for 2 min. Then transferred 800 µL of supernatant to 2ml sterile vial. Then 700 µL of binding buffer; XBA4 was added to the supernatant and vortexed for 5min, 700 µL of lysate was added to the spin column (spin column contains two parts; column tube and collection tube). Then centrifugation was done for 2 min at 10000 rpm and discard the flow through from the collecting tube, this step was repeated to collect all the lysate. Third step is the purification in which 600 µL of XBA5 was added to the spin column and centrifuged for 10000rpm for 2 min and the flow through were discarded and again the empty spin was centrifuged for 5 min at 10,000rpm and the spin column was placed into a new sterile vial. Fourth step elution was done by adding 30 µL of elution buffer; XBA7and incubated for 2 min and centrifuged for 10000rpm for 5 min.

5.5 DNA QUANTIFICATION

In order to check the DNA concentration, a device called Qubit fluorometer is used. For this, 199 µL of high sensitivity buffer was added to 1 µL of DNA and pulse vortexed. Placed it

into Qubit fluorometer and the amount of DNA were noted. Isolation of *E. coli*, and *P. aeruginosa* were done separately by the same process.

5.6 PCR

5.6.1 Single plex PCR

Polymerase chain reaction is a method to produce lots of copies of DNA. There are three steps in PCR: Denaturation, Annealing, Extension. Here the kit used for PCR was Carmin kit. Into the PCR vial 19.5µL of nuclear free water was added, 1µL each reverse and forward primers, 1µL DNA to be amplified, and 12.5µL master mix was added, mix the content using 100µL pipette. Break the bubbles formed by tapping the vial. Place them inside the PCR and run by setting the thermal profile. For *E. coli* the annealing temperature is 55 degree Celsius and that of *P. aeruginosa* is 54 degree Celsius, both done separately.

5.7 MULTIPLEX PCR

This technique amplifies multiple DNA simultaneously, here kit used were Takara. 7µL of buffer,6µL of DNTP, 1µL each of forward and reverse primer of *E. coli* and *P. aeruginosa* and 1µL each of its DNA .0.25µL of enzyme, 30.75µL of nuclease free water was added into the PCR vial, mixed and placed in PCR and run at 54 degree Celsius of annealing temperature.

5.8 GEL ELECTROPHORESIS

Agarose gel is prepared by adding 2g of agarose in 100ml buffer, and kept for few hours after placing the comb. Removed the comb and transferred the gel into chamber. The buffer used here was Tris borate EDTA.

The sample after PCR, is loaded to well to separate DNA. Ladder and sample are mixed with tracking dye, and visualized through UV light.

5.9 DNA ISOLATION OF URINE SAMPLE

100µL of urine sample was taken in a vial and centrifuged at 11000 rpm for 2 min, the pellet was collected and to this 180µL of tissue lysis buffer (ATL)and 20µL of proteinase k was added, vortexed for few min and was kept in dry bath. After complete lysis 200µL of lysis buffer (AL) is added and kept in dry bath for 10 min. Then 200µL of ethanol is added, mixed and transferred to spin column, centrifuged at 8000rpm for 1 min. After this the sample was washed using 500µL of AW1 buffer and centrifuged at 8000rpm for 1min and then add 500µL AW2 buffer and again centrifuged at 1400rpm for 3 min. the next step was to add 200µL of elution buffer and then incubated for 1 min and centrifuged at 8000rpm and was placed into a Qubit fluorometer and the quantity of DNA was noted.

5.10 PCR ANALYSIS OF URINE SAMPLE

Here the kit used were Takara. 7µL of buffer,6µL of DNTP, 1µL each of forward and reverse primer of *E. coli* and *P. aeruginosa* and 1µL of DNA isolated from the urine sample. 0.25µL of enzyme, 30.75µL of nuclease free water was added into the PCR vial, mixed and placed in PCR and run at 54 degree Celsius of annealing temperature.

5.11 GEL ANALYSIS

Agarose gel is prepared by adding 2g of agarose in 100ml buffer, and kept for few hours after placing the comb. Removed the comb and transferred the gel into chamber. The buffer used here was Tris borate EDTA.

The urine sample after PCR, is loaded to well to separate DNA. Ladder and the sample were mixed with tracking dye, and visualized through UV light.

6. RESULT

Multiplex PCR panel for the simultaneous detection of *E. coli* and *P. aeruginosa* is developed through the steps mentioned in methodology. The result obtained in each step are:

6.1 SAMPLE COLLECTION

Sample which were already present in the lab is used which is shown in the figure 1 and 2.

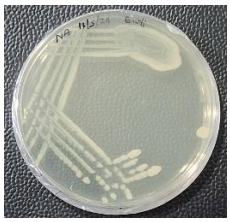

Fig 1: Streak plate of *E. coli*

Fig 2: Streak plate of *P. aeruginosa*

6.2 PRIMER DESIGNING AND SYNTHESIS

In the present study, the first step was primer designing, here the primers for *E. coli* and *P. aeruginosa* were successfully designed by using the tool NCBI and PRIMER BLAST. The virulent gene of *E. coli* and *P. aeruginosa* is found and they are eae and which is shown in the fig 3 and 4.

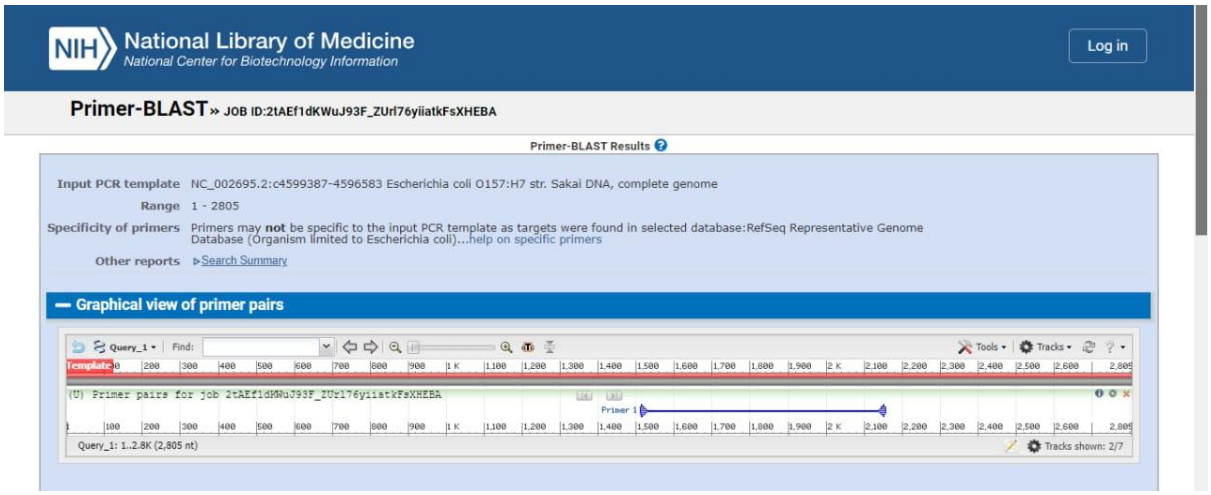

Fig 3: PRIMER BLAST result of *E. coli*

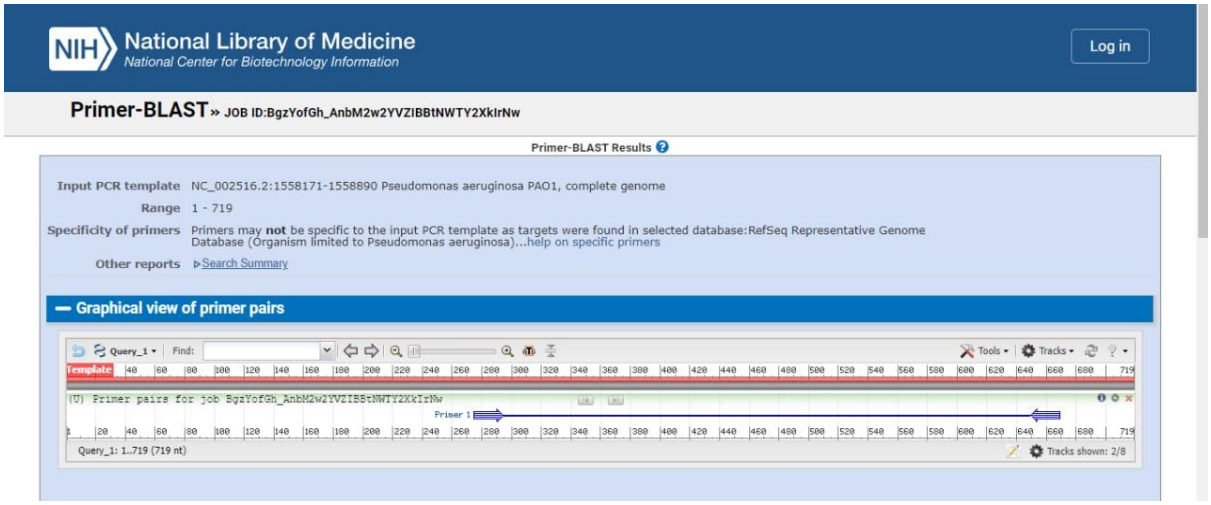

Fig 4: PRIMER BLAST result of *P. aeruginosa*

Product length of both the bacteria *E. coli* and P. *aeruginosa* is found to be 646 and 395 respectively. Which satisfies all the parameters of a PCR primer figure (5 and 6.) or else it may cause nonspecific amplification.

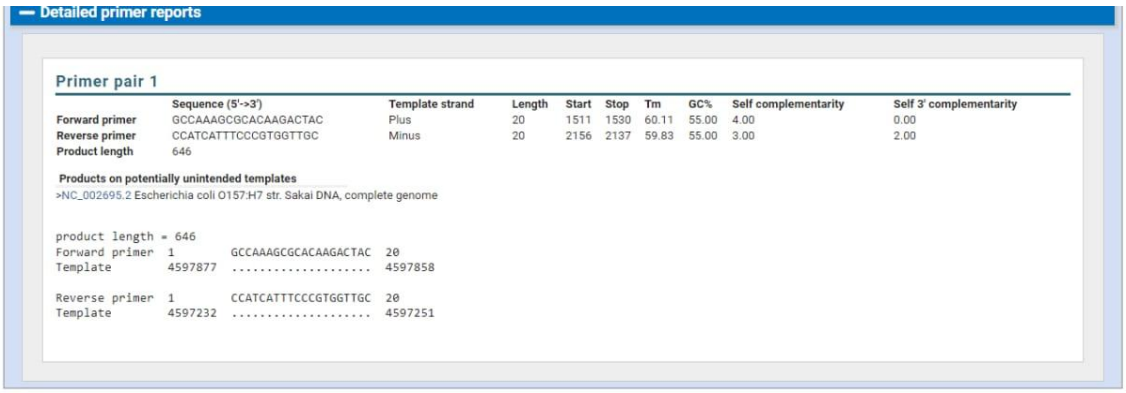

Fig 5: product length of *E.coli*

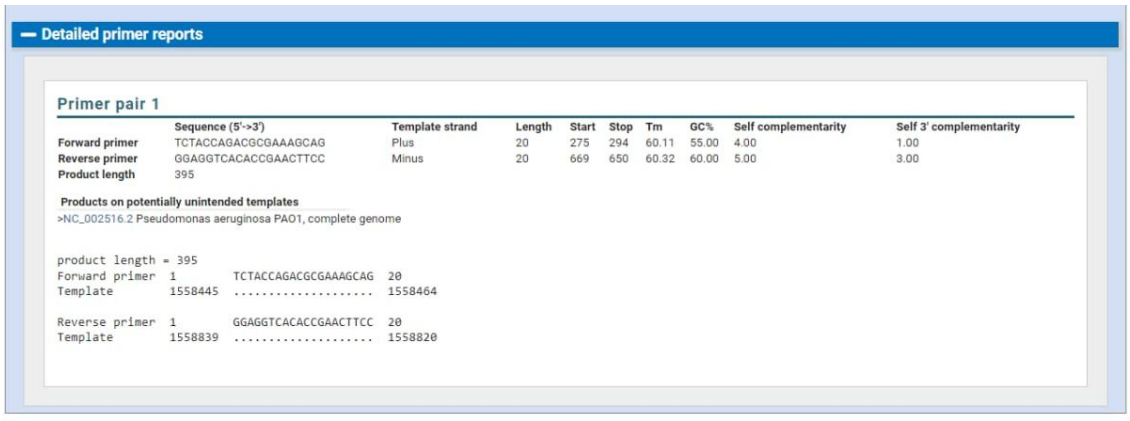

Fig 6: product length of *P. aeruginosa*

6.3 DNA ISOLATION

Second step was isolation of DNA. For this, culture of *E. coli* and *P. aeruginosa* were present in the lab. The kit used here for isolation is XPLOREGEN and the concentration is measured using Qubit Fluorometer, and the result is 16.4ng/µL and 33.2 ng/ µL respectively. (fig 7 and 8). Which is quite sufficient for reliable PCR to take place.

Fig 7: sample concentration of *E.coli*

Fig 8: sample concentration of *P. aeruginosa*

6.4 PCR REACTION AND GEL ANALYSIS

Single plex PCR were done by mixing DNA isolates with the respective forward and reverse primers. Annealing temperature used for *E. coli* and *P. aeruginosa* is 54 and 55 degree Celsius. From this using the annealing temperature of 54-degree Celsius multiplex PCR were

successfully developed to amplify both *E. coli* and *P. aeruginosa* in a single reaction products were analysed using agarose gel electrophoresis, and the presence of distinct amplicons confirmed the successful amplification of both target genes. The expected product sizes were:

- *E. coli* formed band in between 600-700 bp (fig.9)
- *P. aeruginosa* formed band in between 300-400bp (fig.10)
- In multiplex reaction two bands were formed near to its product length. (fig.11)

Fig 9: gel amplification of *E. coli*

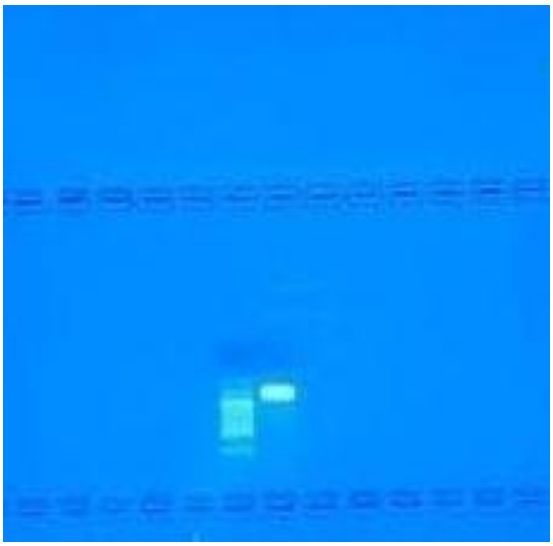

Fig 10:gel amplification of *P.aeruginosa*

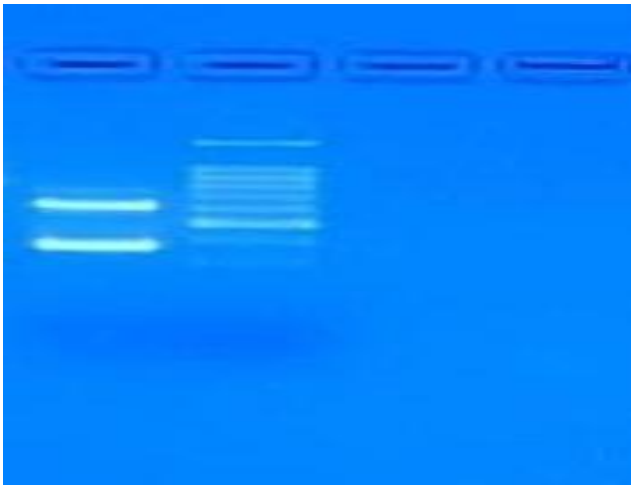

Fig 11: gel amplification of *E.coli* and *P.aeruginosa* by multiplex PCR

6.5 CLINICAL SAMPLE TESTING

The multiplex PCR panel was tested on clinical urine sample. Sample has amplified successfully and formed band near the 300-400bp, which shows the presence of and *P. aeruginosa.* shown in the figure given below (fig 12)

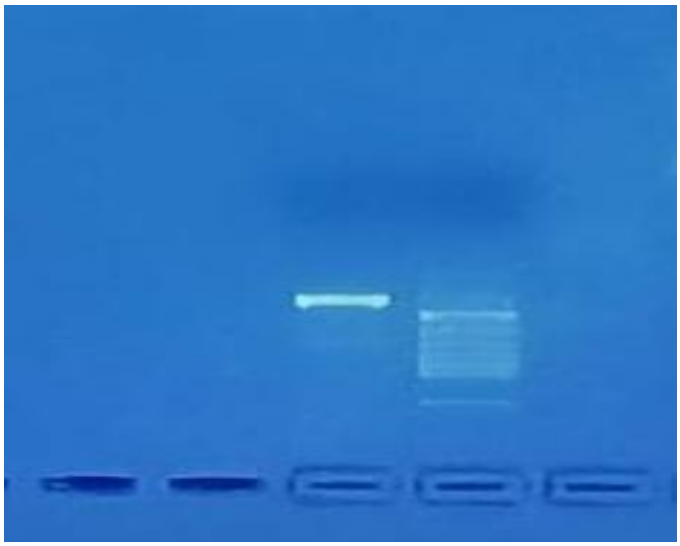

Fig 12: gel amplification of urine sample

The multiplex PCR panel developed for the simultaneous detection of *E. coli* and *P.aeruginosa* in urine samples was successfully optimized and validated. The assay demonstrated high specificity, sensitivity, and accuracy. This multiplex PCR panel offers a promising alternative for rapid, reliable, and simultaneous detection of *E. coli* and *P.aeruginosa* in clinical settings, which could improve the diagnosis and management of urinary tract infections.

7. DISCUSSION

E. coli and *P. aeruginosa* are bacteria which cause dangerous infectious disease by invading into the body and destroying the immune system. In this study they are isolated and detected in fastest method. Here Multiplex PCR panel is developed for the simultaneous detection of pathogens in urine sample which is an advanced technique in the molecular diagnostic field. It lets the amplification of multiple target genes in single reaction which improves the diagnostic speed, accuracy and specificity.

Several research papers have explored the value of multiplex PCR in the detection of multiple pathogens in clinical settings. For instance, a study by Angela.M.Caliendo ,(2011) on respiratory pathogens identified that the multiplex PCR is a rapid and sensitive technique for the detection of pathogens. Their findings align with our study that this method is rapid over the conventional method.

A study conducted by Safdar et al;(2023) says about the challenges faced during the development of multiplex PCR. And suggested directions for future study and improvement in this technology. In our study the main challenges faced during the experiment is, that both the pathogens share some similarities in genetic marker which can lead to nonspecific amplification and primer dimer formation. This has been tackled up by selecting highly conserved region which is specific to each pathogen. Although previous studies have made significant progress in developing multiplex PCR assays for UTI pathogens, food borne pathogens. It also has to deal with issues including reaction condition optimization, product separation and detection, method validation and quality control, and the ethical and societal ramifications of the outcomes. It can be enhanced by using new probe technologies, amplification techniques, detecting systems, microfluidic platforms, point-of-care devices. It can also be combined with other techniques, such as microarrays, next-generation sequencing, digital PCR, immunomagnetic separation, aptamer-based capture, CRISPR-Cas systems, lateral flow assays, electrochemical biosensors, or smartphone-based readers. Finally, multiplex PCR has great potential for future applications in detecting pathogens and for diagnosing in faster way.

8. CONCLUSION

The development of multiplex PCR panel for the simultaneous detection of pathogens such as: *E. coli* and *P. aeruginosa* in urine samples is an advanced technology. Normally this is done by microbiological methods which is time consuming, and need effort for the detection. This panel is created by targeting the specific genes, eae for *E. coli* and toxA for *P. aeruginosa.* Both the genes were amplified by single plex PCR reaction and then the multiplex PCR is done which successfully amplified both the pathogens. Clinical sample of urine is detected by the multiplex PCR panel which showed the presence of *P. aeruginosa.* It is an opportunistic pathogen that can cause infections in individuals with compromised immune systems or underlying medical conditions, such as diabetes, catheter use, or recent surgeries. This is a fast, accurate and cost-effective, alternative to traditional diagnostic methods, which often suffers from limitations in sensitivity, specificity and treatment time.

In addition to its diagnostic potential, the multiplex PCR assay also holds significant implications for improving the efficiency of microbiological laboratories. It reduces the workload associated with multiple individual tests, cuts down on reagent usage, and allows for the simultaneous detection of multiple pathogens, which is particularly beneficial in settings with limited resources.

Ultimately, the multiplex PCR assay developed in this study has the potential to become a valuable tool in routine clinical diagnostics, guiding appropriate antimicrobial therapy and helping prevent the development of antibiotic resistance through early and accurate pathogen identification. Further studies and optimization could expand this approach to include a broader range of pathogens, paving the way for more comprehensive diagnostic panels in clinical microbiology. The main benefits of multiplex PCR in comparison to traditional are they can detect the pathogens simultaneously, higher sensitivity and specificity, reduction in sample volume, cost effective, rapid detection and result. Drawbacks are: complexity in assay designing, risk of false positives and negatives, increased risk of contamination, expensive equipment and reagents, limited detection of pathogens.

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