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MOLECULAR IDENTIFICATION OF MULTIDRUG RESISTANT ESCHERICHIA COLI ISOLATED FROM WATER SOURCES IN CENTRAL KERALA

Clinical Microbiology

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ABSTRACT

The present study aimed to investigate the presence of virulence factor genes and multidrug resistance (MDR) genes in eight selected Escherichia coli isolates obtained from water sources within Central Kerala, India. Genomic DNA was extracted from the bacterial isolates using the magnetic bead-based extraction method. Selected genomic DNA was sequenced and BLAST analysis was conducted to confirm the identity of the isolates. The extracted DNA was then subjected to multiplex PCR assays using gene-specific primers to detect the presence of virulence factor genes, papC, sfa, and afa. The PCR amplicons were visualized and confirmed by agarose gel electrophoresis. Furthermore, plasmid DNA was prepared using the alkaline lysis method to analyse the presence of MDR genes, namely NDM, OXA, and CTX-M. The isolated plasmids were analysed using gel electrophoresis, and their banding patterns were observed. Finally, Sanger sequencing was conducted to obtain the nucleotide sequences of the amplicons, followed by BLAST analysis to identify sequence similarities with known genes in the NCBI database. Phylogeny was conducted MDR genes to understand their evolutionary relationship. The results of this study will provide valuable insights into the virulence potential and antibiotic resistance profiles of the E.coli isolates. This information is essential for understanding the pathogenicity of these strains and for the development of effective strategies to combat E.coli infections.

KEYWORDS

Multidrug resistance, Escherichia coli, Virulence factor genes, Multiplex-PCR, Phylogeny

1. INTRODUCTION

The discovery of penicillin in 1928 opened the doors for the subsequent discovery and commercialization of numerous antibiotics (Jovanovic et.al., 2021). Over time, the production of antibiotics has increased dramatically, reaching an estimated global scale of around 100,000 tons annually (Cycon et.al., 2019). However, the widespread use of antibiotics has had a significant impact on the bacterial life cycle, leading to the emergence of antibiotic-resistant pathogens. Some of these pathogens have developed resistance to multiple antibiotics and chemotherapeutic agents, a phenomenon known as multidrug resistance.

One notable example of multidrug resistance is methicillin-resistant Staphylococcus aureus (MRSA), which is resistant to methicillin, a drug developed to treat penicillin-resistant S. aureus (Kejela & Bacha, 2013). Additionally, there have been reports of Enterococcus acquiring transferable resistance to vancomycin, making it one of the most dangerous strains (Moellering, 1998). In response to the discovery of MRSA, researchers developed new antibacterial to combat the resistance. However, the emergence of completely resistant gram-negative bacteria has become a concern as the production of new antibiotics has slowed down in the global market. Currently, there are no agents available to effectively treat these strains.

Bacteria develop multidrug resistance through various mechanisms, including the acquisition of resistance genes on plasmids or transposons. These genes encode specific functions, such as efflux pumps that transport multiple drugs across the bacterial cell membrane (Alekshun & Levy, 2007). The emergence of resistance in S. aureus against penicillin was facilitated by plasmid-encoded β-lactamase, an enzyme that breaks down the β-lactam ring in antibiotics. This led to the development of TEM β-lactamase, which exhibited multidrug resistance (Bush & Bradford, 2020). Third-generation cephalosporins were introduced to combat highly resistant strains, and fourth-generation cephalosporins were developed to resist hydrolysis by enzymes like AmpC. However, the widespread use of antibiotics has exerted selective pressure, favouring the spread of highly resistant plasmids carrying mutant enzymes like TEM and SHV, known as extended spectrum β-lactamases (ESBLs). Among ESBLs, CTX-M is particularly problematic due to its rapid and extensive transfer. In *E.coli* strains causing diseases, adhesion to host cells is facilitated by genes such as sfa, afa, and pap. The pap gene encodes the pilus rod, while the afa operon is involved in afimbrial adhesion, and the sfa gene codes for mannose-resistant adhesions. These adhesion-encoding genes are prevalent in *E.coli* strains associated with urinary tract infections (Le Bouguenec et.al., 1992).

Microorganisms acquire multidrug resistance through horizontal gene transfer, often on plasmids, carrying MDR genes such as NDM, OXA, and CTX-M. NDM produces Metallo beta-lactamases that cleave the β-lactam ring in carbapenem antibiotics, exhibiting high resistance. OXA, originally rare, has become more prevalent in causing multidrug resistance. CTX-M confers resistance to the cephalosporin antibiotic cefotaxime (Harris M.et.al., 2023).

In this paper, our focus is on molecular identification of the virulence factor genes sfa, afa, and pap, as well as the MDR genes NDM, OXA, and CTX-M, in *E.coli* isolates that have been previously isolated from urban water sources in Central Kerala, India post 2018 flood.

2. MATERIALS AND METHODS

2.1 Molecular identification of the virulence factor genes

2.1.1 Sample Preparation

Eight isolates (W1, W5, W12, W15, W28, W29, W51, W60) previously identified as MDR *E.coli* collected from water sources in Central Kerala, India were used for the study. The sample was prepared by mixing a loop full of culture of different isolates from the agar plates to nutrient broth media. The mixture was incubated overnight at 37°C.

2.1.2 Genomic DNA isolation

The genomic DNA isolation was conducted in all the 8 isolates by taking 400 µl of an overnight culture. The protocol using the Zymag magnetic bead bacterial DNA extraction kit (ZM15BG-50) involved four main steps: sample lysis, nucleic acid binding, washing, and elution.

2.1.3 16S rDNA-PCR analysis

The isolates were characterized at a molecular level by 16S ribosomal RNA gene (16S rDNA) analysis. PCR amplification was performed to identify the presence of 16S rDNA a reporter gene for the detection of E. coli. To identify the different strains of *E coli*, universal primers specific to *E.coli* were used (Table 1). A 30 cycle PCR protocol used for amplifying the 16S rDNA involves the following steps: Initial Denaturation at 94.0°C for a duration of 5 minutes. Denaturation at 94.0°C for 45 seconds. Annealing at 52°C for 45 seconds. Extension at 72°C for 1 minute 30 seconds. Final Elongation at 72°C for 8 minutes. The DNA where visualized by agarose gel electrophoresis, sequenced using Sanger's method and were analysed by BLAST as mentioned in sections 2.3 and 2.4.

2.1.4 Multiplex-PCR analysis of virulence factor genes

The virulence factor genes (sfa, afa, papC) in *E.coli* were amplified by multiplex PCR from genomic DNA using gene-specific primers. PCR was carried out in a final reaction volume of 25 µl in 200 µl capacity thin wall PCR tube. Details of primers used for virulence factor genes PCR is given in Table 1. The PCR protocol designed for amplifying the target DNA involves denaturation of the DNA at 94.0°C for 30 seconds. Annealing was performed at 63°C for 30 seconds and extension was done at 68°C for 30 seconds. The entire step was repeated for 30 cycles. An initial denaturation was given at 95.0°C for a

duration of 12 minutes and a final elongation was performed at 72°C for 10 minutes in order to ensure the maximum amplification. The amplified PCR product were sequenced using Sanger's method and analysed by BLAST. The amplicons were visualized by agarose gel electrophoresis the sequences were analysed by NCBI-BLAST as mentioned in sections 2.3 and 2.4.

2.2. Molecular identification of MDR genes by PCR

2.2.1 Plasmid isolation

The extraction of plasmid was carried out by the alkaline lysis method and subjected to gel electrophoresis and documentation. The isolated plasmid DNA of *E.coli* was amplified for MDR genes.

2.2.2 PCR analysis of MDR genes

MDR genes NDM, OXA, and CTX-M were identified using PCR for the eight isolates selected. The primers used for amplification of the MDR genes NDM, OXA, and CTX-M are provided in Table 1. Separate PCR analysis were carried out for the three genes and the respective protocols followed are given below. The PCR protocol for NDM consisted of the following steps: Initial denaturation at 95°C for 5 minutes. Denaturation at 94°C for 1 minute. Annealing at 56°C for 30 seconds. Extension at 72°C for 45 seconds. Steps 2 to 4 were repeated for a total of 35 cycles. Final elongation at 72°C for 8 minutes. The PCR protocol for OXA consisted of the following steps: Initial denaturation at 95°C for 5 minutes. Denaturation at 94°C for 1 minute. Annealing at 55°C for 40 seconds. Extension at 72°C for 45 seconds. Steps 2 to 4 were repeated for a total of 35 cycles. Final elongation at 72°C for 8 minutes.

The PCR protocol for CTX-M consisted of the following steps: Initial denaturation at 95°C for 5 minutes. Denaturation at 94°C for 1 minute. Annealing at 58°C for 45 seconds. Extension at 72°C for 45 seconds. Steps 2 to 4 were repeated for a total of 35 cycles. Final elongation at 72°C for 8 minutes. The amplicons obtained where visualized by agarose gel electrophoresis, sequenced using Sanger's method and were analysed by BLAST as mentioned in sections 2.3 and 2.4.

2.2.3 Phylogeny

Phylogeny analysis of selected isolates were conducted for the MDR gene. The BLAST analysis identified representative sequences with similarities, which were retrieved and aligned using a multiple alignment program. The resulting multiple alignment file was then utilized to generate a tree using MEGA-X software.

2.3 Agarose Gel Electrophoresis

1.5% Agarose was used for gel electrophoresis. The images of ethidium bromide-stained bands were digitized using a gel documentation system (Biorad, USA).

2.4 Sequencing and BLAST analysis

The amplicons obtained from the PCR were analysed using the Sanger sequencing method, which involves the use of chain-terminating inhibitors to obtain DNA sequences. The obtained sequence was then subjected to BLAST analysis, utilizing the NCBI-BLAST tool, to perform a search for similarities with other sequences in the database.

3. RESULTS AND DISCUSSION

3.1 Molecular identification of the virulence factor genes

Genomic DNA was successfully isolated using magnetic bead based technology from 8 isolates which were earlier identified as MDR *E.coli* (Fig 1A). The isolates were named W1, W5, W12, W15, W28, W29, W51 and W60. The isolates were subjected to PCR for the presence of MDR genes, yielded amplicons of 1500bp ((Fig 1B). The isolate W15 was sequenced and was analysed by BLAST. It showed 100% similarity to *E.coli* (Fig 1C). The isolates were all subjected multiplex PCR for virulence factor genes sfa, afa and papC (Fig 1D). It was observed that all the isolates tested positive for all the genes tested indicating high virulence for all the genes. Amplicons were sequenced. Representative sequence from one each from set of three different genes were subjected to BLAST analysis. This showed 100% similarity with that of their respective genes found in other *E.coli* strains in the NCBI repository.

Virulence genes play a crucial role in the pathogenicity of bacteria by enabling them to colonize, invade, and cause disease in the host. Adhesion genes, such as sfa, pap, and afa, are specific types of virulence genes that encode adhesins—proteins or structures that facilitate the attachment of bacteria to host tissues. Detection of virulence factor genes sfa, afa and papC in *E.coli* has been documented immensely in recent years. In a study conducted by Feria et.al., 2001, 118 Escherichia coli isolates from dogs and cats with urinary tract infection (UTI) were tested. A multiplex polymerase chain reaction showed the presence of these genes in the range 43% pap, 57% sfa and 1% afa, Detection of these genes are commonly reported in uropathogenic *E.coli* isolated from UTI patients (Rahdar et.al., 2015, Yun et.al., 2023). However, the simultaneous occurrence of all these genes in *E.coli* isolated from water sources shows the alarming nature of antibiotic resistance.

3.2 Molecular identification of MDR genes

Plasmid DNA was successfully isolated from 8 isolates W1, W5, W12, W15, W28, W29, W51 and W60 (Fig 2A). It was observed that all the isolates were positive for NDM and OXA genes, while CTX-M genes were found in all but one isolate which is W1 (Fig 2B, 2C, 2D). The amplicons were sequenced. Representative sequence from one each from set of three different genes were subjected to BLAST analysis. This again showed 100% similarity to the respective genes found in the NCBI repository for *E.coli*. Phylogeny conducted for three genes from different isolates gave an insight into the evolutionary relationships of these genes (Fig 3,4,5).

NDM and OXA genes are types of carbapenemase genes that encode enzymes capable of breaking down carbapenem antibiotics, rendering them ineffective against bacterial infections. These genes contribute to the development of resistance in bacteria, particularly against carbapenem antibiotics, which are considered as last-resort treatments for many multidrug-resistant infections.

CTX-M MDR genes are of significant concern due to their ability to rapidly disseminate and transfer between bacterial strains. This has led to the emergence of strains with extensive drug resistance, making treatment options limited and challenging. The spread of CTX-M MDR genes underscores the importance of infection control measures, antimicrobial stewardship, and the development of new treatment strategies to combat the growing threat of multidrug-resistant bacteria. The presence of these genes in bacterial strains poses significant challenges in the treatment of infections, as they limit the effectiveness of available antibiotics. Understanding the mechanisms and prevalence of these resistance genes is crucial for implementing appropriate infection control measures and developing novel therapeutic approaches to combat multidrug-resistant bacteria.

Co-occurrence of MDR genes NDM, OXA and CTX-M has been widely reported in recent years. A recent study indicated the presence of these genes in an *E.coli* strain isolated from the sputum samples of a patient effected by pneumonia (Nukui et.al., 2019). Another study indicated the predominance of CTX-M in *E.coli* isolated from the backwaters of Vembanad lake, Kerala (Vaiyapuri et.al., 2021). Another relevant study indicated the occurrence of these genes in carbapenam resistant Enterbacteriaceae in water bodies close to hospital settings in India (Kalasseril et.al., 2020).

4. CONCLUSION

Uropathogenic *E.coli* (UPEC) is a specific pathogenic strain that causes severe infections, particularly in individuals at risk. Its prevalence in the environment is significant, primarily due to contamination of water bodies by human and animal waste. Factors such as poor community or personal hygiene and exposure to wastewater contribute to the increased prevalence and spread of UPEC. The environmental prevalence of uropathogenic *E.coli* underscores the importance of addressing issues related to contamination and promoting good hygiene practices to mitigate its spread.

In this study, genomic DNA was extracted to identify virulence factor genes, specifically adhesion genes, which are naturally present in the chromosomes of *E.coli*. Through multiplex PCR analysis, it was observed that all the tested MDR isolates contained these adhesion genes. Further confirmation was obtained through BLAST analysis, which showed that the gene sequences identified in the isolates were 100% similar to the respective genes in E. coli. Additionally, PCR analysis was performed to identify MDR genes, namely NDM, OXA, and CTX-M, which were found in all eight isolates tested. This finding aligns with the observed multiple drug resistance exhibited by these isolates. The BLAST analysis of the gene sequences further supported this, showing 100% similarity to the reported MDR genes in E. coli.

The presence of more than two antibiotic resistances in a single isolate defines multiple drug resistance. Based on molecular studies conducted, it can be concluded that the identified isolates are indeed multiple drug-resistant. Overall, this study provides evidence of the presence of adhesion genes and MDR genes in the tested isolates, indicating their potential for causing infections and their resistance to multiple antibiotics.

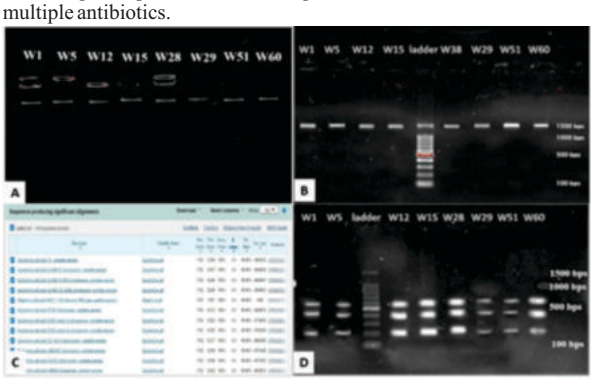

Fig 1.A. Genomic DNA isolation of 8 isolates**. B**. 16S PCR amplification of the isolates **C.**BLAST analysis of W51 isolate sequence **D.** Multiplex PCR of virulence factorgenes

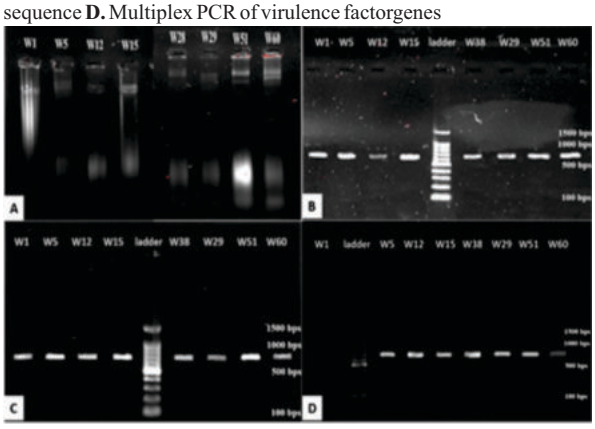

Fig 2. A. Plasmid isolation of 8 isolates B. PCR amplification of the NDM genes **C.** PCR amplification of the OXA genes **D.** PCR amplification of the CTX-M genes

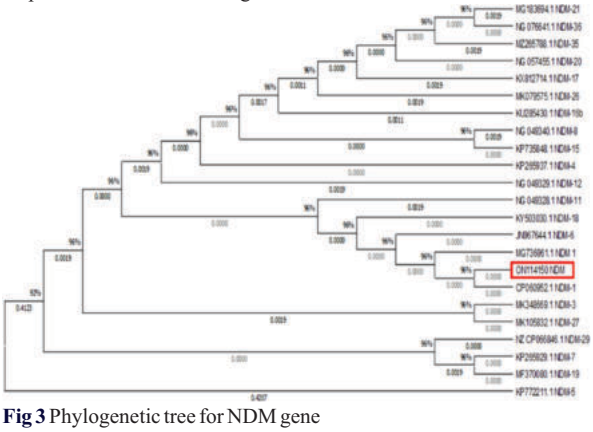

Fig 3 Phylogenetic tree for NDM gene

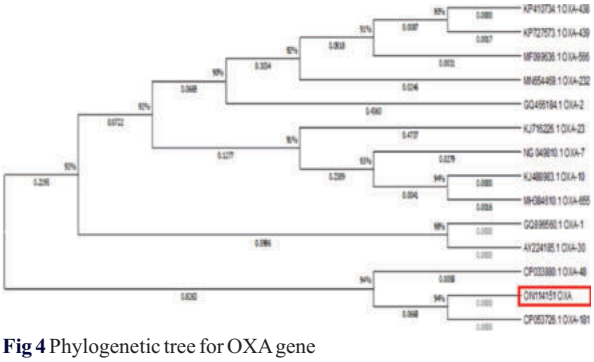

Fig 4 Phylogenetic tree for OXA gene

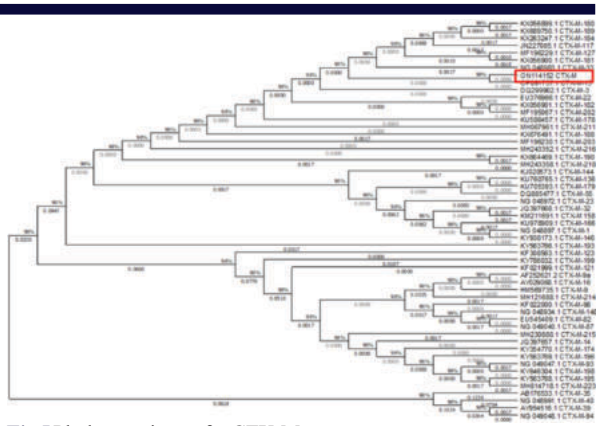

Fig 5 Phylogenetic tree for CTX-M gene

Table 1: Primer sequences for the PCR tests conducted

Primer/ Gene Name	Primer sequence		Product Size
16S rDNA	F	AGAGTTTGATCMTGG	1500bp
	R	ACCTTGTTACGACTT	
papC	F	GTGGCAGTATGAGTAATGACCGTTA	205 bp
	R	ATATCCTTTCTGCAGGGATGCAATA	
sfa	F	CTCCGGAGAACTGGGTGCATCTTAC	410 bp
	R	CGGAGGAGTAATTACAAACCTGGCA	
afa	F	GGCAGAGGGCCGGCAACAGGC	594 bp
	R	GTGGCAGTATGAGTAATGACCGTTA	
NDM	F	GGTGCATGCCCGGTGATC	661 bps
	R	ATGCTGGCCTTGGGGAACG	
OXA	F	TTGGTGGCATCGATTATCGG	744 bps
	R	GAGCACTTCTTTTGTGATGGC	
CTX-M	F	TTAGGAARTGTGCCGCTGYA	688 bps
	R	CGATATCGTTGGTGGTRCCCAT	

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