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Review Article

MOLECULAR CHARACTERIZATION OF MULTIDRUG RESISTANT *ESCHERICHIA COLI* ISOLATED FROM URINARY TRACT INFECTIONS

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ABSTRACT

Objective: Molecular Characterization of Multidrug Resistant *Escherichia coli* Isolated from Urinary Tract Infections by RAPD analysis. Random Amplification of Polymorphic DNA (RAPD) is a method of DNA fingerprinting. It is called fingerprinting because the nucleotide sequence in the DNA of each individual organism varies even within the same species and hence generates a unique pattern upon amplification using primers that bind randomly in the genome.

Methods: In this study Ten *Escherichia coli* samples isolated from the urine of several patients showing symptoms of urinary tract infection were collected from a Diagnostic Centre in Bangalore. The genus and species were confirmed by biochemical characterization according to Bergey's Manual for Characterization of Bacteria followed by PCR confirmation and constructing dendrogram based on primers used. The confirmed specimens were subjected to antibiotic sensitivity test using Kirby-Bauer Disk Diffusion Susceptibility Test protocol. The sensitivity of *E. coli* to a range of antibiotics including Nalidixic acid, Gentamycin, Trimethoprim and Sulfamethoxazole used in treatment of urinary tract infections was checked. Six multidrug resistant varieties of *E. coli* were identified. The genomic DNA of these bacterial samples was isolated using phenol chloroform extraction method. The DNA was estimated qualitatively using agarose gel electrophoresis and quantitatively using Nano-drop spectrophotometer. Using four decamer oligo-nucleotides as single primers in Polymerase Chain Reaction (PCR), DNA fingerprinting, dendrogram analysis and genetic similarity matrix were estimated, revealing variations between selected six strains of *E. coli*.

Result: Amongst primers used, D-20 has shown clear distinctive band pattern and shows that samples 1 and 3 are closely related to each other and distantly related to 4 and 2. Samples 5 and 6 are closely related to each other but very distantly related to other samples phylogenetically.

Keywords: *E.coli*, RAPD, PCR, Antibiotic resistance

INTRODUCTION

Multiple drug resistance organisms (MDRO), are defined as resistant to minimum one antibiotic agent in more than two antimicrobial classes [1]. It remains as a major health concern in the health care institutions like hospitals [2, 3, 4, 5]. These MDR pathogens which results from the intensive use of antibiotics are capable of producing the extended-spectrum beta-lactamases (ESBLs) [6, 7]. It has been known that these bacterial enzymes (ESBLs) that confer resistance to many antibiotic classes are sometimes undetected using the conventional tests and thereby resulting in treatment failure [8, 9]. However, prior colonization with these ESBL pathogens is a predisposing risk factor for the development of the infection, the pathogens virulence factors also play an important role [10, 11, 12].

Escherichia coli, a member of the Enterobacteriaceae family of bacteria, is one of the common pathogen that causes community- and hospital-acquired urinary tract infections (UTIs) [12]. Virulence factors for *E.coli* infection assist in colonization, proliferation and causing severe infection by interacting with its host cells. Virulence in association with resistance could potentially affect the outcome of the *E.coli* infection. Although the virulence factors for *E.coli* are extensively studied, the prevalence of VFs in a particular clinical condition and patient population is necessary for the prevention of *E. coli* infections [13]. Consequently, the aim of the present study was to evaluate MDR *E.coli* strains isolated from UTIs. The study also aims to assess the association between the phylogeny, antimicrobial resistance, and virulence among *E. coli* isolates from UTIs.

MATERIALS AND METHODS

Materials

Culture of the *E.coli* strains, sterile loops, microscopic slides, compound microscope, crystal violet, Gram's iodine, saffranin, and ethanol ordered from Vasa Scientific Pvt Ltd, Bangalore, India.

Sample Collection

Escherichia coli on MacConkey agar isolated from suspected urinary tract infection specimens were collected from Doctor's Diagnostic Centre in Kalyan Nagar, Bangalore.

Isolation and Screening of *Escherichia coli (*Eosin Methylene Blue Agar)

For the isolation of pathogenic *E.coli*, EMB agar was prepared and autoclaved at 121° C at 15 lbs/ sq. inch for 15 minutes. After sterilization, media was poured on the appropriate sterilized Petri dishes and allowed to solidify. After the solidification of the media, clinical samples were streaked and incubated at 37° C for 24 hrs.

Culturing On Nutrient Agar

The nutrient agar media was prepared in conical flask and autoclaved at 121° C at 15 lbs/ sq.inch for 15 minutes. After sterilization, the media was poured on the appropriate sterilized Petri dishes and allowed to solidify. After solidification, plates were inoculated with *E.coli* and incubated at 37°C for 24 hrs.

Identification of the Organism

Gram Staining

The colonies were isolated on the basis of the clear zone, morphology and biochemical tests i.e. gram staining was performed. The smear containing the isolate was prepared, air dried and heat fixed by passing it through the flame of the Bunsen burner and allowed to cool.

The slide was flooded with a crystal violet solution and allowed to stand for 1 minute, followed by Gram's iodine solution, 95% alcohol for 20 seconds, and saffranin solution for at least one minute. Slide was washed briefly with tap water (not longer than 5 seconds) after

each step. Slide was dried and observed under the microscope first under high power lens and then under oil immersion lens.

Biochemical Characterization

Lactose peptone broth with phenol red

Identification of microorganism was done according to Bergey's Manual of determinative bacteriology by using different mediums for the isolated and selected bacteria for their biochemical characterization. Lactose peptone broth prepared in several tubes containing inverted Durham's tubes. Phenol red indicator added, sterilized at 121° C and 15lbs pressure for 15 minutes, followed by inoculation of culture in each tube and incubated at 37° C for 24 hours.

Indole production test

Tryptone broth was prepared and autoclaved, followed by inoculation of culture and incubation at 37° C for 24 hours. Development of cherry red color on addition of Kovac's reagent would indicate a positive result.

Citrate utilization test

Test tubes containing Simon's citrate agar were autoclaved and followed by inoculation of culture and incubation at 37° C for 24 hours. Change of color of the slant form green to blue would indicate a positive result.

Antibiotic susceptibility testing

Mueller-Hinton agar was prepared, autoclaved and poured into petriplates aseptically. 100 µl of 24 hour old bacterial cultures were inoculated in each plate and spread evenly to form a lawn culture. Solutions of concentration 30µg/ml of myriad antibiotics were made. Whatman filter paper discs soaked in the antibiotic solutions were placed on the inoculated plates and incubated at 37° C for 24 hours. Growth and zone of inhibition after 24 hours were recorded.

RESULTS

Escherichia coli cultures collected from the diagnostic centre were first subcultured on nutrient agar slants and further subjected to biochemical characterization (Indole test and lactose fermentation test) to confirm the genus and species. Confirmed samples were then subjected to Antibiotic Sensitivity Testing using the antibiotic discs. The DNA of the resistant strains were then isolated and subjected to PCR amplification and genetic variability study using Random amplification of Polymorphic DNA.

Fig.1: *Escherichia coli* showing green metallic sheen on EMB Agar

Antibiotic Sensitivity Test

Antibiotic Sensitivity Test was performed for all ten *Escherichia coli* samples with 9 antibiotics used in treatment of urinary tract infection to determine the resistance pattern of the strains. Strains showing zone of inhibition less than 15 mm in diameter were considered resistant. The strains which were resistant to more than 6 antibiotics were considered to be multi drug resistant.

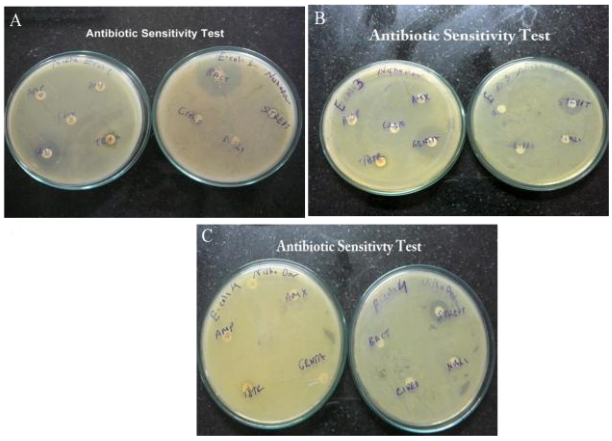

Fig.2: Antibiotic Sensitivity Test Result for six samples (A) *E. coli*-1, *E. coli*-3; (B) *E. coli*-4, *E. coli*-23; (C) *E. coli*-459, *E. coli*-523 treated with different antibiotics like, ampicillin, amoxycillin, bactrim, ciprofloxacin, cloxacillin, gentamycin, nalidixic acid, streptomycin, tatracyclin respectively were incubated at 37° C for 24hrs.

Table 1 Antibiotic Sensitivity Test Result for six *E. Coli* samples (R – Resistant; mm- Diameter of zone of clearance).

	E.coli 1	*E.coli 3*	*E.coli 4*	*E.coli 23*	*E.coli 459*	*E.coli 523*
Ampicillin	R	R	R	R	R	R
Amoxycillin	R	R	R	R	R	R
Bactrim	30 mm	33 mm	R	R	R	R
Ciprofloxacin	R	30 mm	R	R	R	R
Cloxacillin	R	R	R	R	R	R
Gentamycin	18 mm	R	R	R	R	R
Nalidixic acid	R	18 mm	R	R	R	R
Streptomycin	R	33 mm	15 mm	15 mm	R	R
Tetracyclin	R	R	R	R	R	R

As shown in the **figure.2**, *E.coli* 1 was resistant to seven out of nine antibiotics used , *E.coli* 3 was resistant to five out of nine antibiotics used in the study,whereas, *E.coli* 4 and 23 were resistant to eight out of nine antibiotics used in the study, *E.coli* 459 and *E. coli* 523 were resistant to all the antibiotics used in the study **(Table.1).**

RAPD Analysis and Phylogenetic Analysis using Dendrogram

Primer D-18: Six multi drug resistant *E.coli* strains were used for RAPD analysis and the PCR products obtained were run on 1% agarose gel as shown in the **figure.3** and the dendrogram of the RAPD product with primer D-18 shown in the **figure. 4**.

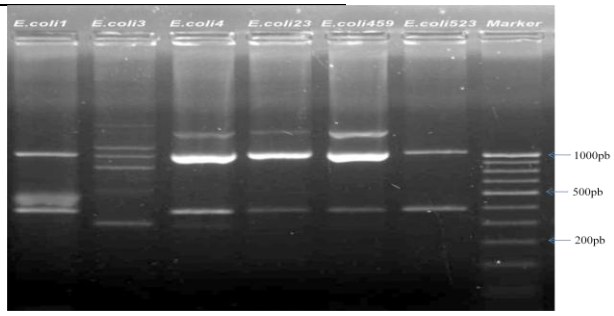

Fig.3 Gel for RAPD product obtained upon amplification with primer D-18

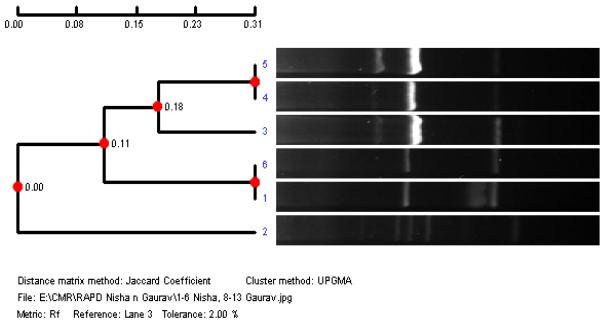

Fig.4 Dendrogram of the RAPD product with primer D-18

(1)	1	100.00	50.0	50.00	66.67	66.67	100.00
(2)	3	50.00	100.00	25.00	33.33	33.33	50.00
(3)	4	50.00	25.00	100.00	75.00	75.00	50.00
(4)	23	66.67	33.33	75.00	100.00	100.00	66.67
(5)	459	66.67	33.33	75.00	100.00	100.00	66.67
(6)	523	100.00	50.00	50.00	66.67	66.67	100.00

Similarity Matrix Calculated by: Jaccard Coefficient

The phylogenetic tree produced from the UPGMA average cluster using Primer D-18 Matrix analysis shows that, the close relatedness between *E. coli* 1 and *E. coli* 523. *E. coli* 23 and *E. coli* 459 are related to each other while being distantly related to the other samples.

Primer D-20: Six multi drug resistant *E.coli* strains were used for RAPD analysis and the PCR products obtained were run on 1% agarose gel as shown in the figure 5 and the dendrogram of the RAPD product with primer D-20 shown in the **figure 6**.

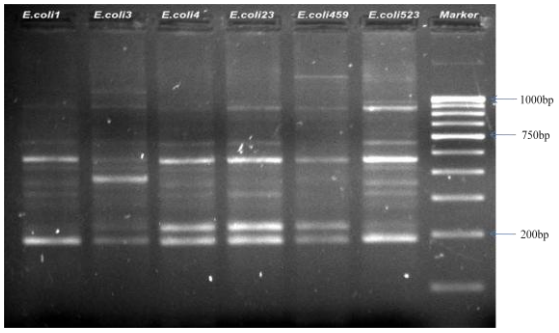

Fig.5: Gel for RAPD product obtained upon amplification with primer D-20

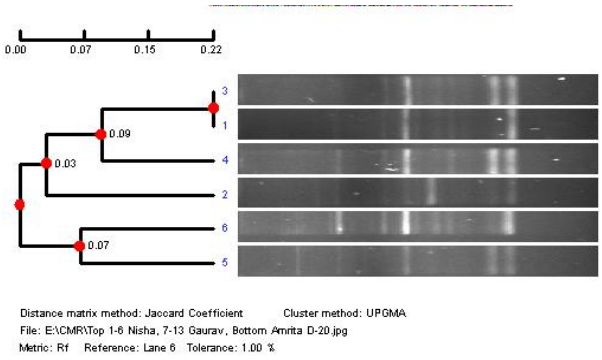

Fig.6: Dendrogram of the RAPD product with primer D-20

The phylogenetic tree produced from the UPGMA average cluster analysis shows close relatedness between *E. coli* 1, *E. coli* 4 but distantly related to the other samples.

Primer T-7: Six multi drug resistant *E.coli* strains were used for RAPD analysis and the PCR products obtained were run on 1% agarose gel as shown in the figure 7 and the dendrogram of the RAPD product with primer D-20 shown in the **figure 8.**

1	1	100.00	62.50	100.00	75.00	40.00	70.00
2	3	62.50	100.00	62.50	62.50	44.44	60.00
3	4	100.00	62.50	100.00	75.00	40.00	70.00
4	23	75.00	62.50	75.00	100.00	55.56	70.00
5	459	40.00	44.44	40.00	55.56	100.00	70.00
6	523	70.00	60.00	70.00	70.00	70.00	100.00

Similarity Matrix Calculated by: Jaccard Coefficient

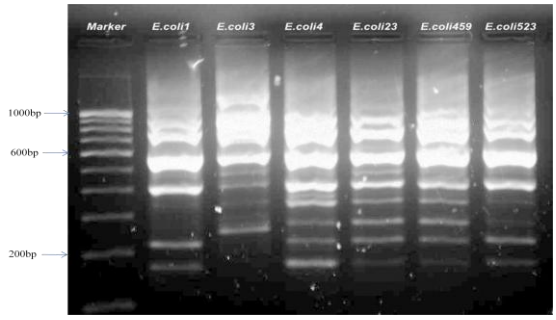

Fig.7 Gel for RAPD product obtained upon amplification with primer T-7

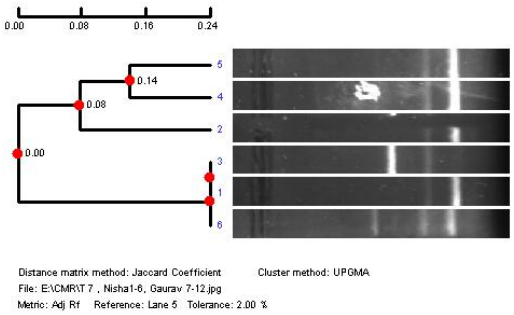

Fig.8 Dendrogram of the RAPD product with primer T-7

1	1	100.00	66.67	100.00	50.00	40.00	100.00
2	3	66.67	100.00	66.67	75.00	60.00	66.67
3	4	100.00	66.67	100.00	50.00	40.00	100.00
4	23	50.00	75.00	50.00	100.00	80.00	50.00
5	459	40.00	60.00	40.00	80.00	100.00	40.00
6	523	100.00	66.67	100.00	50.00	40.00	100.00

Similarity Matrix Calculated by: Jaccard Coefficient

The phylogenetic tree produced from the UPGMA average cluster analysis shows close relatedness between *E. coli* 1, *E. coli* 4 and *E. coli* 523 and distantly related to the other samples.

Primer W-2: Six multi drug resistant *E.coli* strains were used for RAPD analysis and the PCR products obtained were run on 1% agarose gel as shown in the figure 9 and the dendrogram of the RAPD product with primer D-20 shown in the figure 10.

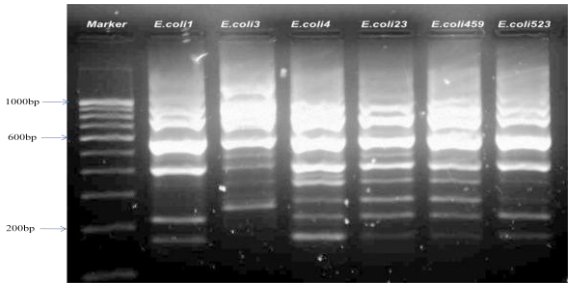

Fig.9 Gel for RAPD product obtained upon amplification with primer W-2

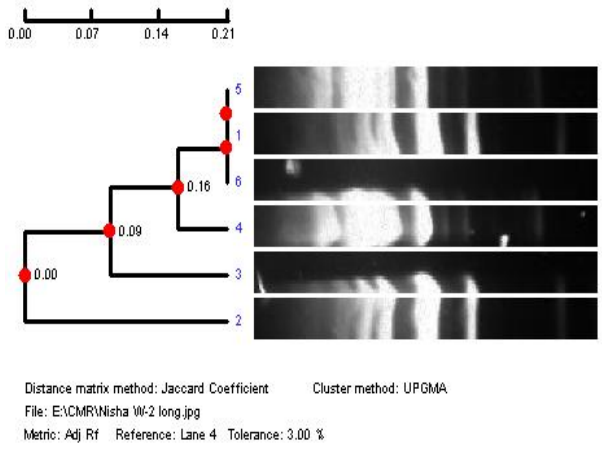

Fig.10 Dendrogram of the RAPD product with primer W-2

1	100.00	60.00	77.78	90.00	100.00	100.00
3	60.00	100.00	40.00	70.00	60.00	60.00
4	77.78	40.00	100.00	70.00	77.78	77.78
23	90.00	70.00	70.00	100.00	90.00	90.00
459	100.00	60.00	77.78	90.00	100.00	100.00
523	100.00	60.00	77.78	90.00	100.00	100.00

Similarity Matrix Calculated by: Jaccard Coefficient

The phylogenetic tree produced from the UPGMA average cluster analysis shows close relatedness between *E. coli*1, *E. coli* 459 and *E. coli* 523, while being distantly related to the other samples.

DISCUSSION

In the current study, most of the isolates were resistant to the first-line antibiotics including ampicillin, amoxicillin, and cloxacillin. In addition, resistance to tetracycline, which is considered as broad spectrum antimicrobial, commonly occurs among *E.coli* strains except two strains (*E.coli 3* & *E.coli* 23) isolated from UTIs. It is important to note that one *E.coli* strain (*E.coli* 523) in the present study is resistant to all the nine drugs including the commonly prescribed and the broad spectrum antibiotics. This raises an issue on the therapeutic options for these multidrug resistant *E.coli* strains to prevent the severe infection.

A study by Yilmaz N 2009 which assessed the antibiotic susceptibility patterns of *E. coli* strains isolated from patients with UTI, in Izmir, Turkey, found that a significant proportion of the *E. coli* isolates were resistant to most antibiotics except amikacin. In the present study, most of the studied *E.coli* isolates except *E.coli* 1 and *E.coli* 523 were susceptible to streptomycin. These results provide information that may assist in formulating the antimicrobial drugs against the *E.coli* pathogen that cause UTIs [14]. A study by Pobiega M et al which studied the prevalence of MDR Escherichia coli and ESBL pathogens isolated from asymptomatic bacteriuria and urinary tract infections found that a large proportion of isolates were resistant to ciprofloxacin (32.7%, n=33). While among 45 isolates (44.5%), the *E.coli* was resistant to trimethoprim/sulfamethoxazole. The study also demonstrated the lack of association between the number of VF genes and phylogeny in *E.coli* [15].

A study by Hannah EL et al in which the antimicrobial-resistant and antimicrobial-susceptible *E. coli* isolates from retail meats and from human stool and clinical specimens from a single rural U.S. community were compared for polymerase chain reaction (PCR)-defined phylogenetic group (A, B1, B2, or D) and virulence genotype. Meat and human isolates from the same phylogenetic group with similar virulence profiles underwent sequential two-locus sequence analysis, random amplified polymorphic DNA (RAPD) analysis, and pulsed-field gel electrophoresis (PFGE) analysis. Phylogenetic analysis of *E.coli* strains isolated from the human and meat source suggested some degree of commonality and thereby supported the potential role of retail meat in disseminating the resistant and virulent *E.coli* in humans [16].

In the present study, RAPD patterns for the genomic DNA of *E.coli* strains including *E.coli* 1, *E.coli* 3, *E.coli* 4, *E.coli* 23, *E.coli* 459 and *E.coli* 523 isolated from UTIs, which are resistant to six drugs of all the nine antibiotics, were analyzed for polymorphism. The Random primer used for amplification produced 13 scorable bands. Phylogenetic tree was created by the unweighted pair-group method arithmetic (UPGMA) average cluster analysis. Genetic fingerprinting and phylogenetic diversity was determined by converting RAPD data into a Jaccard similarity matrix and analyzed by UPGMA to produce a phylogenetic tree. In this study, the RAPD data was recorded in to similarity matrix and UPGMA to produce a Phylogenetic tree. From the results obtained, it was found that samples *E.coli* 1 and *E.coli* 4 are closely related to each other and distantly related to *E.coli* 23 and *E.coli* 3. Samples *E.coli* 459 and *E.coli* 523 are closely related to each other but very distantly related to other samples phylogenetically.

CONCLUSION

The current study which demonstrated the prevalence of MDR *E.coli* isolated from urine samples collected from a diagnostic centre in Bangalore suggest the need for an extensive precautions to MDRO. These findings also emphasize the necessasity for further research on epidemiology of MDR E.coli including virulence factors associated with infections.

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