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RAPD pattern, virulence nature and plasmid profile of MDR uropathogenic *Escherichia coli*

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ABSTRACT

UPEC is responsible for morethan 90% of UTI. Conventional and molecular characterizations are essential for epidemiological surveillance as well as proper management of diseases. Virulence factors, antibiotic susceptibility, RAPD pattern, plasmid profile of the E. coli isolated from UTI cases were studied using standard methods. Antibiotic susceptibility pattern of 127 uropathogenic E. coli revealed 11 specific resistance patterns with 96.7% resistance to erythromycin and cefpodoxime. Twenty three strains were resistance to all antibiotics tested and revealed the presence of virulent genes like fimH, hly and kps. All the strains showed six clustered RAPD pattern. All the tested isolates harboured plasmid. UPEC also showed the presence of tem, oxa, shv and ctx_m genes. Antibiotic resistances were due to plasmid which is evidence in plasmid curing study. This study concludes the relationship between plasmid profile, virulence, antibiotic resistance and relatedness of organisms in a community.

Keywords: UTI, UPEC, Escherichia coli, UPEC, virulence, plasmid

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INTRODUCTION

Urinary tract infection is a common infection and more prevalent all over the world [1]. In India, it is one of the most common causes of morbidity and mortality, affecting all age groups across the life span [2]. *Escherichia coli (E. coli)* are present in the gastrointestinal tract as a normal flora and are the common cause of community as well as hospital acquired infections of UTI [3]. In human, *E. coli* associated with extra intestinal disease are termed as extra intestinal pathogenic *E. coli* (ExPEC). These strains related to UTI are called UPEC [4]. The uropathogenic *Escherichia coli* (UPEC) strains are responsible for 70-90% UTI. In recent years, incidence of cephalosporins, fluoroquinolones and trimethoprim resistant *E. coli* causing UTIs shows special clinical importance, because they cause multiple virulence and are not responding to common therapeutic applications. Biofilm forming ability of the *E. coli* protects the bacteria against high antimicrobial concentration and phagocytosis. Detection of virulent factor producing strains is relevant for the design of adequate control measures for UPEC infection. Motility, adherence and biofilm formation are from the primary steps in bacterial pathogenesis and in the development of antimicrobial resistance [5]. CDC recommended the use of short term antibiotics for the treatment of UTI. Therefore it is important that the susceptibility data of major uropathogens should be known. Hence in the present study antibiotic sensitivity pattern of the uropathogens were assessed. Multidrug resistance properties of the pathogens are plasmid mediated phenomenon [6]. Pathogenic entry to the host cells are mediated by virulent factors like bacterial enzymes, fimbriae, pili, flagellin, urease, the hemolysin HmpA, the IgA metallo protease ZapA and extended spectrum β-lactamases (ESBLs) [7]. Having known the incidence of UTI, prevalence of uropathogens, its virulence, antibiotic susceptibility, the present study was undertaken to determine the virulence factor gene and genetic variability among the uropathogenic *Escherichia coli*.

MATERIALS AND METHODS

Isolation and identification of Uropathogens

Uropathogenic *Escherichia coli* were isolated from the mid stream urine samples from UTI cases. Four hundred and ninty eight samples were collected over a period of one year. UPEC strains were isolated and differentiated using selective cum differential media like Eosin Methylene Blue Agar, Mac Conkey Agar, SS agar, XLD agar, Haektoein enteric agar and Hi Chrome UTI agar (Hi media, Mumbai, India). Isolates were identified by conventional methods [8].

Assessment of antibiotic sensitivity pattern of Uropathogenic *Escherichia coli*

All UPEC isolates (n=127) were subjected to antibiotic susceptibility test by disc diffusion mehod [9]. Antibiotics used in this study are Gentamycin (Gen), Ciprofloxacin (CF), Amikacin (Ak), Erythromycin (E), Co-trimoxazole (Co), Nalidixic acid (Na), Tetracycline (T), Ceftriaxone (CZX), Cephalosporins (CE) and Cefpodoxime (CPD).

Multiplex PCR for the Identification of Multidrug Resistance Isolates

All the available partial and full-length gene sequences of resistance gene were determined according to Shalini *et al.*, [10] protocol with some modification. The standard primers for *shv*, ctx_m and *tem* were obtained from Sigma, India and used for PCR amplification.

Amplification of virulence factors from *E.coli* by multiplex PCR

Virulence genes like fimH, hlyA, kps, pap and cnf were detected by gene amplification method using multiplex PCR. Primer sequence used designed by Yamamoto *et al.*, [11] and Johnson [12].

RAPD analysis [13]

RAPD profiles of the amplified DNA of the uropathogenic isolates were studied using ten OPA primers described below 1. 5 -TCC CAG CAGT- 3; 2. 5 -GTC GTC GTCT- 3; 3. 5 -ACG GGA CCTG-3; 4 .5 -GTT AGT GCGG- 3; 5 .5 -GTG GCC GATG- 3; 6. 5 -AGA GCG TACC- 3; 7. 5 -CCT GGG TCAG- 3; 8. 5 -GGC GAG TGTG- 3; 9. 5-CAATGCGTCT-3 and 10. 5′-AGAAGCGATG-3. Each polymerase chain reaction mixture consists of 2 µl of template DNA, 1 µl of 1.6 micromolar solution of primer , 10 µl 2 X PCR master mixes (Promega, USA) and made up to 20 µl with molecular grade water. Amplification was performed in a Bangalore Genei thermocycler.

Assessment of virulent features of Uropathogenic *Escherichia coli*

Assessment of virulent factors will provide the nature of pathogens and helps to take specific precautions to handle the potent pathogens. Biofilm study and Beta lactamase production was assayed using the standard methods [14, 15].

Plasmid profile of *E.coli*

Plasmid DNA was extracted by alkaline lysis method of plasmid preparation [16]. Extracted plasmids are separated gel electrophoresis using agarose gel of 0.8% to identify the number of plasmid copies present in different isolates and the nfragments were stained with ethidium bromide and they were visualized by UV-Trans illumination. Standard DNA molecular weight markers were used to estimate the Plasmid size.

Plasmid curing

The tested multi-resistant isolates were cured from their own plasmids by growing them in elevated temperature at 43^{o}C [17]. Thereafter, an appropriate dilution of cured bacterial cultures were spread on Muller Hinton agar plates and incubated at 37^{o}C. Five random single colonies were picked up and tested for their sensitivity against tested antibiotic and PCR for determination of ESBL genes.

RESULTS AND DISCUSSION

Urine samples were collected from clinically evident cases of UTI. *Escherichia coli* were identified using conventional microbiological methods. Among 498 urine samples 127 pure isolates of E. coli were detected. Urinary tract infection is one among the commonest infectious inflammatory disease and responsible for more than 8 billion hospital visits in India [1, 18, 19]. It affects all age groups across the lifespan [20]. Though various factors associated with the incidence of UTI, bacteria play a major role [21]. *E. coli* is one of the major predominant pathogen of UTI. Our results are in line with the findings of Siedelman *et al.*,[22], Walters *et al.*, [23], Yamamichi *et al.*, [24], Acarya *et al.*, [21], Sharma *et al.*, [25].

All the 127 pure *E. coli* isolates were subjected to antibiotic sensitivity assay by disc diffusion method. Out of 127 isolates, maximum number of (n=123) organisms were resistant to erythromycin and cefpodoxime (96.9% each)

followed by gentamycin and amikacin (89.8%). Similarly other isolates were also resistant to multiple numbers of antibiotics (Table 1). All the isolates were considered as multiple drug resistant uropathogenic *E. coli*. Siedelman *et al.,* [22] and Walters *et al.,* [23] reported that 76.5% of community acquired UT infections were due to *E. coli*. Among them, 60.6% of *E. coli* were ESBL producers i.e.. multidrug resistant isolates.

Table 1-Antibioticsensitivity pattern of uropathognic *E. coli* (n=127)

S. No.	Antibiotics	Number of Resistant isolates	Resistant
1	Gentamycin (G)	114	89.8
2	Ciprofloxacin (C)	113	88.9
3	Amikacin (Ak)	114	89.8
4	Erythromycin (E)	123	96.9
5	Cotrimaxozole (Co)	090	70.9
6	Nalidixic Acid (NA)	090	70.9
7	Tetracycline (T)	118	92.9
8	Ceftriaxone (CZX)	104	81.8
9	Cefpodoxime (CPD)	123	96.9
10	Cephalosporin (CE)	084	66.1

Antibiotic resistance pattern of multidrug resistant isolates revealed that all the 127 UPEC isolates belonged to eleven different patterns of antibiotic resistance. None of the organisms were susceptible to all the antibiotics tested (Table 2).

Table 2- Antibiotic resistance pattern of *E. coli* isolates

S. No	Resistance patterns
1	E, NA, T, CZX, CPD
2	G, A, T, CZX, CPD
3	A, E, NA, T, CPD, CE
4	CF, A, E, CO, NA, T, CZX, CPD
5	G, CF, A, E, CO, T, CZX, CPD, CE
6	G, CF, A, E, CO, NA, CZX, CPD, CE
7	CF, A, E, CO, NA, T, CZX, CPD, CE
8	G, CF, A, E, CO, NA, T, CZX, CPD, CE
9	G, CF, A, E, CO, NA, T, CZX, CPD
10	G, CF, A, E, NA, T, CZX, CPD, CE
11	G, CF, A, E, CO, NA, T, CZX, CPD, CE

Biofilm and β lactamase production ability is the major virulence determinant of uropathogens. Among the 11 isolates six isolates possess ESBL producing ability (E3, E7, E8, E16, E33 and E64). Biofilm formation is one of the major virulence factors of urinary pathogens of the present study, except E1 and E32 all the other strains possess biofilm producing ability (Table 3). UPEC exhibits multiple numbers of virulence factors. It facilitates colonization of *E. coli* in the bladder [26]. Virulence factors are responsible for the pathogenic potential of *E. coli* strains [27]. Our results were in line with the report given by Markoviae *et al.,* [28], who stated that almost 60% of isolates produced two or three virulence factors and only 3.8% produced none of the virulence factors.

Table 3-Virulence features of UPEC isolates

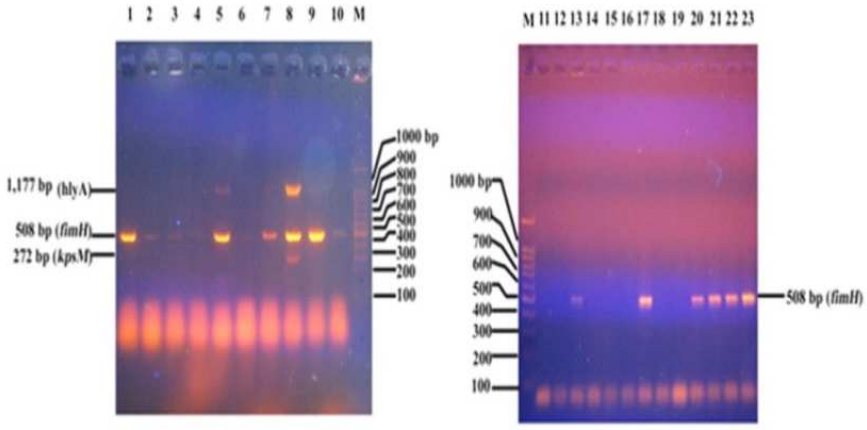
S. No	Isolate name	ESBL	Biofilm
1.	E1	Negative	Negative
2.	E3	Positive	Positive
3.	E7	Positive	Positive
4.	E8	Positive	Positive
5.	E13	Negative	Positive
6.	E16	Positive	Positive
7.	E32	Negative	Negative
8.	E33	Positive	Positive
9.	E64	Positive	Positive
10.	E66	Negative	Positive
11.	E88	Negative	Positive

Amplification of virulence genes by multiplex PCR

*Fim*H gene represents Type 1 fimbriae, adhesive subunit, similarly *kps* represents Capsule, *pap*C is a fimbrial gene and *hlyA* is a Haemolysin toxin protein. All the related 23 multi drug resistant *E.coli* were also subjected to evaluation based on multiplex PCR targeting 5 varying virulent genes (*fimH, hlyA, kps, pap* and *cnf*). The five different sets of primers involved in the study revealed the presence of three varying virulence genes such as such as *fim, hly* and *kps* among the isolates. No *pap* and *cnf* genes were identified on the isolates indicating the absence of

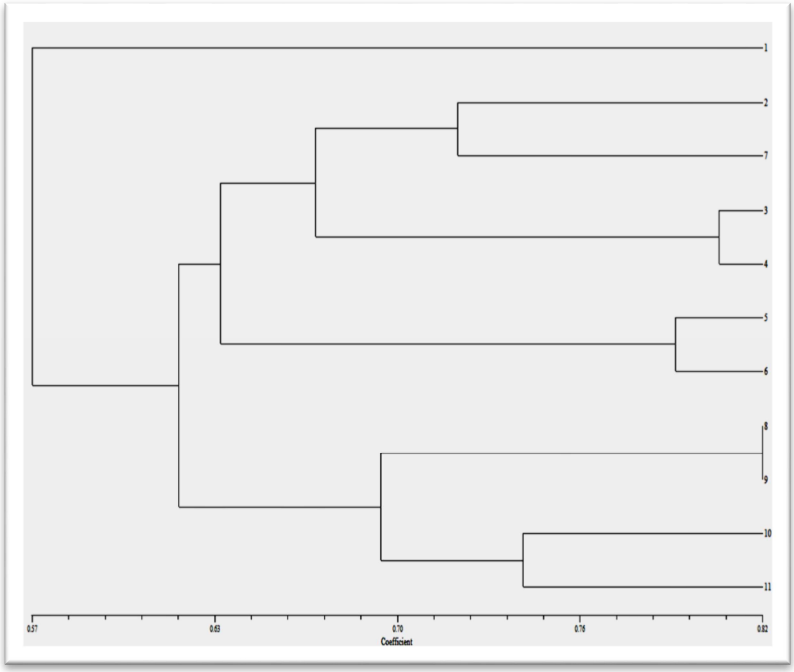
these genotypes among the test isolates (Figure 1). Out of the 23 strains, two strains showed *kps* gene, 15 strains had *fim H* whereas 3 strains harboured *hlyA* gene. This also indicated virulence gene in chromosome / plasmid is responsible for virulence.

Figure 1- Virulence genes of UPEC



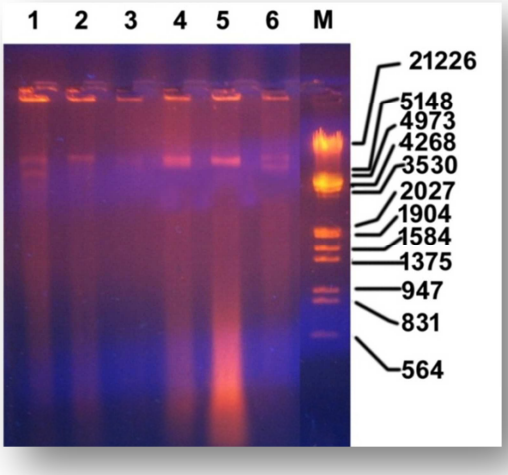
Genomic DNA of 11 bacterial isolates was successfully amplified and the genomic DNA was subjected to amplification randomly using ten different primers which revealed different pattern. Cluster analysis of RAPD profile of the genomic DNA produced a specific pattern of dendrogram. On evaluating genetic profile using cluster method, it shows 6 clusters, there by confirming genetic variation among the isolates. Similarity index of *E. coli* population revealed that none of the isolates were 100% similar with their genetic relatedness. Based on these pattern, six strains were selected for further study. RAPD is a simple and widely used method for strain differentiation, since it does not require any specific knowledge of the DNA sequences in the target organism [29]. Haryani *et al.*, [30] found that 4 RAPD profiles among seven studied *Enterobacter cloacae.* This study clearly indicated that the place of survival and community setup also responsible for the transfer of infectious agents. None of the strains showed 100% similar RAPD pattern.

Figure 2- Dendrogram pattern of UPEC isolates derived from cluster analysis



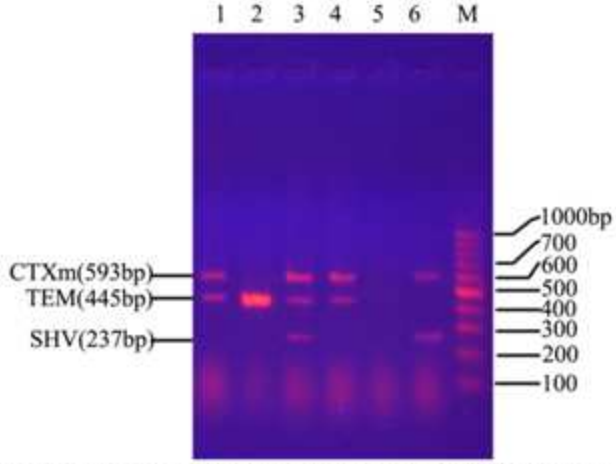
Plasmid assessment revealed the presence of plasmids in all the strains and the results were compared with antibiotic resistance pattern. The highest antibiotic resistance isolates had a higher number of plasmid bands. In this study all the isolates harboured plasmids. The strain E3, E8 and E64 harbored 2 plasmids bands, and remaining isolates had one plasmid The plasmid size ranges from 3530bp to above 4973 bp (Figure 3).

Figure 3 - Plasmid Profile of UPEC isolates



Antibiotic resistance of bacteria could be due to specific genes like TEM, SHV, OXA, CTX_M. Results of amplification of a resistance gene revealed that TEM gene was found in E3, E7, E8 and E16. CtxM gene was found in all the isolates except E7 isolate. E8 and E64 isolates only having SHV gene, whereas none of the organism showed the availability of OXA gene, which was also evident in figure 2. The isolate E8 showed three antibiotic resistance gene. $CTX\text{-}_M$ gene was found in 5 isolates, TEM was found in 4 isolates. Bedenic *et al.*, [31] reported that TEM gene was detected in 28 % of the isolates, SHV gene in 74 % and CTX-M gene was detected in only 2.5% isolates. CTX – M was a major reason for antibiotic resistance were reported by Lepeule *et al.*, [32] and Randall *et al.*, [33] from England , Titelman *et al.*, [34] from sweeden, Bourjilat *et al.*, [35] from Morocco, Narciso *et al.*, [36] from Portugul, Chouchani *et al.*, [37] from Tunisia and Akram *et al.*, [38] from India.

Figure 4-Assessment of ESBL genes in UPEC isolates



Lane 1-E3, Lane 2-E7, Lane 3-E8, Lane 4-E16, Lane 5-E33, Lane-6-E64

Lane- M- 100bp DNA lader

Plasmids could be a reason for major drug resistance and in some bacterial drug resistance is borne in the genomic DNA. In the present study, E33 isolate loses its resistance to gentamycin, ciprofloxacin, amikacin, erythromycin, cotrimaxozole, tetracycline and cephodaxime from 90% to 20% of plasmid curing. Similarly E64 loses its resistance from 100% to 50%, E3 90% to 20%; E8 80% to 20%. Very low resistance conversion was noted in the isolate E7 strain (50% to 20%). Antibiotic resistance of the bacteria could be due to a specific genes like TEM, SHV, OXA, CTX_M. After plasmid curing, pure isolates were subjected for the amplification of resistance gene. Results of amplification of resistance gene revealed that none of the resistance gene were found in all the UPEC strains. It was also indicated that all the isolates becomes ESBL negative trait (Table 4 and 5). Plasmid is one of the most important known mediators in facilitating the fast spreading of antibiotic resistance among bacteria [39]. Our result is in agreement with the findings of Shahid *et al.*, [40] and Oppegaard *et al.*, [41], as they have isolated single plasmid of 48.5 kb and 65 kb in MDR isolates of *Pseudomonas aeruginosa* and lactose-fermenting Coliform, respectively.

This study clearly depicted that drug resistance and virulence nature were due to the available plasmids. plasmid is cured using elevated temperature (45°C), which results in loss of the plasmid. Fortina and Silva, [42] obtained curing of 14.3 kb plasmid in *Lactobacillus helveticus* strain ILC 54 at 45°C. The plasmid cured cells became sensitive to all previously resistant antibiotics, which revealed that antibiotic resistance marker genes were located in plasmid [43]. It is clear from Elias *et al.*, [44] that the elevated temperature has a remarkable effect on all antibiotic resistance conferred by the bacterial isolates.

Table 4Antibiotic resistance patterns of UPEC isolate after plasmid curing

S. No	Isolates	% of resistance Before Plasmid Curing	% of resistance after plasmid Curing
1	E3	80	20
2	E7	50	20
3	E8	80	20
4	E16	90	40
5	E33	90	20
6	E64	100	50

Table 5 - Amplification of ESBL genes before and after Plasmid curing

S. No	Isolate	Before curing					After curing				
		TEM	SHV	OXA	CTXm	ESBL	TEM	SHV	OXA	CTXm	ESBL
1	E3	+	-	-	+	+	-	-	-	-	-
2	E7	+	-	-	-	+	-	-	-	-	-
3	E8	+	+	-	+	+	-	-	-	-	-
4	E16	+	-	-	+	+	-	-	-	-	-
5	E33	-	-	-	+	+	-	-	-	-	-
6	E64	-	+	-	+	+	-	-	-	-	-

CONCLUSION

E. coli is one of the most predominant pathogen of UTI infection in patients of Namakkal district, Tamil Nadu, India. *E. coli* possess multiple virulent factors and were MDR-ESBL pathogens, which are difficult to treat. The variation in antibiotic resistance pattern among the uropathogenic isolates was confirmed by the variation in RAPD pattern among the isolates. This genetic polymorphism among the isolates makes it difficult to choose a common antibiuotic therapy for antibiotic for the bacterial isolates. Further studies on the identification of conserved region in the virulence gene may help to design a common drug which may be able to compat the genetically polymorphic isolates. All the *E. coli* pathogens were isolated from multiple sources and possess variable number of plasmids, which are transmitted between clones. Empirical antibiotic treatment becomes more difficult due to the emergence of Multidrug resistance (MDR) among uropathogens. Alternate and modified strategy of antibiotic selection should be considered to overcome the problems of MDR.

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