

Molecular typing of enteropathogenic *Escherichia coli* from diarrheagenic stool samples

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ABSTRACT

Background: Acute diarrhoea is a leading cause of mortality in the developing countries. Enteropathogenic *Escherichia coli* (EPEC) were originally serogroup-defined *E.coli* which were associated with infantile diarrhoea. Hence, only serotyping was used for the discrimination of EPEC. Molecular typing methods, due to their higher discriminating ability, may help in the better characterization of the EPEC isolates and these have been used worldwide. However, the molecular typing of the EPEC strains has not been reported from this part of the country. Hence, this study was undertaken with the following aims and objectives.

Aim and objectives: This study was aimed at subjecting the EPEC isolates from the stool samples to molecular typing methods like the Random Amplification of Polymorphic DNA (RAPD) and Enterobacterial repetitive intergenic consensus sequences (ERIC) polymerase chain reaction (PCR). The results of these typing methods were compared with those of conventional methods like antibiogram and serotyping to study their similarities and differences.

Materials and Methods: *E.coli* strains (n=35), which were isolated over a period of two years from diarrheagenic stool samples (n=100), were subjected to antibiotic susceptibility

testing by the disc diffusion method. The EPEC strains which were confirmed by PCR were serotyped at the National Salmonella and Escherichia Centre, Kasauli, India. The EPEC strains were subjected to molecular typing methods like RAPD and ERIC PCR.

Results: Among the 35 *E.coli* isolates, 25 belonged to the serogroup O101 and they were positive for the *eae* gene. Among these, one of the *eae* positive isolates was also positive for the EHEC *hlyA* gene; five isolates were of the O111 serotype and they had both the *eae* and the *bfp* genes; there were five nontypeable strains which were negative for all the virulence genes which were tested. The non typeable *E.coli* strains were sensitive to all the antibiotics were tested, except ampicillin. Two EPEC isolates which belonging to the serogroup O111, showed genetic similarity in both RAPD and ERIC PCR.

Conclusion: EPEC isolates which belonged to same serogroup were found to be highly diverse, as shown by their differing antibiotic susceptibility patterns and by their ERIC PCR and RAPD profiles. The genetic similarities which were observed among few EPEC strains indicated a common ancestral origin or source.

Key Words: Enteropathogenic *E.coli*, RAPD, ERIC PCR, Molecular typing

INTRODUCTION

Diarrheagenic *E.coli* include enteropathogenic *E.coli* (EPEC), enterotoxigenic *E.coli* (ETEC), enteroinvasive *E.coli* (EIEC), enteroaggregative *E.coli* (EAEC) and shiga toxin producing *E.coli* (STEC) [1]. EPEC, in addition to their ability to induce attaching and effacing (AE) lesions, also possesses a large EPEC adherence factor (EAF) plasmid and the cluster of genes that encode the bundle-forming pili (BFP) [2,3]. The EPEC strains which carry the *eae* gene but lack the EAF plasmid and the *stx* gene are described as atypical EPEC. The atypical EPEC is more closely related to the STEC, which appears to be an emerging pathogen [4].

Serotyping alone is insufficient for the strain differentiation of the diarrheagenic *E.coli*, as it has already been done in many clinical microbiology laboratories. The study of the genetic relatedness by molecular typing methods like RAPD and ERIC PCR has contributed to the assessment of the pathogenic diversity of the *E. coli*, the relationships between the serotypes and the virulence

properties of the strains [5]. The molecular typing of the EPEC strains has not been reported so far, from this part of the country. Hence, *E.coli* isolates from stool samples were studied for their antibiograms and serotypes and they were characterized further by using RAPD and ERIC PCR.

MATERIALS AND METHODS

Bacterial strains: *E.coli* strains (n=35) which were isolated over a period of two years from diarrheagenic stool samples (n=100), which were received for routine culture sensitivity testing at the Department of Microbiology, Kasturba Medical College Hospital, Mangalore India, were included in the study, with the approval of the institutional ethics committee.

Isolation of *Escherichia coli* from the stool samples:

The stool samples were microscopically screened for pus cells, RBCs and the ova and cysts of parasites. The culture media and the antibiotic supplements which were used in the study were procured from Hi-Media Laboratories, Mumbai. All the stool samples

with pus cells and RBCs were cultured to isolate *E. coli* as well as other enteric pathogens [6]. Briefly, 4-5 loopfuls of the stool sample were added to 10 ml of modified tryptic soy broth (TSB containing 20 mg/l novobiocin), selenite F broth (SFB) and alkaline peptone water (APW) and these were incubated for 6-8 h at 37°C. Enrichment cultures in modified TSB were streaked onto sorbitol MacConkey's agar (SMAC) and MacConkey's agar.

The SFB and APW were subcultured onto MacConkey's agar, deoxycholate citrate agar (DCA) and thiosulphate citrate bile salt sucrose (TCBS) agar and these were examined for the presence of other enteric pathogens like, *Salmonella* spp., *Shigella* spp., *Vibrio* spp. and *Aeromonas* spp. The plates were incubated for 18-24 h at 37°C. The sorbitol fermenting, sorbitol non-fermenting, lactose fermenting and lactose non-fermenting colonies were picked up and identified biochemically [7].

Detection of the EPEC and the STEC virulence genes by PCR

Extraction of DNA from the E.coli isolates [6]: The biochemically confirmed *E.coli* isolates were further tested for the presence of the virulence genes of EPEC and STEC like, the *eaeA*, *bfp*, *stx1*, *stx2*, *rfb O157*, and the EHEC *hlyA* genes. The *E.coli* isolates (3 to 4 colonies) were emulsified in sterile distilled water. Phenol and chloroform were added in a 1:1 ratio. This mixture was vortexed and centrifuged at 5000 g. The aqueous layer was transferred into a fresh tube. The DNA was precipitated with ethanol by keeping at -20°C for 1-2 hour, after which it was centrifuged and the pellet was re-suspended in sterile PCR grade water. It was centrifuged again and the supernatant was used as DNA for all the PCR reactions.

The primers which were used for the detection of the EPEC and the STEC virulence genes [8, 9]:

stx1: <F - ACACTGGATGATCTCAGTGG; R - CTGAATCCCCCTC-CATTATG>

stx2: <F - CCATGACAACGGACAGCAGTT; R - CCTGTCAACT-GAGCAGCACTTTG>

rfb O157: <F - AAGATTGCGCTGAAGCCTTTG; R - CATTG-GCATCGTGTGGAC>

EHEC hlyA: <F - ACGATGTGGTTTATTCTGGA; R - CTTACAGT-CACCATACATAT>

eaeA: <F - GACCCGGCACAAGCATAAGC; R - CCACCTGCAG-CAACAAGAGG>

and *bfp*: <F - TCTTGCTTTGATTGAATCTGCA; R - GTAAAATCGT-TGAGTCCAATCCA>

The PCR reaction included initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1.5 min, extension at 72°C for 1.5 min and a final extension at 72°C for 5 min. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide [10] and photographed. The lysates which were positive for only the *eae* gene and negative for the other STEC genes were further tested for the presence of the *bfp* gene by PCR, at an annealing temperature of 56°C for 1 min. In all the PCR reactions, the *E.coli* reference strains, EDL933 and E2348/69 were used as positive controls for STEC and EPEC respectively. The *E.coli* ATCC 25922 strain was used as a negative control.

Antibiotic susceptibility testing: Antibiotic susceptibility testing was performed by the disk diffusion method and it was interpreted as per the Clinical and Laboratory Standards Institute (CLSI) standards [11]. Different antibiotic disks like ampicillin, ceftazidime, ce-

photaxime, ciprofloxacin, ceftriaxone, cefuroxime, chloramphenicol and gentamicin (Hi-Media Laboratories, Mumbai) were used. *E.coli* ATCC 25922 was used as the quality control strain.

Serotyping of the E.coli isolates: All the *E.coli* isolates from the stool samples, which were tested for the virulence genes by PCR, were serotyped at the National Salmonella and Escherichia Centre, Kasauli, India.

Molecular typing by RAPD and ERIC PCR:

Two custom-synthesized, decamer, random primers, R1 (5'GCGATCCCCA3') and R2 (5'CAGCACCCAC3'), which were procured from Bangalore Genei, Bangalore, were used for the RAPD reaction. The DNA amplification and the detection of the amplified product were done according to the methods of Neilan [12], and Sambrook et al [10]. The amplifications were performed in a 25 µl reaction mixture which consisted of genomic DNA, 1× reaction buffer, 100 µM of dNTPs, 0.2 µM of the single random primer, 2.5 µM of MgCl₂ and 1U of Taq polymerase. The amplification reaction conditions were: initial delay at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 min and the final delay at 72°C for 10 min. The amplified product was resolved on a 2% agarose gel, stained with ethidium bromide and photographed.

The custom-synthesized ERIC primers, ER-1 <5'ATGTAAGCTCCTGGGGATTCAC3'> and ER-2 <5'AAGTAAGTGACTGGGGTGAGCG3'> were used for ERIC PCR. The DNA amplification and the detection of the amplified product were done according to the methods of Dallacosta et al [13]. The PCR amplifications were performed in 25 µl volumes which contained 5 mM of MgCl₂, 2 U of Taq polymerase, 0.4 mM (each) of dNTPs, 2 µl of crude template DNA, and 25 pM of the ER-1 or ER-2 primer. The reaction mixture was initially denatured for 2 min at 94°C, subjected to 35 cycles of denaturation at 94°C for 30s, annealed at 60°C for 1 min, extended at 72°C for 4.5 min and finally extended at 72°C for 5 min. The amplified PCR products were resolved by electrophoresis on a 2% agarose gel and photographed [10].

RESULTS

Among the 100 stool samples which were screened, 35 samples showed the growth of *E.coli*, two samples had *Salmonella typhimurium*, one had *Shigella sonnei* and one sample each had *Vibrio parahaemolyticus* and *Aeromonas hydrophila*, as shown in [Table/ Fig 1].

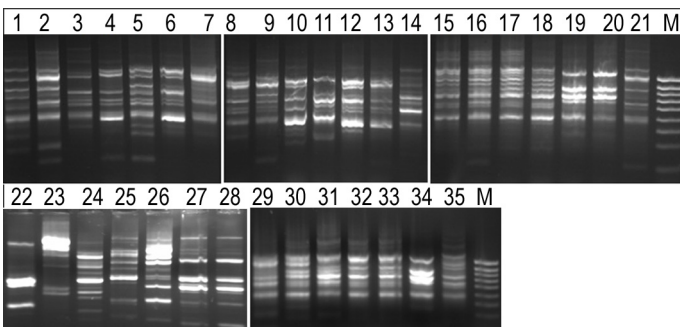
PCR which was performed on the 35 biochemically confirmed *E.coli* strains for the detection of various STEC and EPEC genes

Sl. No.	Type of microorganism	Number
1	<i>Aeromonas hydrophila</i>	01
2	<i>Escherichia coli</i>	35
3	<i>Salmonella typhimurium</i>	02
4	<i>Shigella sonnei</i>	01
5	<i>Vibrio parahaemolyticus</i>	01
	Total	40

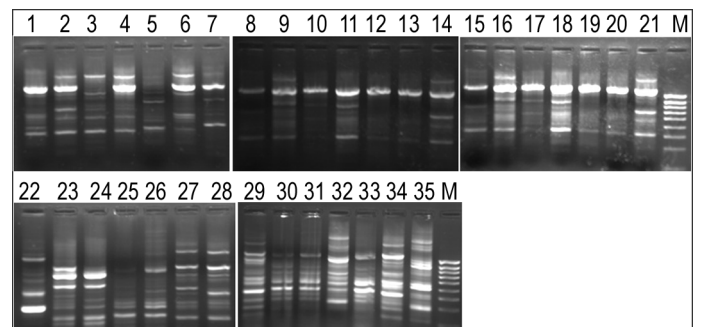
[Table/Fig-1]: Bacterial isolates from stool samples

Antibiotics tested	Serotypes of <i>E.coli</i>								
	O111 (n=5)			O Untypeable (n=5)			O101 (n=25)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ampicillin	2 (40)	-	3 (60)	2 (40)	-	3 (60)	7 (28)	2 (08)	16 (64)
Ceftazidime	4 (80)	-	1 (20)	5 (100)	-	-	16 (64)	3 (12)	6 (24)
Cefotaxime	4 (80)	-	1 (20)	5 (100)	-	-	13 (52)	-	12 (48)
Ciprofloxacin	2 (40)	-	3 (60)	5 (100)	-	-	9 (36)	-	16 (64)
Ceftriaxone	4 (80)	-	1 (20)	5 (100)	-	-	13 (52)	-	12 (48)
Cefuroxime	3 (60)	-	2 (40)	5 (100)	-	-	10 (40)	3 (12)	12 (48)
Chloramphenicol	4 (80)	-	1 (20)	5 (100)	-	-	10 (40)	3 (12)	12 (48)
Gentamicin	4 (80)	-	1 (20)	5 (100)	-	-	17 (68)	1 (04)	7 (28)

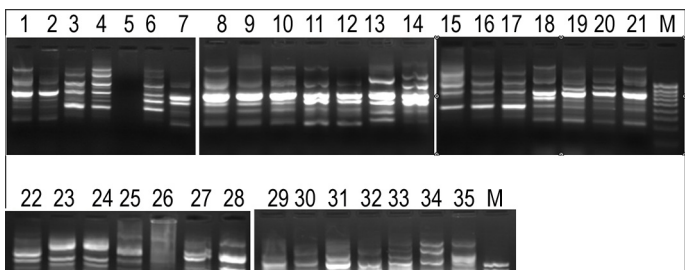
[Table/Fig-2]: S=sensitive; I=intermediate; R=resistant Antibiotic susceptibility pattern of *E.coli* isolates



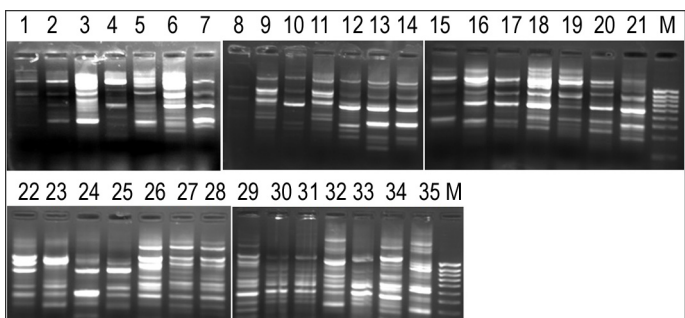
[Table/Fig-3]: Gel picture showing RAPD fingerprint pattern of different EPEC strains with R1 primer



[Table/Fig-6]: Gel picture showing ERIC fingerprint pattern of different EPEC strains with ER-2 primer



[Table/Fig-4]: Gel picture showing RAPD fingerprint pattern of different EPEC strains with R2 primer



[Table/Fig-5]: Gel picture showing ERIC fingerprint pattern of different EPEC strains with ER-1 primer

showed 24 strains to be positive for only the *eae* gene. One was positive for both the *eae* and the EHEC *hlyA* genes, five isolates had both the *eae* and the *bfp* genes and others (n=5) were negative for all the EPEC and the STEC genes which were tested. The

E.coli strains which carried the *eae* gene, but lacked the *bfp* and the STEC genes, were considered as atypical EPEC and the *E. coli* isolates which carried both the *eae* and the *bfp* genes were considered as typical EPEC. The *E.coli* isolates with both the *eae* and the EHEC *hlyA* genes were considered as STEC.

Twenty four *E.coli* isolates which were positive for only the *eae* gene and one isolate that was positive for the *eae* and the EHEC *hlyA* genes belonged to the serogroup O101. Five isolates which were positive for both the *eae* and the *bfp* genes belonged to the O111 serogroup. Five isolates which were negative for the EPEC and the STEC genes were serologically untypeable.

The antibiotic susceptibility pattern of the 35 *E.coli* isolates is shown in [Table/Fig 2].

The thirty five strains of the *E.coli* isolates which belonged to the different O serogroups were typed by RAPD by using the R1 and R2 primers, which generated 28 and 29 profiles respectively [Table/Fig 3 and 4]. With the R1 primer, the atypical EPEC which belonged to O101 (lanes 15, 16 and 17) and the typical EPEC (*eae* and *bfp* positive) strains of the serogroup O111 (lanes 27 and 28) showed genetic similarities among themselves. With the same primer, two typical EPEC strains of O111 also showed similarities with 3 untypeable strains of *E.coli* [lanes 29 to 33 in Table/Fig 3].

With the R2 primer, five atypical EPEC strains (lanes 15 to 17; lanes 23 and 24), two typical *E.coli* strains (lanes 27 and 28) and two untypeable *E.coli* (lanes 32 and 33) showed similarities among them-

selves [Table/Fig 4].

The thirty five strains of the *E.coli* isolates which belonged to the different O serogroups, which were typed by the ERIC primers, ER-1 and ER-2 generated 28 [Table/Fig 5] and 25 [Table/Fig 6] profiles respectively. Two strains which belonging to O101 [Table/Fig 5 lanes 13 and 14; lanes 15 and 17] showed similarities among themselves. Two typical *E.coli* strains of O111 showed similarities in their banding pattern [lane 27 and 28 in Table/Fig 5 and 6] with both the ERIC primers.

DISCUSSION

The epidemiological significance of each *E.coli* category may vary with the geographical area. In the present study, the *E.coli* isolates which were positive for the *eae* genes alone predominated (24 out of 35). Earlier studies referred to the EPEC strains which carried the *eae* gene, but lacked the *bfp* and the STEC genes, as atypical EPEC and the *E. coli* isolates which carried both the *eae* and the *bfp* genes as typical EPEC [3,4]. Even in the present study, the *E.coli* isolates which were positive for only the *eae* genes and negative for other genes could be considered as atypical EPEC. This study also showed that atypical EPEC was more commonly isolated than typical EPEC and the same was found to be true in a recently published Indian study from Kolkata [14]. However, these *eae* positive, atypical *E.coli* require further studies with regards to their virulence and epidemiological significance. Among the 25 *eae* positive *E.coli*, one isolate was also positive for the EHEC *hlyA* gene (virulence gene specific for EHEC) and it belonged to the serogroup O101. Hence, this isolate was considered as STEC other than *E.coli* O157.

The detection of EPEC by serological screening for certain *E.coli* O-serogroups is still the method of choice in most of the clinical diagnostic laboratories worldwide. The diversity of the serotypes which was found in the EPEC group discouraged the use of serodiagnostic methods for their detection [15,16]. Even in the present study, it was found that the *E.coli* isolates which belonged to the O101 serotypes (n=25) included one strain of STEC and 24 strains of atypical EPEC. If the detection of the virulence genes was not done, this STEC isolate would have been missed.

The therapeutic options vary depending on the type of the *E.coli* infection. Many investigators have documented a high prevalence of antimicrobial resistance among the EPEC strains in different parts of the world [17]. In the present study, the resistance was seen more commonly in the typical EPEC which belonged to the serogroup O111 than in the atypical EPEC strains, which was in agreement with the findings of earlier studies. The untypeable *E.coli* strains were resistant to only ampicillin. The increasing antimicrobial resistant phenotypes which were seen in the human isolates could be due to the indiscriminate use of antimicrobials in the clinical practice.

It was interesting to note that the typical EPEC O111 strains [lanes 27 and 28 in Table/Figs 3 and 4] showed genetic similarities among themselves with both the R1 and the R2 primers. Earlier studies had used RAPD and multilocus enzyme electrophoresis to show the existence of the two evolutionary divergent groups in EPEC: one was genetically and serologically very homogeneous, while the other harboured the EPEC and the non-EPEC serotypes [18]. A similar result was observed in the present study, wherein, molecular typing could show the differences in banding patterns among the isolates which belonged to the same serogroup [Table/Fig 3 and 4] and the similarity among the isolates which belonged to the

same serogroup [lanes 27 and 28 in Table/Fig 3 and 4].

The ERIC sequences were found to be useful targets for molecular typing [13]. The different profiles which were observed in the present study appeared to be due to the differences in the ERIC sequences and due to the differences in the inter-ERIC distances. Earlier studies had indicated the similarity of the strains of *E.coli* which were isolated from the soil and vegetables which were irrigated by treated wastewater, by using ERIC PCR [13,19]. Two typical EPEC which belonged to the serogroup O111 which showed a similarity in their banding patterns [lanes 27 and 28 in Table/Fig 5 and 6] with both the ERIC primers also showed a similarity in RAPD with both the R1 and the R2 primers [lanes 27 and 28 in Table/Fig 3 and 4]. Hence, this study indicated that RAPD and ERIC PCR may be used as tools for the differentiation of the *E.coli* isolates which belonged to the same and different serogroups.

The diarrheagenic *E.coli* strains are not routinely sought as stool pathogens in many clinical laboratories. This study highlights the importance of the routine identification and the characterization of all the *E.coli* which were isolated from diarrheagenic stool samples. Molecular typing revealed that the typical and atypical EPEC which belonged to the different O serogroups corresponded to the different genetic clusters. The genetic similarities which were observed among the two typical EPEC strains in both RAPD and ERIC PCR indicated a common genetic origin or a common source. Further characterization is needed to prove the pathogenic potential of the untypeable *E.coli* strains (negative for virulence genes) which were isolated from the stool samples in the present study.

To the best of our knowledge, molecular typing has not been used in this part of the country to discriminate *E.coli* isolates from stool samples. However, an attempt was made in the present study, to study the genetic similarities and the differences among the various serogroups of the *E.coli* isolates from stool samples. Further studies which involve more number of samples and better discriminating molecular techniques are necessary to know the genetic similarities and the differences among the different diarrheagenic *E.coli* and commensal *E.coli*.

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