

Differentiation of Enterotoxigenic and Enteropathogenic *Escherichia coli* Isolated from Humans and Calves by rep-PCR

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ABSTRACT

To determine the virulence markers of enterotoxigenic *E. coli* [ETEC] and enteropathogenic *E. coli* [EPEC], 224 stool specimens from diarrheic humans and 60 diarrheic calves were examined by PCR. This study was conducted to achieve a better understanding of the genetic relationships and evolutionary patterns within *E. coli* pathotypes isolated from humans and calves. Stool specimens from 224 diarrheic humans and 60 diarrheic calves were cultured for *E. coli*. The samples were obtained from North West of Iran. The rep-PCR fingerprint technique, which performed with primer BOX A1R, was used for discriminating between human and calf sources. A total of 25 ETEC and EPEC strains isolated from humans and calves. The discriminant analysis showed an Average Rate of Correct Classification [ARCC] of 97% for ETEC and 94.5% for EPEC isolates. This result reveals that the rep-PCR fingerprint technique with primer BOX A1R may be an effective technique for discriminating and grouping *E. coli* isolates, and can be employed as a source following tool for differentiation and identification of host sources.

Key words: ETEC, EPEC, Human, Calves, rep-PCR.

INTRODUCTION

Diarrhoeal disease is a significant worldwide problem, especially in the developing countries¹. Diarrheagenic *Escherichia coli* are one of the most important etiologic agents of diarrhea, and a major agent of diarrhea leading to high morbidity and mortality, mostly among children in the developing countries^{2,3}. *E. coli* pathotypes are important pathogens in bovine neonates and the main cause of economic loss on farms^{4,5}. Cattle and sheep, are main reservoirs for the transmission of pathogenic *E. coli* to humans through the food chain^{6,7}.

Based on their specific virulence factors and different epidemiological and clinical features, they are divided into enterotoxigenic *E. coli* [ETEC], enteropathogenic *E. coli* [EPEC], enteroaggregative *E. coli* [EAEC], enteroinvasive *E. coli* [EIEC], enterohemorrhagic *E. coli* [EHEC] and diffusely adherent *E. coli* [DAEC]^{1,8,9}.

The identification of pathogenic *E. coli* requires finding agents that determine the virulence of these organisms; it cannot be based only on cultural and biochemical criteria. Polymerase Chain Reaction [PCR] is a usually used method based on the virulence-associated factors or genes. It is

capable of identifying *E. coli* pathotypes as well as other non-*E. coli* pathogens. With the finding of PCR, it has become feasible to detect virulence genes in bacterial isolates, allowing the fast and reliable recognition of pathogenic *E. coli*¹⁰.

In order to find effective control plans, it is necessary to define the source of fecal pollution and clonal relatedness of diarrheagenic *E. coli*^{11,12}. Nowadays, there are a number of DNA analysis techniques for discriminating between human and animal origins of fecal contamination. These methods include pulsed-field electrophoresis [PFGE], ribotyping, ribosomal DNA heterogeneity, and repetitive extragenic palindromic-PCR [rep-PCR]. There have been several works of analytical techniques for finding human and animal origins of fecal contamination¹³.

This study was conducted to achieve a good perception of the genetic relationships and evolutionary patterns within *E. coli* pathotypes isolated from humans and calves. The rep-PCR DNA fingerprint, which uses repetitive extragenic DNA sequences, was examined as a method for grouping the EPEC and ETEC isolates. This method is simple, fast and less expensive than PFGE, and has proven to work well to distinguish between species of *E. coli* pathotypes.

MATERIALS AND METHODS

Bacterial sources and isolation

Stool specimens from 224 diarrheic humans and 60 diarrheic calves were cultured for *E. coli*. The isolates were obtained from North West of Iran. The samples were collected using sterile rectal swabs, inoculated in Cary Blair medium tubes, and carried to the laboratory. They were cultivated on MacConkey agar (Merck, Germany) and incubated at 37°C for 24 h, two to three lactose positive per plate were selected and submitted to the biochemical tests. The isolates were stocked in trypticase soy broth supplemented with 20% glycerol at -80°C for further procedures.

DNA extraction

Bacterial DNA extraction was taken by using the Promega DNA extraction kit (A11125,

USA). The extracted DNA was used as a template for PCR according to the described protocols.

Detection of virulence genes by PCR

The DNA templates were subjected to polymerase chain reaction with appropriate primers (Table 1) for detection of the following virulence factors: *eae* and *bfp* for EPEC, *lt* and *st* for ETEC labile and stable toxins, respectively. The PCR assays were accomplished in a 25 µl reaction mixture, consisting of 2X PCR Master Mix (2X concentrated solutions of *Taq* DNA polymerase, reaction buffer, MgCl₂ and dNTPs) with a BioRad T100™ thermal cycler. 2X PCR Master Mix (CinnaGen Inc.) contain all components for PCR, except DNA template and primers. Primers were provided by GeNetBio Inc. (Korea).

A duplex-PCR was optimized for detection of ETEC. The PCR conditions for *lt* and *st* amplification were: predenaturation at 95°C for 5 min for one cycle followed by 35 cycles of 95°C for 45 sec, 49°C for 45 sec, 72°C for 45 sec and final extension at 72°C for 7 min. The second duplex-PCR was standardized for detection of EPEC. The PCR conditions for *eae* and *bfp* determinants of EPEC were: predenaturation at 95°C for 3 min for one cycle followed by 38 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 1min and final extension at 72°C for 10 min. Amplified PCR products were observed after electrophoresis on 1% agarose and staining with safe dye. The PCR products were visualized under UV transilluminator and photographed.

The rep-PCR condition

The rep-PCR assay was carried out with 50 ng template DNA and 2 µM BOX A1R primer (52-CTA CGG CAA GGC GAC GCT GAC G-32)¹⁴. The rep-PCR amplifications were performed with predenaturation at 95°C for 2 min 35 cycles including of denaturation at 94°C for 30 sec and 55°C for 1 min, and annealing at 65°C for 1 min, followed by a single step extension at 65°C for 8 min. The separation of amplified DNA fragments was achieved by electrophoresis on 1% agarose gel with 1kb and 100bp DNA ladder (GeNetBio Inc. Korea). The gels were stained with safe dye, and imaged under UV illumination.

RESULTS

Diarrheagenic *E. coli* was isolated from 26.8% of the humans with diarrhea and 83.3% of the calves with diarrhea. Distribution of ETEC and EPEC is shown in Table 2. We found the incidence of ETEC, typical EPEC and atypical EPEC pathotypes in humans as 16.6%, 1.6%, 3.3%, and in calves were 4%, 8%, 12%, respectively. Fig. 1 shows the typical fingerprint for *E. coli* isolates by using rep-PCR carried out with BOX A1R primer. All strains were studied in terms of the electrophoretic profiles of their DNA fragments. The fingerprinting generated separate bands ranging in size from 500 to 2000 bp. The band patterns of *E. coli* pathotypes from humans and calves were very similar, and it was deduced that the strains were closely related.

The rep-PCR fingerprints were handily determined and statistical analysed with NTSYS-pc program (Ver.2.2). The dendrogram in Fig. 2 shows the clonal structure and the genetic

relationships of the 25 strains isolated from humans and calves. It was generated using Jaccard similarity coefficients and UPGMA algorithm, and produced two major clusters (A, B). Cluster A is genetically homogenous including the majority of ETECs obtained from humans. In contrast to cluster

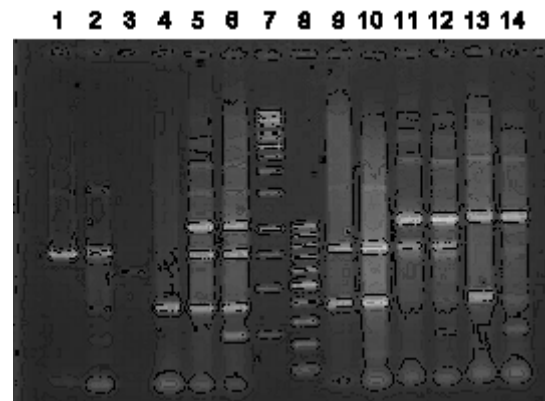


Fig. 1: rep-PCR DNA fingerprint patterns of *E. coli* obtained from humans and calves. Lane 7: DNA molecular size marker (1kb ladder), Lane 8: DNA molecular size marker (100bp ladder)

Table 1: Primers used for detection of ETEC and EPEC virulence genes

Target organism	Target genes	Gene location	Primer sequences (5'!3')	Product size
ETEC	<i>st</i>	Plasmid	ATT TTT ATT TCT GTA TTA TCT T CAC CCG GTA CAT GCA GGA TT	190
	<i>lt</i>	Plasmid	GGC GAC AGA TTA TAC CGT GC CGG TCTCTA TAT TCC CTG TT	450
EPEC	<i>eae</i>	Chromosome	AGG CTT CGT CAC AGT TG CCA TCG TCA CCA GAG GA	570
	<i>bfp</i>	Chromosome	AAT GGT GCT TGC GCT TGC TGC GCC GCT TTA TCC AAC CTG GTA	326

Table 2: Distribution of positive ETEC and EPEC samples in calves and humans

Target organism	Calves No (%)	Humans No (%)	Virulence genes in Calves isolates (%)	Virulence genes in humans isolates (%)
ETEC	2 (4%)	10 (16.6%)	<i>lt</i> (2%) <i>st</i> (0.0%) <i>lt, st</i> (2%)	<i>lt</i> (10%) <i>st</i> (1.6%) <i>lt, st</i> (5%)
tEPEC	4 (8%)	1 (1.6%)	<i>eae, bfp</i> (8%)	<i>eae, bfp</i> (1.6%)
aEPEC	6 (12%)	2 (3.3%)	<i>eae</i> (12%)	<i>eae</i> (3.3%)

A, cluster B is more heterogenous, and includes the remaining 18 strains (72%), which have different subclusters. These subclusters contain both human and calf ETECs and EPECs.

The strains were manually assigned to the correct group, and a Jackknife analysis was used to determine how precisely the similarity coefficients predicted the source group. According to findings of the present study, humans ETEC, calves ETEC, humans EPEC and calves EPEC were highly classified with rate of correct classification [RCC] of 96%, 98%, 94%, and 95%, respectively. Also, humans and calves isolates were

highly classified with an average rate of correct classification [ARCC] of 97% for ETEC and 94.5% for EPEC isolates. It was deduced that both cluster and Jackknife analyses are suitable statistical methods for bacterial source tracking.

Matrix correlation, a clustering method based on correlation and dependence, can be used to investigate any statistical relationship between multiple random variables at the same time and it is very useful to find the most correlated variables in a data table having the correlation coefficient between each variables. The correlation coefficient, denoted by *r*, values between -1.0 to +1.0. The closer

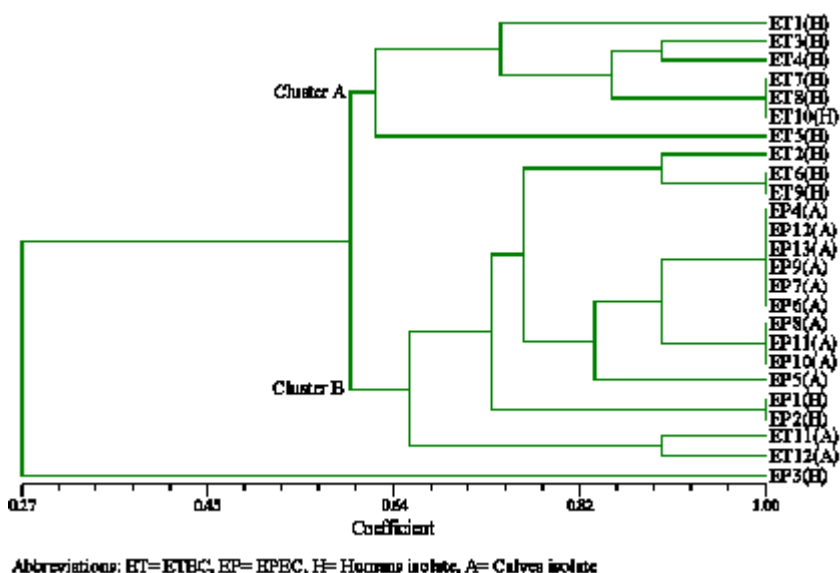


Fig. 2: Dendrogram illustrates the clonal structure and the genetic relationships of the 25 strains isolated from humans and calves

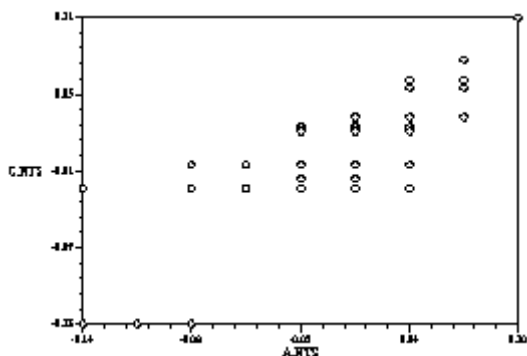


Fig. 3: Plots 2-way scatter diagrams of rows or columns of matrix

r is to +1 or -1, the more strong relation the two variables have. If *r* is close to 0, it means there is no linear correlation between the variables¹⁵. In present study, tests for association were generated by Matrix correlation plot using an option that accounted for comparison of similarity matrices (Fig. 3). According to the results, correlation coefficient is 0.8. It means that variables are closely related.

DISCUSSION

ETEC and EPEC are two of the six pathogenic *E. coli* identified in our study. Among the recognized *E. coli* pathotypes, ETEC is very

important and usual, particularly in the developing countries¹⁶. ETEC can be dangerous due to serious fluid loss and critical dehydration. Beyond its burden in endemic areas, ETEC is the main reason of diarrhea in travelers to developing countries^{6,9}. ETEC also causes diarrhea in new born calves and it is one of the main causes of economic loss in farms⁵. EPEC strains have *eae* gene but do not produce Shiga toxin, which can cause diarrhea in humans and different animal species¹⁷. The results further showed the significance of ETEC as the reason of human diarrhea in the analyzed area the North West of Iran. Similar results have been reported by Tornieporth *et al.*,¹⁸ and Vilchez *et al.*,¹. Our findings are the documented results by Bueris *et al.*, in Salvador, Bahia¹⁰. However, in a study carried out by Osman *et al.*,⁷ the results obtained from the calve diarrhea were in contrast to our findings.

RCC values of the present study are within the range of RCC values showed by Carson *et al.*,¹⁹ and Dombek *et al.*,¹². But, they were higher than the values found by Seurink *et al.*,²⁰ and Mohapatra *et al.*,¹¹. The ARCC obtained in present work is different in comparison with the results described by Mohapatra *et al.*, and Carson *et al.*, with *E. coli* isolates from human and animal sources^{11,19}. Also the results of our study were comparable to the other studies, worked with fecal *E. coli* isolates from different host groups^{12,20,21}.

Up to now, there is no definite standard of precision available to bacterial source tracking methods. Any Classification Technique with a rate of correct classification from 60%-70% is considered to be useful for the fecal pollution control authorities to prevent more infections¹¹.

Based on the discriminatory power and higher RCC, BOX A1R- PCR was found to be appropriate technique for discrimination of fecal *E. coli* isolates from different sources. BOX A1R- PCR genomic fingerprinting, which is fast, low cost and easy, may be used as a supplementary molecular tool for detection of fecal *E. coli* origins. Latter surveys should be carried out to determine efficacy of BOX A1R-PCR with fecal *E. coli* isolates from other possible sources of fecal contamination as well as with other usual techniques, such as ribotyping and antibiotic resistance analysis before its application in field studies⁷. Our study is the first and most comprehensive report on genetic relationship in ETEC and EPEC pathotypes isolated from diarrheic calves and humans in Iran. It was deduced that, calves could be a source of infection to humans as the major pathogens previously explained and associated with severe disease in humans.

In conclusion, our results indicate that rep-PCR is able to discriminate between ETEC and EPEC strains. We believe that rep-PCR can be an appropriate method for differentiating and clustering the *E. coli* isolates. This method could further be helpful for determining the sources of closely associated *E. coli* strains obtained from environmental samples.

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