

Virulence Characteristics, Antibiotic Resistance Patterns and Molecular Typing of Enteropathogenic Producing *Escherichia coli* (EPEC) Isolates in Eastern Province of Saudi Arabia: 2013–2014

Lamya Zohair Yamani , Nasreldin Elhadi 

Department of Clinical Laboratory Science, College of Applied Medical Sciences, Imam Abdulrahman Bin Faisal University, Dammam, Kingdom of Saudi Arabia

Correspondence: Nasreldin Elhadi, Department of Clinical Laboratory Science, Imam Abdulrahman Bin Faisal University, Dammam, Kingdom of Saudi Arabia, Email nmohammed@iau.edu.sa

Introduction: The Kingdom of Saudi Arabia has limited data on enteropathogenic *Escherichia coli* (EPEC). Therefore, this study was undertaken to contribute to EPEC surveillance and investigate the molecular epidemiology of EPEC strains that have been implicated in human infection in King Fahd Hospital of the University (KFHU) between 2013 and 2014 in the Eastern Province of Saudi Arabia.

Methods: A total of 60 non-duplicate *E. coli* isolates associated with human gastroenteritis were included in this study. They were characterized using PCR to determine virulence genes, antimicrobial resistance patterns, and enterobacterial repetitive intergenic consensus (ERIC-PCR).

Results: Among the 60 strains, 20% of those examined were positive for the intimin *eae* and *bfpA* genes and identified as typical EPEC (tEPEC). Furthermore, 44 of *E. coli* strains tested positive for the *eae* gene only and revealed a high occurrence rate of 73.3% of atypical EPEC (aEPEC) within the overall examined strains. All strains were positive for the EAST1 gene, and none tested positive for the *stx* gene. More than 70% of EPEC strains were multi-drug resistant (MDR) and aEPEC strains with the highest proportion of this feature of MDR. ERIC-PCR fingerprint revealed a total of 19 ERIC types with eight related distinct clusters and a similarity rate cut-off with $\geq 90\%$ homology from the identified isolates.

Conclusion: A high antibiotic resistance rate was reported for first-line antibiotics, such as ampicillin, tetracycline, nalidixic acid, trimethoprim-sulfamethoxazole, noroxin, and ciprofloxacin.

Keywords: EPEC, virulence, antimicrobial resistance, MDR, ERIC-PCR

Introduction

The uniqueness of *Escherichia coli* (*E. coli*) lies in its versatile nature compared to many microorganisms. It is an important member of the normal intestinal flora, where the colonization of the gastrointestinal tract begins within a few hours after birth.¹ The apparent coexistence between these commensals and hosts has led to mutual benefits. They have been observed to colonize the mucosal membranes of the colon, forming niches in that area. Their presence has rarely been shown to cause disease unless the host was immunocompromised or in instances where the barriers of the intestinal tract have been defective or breached.² *E. coli* strains that have been known to cause infections were found to possess highly potent virulence attributes that support their adaptation within new niches causing extensive complications, leading to a diseased state. Around the world, deaths of children under the age of 5 years have been credited to diarrheal diseases in underdeveloped countries, where the highest implication has been due to diarrheagenic *E. coli* (DEC).^{3–5} DEC strains were quite difficult to detect as they had major similarities with their normal counterpart and were unable to be distinguished from one another until PCR techniques made it possible

to detect virulence genes found only within the pathogenic strains.⁶ Extraintestinal pathogenic *E. coli* (ExPEC) has been observed to cause infections related to diarrheal/enteric disease, meningitis or sepsis, and urinary tract infections.⁷ These ExPEC are results of successful combinations with diverse virulence factors that persist within the host system.⁷ All *E. coli* pathotypes can be grouped together based on shared O lipopolysaccharide (LPS) and H flagellar antigens, which make up the serogroup O antigen, or individual serotypes related to O and H antigens.^{3,8}

Enteropathogenic *E. coli* (EPEC) falls under the pathotype category of intestinal pathogens. It was the first to be discovered, as there was a large outbreak of infant diarrhea in the United Kingdom. The causative agent for the outbreak was not well understood, but strain isolation was performed, and since then, many advances have been made pertaining to EPEC mode of transmission and pathogenesis cycles.^{5,7,9} EPEC infections have characteristic intestinal histology that is known as “attaching and effacing”. The strains are observed to attach themselves to the epithelial cells of the intestine, causing drastic cytoskeletal changes leading to polymerization of the actin filaments.¹⁰ The intestinal microvilli become effaced, and the pathogenic bacteria will be able to move, causing infectious spread. The ability of this strain to have such characteristic histology lies within its pathogenicity island, locus of enterocyte effacement (LEE). LEE encodes a protein intimin, which is needed for EPEC attachment. EPEC attaches to epithelial cells within the intestine.^{7,11} This site also encodes the *eae* gene. EPEC strains can be divided into either “typical” or “atypical” strains. This division is based on whether they have the EPEC adherence factor (EAF) plasmid.¹² This plasmid encodes a type IV pilus named the bundle-forming pilus (BFP) needed for bacterial strain adherence to intestinal epithelial cells.¹³ Atypical EPEC (aEPEC) does not have this EAF plasmid. Thereby, strains that possess both *eae+* *bfpA+* are classified under typical EPEC (tEPEC), while those that are *eae+* but lack *bfpA-* are said to be aEPEC. aEPEC has been isolated more frequently in diarrheal cases from developed countries, whereas; tEPEC isolation has been found more often in developing countries.^{4,14}

An increasing concern relates to antibiotic resistance with increased use as a method of treatment, as there have been clinical reports of an increased occurrence of resistance to third-generation cephalosporin bacteria in Saudi Arabia.^{15,16} One study looking at drug resistance foretold that approximately ten million people would suffer fatally from antibiotic-resistant diseases annually by the year 2050. *E. coli* infections are among the bacterial infections that have high death predictions by antibiotic-resistance.¹⁷ Another group advised that infections caused by *E. coli* strains should not be limited to antibiotic usage as a treatment method to prevent emerging antibiotic resistance with high risks of severe complications leading to hemolytic uremia. *E. coli* strains that are either pathogenic or non-pathogenic have developed ways to assist in their resistance to antibiotics by utilizing diverse mechanisms. In Saudi Arabia, EPEC outbreaks have been very limited or seldom reported.¹⁸ Studies describing the clinical prevalence of diarrhea among Hajj pilgrims have reported infectious spread and diarrhea that were associated with *E. coli* strains. EPEC attributed to diarrheal during the 2013 and 2016 Hajj pilgrimages.¹⁹ Therefore, this study was undertaken to contribute to EPEC surveillance and investigate the molecular epidemiology of EPEC isolates that have been implicated in human infection between 2013 and 2014 in the Eastern Province of Saudi Arabia.

Materials and Methods

Bacterial Strains Al-Khobar, Saudi Arabia

A total of 60 non-duplicate *E. coli* isolates which were previously isolated from humans showing gastroenteritis symptoms of diarrhea between 2013 and 2014 were characterized in this study. These isolates were collected from the culture collection of the microbiology laboratory at KFHU, Al-Khobar, Saudi Arabia. Clinical information on patients from which the isolates of EPEC were isolated was unavailable. The collected bacterial isolates were further inoculated onto MacConkey agar medium and followed by 18–24 h incubation at 37°C in order to isolate pure *E. coli* colonies. Isolated colonies of EPEC isolates were further tested using standard biochemical tests and API20E (BioMérieux, France) for validation. Isolates were further examined for EPEC virulence gene signatures using PCR.

Table 1 Primers Used in This Study

Target Gene	Primer Sequence (5'-3')	Amplicon Size (bp)	Reference
<i>eae</i>	Forward: TCAATGCAGTTCGGTTATCAGTT Reverse: GTAAAGTCCGTTACCCCAACCTG	482	[20]
<i>bfp</i>	Forward: GGAAGTCAAATTCATGGG GGTAT Reverse: GGAATCAGACGCAGACTGGTAGT	300	[20]
<i>stx1</i>	Forward: CAGTTAATG TGGCGAAGG Reverse: CACCAGACAATGTAACCGCTG	348	[20]
<i>stx2</i>	Forward: ATCCTATTCCCG GGA GTTTACG Reverse: GCGTCATCGTATACACAG GAGC	584	[20]
EAST1	Forward: TGCCATCAACACAGTATATCCG Reverse: ACGGCTTTGTAGTCCTTCCAT	102	[21]

DNA Extraction and PCR Assay

Pure isolated colonies of EPEC isolates were cultured in 5 mL of Luria Bertani (LB) broth and incubated overnight at 37°C. Then, 1.0 mL of incubated cultures were centrifuged at $10,000 \times g$ for 2 min, and pellets were re-suspended in 0.5 mL sterilized distilled water. All isolate pellets were vortexed for 2 min and boiled at 100°C for 15 min. Furthermore, the boiled tubes were centrifuged at $12,000 \times g$ for 5 min, and supernatant DNA was transferred into 1.2 mL sterilized tubes and stored at -20°C until used for PCR assay of virulence genes and molecular typing of EPEC isolates. The extracted DNA was used as a template in all PCR reactions to screen EPEC virulence gene markers using the previously described protocol,^{20,21} as presented in Table 1, and positive controls were included in each PCR run.

Antimicrobial Susceptibility Testing

The antimicrobial agent susceptibility testing for all EPEC isolates was examined following the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.²² The antimicrobial disks used were ampicillin (AM, 25 µg), aztreonam (ATM 30 µg), augmentin (AUG 30 µg), cefotaxime (CTX 30 µg), chloramphenicol (C 30 µg), cephalothin (KF 30 µg), ciprofloxacin (CIP 5 µg), cefepime (FEP 30 µg), gentamicin (GM 5 µg), nalidixic acid (NA 30 µg), noroxin (NOR 10 µg), piperacillin PRL (100 µg), streptomycin (S 10 µg), trimethoprim-sulfamethoxazole (SXT 25 µg) and tetracycline (TE 30 µg). All the antibiotic disks were obtained from Oxoid (Basingstoke, Hampshire, UK). The zone of inhibition diameter was measured in mm using Vernier calipers and results were interpreted as sensitive (S), intermediate (I) and (R) resistant according to CLSI breakpoints and for pathogenic *E. coli* with the help of protocol published elsewhere.²² The strain of *E. coli* ATCC 25922 was used as a control for antibiotic susceptibility testing.

ERIC-PCR Typing

The ERIC2 primer ('5-AAGTAAGTGACTGGGGTGAGCG-3') was used for typing EPEC isolates as described previously.^{23,24} The Gelj software²⁵ was used for genetic similarity analysis. The construction of the dendrogram clusters and dice correlation coefficient calculations were obtained using the unweighted average pair group method (UPGMA). Optimization and band position tolerance were adjusted to 1%. The similarity coefficient of 90% cut-off was used for cluster groups.

Statistical Analysis

The antibiotic susceptibility testing results for resistance, intermediate, and sensitivity were observed, and the difference between the groups (tEPEC, aEPEC and non-EPEC) was investigated using the Kruskal–Wallis test. Following the test, post hoc comparisons between groups were performed using the Mann–Whitney *U*-test with Bonferroni corrected *p*-values. The analysis was completed using PAST statistical software.²⁶

Table 2 Distribution of Virulence Genes in EPEC Isolates

Virulence Gene Profile	No. (%) of EPEC
<i>eae</i> ⁺ <i>bfpA</i> ⁻ (atypical EPEC)	44 (73.3)
<i>eae</i> ⁺ and <i>bfpA</i> ⁺ (typical EPEC)	12 (20%)
<i>eae</i> ⁻ <i>bfpA</i> ⁻	4 (6.7)
<i>Stx1</i> , <i>Stx2</i>	0
EAST I	60 (100%)

Results

Occurrence of Virulence Genes

As presented in Table 2, 12 (20%) and 4 (6.7%) of the examined isolates tested positive for intimin *eae* and *bfpA* genes and were identified as tEPEC and negative isolates for both genes were identified as non-EPEC, respectively. The most prevalent among the examined *E. coli* isolates was aEPEC, with occurrence rate of 44 (73.3%), as shown in Table 2. Despite the fact that all 60 isolates tested positive for the EAST1 gene, no *stx* gene was found positive (Table 2).

Antibiotic Resistance Patterns

Results of antibiotic susceptibility testing for 15 types of antibiotics are presented in Table 3. The overall antibiotic resistance prevalence rates among the 60 strains of *E. coli* were: ampicillin (63.3%), tetracycline (60%), nalidixic acid (55%), trimethoprim-sulfamethoxazole (48.3%), noroxin (36.7%), and ciprofloxacin (35%). Among the categories of EPEC, aEPEC isolates were observed with high resistance > 35% for ampicillin, tetracycline, nalidixic acid, and trimethoprim-sulfamethoxazole (Table 3). Findings of these studies demonstrated that 70% of investigated isolates were found to be MDR (Figure 1). Table 3 shows the resistance of the three groups of tEPEC, aEPEC, and non-EPEC isolates that were compared using the Kruskal–Wallis test. The test showed significant differences between the three groups of tEPEC, aEPEC, and non-EPEC strains. Similarly, for intermediate and sensitive, the difference between the three groups was significant. For resistance, the post hoc comparison showed that Bonferroni corrected p-values were significant for tEPEC vs aEPEC and tEPEC vs non-EPEC. For intermediate strains, the post hoc comparison showed that only tEPEC vs non-EPEC was significant. For sensitive isolates, all three post hoc tests were significant (Table 3).

ERIC Typing

The ERIC products ranged in size between 250 to 2500 bp, and DNA fingerprint patterns ranged between 5 to 13 bands among analyzed isolates (Figure 1). The ERIC typing method was able to genotype all isolates from humans between 2013 and 2014 of tEPEC, aEPEC, and non-EPEC into detailed cluster groups generated by the Gelj software (Figure 1). Before clustering isolates with a high degree of DNA fingerprints homology can be identified and labeled, a cut-off value of 90% genetic similarity was defined (Figure 2). Based on the ERIC typing dendrogram, 19 ERIC types and eight related clusters (A to H) with ≥ 90% homology of isolates were identified (Figure 2). At the same time, 11 (18.3%) isolates had individual, different ERIC fingerprint type lineages (1 to 7 and 9 to 12). Cluster F, G, and H comprised 58.3% of examined isolates of EPEC in this study. The highest number of tEPEC and aEPEC isolates of the year 2013 were grouped in cluster H, and two isolates of non-EPEC were grouped together in cluster A. The ERIC-PCR dendrogram in this study showed high genetic diversity, and there was no correlation observed between the phylogenetic relationship among the 60 isolates of EPEC. However, the result indicates a variation in tEPEC, aEPEC, and non-EPEC isolates is implicated in the human infection between 2013 and 2014 in Eastern Province of Saudi Arabia. Based on the obtained results, ERIC-PCR is a more suitable discriminative DNA fingerprinting method to analyze the genetic diversity of investigated EPEC isolates in this study.

Table 3 Antibiotic Susceptibility of Typical, Atypical EPEC and Non-EPEC Isolated from Patients with Diarrhea

Antibiotic Agent	Isolates No. (%) for											
	Resistance			p-value	Intermediate			p-value	Sensitive			p-value
	tEPEC	aEPEC	non-EPEC		tEPEC	aEPEC	non-EPEC		tEPEC	aEPEC	nonEPEC	
AM	7 (11.7)	30 (50)	1 (1.7)	Kruskall-Wallis, H (corrected)=26.84 p= 0.001 Post-hoc: tPEC vs aEPEC: p=0.00027 tPEC vs non-EPEC: p=0.1441 aPEC vs non-EPEC: p= 0.001	0	0	0	Kruskall-Wallis, H (corrected)=10.18 p=0.00616 Post-hoc: tPEC vs a-EPEC: p=0.008591 tPEC vs non-EPEC: p= 1 aPEC vs non-EPEC: p=0.01768	5 (8.3)	14 (23.3)	3 (5)	Kruskall-Wallis, H (corrected)=39.48 p=0.001 Post-hoc: tPEC vs aEPEC: p=0.0001 tPEC vs non-EPEC: p=0.001 aPEC vs non-EPEC: p= 0.001
ATM	0	7 (11.7)	0		0	3 (5)	0		12 (20)	34 (56.7)	4 (6.7)	
AUG	1 (1.7)	2 (3.3)	0		0	1 (1.7)	0		11 (18.3)	41 (68.3)	4 (6.7)	
CTX	0	11 (18.3)	0		0	0	0		12 (20)	33 (55)	4 (6.7)	
C	0	11 (18.3)	0		1	2 (3.3)	1 (1.7)		11 (18.3)	31 (51.7)	3 (5)	
KF	1 (1.7)	11 (18.3)	0		2	1 (1.7)	0		9 (15)	32 (53.3)	4 (6.7)	
CIP	4 (6.7)	16 (26.7)	1 (1.7)		0	1 (1.7)	0		8 (13.3)	27 (45)	3 (5)	
FEP	0	1 (1.7)	1 (1.7)		0	2 (3.3)	0		12 (20)	41 (68.3)	3 (5)	
GM	0	6 (10)	0		0	0	0		12 (20)	38 (63.3)	4 (6.7)	
NA	6 (10)	26 (43.3)	1 (1.7)		0	0	0		6 (10)	18 (30)	3 (5)	
NOR	4 (6.7)	18 (30)	0		0	1 (1.7)	0		8 (13.3)	25 (41.7)	4 (6.7)	
PRL	1 (1.7)	14 (23.3)	0		0	1 (1.7)	0		11 (18.3)	29 (48.3)	4 (6.7)	
S	4 (6.7)	13 (21.7)	1 (1.7)		0	0	0		8 (13.3)	31 (51.7)	3 (5)	
SXT	5 (8.3)	22 (36.7)	2 (3.3)		0	0	0		7 (11.7)	22 (36.7)	2 (3.3)	
TE	6 (10)	29 (48.3)	1 (1.7)		0	0	0		6 (10)	15 (25)	3 (3.3)	

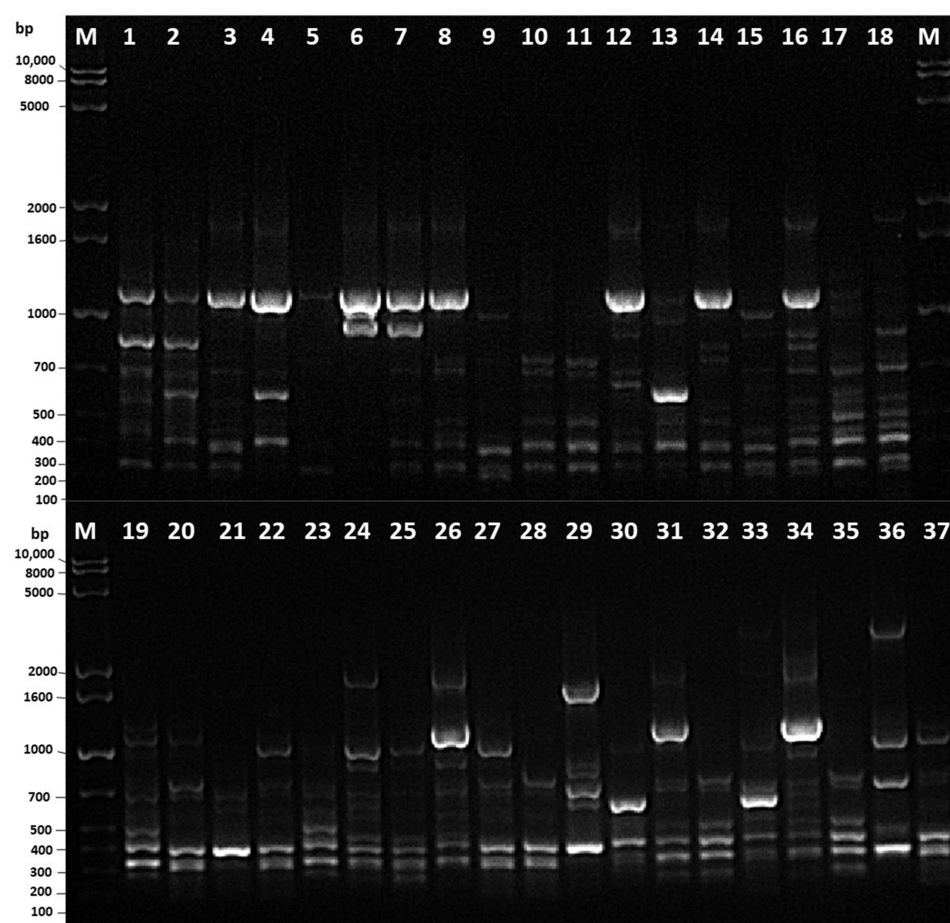


Figure 1 Representative gel electrophoresis of ERIC-PCR using ERIC2 primer. Lane 1: molecular mass ladder and lanes 2 to 20: isolates of typical and atypical EPEC from humans in the Eastern Province of Saudi Arabia.

Discussion

EPEC is an important pathogen among the DEC pathotypes and is a known causative agent of persistent diarrhea in children and adults worldwide.^{4,9} Strains of EPEC isolated from fecal specimens during sporadic and emerging cases are divided into tEPEC and aEPEC.^{14,27,28} The tEPEC is usually responsible for causing acute diarrhea in infants and children in developing countries, while the aEPEC is a causative agent of infection in children and adults worldwide.^{4,29,30} In this study, we investigated the frequency of EPEC categories, virulence genes, antibiotic resistance patterns, and genetic relationships among EPEC isolates implicated in the human infection between 2013 and 2014 in Eastern Province of Saudi Arabia. Few studies conducted in Saudi Arabia have been documented regarding DEC pathotypes implicated in human infections.³¹ Our study is consistent with several recent epidemiological studies that have reported an increased emergence and identification of aEPEC among EPEC infections, and there is an association with diarrheal outbreaks in both developing and developed countries.^{30,32–38}

All strains in this study were positive for the EAST1 gene and negative for the *stx* gene (Table 2). The EAST1 gene is originally recognized in strains of an enteroaggregative *Escherichia coli* (EAEC) isolated from a Chilean children's stool specimen while suffering diarrhea.³⁹ Our study is in concordance with several studies that have proven that gene encoding EAST1 is not restricted to EAEC and broadly distributed among DEC pathotypes and other human enteric pathogens.^{40–43} Strains expressing the EAST1 gene have been associated with inducing diarrhea in children and adults.^{41,44} Although some strains of *E. coli* isolated from human diarrheal cases were lacking the known virulence determinants other than the EAST1 gene thus making it a potential virulence factor.^{41,45} During the second international

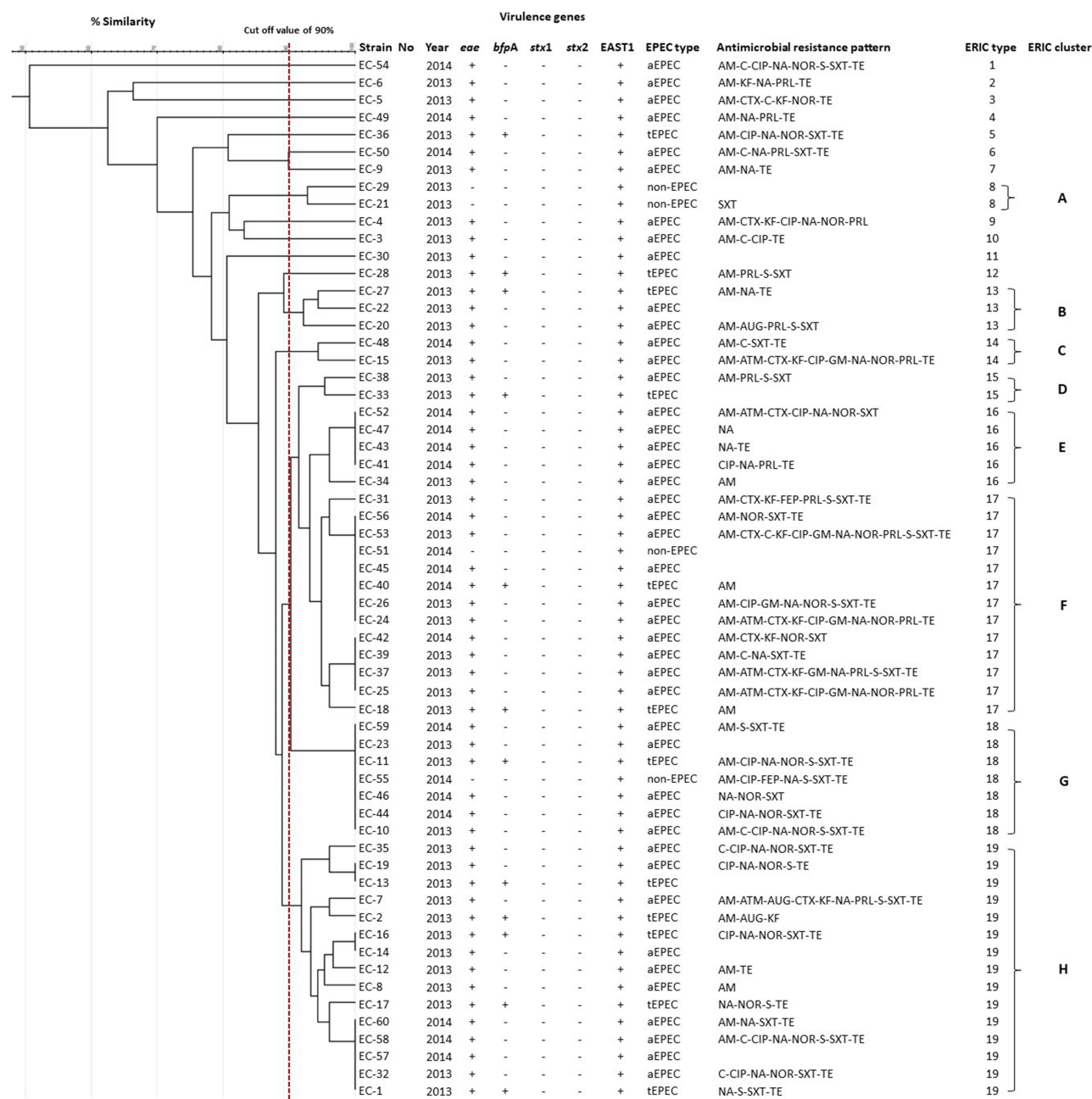


Figure 2 UPGMA dendrogram of the genetic relationships among 60 isolates of EPEC isolated from humans between 2013 and 2014 in the Eastern Province of Saudi Arabia. The arrow above percentage similarity scale indicates the cutoff value of 90% for cluster analysis.

symposium on EPEC conducted in 1995 in São Paulo, Brazil, most of the participants had agreed and defined EPEC as DEC that produces characteristic histopathology known as attaching and effacing (A/E) on intestinal cells and not expressing Shiga-toxin genes.¹⁴ However, the tEPEC strains harbor a virulence plasmid known as the EPEC adherence factor (EAF) plasmid.³⁰ This plasmid is encoded by the bundle forming pilus (bfp), which is responsible for its adherence to intestinal epithelial cells. The presence of this EAF plasmid is an important marker to differentiate between strains of tEPEC and aEPEC.^{14,27,30}

Among overall isolates of EPEC analyzed in this study, more than 80% were resistant to at least one of the commonly prescribed antibiotics. These isolates were more often resistant to the first-line antibiotics such as ampicillin, tetracycline, nalidixic acid, and sulfamethoxazole-trimethoprim, and this might indicate the misuse of prescribed antibiotics in some

countries for the treatment of enteric infections caused by Gram-negative bacteria.³⁵ Moreover, 70% of EPEC isolates in this study (Figure 2) were classified and identified as MDR based on the most frequent method used to characterize bacterial strains as MDR if they are resistant to three or more antimicrobial agents.^{46–48} Similarly, a hospital-based study from China and Mexico reported a high prevalence of drug resistance among aEPEC isolated from children and adult patients with acute diarrhea, and the majority of strains were resistant to cephalosporins, fluoroquinolones and trimethoprim-sulfamethoxazole.^{35,37,49,50}

The analysis of ERIC fingerprinting patterns results by using Dice coefficient and UPGMA revealed that the genetic similarity relationship rate among EPEC isolates rate was 81.7% when clustered with the similarity between 90 to 100% (Figure 2). Whereas 11 (18.3%) isolates of EPEC had less than 90% similarity, as presented in ERIC dendrogram analysis (Figure 2). Our study agreed with a study conducted elsewhere,⁵¹ showing that strains with a similarity rate below 90% were considered genetically unrelated. The results of ERIC fingerprinting patterns generated using the ERIC2 primer in this study have indicated the potential and usefulness of ERIC-PCR as a molecular typing tool method for epidemiological investigation and genetic analysis of EPEC isolates. Overall, ERIC-PCR DNA fingerprinting has good discriminatory power and revealed genetic diversity among EPEC isolates investigated from humans in Eastern Province of Saudi Arabia.

Conclusion

To our knowledge, this is the first study to demonstrate detailed characterization of human EPEC isolates in Saudi Arabia. Genotyping for virulence gene signatures of EPEC revealed that most of the isolates were positive for *eae* gene and very few isolates were concurrently positive for *eae* and *bfp* genes. The majority of EPEC infections which occurred in Eastern Province of Saudi Arabia between 2013 and 2014 were caused by subtype “aEPEC” and these findings are in agreement with global trends of EPEC human infections. A high antibiotic resistance rate was reported for first-line antibiotics, such as ampicillin (63.3%), tetracycline (60%), nalidixic acid (55%), trimethoprim-sulfamethoxazole (48.3%), noroxin (36.7%), and ciprofloxacin (35%). The majority of EPEC isolates were MDR and aEPEC with the highest proportion of this feature of MDR. The results of the ERIC-PCR revealed high genetic similarity among the EPEC strains and demonstrated that ERIC-PCR is a rapid method for studying the clonal variation within EPEC associated with human gastroenteritis. This study recommends further future molecular epidemiology studies of EPEC to understand the epidemiology of EPEC by comparing EPEC isolates of human and food origin to trace the sources of human infection in Saudi Arabia.

Abbreviations

DEC; diarrheagenic *E. coli*, LPS; lipopolysaccharide, LEE; locus of enterocyte effacement, EAF; *E. coli* adherence factor, *eae*; *E. coli* attaching and effacing, *bfp*; bundle-forming pilus, *stx*; Shiga toxin, EAST-1; enteroaggregative heat stable enterotoxin 1, EPEC; enteropathogenic *E. coli*, aEPEC; atypical EPEC, tEPEC; typical EPEC, ERIC-PCR; enterobacterial repetitive intergenic consensus, PCR; polymerase chain reaction, MDR; multi-drug resistant, KFHu; King Fahd Hospital of the University, CLSI; Clinical and Laboratory Standards Institute, LB; Luria Bertani, AM; Ampicillin, ATM; Aztreonam, AUG; Augmentin, CTX; Cefotaxime, C; Chloramphenicol, KF; Cephalothin, CIP; Ciprofloxacin, FEP; Cefepime, GM; Gentamicin, NA; Nalidixic acid, NOR; Noroxin, PRL; Piperacillin, S; Streptomycin, SXT; Trimethoprim-sulfamethoxazole, TE; Tetracycline, S; sensitive, I; intermediate, R; resistant, CLSI; Clinical Laboratory Standards Institute, ATCC; American type culture collection, UPGMA; unweighted pair group method with arithmetic average.

Ethical Approval

This study was approved by the Institutional Review Board (IRB) of Imam Abdulrahman Bin Faisal University (IRB approval number: IRB – 2022 –03 –220).

Disclosure

The authors report no conflicts of interest in this work.

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