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Research article

Antibiotic resistance and molecular characterization of enteroaggregative *Escherichia coli* isolated from patients with diarrhea in the Eastern Province of Saudi Arabia

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ABSTRACT

Aim: To investigate presence of Enteroaggregative *Escherichia coli* (EAEC) in patients suffering with diarrhea by targeting the *pCVD*432 (*pAA)* gene using PCR.
Methods: There were 63 non-duplicate isolates of *E. coli* isolated from diarrheal cases in teaching hospital in Eastern Province of Saudi Arabia between May 2013 to July 2014. All *E. coli* strains were examined for antibiotic susceptibility testing and polymerase chain reaction (PCR) for detection of virulence gene markers for EAEC.
Results: Of the 63 *E coli* strains that were reported with diarrheal cases, 35 (55.6%) EAEC were tested positive for *pCVD*432 gene and *agg*R gene was present in 19 (54.3%) strains. All strains tested positive for *pCVD*432 and *agg*R genes were classified as typical EAEC (tEAEC). EAEC revealed resistance to tetracycline, ampicillin, nalidixic acid, trimethoprim sulfamethoxazole, ciprofloxacin, streptomycin, noroxin, and piperacillin.
Conclusion: EAEC was detected for the first time, among Saudi patients with diarrhea in this region of Saudi Arabia. The reported antibiotic resistance in this study is considered high among isolated EAEC strains to routinely prescribed antibiotics in our area.

1. Introduction

Enteroaggregative *Escherichia coli* (EAEC) is among the most remarkable heterogeneous strains of emerging *E. coli* that are responsible from causing persistent watery diarrheal cases in children and adults worldwide [1]. EAEC was determined to be a diarrheal enteric pathogen due the distinctive features of aggregative adherence (AA) pattern to HEp-2 cells in culture as well as its ability to form a "stacked-brick" adherence pattern, which is harboring a 60-MDa plasmid (*pAA*) [2]. However, the HEp-2 cell assay test is remaining as the gold standard technique for the confirmation of EAEC [3, 4, 5]. Therefore, this test is a diagnostic assay that is not suitable for most laboratories worldwide because it is too laborious and need to be carried out only in reference research laboratories with cell culture, it requires cell culture setup, [6]. At the molecular level, polymerase chain reaction (PCR) is most suitable and reliable techniques for identification of virulence gene markers of EAEC in suspected diarrheal cases.

The *pAA* plasmid in EAEC is encoding AA fimbriae (AAF) from I to IV genes [7]; antiaggregating protein dispersin (app); entroaggregative heat stable enterotoxin 1 (EAST-1) also known as *astA*) [8] and the gene encoding the transcriptional activator of virulence genes (AggR) [9]. The aggR gene is playing significant role in adherence and pathogenesis of EAEC, however, any strain harboring *aggR* gene is considered typical EAEC (tEAEC) strain [9]. The AggR promotes the expression of plasmid harboring the virulence factors, since its role as a transcriptional activator [7]. The surveillance of antibiotic resistance is very important in bacterial isolates due to continuous application of antibiotics in treatment of enteropathogenic infection and cephalosporins and fluoroquinolones are the most common used. Continuous surveillance and documentation of antibiograms resistance patterns will provide useful information to treatment and control the spread of antibiotic resistance.

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In Saudi Arabia, there is no information on the prevalence of EAEC and other diarrheagenic *E. coli* (DEC) pathotypes isolates and their antimicrobial resistance pattern. Therefore, the aims of this study were as follows: 1) to investigate the pAA plasmid gene in *E. coli* strains isolated from inpatients and out patients with cases of diarrhea by using PCR; 2) to analyze the strains harboring *pAA* plasmid for the prevalence of virulence genes that are associated with EAEC; and 3) to carry out antibiotic susceptibility to find out the resistance pattern in all *E. coli* strains, and to analyze all strains using enterobacterial repetitive intergenic consensus (ERIC-PCR) to track clonal relationship.

2. Materials and methods

2.1. Fecal samples strains

There were 63 strains of *E. coli* isolated and collected from children and adults patients with diarrheal episodes in King Fahd teaching hospital in Al-Khobar between May 2013 and July 2014. Among the 63 *E coli* strains included in this study, 52 and 11 strains were isolated from adults and children, respectively. All data concerning the patients age, gender, and month of isolation were recorded in this study. The collected strains of *E. coli* isolated from diarrheal specimens were identified using the VITEK 2 system (Bio-Merieux) and standard methods [10]. PCR was used to screen all *E. coli* strains for EAEC virulence gene markers.

2.2. EAEC assay by PCR

The obtained strains of *E. coli* isolated from patients with diarrheal episodes were further cultured in on McConkey agar. The lactose-fermenting Gram-negative colonies of obtained *E. coli* were tested using standard biochemical tests for confirmation of *E. coli*. The isolated purified colonies on selective agar were cultured onto Tryptic soy agar plates (TSA, Oxoid) and tested for indole production using API 20 E strips and others biochemical tests. Bacterial genomic DNA was extracted by using boiling method as described elsewhere [11]. Three colonies of each *E. coli* strain from the TSA plates were mixed with 300μl of nuclease free water in micro-centrifuge tubes and boiled in water bath for 10 min. The boiled bacterial suspensions were centrifuged at 10,000 *g* for 5 min and the supernatants were used as DNA templates for PCR [11]. Primers to examine the virulence markers are presented in Table 1. All strains of *E. coli* strains were examined for the *pCVD*432 gene [12] using PCR. All positive strains of *E. coli* for *pCVD*432 were screened for *aggR* [13], *aap* [14], *astA* [15], and *aaf/II* [16] and positive controls were included in each PCR run.

2.3. Molecular typing

The ERIC-PCR DNA fingerprinting method with repetitive primer sequence and amplification conditions, as described elsewhere [17, 18] was used to analyze *E. coli* strains for clonal relationship. The obtained ERIC fingerprint profiles were clustered by using Dice coefficients and the unweighted average pair group method (UPGMA) with position tolerance of 1%. The ERIC electrophoresis agarose gels images of DNA fingerprints were constructed with the use of Gel Compar II software (Applied Maths, St-Martens-Latem, Belgium).

2.4. Antibiotic susceptibility testing

All 63 strains of *E. coli* were examined by using disc diffusion method for 20 antimicrobial agents (Oxoid, England) [19]. The tested antibiotic agents were: ampicillin (AM, 25 μg); amikacin (AK, 30 μg); augmentin (AUG, 30 μg); aztreonam (ATM, 30 μg); cephalothin (KF, 30 μg); cefotaxime (CTX, 30 μg); ceftazidime (CAZ, 30 μg); cefepime (FEP, 30 μg); cefoxitin (FOX, 30 μg); chloramphenicol (C, 30 μg); colistin (CT: 30 μg); ciprofloxacin (CIP, 5 μg); gentamicin (GM, 5 μg); nalidixic acid (NA, 30 μg); neomycin (N, 30 μg); noroxin (NOR, 10 μg); piperacillin (PRL, 100 μg); streptomycin (S, 10 μg); trimethoprim sulfamethoxazole (SXT, 25 μg); and tetracycline (TE, 30 μg). The reference control strain of *E. coli* ATCC25922 was used while doing antibiotic susceptibility testing.

2.5. Statistical analysis

A chi-square test was performed to analysis the difference in proportion between antimicrobial resistance results of EAEC and Non-EAEC strains isolated from patients with diarrhea. All calculations were done by using MedCalc software version 17.9.2 (MedCalc Software, BVBA, Ostend, Belgium).

3. Results

There were 63 patients who were included in the present study; 11 (17.5%) samples were from children under 15 years of age and 52 (82.5%) samples were from adults aged 21–85 years. Out of 63 examined isolates of *E. coli* by using PCR, 35 (55.6%) were harboring *pCVD*432 (*pAA*) and 82.9% of these infections occurred during warmer months from May through October, as shown in Table 2. All isolates of *E. coli* were tested positive for the *pCVD*432 gene were examined using PCR to detect *aggR*, *aap*, *aaf/II*, and *astA* virulence genes of EAEC. Among the 35 patients with *pCVD*432 gene, only 19 (54.3%) isolates were positive for the *agg*R gene and were classified as tEAEC. In this study, all the 35 isolates were found positive for the *ast*A gene, and the *aap* gene was found in 33 (94.3%) patients; no single isolate was found positive for the *aafII* gene, as shown in Tables 2 and 3. The most prevalent combination of the virulence factor profile in strains of EAEC were (*aap*, *astA*) and (*aap*, *aggR*, *astA*) as shown in Table 3.

All the 63 diarrheogenic *E. coli* (DEC) isolates that were analyzed using ERIC-PCR were typeable and displayed a unique genotypic pattern, as presented in Figures 1 and 2 (see also Figures S1, S2 and S3). Using the cluster cutoff method, six distinct clusters with 60 strains that were closely related to ERIC fingerprints were identified while a similarity cutoff value of 90% was applied, except for three strains (Non-EAEC6, EAEC15 and EAEC75) that were present in a single cluster (SC), as shown

Table 1. Primers used for detection of virulence markers in this study.

Target gene	Properties of target	Primer sequence	Size of product (bp)	Ref.
PCVD432 (pAA)	Aggregative adherence plasmid	5′-CTG GCG AAAGACTGTATCAT-3′ 5′-CAATGTATAGAAATCCGCTGT T-3′	629	(Schmidt et al., 1995)
aggR	Transcriptional activator	5′-CTA ATTGTACAATCGATGTA-3′ 5′-AGA GTC CATCTCTTT GATAAG-3′	457	(Yatsuyanagi et al., 2002)
aap	Anti-aggregation protein dispersin	5′-CTTGGGTATCAGCCT GAATG-3′ 5′-AACCCATTCGGTTAGAGCAC-3′	310	(Cerna et al., 2003)
astA	Heat stable enterotoxin EAST1	5′-TGCCATCAACACAGTATATCCG-3′ 5′-ACGGCTTTGTAGTCCTTCCAT-3′	102	(Müller et al., 2007)
aaf/II	Aggregative adherence fimbriae	5′-CACAGGCAACTG AAATAAGTCTGG-3′ 5′-ATTCCCATGATGTCAAGCACTTC-3′	378	(Boisen et al., 2008)

in Figure 1 (see also Figure S1). Grouping of DNA fingerprints similarities revealed by ERIC-PCR for all *E. coli* isolates were analyzed constructed and calculated according to algorithm for the unweighted pair group method with arithmetic mean (UPGMA). As shown in Figure 2 (see also Figures S2 and S3 for full images), 12 strains of tEAEC fingerprint profiles were grouped together in clusters 2 and 3 compared with the other clusters Subsequently, 13 strains of tEAEC were isolated from patients' fecal specimens during 2013 while only three strains of tEAEC were isolated during 2014, as shown in Figure 1. Two strains of tEAEC (EAEC71 and EAEC74) were isolated during June and September 2013 and were grouped in cluster ET-6 with strains that were isolated during 2014. Of the 63 strains of *E. coli*, 21 (33.3%) with identical fingerprints were grouped in cluster 6 and most of these strains were isolated in June 2014.

The results of antimicrobial susceptibility testing of all 63 strains were presented in Table 4. EAEC revealed the following high percentages of resistance: tetracycline, 68.6%; ampicillin, 60%; nalidixic acid, 60%; trimethoprim sulfamethoxazole, 42.9%; ciprofloxacin, 40%; streptomycin, 40%; and noroxin, 37.1%. The high percentages of resistance in non-EAEC clinical isolates were ampicillin, 64.3%; tetracycline, 50%; trimethoprim sulfamethoxazole, 46.4%; cefotaxime, 28.6%; nalidixic acid, 28.6%; piperacillin, 28.6%; and cephalothin, 25%. All EAEC isolates were susceptible to amikacin, while all non-EAEC isolates were susceptible to amikacin and colistin. Overall, 68.3% of tested EAEC and non-EAEC isolates were multidrug resistant (MDR) and EAEC showed greater resistance than non-EAEC isolates. As presented in Table 4, most of the *p*-values were more than 0.05 meaning that there is no statistical difference between the proportions. The only exceptions are for cefepime for sensitive (S) category. The two other significant *p*-values are very small with only one case and this is not meaningful. The highest antibiotic resistance patterns were noticed among 68.6% of EAEC isolates and were grouped together in cluster ET-2 and ET-3 while all 63 isolates of EAEC and non-EAEC were typed using ERIC-PCR DNA fingerprinting, as shown in Figure 2.

4. Discussion

The EAEC pathotype is one of subgroup of diarrheagenic *E. coli* (DEC) and its well-known worldwide for causing severe diarrhea in infected children and adults [2]. Nevertheless, EAEC its pathogenicity is

Table 2. Characterization of virulence factors of EAEC strains isolated from patients with diarrhea.

Isolate	Month of isolation	Sex	Age	virulence factors				ERIC-PCR pattern type
				aafII	Aap	aggR	AstA	
EAEC3	December 2013	F	19	-	+	-	+	ET-3
EAEC12	May 2013	F	21	-	+		+	ET-3
EAEC13	June 2013	M	44	-	+	+	+	ET-3
EAEC14	December 2013	M	2	-	-	-	+	ET-5
EAEC15	December 2013	M	68	-	+	-	+	*SC
EAEC20	September 2013	F	55	-	+	+	+	ET-3
EAEC21	September 2013	M	29	-	+	-	+	ET-3
EAEC22	September 2013	F	26	-	+	-	+	ET-3
EAEC25	October 2013	M	25	-	+	-	+	ET-4
EAEC27	December 2013	F	71	-	+	+	+	ET-3
EAEC28	December 2013	M	69	-	+	+	+	ET-3
EAEC29	October 2013	M	71	-	+	+	+	ET-3
EAEC31	July 2013	M	22	-	+	-	+	ET-4
EAEC33	June 2013	M	85	-	+	-	+	ET-1
EAEC41	May 2013	F	2	-	+	-	+	ET-2
EAEC45	June 2013	M	47	-	+	+	+	ET-1
EAEC47	January 2014	F	66	-	+	+	+	ET-5
EAEC55	September 2013	M	61	-	+	-	+	ET-2
EAEC60	May 2013	F	41	-	+	+	+	ET-2
EAEC61	May 2013	M	21	-	+	+	+	ET-2
EAEC62	June 2013	M	45	-	+	+	+	ET-2
EAEC63	October 2013	M	62	-	+	+	+	ET-2
EAEC64	August 2013	F	12	-	+	+	+	ET-2
EAEC65	October 2013	M	39	-	+	+	+	ET-2
EAEC67	May 2013	F	21	-	+	+	+	ET-2
EAEC71	June 2013	F	48	-	+	+	+	ET-6
EAEC74	September 2013	M	31	-	+	+	+	ET-6
EAEC75	May 2013	F	22	-	+	+	+	SC
EAEC80	May 2014	F	30	-	+	+	+	ET-6
EAEC85	May 2014	F	39	-	+	+	+	ET-6
EAEC86	June 2014	F	12	-	+	-	+	ET-6
EAEC87	June 2014	F	27	-	+	-	+	ET-6
EAEC89	June 2014	M	21	-	+	-	+	ET-6
EAEC92	June 2014	F	30	-	+	-	+	ET-6
EAEC95	June 2014	M	4	-	-	-	+	ET-6
No. of positive				0	33	19	35	
% of positive				0%	94%	54%	100%	

* SC, single cluster.

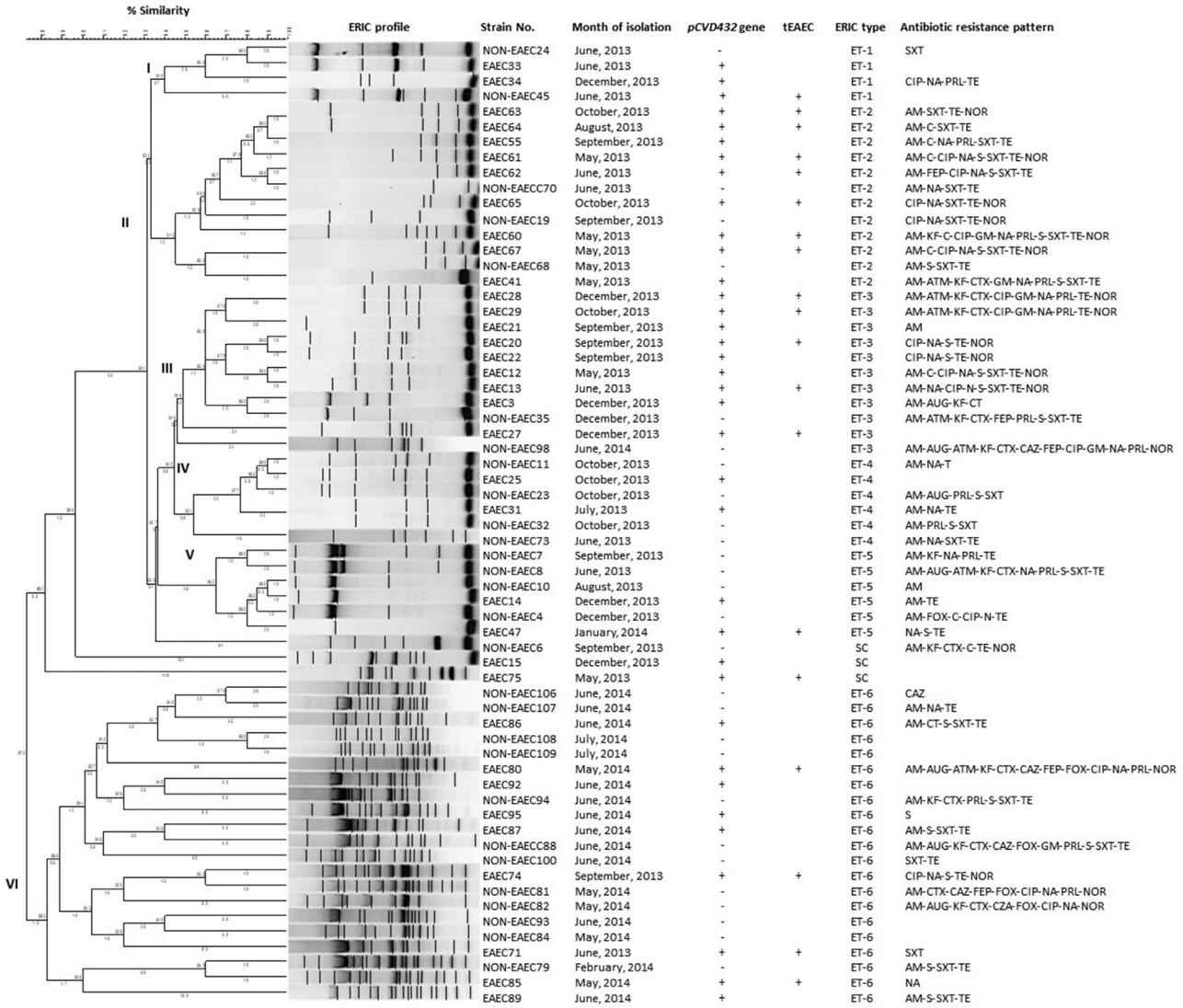
Table 3. Prevalence of virulence genes among EAEC strains isolated from patients with diarrhea.

Virulence gene profile	No (%) of EAEC
aaf/II	0
Aap	33 (94.3)
aggR	19 (54.3)
astA	35 (100)
aap, astA	33 (94.3)
aap, aggR, astA	19 (54.3)

remaining controversial and that due to heterogeneity and clinical relevance of *E. coli* strains [1]. In meta-analysis study conducted by Huang et al., (2006) [20] was reported that EAEC is responsible of causing persistent and chronic diarrhea in different group of populations in developing and industrialized countries.

In meta-analysis study conducted by Huang et al. (2006) [20] was reported that EAEC is responsible of causing acute and persistent diarrhea in different group of populations in developing and industrialized countries, in addition to that, this study confirmed the heterogenicity of EAEC strains and inadequate studies were reported the role of emerging EAEC strains in acute diarrheal illness. The present study also documented the EAEC causes sporadic cases of diarrhea in the Eastern Province of Saudi Arabia. Globally, EAEC shows an alarming increase in resistance to different ranges of antibiotics [21] (Kong et al., 2015). In Saudi Arabia, no published study has reported the situation of EAEC that is associated with diarrhea in terms of molecular characterization and antimicrobial resistance. This study is the first study to report the detection of virulence factors and high rate of antibiotic resistance among the isolated EAEC strains in Saudi patients infected with diarrhea in Saudi Arabia and to provide insight on the current DEC situation.

In this study, 63 fecal specimens from outpatients and inpatients with diarrhea were examined for EAEC. Thirty-five (55.6%) were positive for the *pCVD*432 gene and were identified as EAEC. The DNA probe *pCVD*432 has been developed from the 60-MDa plasmid *pAA* to detect EAEC using PCR [22]. Several studies have reported a high specificity of the *pCVD*432 probe for identification of EAEC [22, 23, 24, 25]. In this study, the HEp-2 cell adherence assay was not used for confirmation of EAEC because this assay experiment is currently performed only in reference research laboratories and it is also laborious to perform. However, in this study, the PCR assay targeting the *pCVD*432 gene was shown to be a more sensitive and reliable molecular assay for the identification of EAEC. Several epidemiological studies have shown that



Strain No.	Month of isolation	*pCVD432* gene	tEAEC	ERIC type	Antibiotic resistance pattern
NON-EAEC24	June, 2013	-		ET-1	SXT
EAEC33	June, 2013	+		ET-1	
EAEC34	December, 2013	+		ET-1	CIP-NA-PRL-TE
NON-EAEC45	June, 2013	+	+	ET-1	
EAEC63	October, 2013	+	+	ET-2	AM-SXT-TE-NOR
EAEC64	August, 2013	+	+	ET-2	AM-C-SXT-TE
EAEC55	September, 2013	+		ET-2	AM-C-NA-PRL-SXT-TE
EAEC61	May, 2013	+	+	ET-2	AM-C-CIP-NA-S-SXT-TE-NOR
EAEC62	June, 2013	+	+	ET-2	AM-FEP-CIP-NA-S-SXT-TE
NON-EAECC70	June, 2013	-		ET-2	AM-NA-SXT-TE
EAEC65	October, 2013	+	+	ET-2	CIP-NA-SXT-TE-NOR
NON-EAEC19	September, 2013	-		ET-2	CIP-NA-SXT-TE-NOR
EAEC60	May, 2013	+	+	ET-2	AM-KF-C-CIP-GM-NA-PRL-S-SXT-TE-NOR
EAEC67	May, 2013	+	+	ET-2	AM-C-CIP-NA-S-SXT-TE-NOR
NON-EAEC68	May, 2013	-		ET-2	AM-S-SXT-TE
EAEC41	May, 2013	+		ET-2	AM-ATM-KF-CTX-GM-NA-PRL-S-SXT-TE
EAEC28	December, 2013	+	+	ET-3	AM-ATM-KF-CTX-CIP-GM-NA-PRL-TE-NOR
EAEC29	October, 2013	+	+	ET-3	AM-ATM-KF-CTX-CIP-GM-NA-PRL-TE-NOR
EAEC21	September, 2013	+		ET-3	AM
EAEC20	September, 2013	+	+	ET-3	CIP-NA-S-TE-NOR
EAEC22	September, 2013	+		ET-3	CIP-NA-S-TE-NOR
EAEC12	May, 2013	+		ET-3	AM-C-CIP-NA-S-SXT-TE-NOR
EAEC13	June, 2013	+	+	ET-3	AM-NA-CIP-N-S-SXT-TE-NOR
EAEC3	December, 2013	+		ET-3	AM-AUG-KF-CT
NON-EAEC35	December, 2013	-		ET-3	AM-ATM-KF-CTX-FEP-PRL-S-SXT-TE
EAEC27	December, 2013	+	+	ET-3	
NON-EAEC98	June, 2014	-		ET-3	AM-AUG-ATM-KF-CTX-CAZ-FEP-CIP-GM-NA-PRL-NOR
NON-EAEC11	October, 2013	-		ET-4	AM-NA-T
EAEC25	October, 2013	+		ET-4	
NON-EAEC23	October, 2013	-		ET-4	AM-AUG-PRL-S-SXT
EAEC31	July, 2013	+		ET-4	AM-NA-TE
NON-EAEC32	October, 2013	-		ET-4	AM-PRL-S-SXT
NON-EAEC73	June, 2013	-		ET-4	AM-NA-SXT-TE
NON-EAEC7	September, 2013	-		ET-5	AM-KF-NA-PRL-TE
NON-EAEC8	June, 2013	-		ET-5	AM-AUG-ATM-KF-CTX-NA-PRL-S-SXT-TE
NON-EAEC10	August, 2013	-		ET-5	AM
EAEC14	December, 2013	+		ET-5	AM-TE
NON-EAEC4	December, 2013	-		ET-5	AM-FOX-C-CIP-N-TE
EAEC47	January, 2014	+	+	ET-5	NA-S-TE
NON-EAEC6	September, 2013	-		SC	AM-KF-CTX-C-TE-NOR
EAEC15	December, 2013	+		SC	
EAEC75	May, 2013	+	+	SC	
NON-EAEC106	June, 2014	-		ET-6	CAZ
NON-EAEC107	June, 2014	-		ET-6	AM-NA-TE
EAEC86	June, 2014	+		ET-6	AM-CT-S-SXT-TE
NON-EAEC108	July, 2014	-		ET-6	
NON-EAEC109	July, 2014	-		ET-6	
EAEC80	May, 2014	+	+	ET-6	AM-AUG-ATM-KF-CTX-CAZ-FEP-FOX-CIP-NA-PRL-NOR
EAEC92	June, 2014	+		ET-6	
NON-EAEC94	June, 2014	-		ET-6	AM-KF-CTX-PRL-S-SXT-TE
EAEC95	June, 2014	+		ET-6	S
EAEC87	June, 2014	+		ET-6	AM-S-SXT-TE
NON-EAECC88	June, 2014	-		ET-6	AM-AUG-KF-CTX-CAZ-FOX-GM-PRL-S-SXT-TE
NON-EAEC100	June, 2014	-		ET-6	SXT-TE
EAEC74	September, 2013	+	+	ET-6	CIP-NA-S-TE-NOR
NON-EAEC81	May, 2014	-		ET-6	AM-CTX-CAZ-FEP-FOX-CIP-NA-PRL-NOR
NON-EAEC82	May, 2014	-		ET-6	AM-AUG-KF-CTX-CZA-FOX-CIP-NA-NOR
NON-EAEC93	June, 2014	-		ET-6	
NON-EAEC84	May, 2014	-		ET-6	
EAEC71	June, 2013	+	+	ET-6	SXT
NON-EAEC79	February, 2014	-		ET-6	AM-S-SXT-TE
EAEC85	May, 2014	+	+	ET-6	NA
EAEC89	June, 2014	+		ET-6	AM-S-SXT-TE

Figure 1. ERIC-PCR profiles and antibiotic resistance patterns of 63 DEC strains isolated from patients with diarrhea in KFHU (See supplementary Figure 1 for full image).

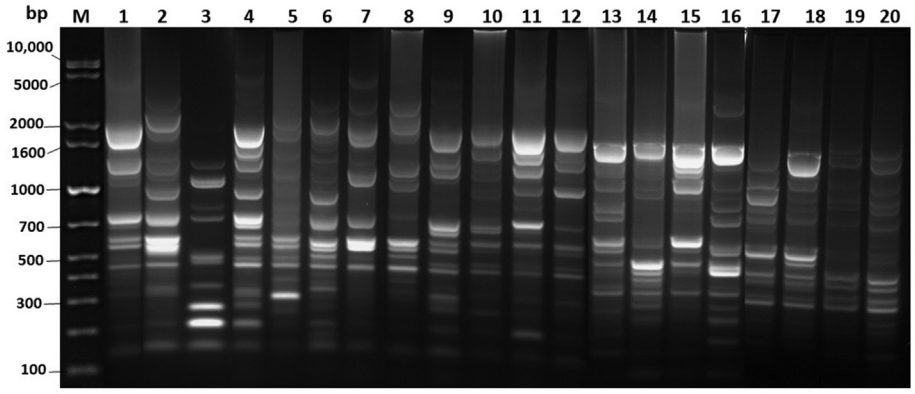


Figure 2. Representative ERIC profiles (See supplementary Figures 2 and 3 for full images). Lanes: M, molecular mass marker (GelPiolt 1kb Plus ladder ranging from 100 to 10000 bp); 1, EAEC71; 2, EAEC74; 3, EAEC75; 4, Non-EAEC; 5, EAEC80; 6, Non-EAEC81; 7, Non-EAEC82; 8, Non-EAEC84; 9, EAEC85; 10, EAEC86; 11, EAEC87; 12, Non-EAEC88; 13, EAEC92; 14, Non-EAEC93; 15, Non-EAEC94; 16, EAEC95; 17, Non-EAEC106; 18, Non-EAEC107; 19, Non-EAEC108; 20, Non-EAEC109.

Table 4. Antimicrobial susceptibility of EAEC and Non-EAEC strains isolated from patients with diarrhea.

Antimicrobial agent	Isolates no. (%) for								
	₫R		p-value	ϵI		p-value	ϵS		p-value
	EAEC	Non-EAEC		EAEC	Non-EAEC		EAEC	Non-EAEC	
Ampicillin	21 (60)	18 (64.3)	0.9561	0	0	-	14 (40)	10 (35.7)	0.8319
Amikacin	0	0	-	0	0	-	35 (100)	28 (100)	-
Augmentin	2 (5.7)	5 (17.9)	0.4378	0	1 (3.6)	-	33 (94.3)	22 (78.6)	0.1826
Aztreonam	4 (11.4)	3 (10.7)	0.2354	1 (2.9)	4 (14.3)	0.1599	30 (85.7)	21 (75)	0.5488
Cephalothin	7 (20)	7 (25)	0.6774	1 (2.9)	3 (10.7)	0.0712	27 (77.1)	18 (64.3)	0.5499
Cefotaxime	5 (14.3)	8 (28.6)	0.9353	0	0	-	30 (85.7)	20 (71.4)	0.3804
Ceftazidime	1 (2.9)	5 (17.9)	0.2551	1 (2.9)	3 (10.7)	0.0712	33 (94.3)	20 (71.4)	0.0573
Cefepime	2 (5.7)	3(10.7)	0.1541	1 (2.9)	2 (7.1)	0.0127	32 (91.4)	15 (53.6)	0.0094
Cefoxitin	1 (2.9)	4 (14.3)	0.1599	0	0	-	34 (97.1)	24 (85.7)	0.2673
Chloramphenicol	6 (17.1)	2 (7.1)	0.4183	3 (8.6)	0	-	26 (74.3)	26 (92.9)	0.1508
Colistin	2 (5.7)	0	-	0	0	-	33 (94.3)	28 (100)	0.5891
Ciprofloxacin	14 (40)	5 (17.9)	0.73	1 (2.9)	0	-	20 (57.1)	23 (82.1)	0.145
Gentamicin	4 (11.4)	2 (7.1)	0.2006	0	0	-	31 (88.6)	26 (92.9)	0.9217
Nalidixic Acid	21 (60)	8 (28.6)	0.2729	0	0	-	14 (40)	20 (71.4)	0.1402
Neomycin	1 (2.9)	1 (3.6)	0.0001	2 (5.7)	0	-	32 (91.4)	27 (96.4)	0.803
Noroxin	13 (37.1)	5 (17.9)	0.827	1 (2.9)	0	-	21 (60)	23 (82.1)	0.1976
Piperacillin	8 (22.9)	8 (28.6)	0.7558	1 (2.9)	0	-	26 (74.3)	20 (71.4)	0.9081
Streptomycin	14 (40)	9 (32.1)	0.9525	0	0	-	21 (60)	19 (67.9)	0.8495
Tetracycline	24 (68.6)	14 (50)	0.4283	0	0	-	11 (31.4)	14 (50)	0.5979
Trimethoprim sulfamethoxazole	15 (42.9)	13 (46.4)	0.8451	0	0	-	20 (57.1)	15 (53.6)	0.8906

₫R, resistance; ϵI, intermediate; ϵS, sensitive.

strains that are positive for the *pCVD*432 gene-positive strains and harboring the *agg*R regulon are characterized as tEAEC pathogens [26]. In this study, 54.3% of strains were identified as tEAEC and they showed different variations to other virulence genes.

In this study, there are different combinations of virulence gene markers were detected in examined strains of EAEC, as presented in Table 3. Among the virulence gene markers, the *ast*A gene was was detected in all 63 strains of EAEC and non-EAEC. A recent and similar study in China reported a high prevalence rate of the *ast*A virulence gene with a detection rate (88%) among EAEC strains that were isolated from clinical fecal specimens [27]. However, our results disagreed with some other studies, which reported that the *ast*A gene is rarely detectable in EAEC strains [24, 28]. The *ast*A gene that encodes the EAST-1 toxin is responsible for causing diarrhea and chloride secretion [29], and also is playing a significant role it was found to be important in the development of prolonged acute and persistent diarrhea [30]. However, the EAST-1 is associated with EAEC and it has been also detected in enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and enterohaemorrhagic *E. coli* (EHEC) strains [28]. In this study, 19 (54.3%) tEAEC were found to be positive for three distinct combinations of virulence genes (*aap*, *agg*R, and *ast*A), and most of the strains were isolated from adult patients, as shown in Table 3. However, our results agreed with those of Huang et al. (2006) [20] who reported and proven that EAEC strains harboring *agg*R, *aap*, and *astA* virulence genes are definitely responsible of causing acute diarrhea in adults.

The adherence of EAEC to the intestinal epithelium is the first step in gut colonization, which requires fimbrial structures that are known as AA factors (AAF) [31, 32]. There are four major variants of the AAF structures in pilin subunits (AAF/I to AAF/IV), and all of them are regulated by the transcriptional activator *agg*R, which is situated on the EAEC virulence plasmid *pAA* [16]. In the present study, the *aaf/II* gene was not detected among all strains of EAEC isolates. The current study results are

consistent with other published epidemiological studies, which reported that most EAEC strains do not express any of the four known AAF variants [33, 34]. This suggests that strains of EAEC with a "stacked-brick" AA lack AAF and might have other adhesion mechanisms [2]. Boisen et al. (2008) [16] demonstrated that a novel aggregative adhesion pilin that was regulated by the *agg*R regulon in the EAEC strains lacks a known AAF and that might be distantly linked to adhesins of Dr family. Several studies have suggested that other additional undiscovered AAF variants may exist and need to be explored [16]. From this study and other results that were reported worldwide, and because of the EAEC heterogeneity, there are no specific common combinations of virulence factor sets that have been found to identify all EAEC strains using the PCR assay [4, 24, 35]. However, the pathogenicity of these bacteria remains controversial [1].

ERIC is a useful chromosomal DNA fingerprinting PCR-based technique for molecular epidemiological characterization of several bacterial pathogens of medical importance. However, the obtained ERIC patterns are easy to evaluate. In this study, the ERIC-PCR typing revealed six cluster patterns, as shown in Figure 2 and Table 3. Our results indicate that ERIC is useful as a molecular tool for typing microbial outbreaks. In this study, we detected high levels of antibiotic resistant EAEC strains that were isolated from Saudi patients with diarrhea, but the percentages of MDR patterns among EAEC and Non-EAEC were 68.6% and 67.9%, respectively, as presented in Figure 2 and Table 4. Overall, EAEC isolates showed greater antibiotic resistance than non-EAEC. Having knowledge about the antimicrobial susceptibility of enteric bacteria that are associated with causing frequent diarrhea such as EAEC and other *E. coli* pathotypes will help to develop useful information for treatment. Our study documented a high level of quinolone resistance among EAEC isolates toward nalidixic (60%) and ciprofloxacin (40%) (Table 4). Therefore, our results are in agreement with a recent study that was performed in Denmark, which reported that 34% of ciprofloxacin resistance in adult patients with diarrhea was caused by EAEC [30].

In this study, the investigated strains of EAEC showed a high rate of resistance to most of the commonly used antibiotics such as tetracycline (68.6%), ampicillin (60%), nalidixic acid (60%), trimethoprim sulfamethoxazole (42.9%), streptomycin (40%), ciprofloxacin (40%), and noroxin (37.1%). Additionally, 68.3% of EAEC and DEC strains showed MDR resistance patterns to more than three antibiotics, as shown in Figure 1. Therefore, our study results are consistent with the findings of similar studies that reported the recent dramatic increase in antibiotic resistance towards most commonly used antibiotics that are used to treat EAEC [30, 36, 37, 38].

5. Conclusion

The high number of MDR EAEC strains that were detected in our study is a cause for concern, and therefore, the establishment of long-term surveillance programs are required to find changes in the spectrum of antimicrobial resistance patterns of EAEC in Saudi Arabia. In this study, resistance of EAEC strains to ampicillin (60%), nalidixic acid (60%), trimethoprim sulfamethoxazole (42.9%), and ciprofloxacin (40%) were extremely high in Saudi patients with diarrhea. Therefore, antibiotics should be prescribed only for patients with severe and persistent diarrhea. In conclusion, the findings of this study confirm that EAEC was detected in children and adult patients with diarrhea and highlights the importance of continuous monitoring for antibiotic resistance in EAEC and another DEC pathotypes.

Declarations

Author contribution statement

Nasreldin Elhadi: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Reem Aljindan: Performed the experiments; Analyzed and interpreted the data.

Khaldoon Alsamman: Performed the experiments.

Amer Alomar, Mohammed Aljeldah: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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