Antimicrobial susceptibility, biofilm formation, and virulence genes among atypical enteropathogenic *Escherichia coli* stool isolates in Tehran, Iran

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ABSTRACT

Background and Objectives: Enteropathogenic *Escherichia coli* (EPEC) strains are emerging pathogens around the world, particularly among pediatric patients in developing countries, such as Iran. This study aims to examine and compare the characteristics of EPEC isolates from patients, who suffer from diarrhea versus isolates from patients without diarrhea.

Materials and Methods: A total of 734 stool specimens [440 diarrheal (D), and 294 non-diarrheal (ND)] were examined. Thirty-six EPEC isolates (26 D, and 10 ND) were recovered by culture on MacConkey agar, followed by biochemical tests. Using PCR assay, eae^+; $stx1^-$ and $stx2^-$ gene profiles of EPEC isolates were confirmed. The antimicrobial resistance was assessed by disk diffusion assay. Biofilm formation was assessed using a standard semi-quantitative microtiter plate assay. Virulence-associated genes, *ehac, espA, fimA, flu,* and *sslE* were detected.

Results: *E. coli* comprised 14% of all isolates were EPEC isolates that showed the highest sensitivity to imipenem (IPM) (100%) and gentamicin (GEN) (89%). However, susceptibility to ciprofloxacin and cotrimoxazole or trimethoprim\sulfa-methoxazole (SXT) was only 28% and 39%, respectively. About 61% of isolates produced Moderate Biofilm (MB), and the frequency of Weak Biofilm (WB) formers (27%) was higher among D and ND isolates, which carried virulence genes more frequently than D isolates.

Conclusion: Preventive measures by public health authorities can thwart the imminent crisis of widespread zoonotic contamination of the food chain in Iran. Our results may help clinicians make optimal therapeutic choices during the treatment of patients with severe EPEC infections, and assist epidemiologists devise policies for effective control of outbreaks.

Keywords: Enteropathogenic *Escherichia coli* (EPEC); Antimicrobial resistance; Biofilm virulence genes

INTRODUCTION

Diarrheal diseases are a major cause of morbidity and mortality among pediatric patients in developed as well as developing countries, such as Iran (1). Enteropathogenic *Escherichia coli* (EPEC) play an important role in complications associated with diarrheal infections by inflicting damage to the small intestine epithelium (2). According to recent researches, aEPEC infections are more prevalent than tEPEC infections in both developed and developing nations (3). aEPEC are frequently associated with diarrhea, and in some countries, they outnumber tEPEC infections. According to studies from 13 developing countries, aEPEC isolates account for 78% of all EPEC cases in children under the age of five (4).

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All EPEC strains carry the LEE gene, which is responsible for inflicting damage to the small intestine epithelium through the formation of attaching and effacing (AE) lesions (5). Typical EPEC (t-EPEC) have a complete set of virulence genes located on chromosomal pathogenicity island (PAI), and a pathogenic plasmid-termed locus encoding the enterocyte effacement (LEE) and EPEC adherence factor (EAF), respectively. However, atypical strains of EPEC (a-EPEC) lack the EAF-harboring operon for bundle type IV pili (*bfp*), while it has the EAF-coding *eae* gene within the LEE chromosomal gene (1).

Generally, EPEC strains are classified by the detection of specific genes, which are also utilized to evaluate their prevalence in various regions of the world (5, 6). Recent epidemiologic studies demonstrate a rise in infections by a-EPEC pathotypes in developing, as well as developed countries (3). The emergence of a-EPEC strains as important pathogens, and the rise in infections is mostly attributed to the consumption of lightly cooked meat products, which serve as vehicles to transmit a-EPEC that colonize cattle and poultry (7). Moreover, the ability of *E. coli* strains to form biofilm has been linked to enhanced survival of EPEC within hostile host environments, as well as complicated chronic diarrhea by allowing colonization of new niches, in addition to increased antimicrobial resistance (8). Virulence-associated genes such as *bfpA* (EPEC only), and *espA* are important in aggregation and micro-colony formation on epithelial cells, as well as abiotic surfaces (9). Type II Secretory System (TTSS) secrets the proteins encoded by *espA* and forms a filamentous structure involved in protein translocation and adhesion (9). Other virulence factors, for instance, type 1 pilus (T1P), flagella, *csgA* (curli fimbriae), antigen 43 (Ag43; *flu*), calcium-binding antigen 43 homolog (*Cah*), and auto-transporter protein of EHEC (*ehaA*) are also implicated in the formation of biofilm by EPEC (9).

Furthermore, unfettered use of antibiotics has been associated with widespread antimicrobial resistance in developing countries (10), therefore information about antibiotic resistance among EPEC are important in selecting the appropriate therapy (11). Although that biofilm formation by *E. coli* has been broadly studied, and EPEC contributes to diarrheal infections complications, few studies have thoroughly investigated EPEC characteristics, and the genes related to biofilm production by EPEC strains. To address the paucity of data regarding traits of EPEC isolates from Iran, we aim to examine and compare the antimicrobial susceptibility and biofilm formation of EPEC strains isolated from patients with diarrhea versus normal individuals. Additionally, we investigate the presence of virulence genes associated with biofilm formation, such as *sslE* (type 2 secreted protein) among the EPEC isolates.

MATERIALS AND METHODS

Patient specimens, and identification of a-EPEC isolates. Between May 2016 and February 2017 in summer and fall seasons, a total of 734 stool samples, comprising 440 diarrheal (D) and 294 non-diarrheal (ND) specimens, were collected from three hospitals affiliated with Tehran University of Medical Sciences (TUMS). Patients with diarrhea were defined as having signs/symptoms of abdominal pain, frequent loose, watery stool, and/or >3 bowel movements per day. Initially, specimens were processed in the microbiology laboratory of mentioned hospitals. After initial isolation, identification of *E. coli* isolates was confirmed by subculture on MacConkey agar plate followed by standard biochemical tests, as described previously (12) at TUMS Medical Microbiology Department (Medical School). In order to identity the enteropathogenic *E. coli* (EPEC), isolates were confirmed by the presence of *eae* gene, and the absence of toxin genes *stx1* and *stx2* (13). Ultimately, a total of 36 EPEC isolates were recovered, which were comprised of 26 (D) and 10 (ND) specimens. All isolates were identified as a-EPEC, as determined by the lack of *bfpA* gene using a specific polymerase chain reaction (PCR) assay.

Determination of antibiotic susceptibility. The antimicrobial susceptibility of isolates to six first-line antimicrobial agents, commonly used against EPEC infections, was determined by disc agar diffusion (DAD) test, which was performed on Muller-Hinton agar (Merck, Germany) plates, according to the Clinical and Laboratory Standards Institute (CLSI) guideline 2018 (14). The antimicrobial agents included trimethoprim\sulfamethoxazole or cotrimoxazole (TSX; 25 µg), gentamicin (GEN; 10 µg), azithromycin (AZT; 15 µg), imipenem (IPM; 10 µg), ciprofloxacin (CIP; 5 µg), and ceftazidime (CTZ; 30 µg). *E. coli* ATCC 25922 was used as a quality control organism.

Semi-quantitative assay for strength of biofilm production. The strength of biofilm (BF) formation was measured semi-quantitatively on 96-well flat-bottom microtiter plates, as previously described (15). Briefly, pure colonies of isolates were suspended in 200 μL of trypticase soy broth (TSB) and diluted to a density of 0.5 McFarland, then added to wells in triplicate. After incubation at 37°C for 24 h, culture supernatants were decanted, wells were washed three times with 250 μL of normal saline (0.90 gm/L NaCl), and biofilms were fixed by addition of 200 μL of methanol. After a 15 min incubation at room temperature (RT), methanol was decanted, wells were air-dried, and then stained with crystal violet (150 μL per well), and incubated for 15 min at RT. The plates were then rinsed 3X with tap water and air-dried. Bound stain in each well was solubilized with 150 μL of 33% (v/v) glacial acetic acid, and the optical density (OD) of samples was measured (570nm) using a Multiskan EX reader (LabSystems, Helsinki, Finland). All samples were run in triplicate, and mock wells with no inoculum (TSB alone) were used as the negative control. The cut-off OD (OD_c) for positive samples was determined as three standard deviations (SD) above the mean OD of the negative control wells. The strength of BF production of isolates was assessed by the following formulas: Strong BF= $OD>(4\times OD_c)$, Moderate BF= $(2\times OD_c)<OD\leq(4\times OD_c)$, Weak BF= $OD_c<OD<2\times OD_c$, and no BF= $OD\leq OD_c$.

Identification of biofilm-associated genes by PCR assay. Table 1 demonstrates specific PCR primers for target genes and their amplicons that have been linked to EPEC pathogenesis; namely, *fimA, sslE, espA, flu, ehaC,* and *bfpA*, as described (16). Amplification conditions for each target gene were as follows; initial denaturation at 95°C for 5min; and 30 cycles of denaturation at 95°C for 35s, and annealing phase at 55°C for 35s, for the *fimA, sslE* primers; 57°C for 35s for *espA, flu,* and *ehaC* primers; and 59°C for 35s for *bfpA* primer. For all primers, the extension phase was carried out at 72°C for 35s, using a final 5 min extension.

Ethics approval and consent to participate. Ethical approval (IR.TUMS.MEDICINE.Rec.1398.121) was obtained from the Ethical Committee of Tehran University of Medical Sciences (TUMS), Tehran, Iran.

Statistical analysis. All statistical analyses, including Chi-square, and Fisher's exact test, were performed using SPSS v 29 for MS Windows, and MS Excel 2022 software, and p<0.05 was considered as significant.

RESULTS

Prevalence and antimicrobial susceptibility of a-EPEC isolates. The overall frequency of *E. coli* pathotypes was 14% (104/747), and surprisingly, while the prevalence of a-EPEC among pathotypes was about 35% (36/104), none of the isolates were identified as typical EPEC. The frequency of a-EPEC among diarrheal (D) and non-diarrheal (ND) isolates was 32% (26/82), and 45% (10/22), respectively; signifying that among ND specimens the incidence of EPEC was 13% higher than D specimens. Table 3 shows that most isolates were recovered from patients younger than one year.

To determine the potential therapeutic effectiveness of six first-line antimicrobial agents against EPEC infections, we compared the susceptibility patterns of EPEC isolates from D versus ND specimens (Fig. 1). Remarkably, 100% of isolates (D and ND) were susceptible to imipenem (IPM); whereas the highest rate of antimicrobial resistance was to CIP (69%; 18/26). Among the D isolates, the rate of resistance to CIP and TSX was identical (69%); however, among the ND isolates, the frequency of CIP resistance was 80% (8/10) and TSX resistance was 40% (4/10). As shown in Fig. 1, while only 31% (8/26) of D isolates were susceptible to ciprofloxacin (CIP), susceptibility to AZT among D and ND isolates was 65% (17/26) and 70% (7/10), respectively. Likewise, D and ND isolates were both highly susceptible to CTZ and gentamicin (GEN), with susceptibility rates of as high as 80%-92% for D isolates and 80% (8/10) for ND isolates. In other words, resistance to GEN and CTZ among D isolates ranged between 8-10% (2-3/26); while 20% (2/10) of ND isolates were resistant to GEN and CTZ.

Genes related to biofilm formation by a-EPEC isolates. As an indicator of the potential pathogenicity of a-EPEC isolates, Fig. 2A demonstrates the strength of biofilm produced by D and ND isolates. Notably, 61% (22/36) of isolates were moderate biofilm (MB) producers, which predominated among D (58%;

Table 1. List of PCR primers used for the identification of a-EPEC isolates and their virulence genes.

| No.	Target	Primer Sequence (5 ⟶ 3)	Amplicon Size (bp)	Reference
1	*eae* (EPEC/EHEC)	F: TCAATGCAGTTCCGTTATCAGTT	482	13
		R: GTAAAGTCCGTTACCCCAACCTG		
2	*bfpA*	F: GGAAGTCAAATTCATGGGGGTAT	800	**
		R: GGAATCAGACGCAGACTGGTAGT		
3	*sxt1*	F: CAGTTAATGTGGTGGCGAAGG	384	13
		R: CACCAGACAATGTAACCGCTG		
4	*sxt2*	F: ATCCTATTCCCGGGAGTTTACG	584	13
		R: GCGTCATCGTATACACAGGAGC		
5	*fimA*	F: GTTCAGTTAGGACAGGTTCG	291	**
		R: GGTTCCGTTATTCAGGGTTG		
6	*flu*	F: GGAATATCCCCGATAACGCC	444	**
		R: GGACTTCTGCACGATAAGCA		
7	*sslE*	F: GAACAGGTTCCAGCCTTTCA	455	**
		R: GTTTCACCAACGATGTGCAG		
8	*espA*	F: CCGTTGTCAGGTTATTCGCT	216	**
		R: TGGCTAATCTTGTGGATGCC		
9	*ehaC*	F: TAATGACGGCAAAGGTGGT	599	16
		R: CATTCATCAGGGAGTTGCT		

** This study
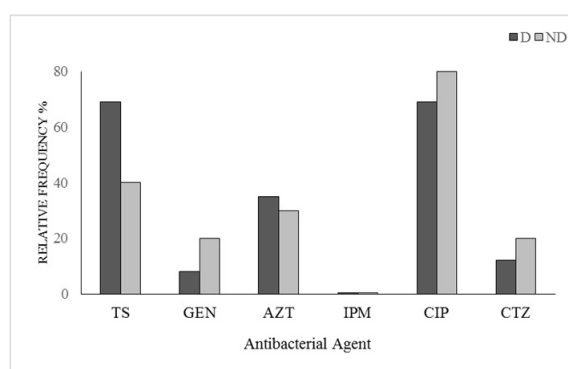
Fig. 1. Relative frequency of antimicrobial resistance among a-EPEC isolates recovered from diarrhea and non-Diarrhea specimens. (SXT= Cotrimoxazole or Trimethoprim\Sulfamethoxazole; GEN= Gentamicin; AZT= Azithromycin; IPM= Imipenem; CIP= Ciprofloxacin; CTZ= Ceftazidime).

15/26) as well as ND (70%; 7/10) isolates; distantly followed by the weak biofilm (WB) forming isolates (22%; 8/36). Remarkably, isolates that formed strong biofilm (SB) were the least common (17%; 6/36) a-EPEC's; which among D and ND isolates showed frequency of 27% and 10%, respectively.

It is worth noting that amongst ND isolates, MB formers (70%) were almost fivefold more frequent than SB formers (15%), whereas among D isolates

WB formers were nearly twice as frequent as SB formers (27% vs. 15%). Interestingly, the frequency of isolation of SB and MB forming a-EPECs was similar among D specimens, which comprised 67% (4/6) of SB formers, and nearly 88% (7/8) of WB forming. However, the weakest biofilm formers were D isolates, though they contained four SB producers. Even though the number of D and ND specimens was not equal, the proportion of SB forming isolates among them was comparable; i.e. 67% (4/6) for D isolates, versus 68% (15/22) for ND isolates. Fig. 2B compares the frequency of virulence genes among both D and ND isolates, and demonstrates that barring *fim*, ND isolates showed a higher frequency of virulence genes than D isolates. The most frequent virulence genes were *ehaC* (78%) and *ssel* (72%); however, among ND isolates, *flu* (100%) was the most frequent virulence gene followed by *ehaC* (80%). Moreover, 50% of D isolates carried *sslE*, while 73% were *fimA+*; whereas *espA* (23%) was the least frequent gene, followed by *flu* (31%), among D isolates. Although among D and ND isolates, the frequency of *ehaC+* was similar, the main virulence gene frequency difference was with *flu* (66%), followed by *fim* (39%).

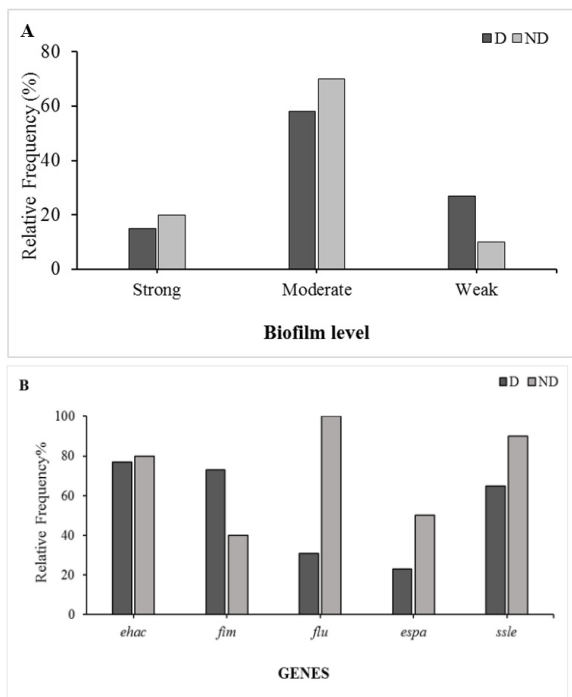**Association of biofilm formation with virulence**

Fig. 2. (A) Comparison of the strength of biofilm produced by a-EPEC isolates recovered from diarrheal versus non-diarrheal specimens; (B) Relative frequency of virulence genes among a-EPEC isolates from diarrhea and non-diarrhea specimens.

genes. Additionally, we compared the frequency of five virulence genes among a-EPEC isolates, and examined whether their presence is associated with strength of biofilm formation (Table 2). By and large, *ehaC* (78%) and *sslE* (72%) were the most frequent virulence genes among all isolates. In contrast, the least frequent gene was *espA*, which was detected in only 17% and 23% of SB and MB isolates, respectively. However, almost 62% of WB isolates were *espA*⁺. Conversely, WB isolates showed the lowest frequency of *flu* (37%); while the frequency of *flu*⁺ isolates among MB, and SB formers was 54% and 50%, respectively. Likewise, among WB and MB forming isolates, the most common genes were *ehaC* (87%), and *sslE* (82%).

As shown in Table 2, about 64% of all isolates carried *fimA* gene; with 75% of WB isolates being *fimA*⁺. However, the *fimA*⁺ distribution was askew; such that only 33% of SB isolates were *fimA*⁺, whereas 68% of MB isolates had this gene. Remarkably, nearly half of SB-forming isolates carried the *flu, sslE,* and *ehaC,* which were the most common virulence genes detected among SB producers.

Table 2. Comparison of relative frequency of virulence genes according to the strength of biofilm produced by the a-EPEC isolates (N=36).

No.	Gene	Total EPEC (N=36) No. (%)	Strong (N=6)	Moderate (N=22)	Weak (N=8)
			EPEC No. (%)		
1	*ehaC*	28 (78)	3 (50)	18 (82)	7 (87)
2	*sslE*	26 (72)	3 (50)	17 (77)	6 (75)
3	*fimA*	23 (64)	2 (33)	15 (68)	6 (75)
4	*flu*	18 (50)	3 (50)	12 (54)	3 (37)
5	*espA*	11 (31)	1 (17)	5 (23)	5 (62)

Table 3. Comparison of frequency of the a-EPEC isolates according to gender and age group.

Age	Male 24 (66.6%)			Female 12 (33.3%)		
	≤ 1yrs	2 to 5 yrs	6 to 10 yrs	≤ 1yrs	2 to 5 yrs	6 to 10 yrs
	10	9	5	7	3	2
	(41.7%)	(37.5%)	(20.8%)	(58.3%)	(25%)	(16.6%)

DISCUSSION

Diarrheagenic *E. coli* is a major cause of morbidity worldwide, and enteropathogenic (EPEC) isolates have emerged as a leading cause of intestinal disease in developing countries (2). While EPEC isolates play an important role in complications of diarrheal infections, knowledge regarding the pathogenic and microbiological characteristics of EPEC isolates from Iran is scarce. Many studies have been conducted to determine the frequency of *E. coli* pathotypes around the world (17). A recent systematic review of the prevalence and antimicrobial resistance patterns of *E. coli* pathotypes in Africa found that EAEC and ETEC were the most commonly documented diarrheagenic *E. coli* pathotypes, while EIEC was reported less frequently (18). A study from Iran in 2017 revealed a startling prevalence of STEC at 50%, with EPEC and EHEC each accounting for 25% of cases (19). According to a broad investigation conducted from 2010 to 2020 on *E. coli* pathotypes in several Iranian regions, STEC and EAEC strains have the highest incidence of *E. coli,* as well as the highest antibiotic resistance (20). The study by Kalantar et al. in Sanandaj (21) had the highest prevalence of EPEC

(59.6%), while the study by Haghi et al. from Tabriz had the lowest (4.2%) (22). Furthermore, recent data demonstrated that atypical EPEC is more widespread than conventional EPEC in both developing and developed countries (23). The incidence of EPEC in Iranian children has been assessed to be 7% (24, 25) to 23% (26) in various studies, whereas the prevalence in Iranian adults has been reported to be 9.9% to 11% (4). Therefore, here we discuss related data to the antimicrobial susceptibility and biofilm formation data pertaining of EPEC isolates, in order to gain further insight into Iran. Importantly, the overall frequency of 14% (104/747) among *E. coli* pathotypes points to no marked change in their prevalence in Iran since 2016, and it is consistent with previous reports (24). Conversely, the 34.6% frequency of a-EPEC is about 10% lower than a 2013 report; but nearly 30% higher than a similar 2018 study (25). The wide variations may partly be explained by the differences in race, age, and regional diet, in each patient population investigated.

Our finding that the prevalence of a-EPEC among ND samples (45%) was about 13% higher than the D samples (32%) suggests that a marked proportion of isolates originated from asymptomatic infected carrier patients. Importantly, data that only a-EPEC were isolated in the present study indicates that human-human transmission is an improbable primary source of most isolates. By the same token, the absence of typical EPEC supports the notion that the likely origin of the isolates has been from zoonotic contamination spillover into the food chain. Likewise, animal source contamination has been the main route of human transmission of resistant EPEC, which ultimately leads to widespread epidemics that cause major challenges for the health care systems (27).

EPEC isolates in Iran are highly resistant to amoxicillin, co-trimoxazole, and cephalosporin antibiotics such cephalexin, cefotaxime, and cefoxitin (26). One a study from Khuzestan, Iran found that EPEC had the highest prevalence of MDR among DEC pathotypes when compared to EAEC and ETEC (28). Remarkably, the broad susceptibility of EPEC isolates to IPM reveals that it may serve as an effective antimicrobial treatment for EPEC infections in Iran. Furthermore, our results also support the notion that antimicrobials GEN, CTZ, and AZT can serve as reliable alternative therapeutic drugs, since most EPEC isolates (both D and ND) also remain predominantly susceptible to these antimicrobial agents. Our data are consistent with a recent report that demonstrates the high sensitivity of EPECs to CTZ and GEN (23). In contrast, neither CIP nor TSX might be used as appropriate choices for the treatment of EPEC infections in Iran, because EPEC isolates show markedly high resistance against these antimicrobial agents.

Based on the observation that clinical isolates of EPEC usually produce stronger biofilm than the environmental isolates, several studies have linked high antibiotic resistance with the ability of EPEC to form strong biofilm, and a greater probability of chronic diarrhea (29, 30). However, we have found that, regardless of specimen type (D, or ND), a majority of EPECs formed MB or WB; and only a few were SB producers (Fig. 2B). This finding implies that perhaps most EPEC infections in Iran ought to respond to treatment without complications, and the development of chronic infection is unlikely. Surprisingly, as Table 2, reveals, a considerable number of WB- forming ND isolates carried all five virulence genes, and in contrast, several SB producers carried fewer virulence genes than WB and MB producers. Furthermore, while a few SB-forming isolates had several virulence genes, there was no apparent association between the ability to form strong biofilm and carrying virulence genes among either D, or ND isolates. Curiously, the majority of isolates (i.e., MB-formation) not only carried all five virulence genes but were susceptible to most test antimicrobials.

Remarkably, except flu, WB producers carried more virulence genes than the SB-producing isolates. The *sslE* gene, which has been shown to play a role in antimicrobial resistance and EPEC biofilm formation as a part of the T2SS virulence factor operon (30), was detected in about 50% of SB-formation, and 77% of MB-formation isolates. Its noteworthy that *sslE* confers the ability to penetrate the mucosa by reducing the intestinal mucus MUC2, MUC3, and hence increases access to epithelial cells by EPEC isolates (31). Similarly, the *flu* gene, which is necessary for biofilm formation and codes Antigen 43 (32), was found in 50% of SB-forming isolates and 54% of MB- forming isolates. This gene plays a role in cell-to-cell binding and aggregation during biofilm formation (33). Approximately, 23% of MB producers had *espA*, which encodes enhancers that are secreted by TTSS causing the adhesion and transport of bacteria inside cells (34). Data show that most

virulence genes are most frequently detected among ND isolates, which produce MB, implying that perhaps these genes are silent during most infections by EPEC, and symptoms (diarrhea) occur only when these genes are expressed. Similarly, this notion might apply to SB producers, which showed that the least frequent virulence genes were *espA* and *fimA* (35).

Our data showed a weak association between virulence genes and biofilm strength among isolates, pointing to the complicated genetic interaction that regulates EPEC biofilm production. The discrepancy between the biofilm phenotype and genotype of some isolates may be due to the intricate regulation of virulence gene expression under in vitro versus in vivo conditions, which can be addressed by testing virulence gene expression under both conditions. Nonetheless, our results warrant a thorough investigation including an in vivo model that examines a wider array of virulence genes and their possible correlation with biofilm production and antimicrobial susceptibility. We acknowledge that including a correlative analysis that cross-matches EPEC traits, such as the expression of biofilm-associated genes with clinical data from patients would make this report more complete. Accordingly, we plan a larger study that will investigate whether virulence gene expression among EPECs correlates with patients' clinical data such as infection symptoms, chronicity, antimicrobial usage, and other biometric data.

In summary, the implications of a widespread zoonotic spillover, evinced by isolation of only a-EPEC strains, underscores the vital responsibility of public health authorities to ensure the safety of beef or poultry, by carrying out continuous and thorough monitoring of the food chain, which includes enforcing rigorous safety regulations. Our findings further underscore the importance of awareness among parents in ensuring food safety and reducing the risk of diarrheal infection among children, who commonly show habitual poor personal hygiene practices.

We conclude that while the prevalence of EPEC has not changed markedly in recent years; the predominance of a-EPEC indicates a worrisome trend of animal source contamination of the food chain by a persistent zoonotic spillover in Iran. While isolates remain mostly sensitive to common antimicrobials like imipenem; data predicts the emergence of highly resistant isolates that may imminently create major challenges in the treatment of a-EPEC infections in Iran. Therefore, prompt preventive measures can thwart this imminent crisis by public health authorities controlling the current widespread zoonotic contamination of the food chain. That most EPEC isolates formed moderate biofilm, advises that for the time being, common treatment of EPEC infections in Iran should not present major challenges for the clinicians. Our findings may help clinicians make optimal therapeutic choices during the treatment of patients with severe EPEC infections in Iran, as well as assist epidemiologists and health care professionals devise policies towards effective control of EPEC outbreaks.

ACKNOWLEDGEMENTS

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