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Dominant EAEC and Widespread Antibiotic Resistance among Diarrheagenic *Escherichia coli* in Children with Acute Diarrhea in Zahedan, Iran

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ABSTRACT

Introduction: Diarrheal disease is a major cause of morbidity and mortality among children under the age of 10 worldwide, particularly in low- and middle-income countries. Diarrheagenic *Escherichia coli* (DEC) is a common cause of gastroenteritis in children. This study investigated the frequency, virulence markers, and antibiotic resistance patterns of DEC in children below 10 years with acute diarrhea in Zahedan, Iran. **Methods**: In this cross-sectional study, 300 *E. coli* isolates were collected from stool samples of children aged below 10 years with diarrhea who presented to hospitals and clinical laboratories in Zahedan. DEC pathotypes were identified using multiplex PCR and confirmed by standard biochemical tests and polyvalent antisera. **Results**: Of the 300 *E. coli* isolates examined, 89 (29.6%) were identified as diarrheagenic *E. coli* (DEC) using polyvalent antisera targeting known DEC pathotypes. Enteroaggregative *E. coli* (EAEC) was identified in 31 isolates (34.83%) based on reaction with antiserum No. 1. Enterotoxigenic *E. coli* (ETEC) was identified in 35 isolates (39.33%) based on reaction with antiserum No. 2. Enteropathogenic *E. coli* (EPEC) was identified in 23 isolates (25.84%) based on reaction with antiserum No. 3 (anti-coli3). Multiplex PCR identified the most common pathotype as EAEC (37.6%), followed by EPEC (21.7%), ETEC (15.9%), and EIEC (11.5%). Statistical analysis revealed no significant correlation between the presence of specific virulence genes (*e.g.*, *eae*, *pcvd432*, *elt*, *est*, and *iaH*) and antibiotic resistance patterns in the DEC isolates. **Conclusion**: Given the distribution of DEC pathotypes among children in Zahedan and their increased antibiotic resistance, antibiotic treatment should be guided by molecular typing and antimicrobial susceptibility testing of isolates, when appropriate.

INTRODUCTION

Comprehensive nationwide studies are warranted in Iran to elucidate the distribution of major *E. coli* pathotypes. These studies should prioritize representative sampling strategies and robust study designs to ensure reliable and generalizable findings. Diarrheagenic *E. coli* (DEC) is a common cause of diarrhea in children under five years of age in developing countries, including Iran, where it constitutes a significant public health burden due to its high prevalence and morbidity. In Zahedan, the prevalence of DEC is likely influenced by a combination of factors, including limited access to safe water, inadequate sanitation, and poor hygiene practices, which contribute to the spread of the bacteria [1-4].

DEC strains can be classified into six main pathotypes based on their distinct virulence factors, serotype associations, and epidemiological and clinical characteristics. The pathotypes include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), and diffuse adhering *E. coli* (DAEC), each characterized by specific adhesins, toxins, and other virulence factors that determine their clinical features [5-8].

Each pathotype is defined by a unique combination of virulence factors, which, in conjunction with host factors, influence the clinical manifestations of disease [5]. ETEC is a leading cause of diarrhea in developing countries,

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particularly among children under two years old, likely due to factors such as contaminated food and water and limited access to healthcare [6]. In children under one year old, EPEC is a leading cause of diarrhea, accounting for approximately 40-50% of *E. coli*-related cases. In the period between 2010 and 2020, STEC has been recognized as a significant cause of severe bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS), particularly among children under five years old. STEC produces a range of virulence factors, including Shiga toxin 1 and Shiga toxin 2, which contribute to its ability to cause severe bloody diarrhea and HUS [9]. EAEC is a significant pathotype associated with a substantial proportion of diarrheal diseases, including endemic and epidemic cases of pediatric diarrhea. EAEC is associated with intestinal inflammation, which can contribute to malnutrition and impaired growth in children, although the relationship is complex and influenced by multiple factors [10, 11]. EAEC and other DEC pathotypes have been identified as important causes of diarrhea in developed countries, contributing to a significant burden of diarrheal diseases [12]. *E. coli* pathotypes are characterized by their ability to cause severe clinical complications, and their resistance to antibiotics is a significant concern, as it involves a complex interplay of factors, including the type of infectious agent, the location of the microbe, and the concentration of the drug, which are influenced by the patient's immune status [13]. The high prevalence of antibiotic-resistant DEC pathotypes in children, estimated to be around 30-50%, poses a significant public health challenge [14]. The prevalence and pattern of antibiotic resistance for each DEC pathotype are influenced by a complex interplay of factors, including geographic location, health conditions, and socioeconomic status [2].

Accurate identification of diarrheagenic *E. coli* pathotypes and antimicrobial susceptibility testing are essential for guiding targeted and effective treatment strategies, as well as informing evidence-based prevention and control measures [15]. DEC pathotypes are identified using commercial O and H antisera, which detect specific somatic and flagellar antigens, or molecular techniques like M-PCR, which offer higher sensitivity and specificity [16].

Given the significance of diarrheagenic *E. coli* (DEC) in causing diarrhea and the scarcity of comprehensive data in developing countries like Iran, this study aimed to address this knowledge gap. The aim of this study was to determine the prevalence and distribution of DEC pathotypes and their specific antibiotic resistance patterns among children with diarrhea in Zahedan, Iran, to inform evidence-based treatment strategies and identify vulnerable populations.

MATERIAL AND METHODS

Sampling and culture. From July 1, 2016, to October 31, 2016, stool specimens (n=300) were collected from children with acute diarrhea (defined as three or more loose or watery stools per day) who were either outpatients or inpatients in three hospitals in Zahedan: Ali ibn Abi Talib Hospital, Bu-Ali Hospital, and Khatamalanbeya Hospital. Diarrhea was defined as a history of three or more loose or watery stools per day, or two or more stools of soft or liquid consistency in the 24 h preceding stool collection. Stool samples were processed within 2 hours of collection. The collected stool samples were initially cultured on McConkey Agar, blood Agar, EMB (Merck, Germany), and XLD (Merck, Germany) to isolate *E. coli*. After 24 h of incubation, the culture plates were examined for the presence of *E. coli* colonies. Presumptive *E. coli* colonies were identified using a combination of biochemical tests, including TSI, SIM, MR-VP, Simmons citrate, urea, and lysine decarboxylase. Identification of *E. coli* isolates was performed using a series of biochemical tests, including fermentation of sugars in TSI medium, indole production and motility in SIM medium, the reaction in MR-VP medium, urea hydrolysis, Simmons citrate utilization, lysine decarboxylation, growth on XLD agar, and growth on EMB medium with a greenish metallic sheen [17].

Isolation of *E. coli* pathotypes by serotyping. *E. coli* strains were serotyped using Sifin *E. coli* polyvalent antisera and a rapid slide agglutination test. Isolated colonies from 18-h cultures on TSI medium were mixed with one drop of antiserum on a glass slide and incubated for 40 seconds before observation. A positive response was defined as agglutination of bacterial cells upon exposure to the antiserum.

Antibacterial susceptibility testing. Antibiotic susceptibility testing was performed according to CLSI M100-S28 guidelines using the following antibiotics: tetracycline (30 µg), chloramphenicol (30 µg), cotrimoxazole (25 µg), cefixime (5 µg), gentamicin (10 µg), ciprofloxacin (5 µg), imipenem (15 µg), cefotaxime (30 µg), ceftriaxone (30 µg), nalidixic acid (30 µg), ampicillin (10 µg). Antibiotic susceptibility was determined by measuring the diameter of the zone of inhibition around each antibiotic disk, with susceptible isolates showing a zone diameter ≥X mm, intermediate isolates showing a zone diameter =Y mm, and resistant isolates showing a zone diameter ≤Z mm, according to CLSI criteria [18].

DNA extraction and multiplex PCR (M-PCR). Total DNA was extracted from *E. coli* isolates using thermal lysis. The bacterial isolates were cultured for 24 h on LB (Lurria-Bertani) agar. One colony was then transferred to 200 µL of sterile water, and the suspension was boiled for 15 min. After the boiling step was complete, the suspension was centrifuged at 13,000 rpm for 10 min. The supernatant was discarded, and the tube was transferred to a laminar flow hood to dry for 30 min. Then, 50 µl of distilled water was added to the tube, which was then stored at -20°C. To assess the quality of the purified DNA, 4 µl of each DNA sample (dissolved in distilled water)

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was electrophoresed on a 1% agarose gel at 80 volts for 45 min. The DNA samples were then stored at -20°C until further use in PCR. The primary PCR mixture was prepared using exact concentrations and volumes of PCR reagents, including $MgCl_2$ (1.5 mM), dNTPs (200 µM), primers (20 pmol), buffer (1X), and distiiled water, according to the WHO guidelines for PCR protocols. The PCR mixture was prepared by experienced laboratory personnel trained in molecular biology techniques [18-20].

The PCR reaction was performed in a final volume of 25 µL. The reaction mixture were contained 1 µL of DNA template, 12 µL of Master Mix (Fermentas, Waltham, Massachusetts, United States), 1 µM of each primer (Table 1), and 10 µL of deionized water (Sigma-Aldrich, USA). PCR assays were performed in a Bio-Rad MJ Mini thermal cycler (T100cycler, Bio-Rad, Hemel Hempstead, UK) with a heated lid for 35 cycles. The PCR products were then analyzed by agarose gel electrophoresis (1% agarose gel stained with ethidium bromide, run for 95 min at 85 volts) for confirmation [18-20].

Table 1. Primers used to detect virulence genes in diarrheagenic *E. coli* by PCR

Target Gene	Primer	Sequence (5´ to 3´)	Size (bp)	Pathotype
eae	F	TTATGGAACGGCAGAGGT	790	EPEC
	R	CTTCTGCGTACTGCGTTCA		
stx	F	ACGAAATAATTTATATGT	900	STEC
	R	TGATTGTTACAGTCAT		
ialH	F	CTGGTAGGTATGCTGAGG	320	EIEC
	R	CCAGGCCAATTATTTCC		
elt	F	TCTATGTGCATACGGAGC	322	ETEC
	R	ATACTGATTGCCGCAAT		
est	F	TAAACAAGTAGAGGTCTTCAAAA	147	
	R	CGGTACAGAGCAGGATTACAACA		
Pcvd432	F	GGCGAAAGACTGTATCAT	630	EAEC
	R	ATGTAGAAATCCGCTGTT		

Statistical analysis. Statistical analysis was performed to determine the correlations between the isolated bacterial strains, antibiotic resistance patterns, and specific clinical (symptom severity, disease duration) and demographic variables. The authors performed the statistical analysis using SPSS software version 19. The chi-square test was used to analyze categorical variables, while Fisher's exact test was used to analyze variables with small sample sizes. A *P*-value <0.05 was considered statistically significant.

RESULTS

Patient demographics. Of the 300 samples collected, 173 (57.7%) were from male patients and 127 (42.3%) from female patients. The age of the patients ranged from under nine years and older.

Table 2. Percentage of *E. coli* isolates positive for virulence genes detected using multiplex PCR and confirmed by antiserum agglutination

Identifiable serogroups	Antiserum	Percentage
O26:K60 O44:K74 O114:K90 O125:K70 O142:k86 O158:K-	Anti-coli1	34.8
O55:K59 O86:K61 O91:K- O111:K58 O119:K69 O126:K71 O127:K63 O128:K67	Anti-coli2	39.3
O25:K11 O78:K80 O103:K- O118:K- O124:K72 O145:K- O157:K- O164:K-	Anti-coli3	25.8

Out of the 300 *E. coli* isolates, 89 (29.6%) were identified as diarrheagenic using multiplex PCR and confirmed by antiserum agglutination. Among these 89 isolates, 31 (34.83%) were identified as serogroup O26:K60, O44:K74, O158:K-, O142:K86, O125:K70, and O114:K90 using antiserum No.1 (anti-coli1). Moreover, 35 (29.33%) isolates were identified as serogroups O55:K59, O128:K67, O127:K63, O126:K71, O119:K69, O111:K58, O91:K-, and O86:K61 using antiserum No. 2 (anti-coli2). Moreover, 23 (25.84%) isolates were identified as serogroups O25:K11, O78:K80, O103:K-, O118:K-, O124:K72, O157:K-, O164:K-, and O145:K- using antiserum No. 3 (anti-coli3).

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Antibiotic susceptibility testing. The prevalence of antibiotic resistance among the identified DEC serotypes was evaluated. The highest prevalence of antibiotic resistance was observed for ampicillin (94.8% of all DEC isolates), tetracycline (87.2% of all DEC isolates), and cotrimoxazole (70.5% of all DEC isolates). The lowest prevalence of antibiotic resistance was observed for imipenem (1% of all DEC isolates) and ciprofloxacin (8.9% of all DEC isolates).

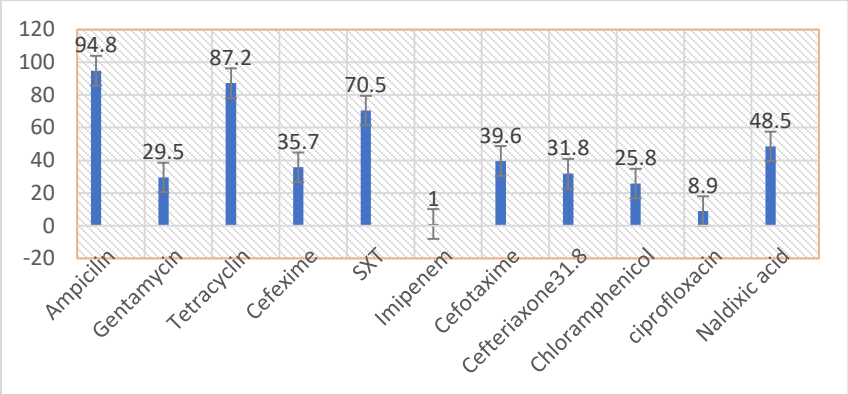

Fig. 1. Antibiotic resistance profile of diarrheagenic *E. coli* (DEC) isolates from children under ten years of age with acute diarrhea in Zahedan, Iran

Multiplex PCR for pathotypes. The amplification of the *PCVD432* gene, encoding a virulence factor of Enteroaggregative *E. coli* (EAEC), resulted in a 630-bp amplicon in 26 (37.6%) of the isolated DEC strains. Additionally, the amplification of the *eae* gene, encoding an adhesin of Enteropathogenic *E. coli* (EPEC), yielded an approximately 790-bp amplicon in 15 (21.7%) of the isolated DEC strains. Among the DEC isolates, 11 (15.9%) carried the *elt* gene, and 9 (13%) carried the *est* gene, both associated with Enterotoxigenic *E. coli* (ETEC). Furthermore, the presence of the *iaH* gene was detected in 8 (11.5%) of the isolates, suggesting their association with Enteroinvasive *E. coli* (EIEC).

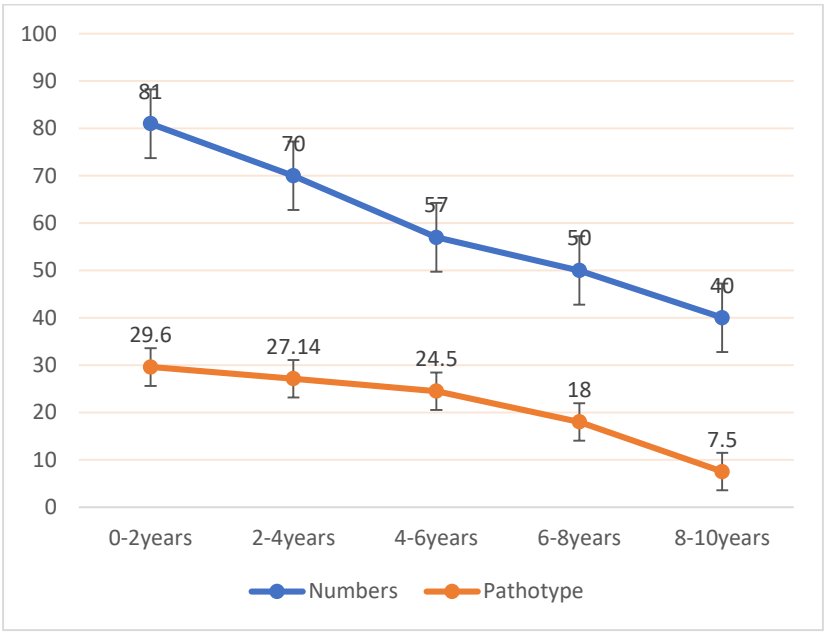

Fig. 2. Frequency distribution and relative frequency of ETEC, EPEC, EAEC, and EIEC (%) by age in children under ten years

Comparing serotyping versus PCR. Serotyping identified 89 isolates as belonging to specific diarrheagenic *E. coli* pathotypes (EAEC, EPEC, ETEC, EIEC), while molecular analysis using PCR detected 69 isolates carrying specific virulence genes. This discrepancy suggests that serotyping may have a lower detection rate for diarrheagenic *E. coli* compared to PCR.

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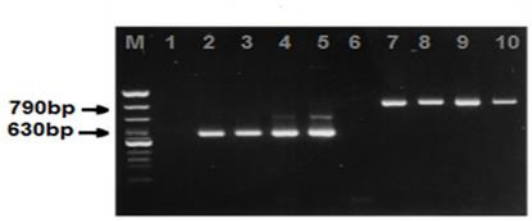

Fig. 3. Multiplex PCR for identification of EAEC and EPEC pathotypes. Lane1: DNA marker, Lane 2-5: Amplicons specific for EAEC virulence factors, Lane 7-10: Amplicons specific for EPEC virulence factors

DISCUSSION

Diarrhea is a common childhood illness caused by a variety of enteropathogens, including bacteria, viruses, and protozoa. *Salmonella*, *Shigella*, *Yersinia enterocolitica*, *Campylobacter*, *Vibrio*, various *E. coli* pathotypes, and other bacteria are among the most common causes of bacterial diarrhea [21-23]. *E. coli* is a significant pathogen that commonly causes watery diarrhea in children under the age of ten, particularly in areas with inadequate access to safe drinking water, such as Zahedan, Iran. Moreover, *E. coli* has long served as a specific indicator of fecal contamination in water microbiology for over four decades [24]. Estimates suggest that *E. coli* diarrheal pathotypes are responsible for 30 to 40% of all diarrhea cases, with approximately 20% necessitating hospitalization [17, 25]. Our study included children with acute diarrhea who were referred to hospitals and the central laboratory in Zahedan. Among the 300 *E. coli* samples analyzed, 89 (29.6%) tested positive for diarrheagenic *E. coli* (DEC) pathotypes (EAEC, EPEC, ETEC, EIEC) through serotyping and molecular methods (PCR), suggesting a higher prevalence of DEC infection compared to previous studies in the region.

Accurate identification of pathogenic strains is crucial. Therefore, the development and utilization of additional molecular techniques, such as PCR and DNA sequencing, are essential for the reliable detection and characterization of specific diarrheagenic *E. coli* pathotypes (EAEC, EPEC, ETEC, EIEC) and their virulence factors [26]. As *E. coli* strains comprise a substantial part of the normal intestinal microflora, distinguishing diarrheagenic *E. coli* strains from the normal microflora requires the use of phenotypic and genotypic tests that target specific virulence factors and adhesins, such as PCR and DNA sequencing [16]. The present study demonstrated a significantly higher prevalence of diarrheagenic *E. coli* (29.6%) in children with acute diarrhea in Zahedan, Iran, compared to previous studies in the region [16].

Furthermore, a significant correlation was discovered between the pathotypes and the month of sampling, with the highest frequency observed during the summer months (June to August), particularly in August. This could be attributed to the warmer temperatures (above 25°C) and increased humidity, which potentially facilitate increased transmission of bacteria and other pathogenic agents through contaminated water and food, as well as via flies and mosquitoes. This increased vulnerability might be attributed to the developing immune system and the lack of protective antibodies in children under 10 years old. Overall, the causes of diarrhea caused by diarrheagenic *E. coli* in developing countries, especially among children, encompass several factors, including limited access to healthcare facilities, inadequate attention to proper handwashing and food handling practices, absence of proper urban and rural water treatment and sewage systems, and underlying health conditions like malnutrition [14].

The antibiotic resistance patterns in *E. coli* isolates differ among specific communities, countries, and geographical regions, influenced by various factors such as agricultural activities [27]. Tetracycline antibiotics are commonly used to treat human infections, particularly bacterial gastroenteritis, due to their relatively low cost and effectiveness, although they can have significant side effects such as gastrointestinal disturbances and allergic reactions. However, the indiscriminate and inappropriate use of tetracyclines has contributed to the emergence and spread of tetracycline-resistant bacteria, including diarrheagenic *E. coli* [28].

The isolates exhibited relatively low levels of antibiotic resistance against imipenem and ciprofloxacin, respectively. Ciprofloxacin is effective against certain gastrointestinal infections caused by *E. coli*, such as traveler's diarrhea. Cefixime can be considered as an alternative therapeutic option for the treatment of *E. coli*-associated diarrhea in pediatric patients when imipenem and ciprofloxacin are ineffective due to resistance. In the present study, the EAEC pathotype was the most prevalent among the diarrheagenic *E. coli* isolates obtained from children with acute diarrhea, as identified by PCR analysis. Furthermore, PCR analysis identified the presence of the *pCVD432* gene, a genetic marker for the EAEC pathotype, in 26 out of 69 isolates (37.6%). Fifteen (21.7%) isolates were identified as EPEC pathotype based on the amplification of the attaching and effacing (*eae*) gene. The presence of the *elt* gene and the *est* gene,

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which are genetic markers for the ETEC pathotype, was detected in 11 isolates (15.9%) and 9 isolates (13%), respectively. Furthermore, 8 strains (11.5%) harbored the *iaH* gene, indicating their characterization as the EIEC pathotype. Nevertheless, none of the diarrheagenic *E. coli* isolates from the children were identified as STEC strains. Significant variations in the frequency of diarrheagenic *E. coli* pathotypes were observed across different months, with the highest prevalence recorded in August, corresponding to the summer season. A statistically significant association was observed between the prevalence of the four diarrheagenic *E. coli* pathotypes (EAEC, EPEC, ETEC, and EIEC) and patient age, with the highest proportion occurring in children under 5 years of age, specifically in infants aged 6-8 months. The higher prevalence observed in infants and young children could be attributed to their increased susceptibility to diarrheagenic *E. coli* infections, likely due to a combination of factors including their developing immune systems, environmental exposure, and host factors. The high burden of diarrheal diseases in developing nations, particularly among children, can be attributed to a complex interplay of factors, including inadequate healthcare infrastructure, poor hand and food hygiene practices, insufficient access to clean water and sanitation, lack of appropriate educational resources, malnutrition, poverty, and environmental factors [27].

In a study conducted by Orni *et al*. (2004), *E. coli* was isolated from 16.9% of the 2,629 children between 1 and 12 years of age with acute diarrhea [29]. Another study carried out in Vietnam in 2002 investigated the prevalence of diarrheal pathogens, which accounted for 27% of all cases examined. Among the identified pathogens, the prevalence of ETEC, EAEC, and EPEC was 6.5%, 15.7%, and 12.2%, respectively [6]. Albert *et al*. (1995) conducted a study on diarrheal and non-diarrheal stool samples collected from children between 1 and 12 years of age in Bangladesh and isolated multiple serotypes, including O125, O127, O114, O126, O55, O128, O142, and O119. Molecular analysis using PCR suggested that most of these serotypes belonged to EPEC strains [30].

In a study conducted in Tabriz, Moghadam *et al*. (2013) identified the most common serotypes using antiserum pool number three, which included O25: K11, O78: K80, O103: K-, O118: K-, O124 K72, O145: K, O157: K-, and O164: K- [16]. In contrast, our study employed antiserum pool number two and identified distinct serotypes.

Haghi *et al*. (2014) reported that children aged 0-5 years had a high prevalence of ETEC pathotype, with a resistance rate of 100% to erythromycin and azithromycin, and a resistance rate of 72.9% to imipenem among children infected with ETEC [31]. In Hamadan, Iran, *E. coli* isolated from children with diarrhea exhibited a high resistance rate to ampicillin and cotrimoxazole, and a relatively low resistance rate to ciprofloxacin [22]. Diarrheagenic *E. coli* (DEC) isolates exhibited the highest resistance rates to tetracycline, ampicillin, amoxicillin, amoxicillin-clavulanic acid, and trimethoprim [27]. Arif *et al*. (2010) developed a multiplex PCR assay for diagnosing four major diarrheagenic *E. coli* pathotypes (ETEC, EPEC, EHEC, and EAEC) by targeting six virulence genes. *E. coli* was identified in 50 fecal samples using standard biochemical tests such as API 20E or VITEK 2. The multiplex PCR assay detected *E. coli* target genes in 19 out of 50 (38%) diarrheal samples, indicating the presence of diarrheagenic *E. coli*. The ETEC group genes (*lt* or *st*) were detected in 26.3% (n=5) of the samples, EPEC group genes (*eae* or *bfp*) were detected in 63.1% (n=12) of the samples, and EAEC group genes (*pCVD*) were detected in 21% (n=4) of the samples. Notably, two samples (10.5%) showed co-infection with EAEC group genes, as well as EPEC and ETEC group genes [32]. Lopez *et al*. (2003) developed a single multiplex PCR assay for identifying gene loci associated with five major diarrheagenic *E. coli* pathotypes, including EPEC, EAEC, ETEC, and STEC. The PCR assay demonstrated specificity, sensitivity, and rapid detection of target *E. coli* isolates in fecal and food samples [21].

The prevalence of diarrhea among children admitted to hospitals in both urban and rural hospitals is significantly associated with the presence of diarrheagenic *E. coli* strains. This association was observed in the present study, which is consistent with previous research findings [33-35]. Due to the limited studies on diarrheagenic *E. coli* strains in developing countries, particularly in Iran, it is crucial to perform diagnostic tests in clinical laboratories to further identify these pathogenic strains.

The multiplex PCR assay demonstrates high analytical sensitivity and rapid and accurate detection of specific gene markers associated with diarrheagenic *E. coli* pathotypes, including EAEC, EPEC, ETEC, and STEC. The simultaneous detection of these pathotypes by the multiplex PCR assay provides a time-saving and cost-efficient alternative that complements conventional phenotypic methods. The multiplex PCR assay can be employed as a valuable tool to complement traditional diagnostic techniques, which are often labor-intensive and cost-prohibitive. Furthermore, due to the increasing antibiotic resistance of these pathotypes, as reported in previous studies, accurate identification and antibiotic susceptibility testing of *E. coli* strains are essential factors in prescribing effective antimicrobial therapy, in conjunction with other critical considerations such as patient history and clinical presentation. Given the increased antibiotic resistance and mortality rates among hospitalized patients, it is crucial to implement accurate and cost-efficient microbiological diagnostic and therapeutic strategies. These strategies can facilitate

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the identification of diarrheagenic *E. coli* strains and inform the selection of effective antimicrobial therapy.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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