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Incidence of Diarrheagenic *Escherichia coli* Pathotypes in Children Suffering from Diarrhea in Tertiary Care Hospitals, Quetta, Pakistan

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Authors' Contribution
ZH performed the experiments, collected the data and wrote the manuscript. AMT designed and supervised the project. KU analyzed the data. TMA compiled results. MZM provided technical help in wet lab. IA, AS and AI helped in data analysis. SR reviewed and finalized the manuscript.

Key words
Diarrhea, *Escherichia coli,* Entero-aggregative *E. coli,* Enterotoxigenic *E. coli*

ABSTRACT

Diarrhea is one of the leading public health problems in under developed countries. In Pakistan, diarrheal cases are treated empirically and, rarely, specific investigations are made. This study was designed to identify the prevalent strains of *Escherichia coli* among children of less than five years of age in Quetta, Balochistan, Pakistan. A total of 200 samples were collected from children suffering with diarrhea. *E. coli* was isolated using standard biological procedures and confirmed by colonial characteristics, biochemical profile and specie specific PCR. Multiplex PCR was applied to differentiate among the five main diarrheagenic *E. coli* pathotypes (enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC). Results show that the virulent EAEC strain of *E. coli* was found to be the most prevalent (35%), while EPEC was identified in 15%, followed by EHEC (8%). Notably, none of the samples was found positive for EIEC. Moreover, ETEC was identified in 11% cases of co-infection. Results showed that children with less than two years of age were affected significantly. However, gender had no significant effect on acquiring diarrhea. In conclusion, pathogen spectrum of pathogenic *E. coli* is EAEC strain. Children under one year of age were affected significantly, and no significant effect was observed when data was stratified on gender base. Further studies are required to investigate the other pathological agents and control parameters should be strengthened to minimize the risks.

INTRODUCTION

Diarrhea accounts for about 10% of all deaths globally in children of less than five years of age and ranked at second position after pneumonia. In Pakistan, 20-30% of total deaths in children of less than 5 years of age are caused by diarrhea (Habib *et al*., 2013). Based on specific virulence factors and other phenotypic characteristics diarrheagenic *E. coli* (DEC) has been categorized into six different pathotypes including enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC) and enterohemorrhagic *E. coli* (EHEC), and shiga-like toxin (STEC). The different DEC has been reported to share 40% of cases of acute diarrhea in children below 5 years (Kaper *et al*., 2004; Kotloff *et al*., 2013; Stritt *et al*., 2012). Although DEC pathotypes are of considerable cause of diarrhea in Pakistan, however, strains are not generally sought, particularly in developing countries like Pakistan, and thus prevalence of diarrhea caused by DEC generally remains unknown.

DEC has been reported involved in outbreaks of gastrointestinal infections in many developing countries. For instance, DEC was found in 23.3% of cases of diarrhea in Mexico (Canizalez-Roman *et al*., 2016). Besides developing countries, other developed countries have also witnessed the epidemics or random cases of gastrointestinal infections, which were mostly food borne associated such as the epidemics of STEC like *E. coli* in Germany in 2011 (Frank *et al*., 2011). Similarly, a report published about the contamination of raw vegetables with STEC *E. coli* in Pakistan report that 34% of vegetables were found positive indicating widespread presence of pathogenic *E. coli* presenting public health threats. Besides human, animals have also been reported to be infected by DEC, which were found multidrug resistant and leading to

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severe diarrhea (Adnan *et al*., 2017).

Despite random reports of *E. coli* recovered from diarrheic patients in Pakistan, molecular characterization and pathotyping of clinical isolates is totally lacking. Such analysis are cruial to understand pathogenicity of *E. coli* isolates and can further help policy makers to devise effective strategy for its control. This study is thus aimed to determine the relative prevalence of five major DEC types in children younger than 5 years of age in Quetta, Pakistan and its virulence.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Ethical Committee of University of Balochistan and all procedures described here were performed as per local institutional ethical guidelines. Informed consent was taken from donors.

Sample collection and microbiology

The samples were collected from Bolan Medical Complex Hospital and Children Hospital Quetta for a period of seven months. Children under the age of five years suffering with diarrhea were included in this study. Patients diagnosed with other infectious disease in addition to diarrhea were excluded from the study. Samples were obtained from rectums of children suffering with diarrhea with help of a sterile cotton swabs. The collected samples were then placed in Cary Blair Transport Medium and transferred to the laboratory for culturing and isolation of *E. coli*. The samples were inoculated and streaked on eosin methylene blue (EMB) and MacConkey agars (Oxoid, UK) for colony formation. After overnight incubation at 37°C, the colonies were observed for characteristic of *E. coli*. The presumable *E. coli* colonies were then streaked onto fresh sterilized nutrient agar (Oxoid, UK) to perform conventional biochemical tests for *E. coli* identification. *E. coli* was identified by positive methyl red and indole test, negative Voges proskauer, citrate and urease tests. All isolates of *E. coli* from each rectal swab showing typical characteristics of colony morphology and biochemical properties were maintained in the laboratory in nutrient broth at 4° C as well as were stocked at -70 °C until further use.

Genomic DNA extraction and differentiation of DEC

Extraction of DNA was carried out by cetyletrimethyle ammonium bromide (CTAB) method as described by Minas *et al*. (2011). The extracted DNA was kept at -70°C until used as a template for PCR. PCR was carried out to confirm *E. coli* by using universal primer of *E. coli* (*uidA*). Conditions for PCR were: 94°C for 5 min for initial template denaturation, 35 cycles at 98°C for 10 seconds for final denaturation, primer annealing at 68°C for 35 seconds and primer extension at 72°C for 45 seconds and finally at 72ºC for 7 min (final extension). Multiplex PCR was used to investigate and categorize isolates into their respective pathotypes/diarrheagenic groups (Persson *et al*., 2007). Total reaction volume of PCRs performed was 90µl, containing 45µl of PCR Master mix (Gene All), 1µl of each set of 11 primers (Macrogen),10µl of DNA template extracted from samples and 15µl molecular grade water. Using multiplex PCR, 11 specific primers (Macrogen) were used to identify the particular *E. coli* pathotypes as shown in Table I. Amplification conditions for multiplex PCR include 95°C (initial template denaturation), 35 cycles at 94°C for 50s (final denaturation), primer annealing starting from 45ºC for 45s with a regular interval increase of 0.5°C after each cycle, till the temperature becomes 60°C, primer extension at 72°C for 1 min and finally at 72ºC for 7 min (final extension). Amplicons were examined by electrophoresis on agarose gel (2% w/v) followed by staining with ethidium bromide

RESULTS

To evaluate the prevalence of DEC in children of less than five years of age, 200 rectal swab samples from children suffering with diarrhea were collected for analysis. The samples were analyzed by conventional and advanced diagnostic methods to document the prevalence of DEC in children suffering with diarrhea. Out of 200 collected samples, 100 samples were found positive for *E. coli* by cultivating on selective EMB agar media. Results demonstrated that positive samples of *E. coli* presented typical colonies with greenish metallic sheen in reflected light with blue black center as shown in Figure 1A. Purified and biochemically confirmed *E. coli* isolates were confirmed by species specific PCR (Fig. 1B).

Samples confirmed as *E. coli* with species specific PCR were further investigated by multiplex PCR to identify the specific type of *E. coli* by amplifying their most common virulent gene (Figure 1C). The multiplex PCR results demonstrated that 35 % of the isolates have the virulent gene *Agg*R (430bp) of EAEC as shown in Table II. Importantly, *Agg*R showed co-infection with EHEC (12%) and EAEC and ETEC (11%). The *eae* virulent gene (229bp) expressed by EPEC was 15% of the samples positive for *E. coli*. The EHEC pathotype expressing Shiga-like toxin 2 (*stx2*) gene (779bp) was amplified in 8% of the total positive samples for *E. coli* and Shiga-like-toxin 1 (*stx1*) gene (614bp) of EHEC was only detected in co-infected samples with *AggR* (EAEC) or combined with *AggR* (EAEC) and *elt* (ETEC).

Table I.- Primer sequences of *E. coli* virulent genes.

Patho-type	Gene	amplicon size(bp)	Primer sequence	Reference
EHEC	Stx 1(F)	614	ACA CTG GAT GAT CTC AGT GG	
	Stx 1(R)		CTG AAT CCC CCT CCA TTA TG	
	Stx 2(F)	779	CCATGA CAA CGG ACA GCA GTT	
	Stx 2(R)		CCT GTC AAC TGA GCA CTT TG	
	HylA(F)	534	GCA TCA TCA AGC GTA CGT TCC	(Ogura *et al*., 2007)
	HylA(R)		AAT GAG CCA AGCTGG TTA AGCT	(Chase-Topping *et al*., 2012)
	PcvD(F)	630	CTG GCG AAA GAC TGT ATC AT	(Nguyen *et al*., 2005)
	PcvD(R)		CAA TGT ATA GAA ATC CGC TGTT	(Pereira *et al*., 2007)
EPEC	bfpA(F)	450	CAC CGT TAC CGC AGG TGT GA	(Rappelli *et al*., 2005)
	bfpA(R)		GTT GCC GCT TCA GCA GGA GT	
	eae(F)	229	CTG AAC CAG ATC GTA ACG GC	(Rappelli *et al*., 2005)
	eae(R)		TGA TAA GCT GCA GTC GAA TCC	
ETEC	elt(F)	322	TCT CTA TGT GCA TAC GGA GC	(Nguyen *et al*., 2005)
	elt(R)		CCA TAC TGA TTG CCG CAA T	
	estl(F)	170	TCT TTC CCC TCT TTT AGT CAGTC	(Rappelli *et al*., 2001)
	estl(R)		CAG CAC AGG CAG GAT TAC	
EIEC	ipaH(F)	619	GTT CCT TGA CCG CCT TTC CGA TAC CGT C	(Sethabutr *et al*., 1993)
	ipaH(R)		GCC GGT CAG CCA CCC TCT GAG AGT AC	
EAEC	AggR(F)	430	AGA CGC CTA AAG GAT GCC C	(Nataro, 2005; Jenkins *et al*., 2005; Sheikh *et al*., 2002)
	AggR(R)		GAG TTA TCA AGC AAC AGC AAT GC	
E.coli	uidA(F)	619	CCA AAA GCC AGA CAG AGT	(McDaniels *et al*., 1996)
	uidA(R)		GCA CAG CAC ATC AAA GAG	

Table II.- Prevalence of *Escherichia coli* (DEC) strains in children suffering with diarrhea.

Escherichia coli (DEC) strains	Total samples	Positive (n)
EAEC	100	35
EPEC	100	15
EHEC	100	08
EAEC+ETEC+EHEC	100	11
EAEC+EHEC	100	12
EPEC+EHEC+EAEC	100	13
EPEC+EHEC	100	06
EIEC	100	00

Similarly, ETEC pathotype expressing *elt* gene (322bp) was only found in samples infected with EHEC and EAEC. Such co-infections were present in 11% of the positive *E. coli* samples. Moreover, *stx2* of EHEC and *AggR* of EAEC were found together in 12% of the positive samples. EPEC co-infection with EHEC and EAEC was found 13%, while EPEC and EHEC co-infection was 6% in all samples analyzed.

To estimate the prevalence of EIEC, specific primers against the virulent gene *ipaH*, (619bp) was employed however no sample was found positive for the said strain (Fig. 1C).

The results of multiplex PCR were further analyzed to dissect the prevalence of DEC in children on basis of gender and age. Of the 100 samples positive for DEC, 49% of them were boys while rests were girls (51%). Majority of the children were less than 12 months (37%) followed by age between 1-2 years (30%), 2-3 years (19%), 3-4 years (6%) and 4-5 years (3%) as shown in Table III. These results indicate that children under five years of age suffering with diarrhea carry high burden of DEC. Importantly, EAEC strain proved to be the most prevalent in area under study.

Table III.- *Escherichia coli* (DEC) strains identified by multiplex PCR and their prevalence sub-divided according to the gender and age of children suffering with diarrhea.

Escherichia coli (DEC) strains	Gender		Age (Years)				
	Male	Female	<1	1-2	2-3	3-4	4-5
EAEC	15	25	12	14	8	1	0
EPEC	9	6	7	4	3	0	1
EHEC	5	3	3	4	1	0	0
EAEC+ETEC+ EAEC	5	6	3	3	2	2	1
EAEC+EHEC	5	7	4	4	3	1	0
EPEC+EHEC+ EAEC	7	6	5	4	1	2	1
EPEC+EHEC	3	3	3	2	1	0	0
EIEC	0	0	0	0	0	0	0

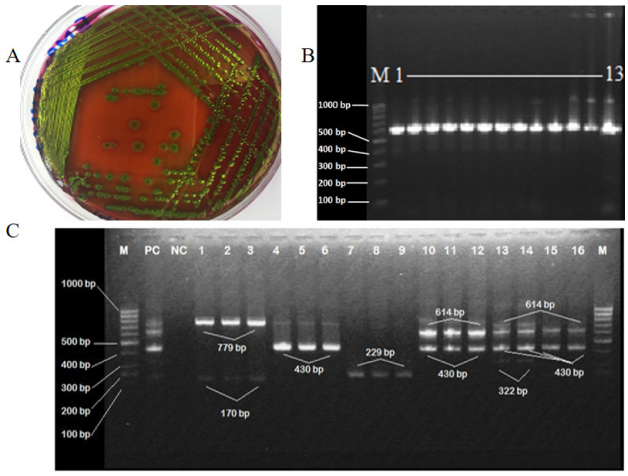

Fig. 1. Isolation and identification of various *E. coli* pathotypes based on virulent gene profile. A, growth of purified *E. coli* isolate on EMB agar; B, representative results of amplification of *uidA* gene of *E.coli* from collected samples: Lanes 1-13 positive amplification of *uidA* gene (630bp). M is molecular weight marker, size of which is demarcated on the left; C, multiplex PCR results showing amplification fragments of different DEC. Lanes 1-3 positive amplification of *stx2* (779bp). 4-6 shows positive amplification of *Agg* (430bp), 7-9 for eae gene (229bp), 10-12 shows amplification of stx1 (614bp) and *Agg* (430bp). Lanes 13-14 denotes the amplification of *stx1*, *Agg* and *elt* genes (614bp, 430bp and 322bp respectively). Lanes 15-16 show amplification of *stx1* (614bp) and *Agg* genes (430bp). M, PC and NC denotes molecular weight marker, positive control and negative control, respectively.

DISCUSSION

In developing countries, the DEC strains are not regularly sought as stool pathogens but treated as per symptoms. Since the infections and complications caused by DEC are critical, especially in infants, proper investigations should be practiced before prescription of any drug. In this study, the low percentage (50%) of *E. coli* obtained from children with diarrhea may be due to the fact that antimicrobial therapy prior to sample collection might have been administered in some cases and it is a known fact that this can reduce the percentage of bacterial enteropathogens isolation (Rohner *et al*., 1997). Our results are in agreement with previous studies and reporting important DEC, which were found in samples obtained from children suffering with diarrhea (Nguyen *et al*., 2005b). In this study four categories of DEC were identified. One of the major emerging pathogens that has been associated with diarrhea is EAEC and it is known to cause persistent diarrhea in children in developing nations. Furthermore, EAEC has been identified in patients infected by human immunodeficiency virus and specially children and travelers in the developing world (Adachi *et al*., 2002; Nataro 2005a; Samad *et al*., 2018). EAEC are a heterogeneous group of bacteria that display a wide range of virulence factors (de Sousa and Dubreuil, 2001). The diarrhea caused by EAEC is watery diarrhea with no blood and manifested by occasional abdominal cramps, but with no fever, and has been documented globally as described earlier consistent with our findings (Rajendran *et al*., 2010). EPEC was found to be the second most prevalent DEC (15%) in this study. Diarrhea caused by EPEC is usually watery that may contain mucus but normally does not necessarily have blood in it. Symptoms include fever, vomiting and dehydration. In infants, typical EPEC is quite common cause of gastroenteritis. ETEC produce heat-labile (LT) and/or heat-stable (STa and STb) toxins that cause diarrhea in humans and animals. Traveler's diarrhea is commonly caused by the exposure of ETEC in these areas (Bern *et al*., 1992). The occurrence of ETEC (11% in the diarrhea group as a mix infection) was lower in our study as reported by studies conducted in other countries (Franzolin *et al*., 2005).

Very less EHEC strains were identified in this study. It has been observed that there is low prevalence of EHEC in developing countries (Franzolin *et al*., 2005; Kimata *et al*., 2005; Moyo *et al*., 2007) which is in agreement with result of our study. EHEC produce verotoxins 1 and 2 (*stx1* and *stx2*) and these strains are related with food-borne diseases predominantly due to consumption of raw shredded meat, uncooked milk or by intake of unclean foods polluted with faeces (Pickering *et al*., 1994).

EIEC is a source of broad range human's infections. Several studies around the globe have reported the low frequencies of EIEC which is also in agreement with our studies (Moyo *et al*., 2007; Nguyen *et al*., 2005b). No work has been done previously in Quetta, Pakistan regarding the use of multiplex PCR to detect DEC. There has been much similarity and sensitivity to our studies concerning the multiplex PCR as assays conducted earlier (Nessa *et al*., 2016; Rajendran *et al*., 2010). In this study, we used only one specific and sensitive multiplex PCR assay that has reduced the number of several assays for gene detection so the time and effort have been saved (Sobel *et al*., 2004). There is a possibility of detecting mixed infections by these assays which involve more than one DEC strain. It is very important to identify DEC accurately so that the spectrum of the disease, the infection source and transmission routs can be understood clearly. By identifying this way the clinician will be able to properly manage and treat the disease. Common antibiotics usually face resistance by strains of DEC. It is therefore very important to rapidly detect the specific pathogen and accomplish the assay within 24 hours. If multiplex PCR assay is used in diagnostic laboratories it will definitely provide a rapid and practical method to identify DEC (Beutin 2006).

CONCLUSION

In conclusion, EAEC is an important pathogen associated with diarrhea was the most prevalent in children from Quetta, Pakistan. Multiplex PCR proved to be specific, sensitive and much rapid method of diagnosis therefore it can be used in diagnostic laboratories to overcome the limitations of traditional techniques.

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Statement of conflict of interest

The authors declare no conflict of interest.

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