ORIGINAL ARTICLE

Screening of Infectious Causes of Diarrhea and Genetic Determination of Diarrheagenic *E. coli* using Multiplex PCR in under 5 Years Children in Egypt

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ABSTRACT

***Background**: Infectious diarrhea represents a life-threatening problem among children in developing countries. **Objectives**: This work aimed to study bacterial, viral and parasitic causes of acute diarrhea; with genetic determination of diarrheagenic E. coli (DEC) in <5 years children. **Methodology:** Stool specimens were collected from 206 diarrheal children. Bacterial agents were isolated and identified by standard microbiological procedures. Multiplex PCR was done for genetic determination of DEC subtypes. ELISA was used for detection of viral and parasitic agents. **Results**: Stool specimens with at least single positive enteropathogen accounted for 98.5% with bacterial, viral and parasitic rates of 98.5%, 42.7% and 25.2%, respectively. Isolated bacteria were DEC (98.5%); Campylobacter (14%), Shigella (3.8%) and Salmonella (1.4%). Rota and Noroviruses showed prevalence of 32.5% and 5.3%, respectively. **Conclusion**: Infectious diarrhea were mostly due to bacterial agents. DEC and Campylobacter were predominant. EAEC and EPEC were the most genetically determined DEC subtypes.*

INTRODUCTION

Globally, diarrheal disease comprises an enormous problem of concern. About two billion cases get affected with diarrhea every year; of whom 1.9 million are children less-than-five-years[1]. It is the second cause of mortality among this age group (after pneumonia), particularly in developing countries[2, 3]. That is primarily related to contaminated water supplies[4], lack of sanitation and education especially in rural areas[5-7]. Moreover, additional factors in children include short-term breastfeeding and under-nutrition[8, 9].

Bacteria and parasites are the most prevalent diarrheal agents in developing countries with the peak of infection in summer seasons[3]. The primary bacterial agents are Campylobacter, Salmonella, Shigella, diarrheagenic E. coli (DEC), Y. enterocolitica and Vibrio. In children less than 5 years of age, DEC with its subtypes is considered the second primary cause of diarrhea following Rotavirus[10]. Viral agents are more dominant in developed countries; particularly those with cold climates[3]. Rota and Noroviruses represent the chief viral agents causing diarrhea in children[4]. Giardia lamblia and Entamoeba histolytica are the most common parasitic agents among children in nurseries and day-care centers[10].

In the present study, we aimed to investigate the infectious causes of acute diarrhea (bacterial, viral and parasitic) with genetic determination of DEC subtypes, among children less than 5 years of age addressing gastroenteritis Outpatient Clinic in our Tertiary Hospital.

METHODOLOGY

This case-series study was conducted on a total number of 206 stool samples collected from children less than five years age suffering from acute diarrhea and presenting to the Gastroenteritis Outpatient Clinic of Abu El-Reesh Pediatric Hospital, Cairo University within one year from July-2016 to June-2017. Acute diarrhea is defined as the increased passage of 3 or more stools per day with decreased consistency than usual for less than 14 days[4]. The study followed the Declaration

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of Helsinki principles and under the Act of the Medical Research Involving Human Subjects, and has been approved by the Ethical Committee of the University mentioned above. Formal consents were obtained from the care givers of the participants who agreed to their child's participation in the study.

Full clinical data about the enrolled children were collected through history taking from parents of the children and clinical examination by the physician. Stool samples were collected using a clean container and transported over Carry Blaire transport medium (Oxoid, England) to the Microbiology laboratory, within 30 minutes, for microscopic examination and culture. The corresponding aliquots were transferred to a −20°C freezer.

Stool examination and culture

Microscopic examination of fresh stool samples was done for detection of red blood cells, pus cells and parasites[11]. For isolation of bacteria, the stool samples were inoculated on MacConkey (MAC), *Salmonella-Shigella* (SS), Hektoen (HE) and Thiosulfate Citrate Bile Salt (TCBS) agar (Difco Laboratories, Detroit, Mich.) and blood agar supplemented with *Campylobacter* selective supplement (Skirrow's) (Oxoid, England)[12]. Except for *Campylobacter*, all cultured agar plates were incubated, aerobically at 35-37° C for 20-24 hours. *Campylobacter* plate was incubated in a microaerophilic environment for 48 hours at 42º C[13]. Suspected enteric pathogens were identified using conventional biochemical testing and confirmed by Analytic Profile Index 20E Identification System (API 20 E)[14].

Virulence genes of DEC

Singly grown *E. coli* in diarrheal cases were highly suggestive of DEC and were tested by Multiplex PCR to determine their subtypes. The sequence of PCR primers used is listed in table 1. The targeted virulence genes included genes coding for enterotoxin production (stable ST and labile LT) in *ETEC*: ST estA 2-4 for STh, estA 1 for STp & elt I for LT[15], pCVD 432 in *EAEC*[16], ipaH and eae for *EIEC/Shigella* and *EPEC*, respectively[17], Stx1 and Stx2 shiga toxin genes in *STEC (EHEC)*[18].

Table 1: Primers used in the multiplex PCR for amplification of DEC genes

Pathogen	Target genes	Primer sequence
ETEC	*estA2-4*	F5'-AATTGCTACTACTATTCATGTTTCAGGAC-3' R5'-TCTTTTTCACCTTTCGCTCAGG -3'
	estA1	F5'-ATGAAAAAGCTAATGTTGGCA-3' R5'-TTAATAACATCCAGCACAGGCA -3'
	eltA1	F5'-CATAATGAGTACTTCGATAGAGGAAC-3' R5'-GAAACCTGCTAATCTGTAACCATCC -3'
EAEC	*pCVD*	F5'-CTGGCGAAAGACTGTATCAT-3' R5'-CAATGTATAGAAATCCGCTGTT -3'
Shigella/EIEC	*ipaH*	F5'-GTTCCTTGACCGCCTTTCCGATACCGTC-3' R5'-GCCGGTCAGCCACCCTCTGAGAGTAC -3'
EPEC	*eae*	F5'-CCCGAATTCGGCACAAGCATA-3' R5'-CCCGGATCCGTCTCGCCAGTA -3'
STEC	*Stx1*	F5'-CAACACTGGATGATCTCAG-3' R5'-CCCCCTCAACTGCTAATA-3'
	Stx2	F5'-ATCAGTCGTCACTCACTGGT-3' R5'-CTGCTGTCACAGTGACAAA-3'

ETEC: Enterotoxogenic *E. coli*, ***EPEC***: Enteropathogenic *E. coli*, ***EAEC***: Enteroaggregative *E. coli*, ***STEC***: Shiga toxin producing *E. coli.*

Antibiotic susceptibility testing

Antimicrobial drug susceptibility for enteric pathogens was tested by Kirby–Bauer disc diffusion method and interpreted in accordance with the clinical and laboratory standards institute 2016[19]. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains for susceptibility studies as recommended.

Screening for viral and parasitic causes

ELISA technique was used for detection of the inquired viral and parasitic antigens in stool[14,20]: *Rotavirus*, *Norovirus*, *Adenovirus* and *Astrovirus* [IDEIATM Oxoid (K602011-2) UK]; *E. histolytica* II (catalog No 30404), *Giardia* II (catalog No 30405) and *Cryptosporidium* II (catalog No 30406) [Wampole TechLab, UK].

Statistical methods:

Statistically, data were described as mean ± standard deviation (± SD), and range, or frequencies (number of cases) and percentages when appropriate. Comparison between the study groups was made using Chi-square (X^2) test. P values less than 0.05 was considered

statistically significant. SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows was used for statistical calculations.

RESULTS

The enrolled children were 135 males and 71 females. The age ranged from 26 days to 5 years, with a median of 11 month. The weight of patients ranged from 2.5- 18 kg at time of sample collection, with a median 6 Kg. Eighty two cases were of urban origin, while 124 cases were of rural origin. Comparison of residence among type of infection revealed a statistically non-significant difference.

Regarding the isolated bacterial enteropathogens, 98.5% of diarrheal cases (203/206) had DEC, classified according to determined virulence genes into *entero-aggregative E. coli (EAEC in* 99/203*), entero-toxigenic E. coli (ETEC in* 51/203*), entero-pathogenic E. coli (EPEC in* 37/203*) and entero-invasive E. coli (EIEC in* 16/203*) (*Figure 1). The 51 *ETEC* were distributed into 23 stable toxin (ST: STp /STh) producing *ETEC* harboring *estA2-4/estA1*, 13 Labile toxin (LT) producers harboring *eltI* genes and 15 *ETEC* producing ST and LT carrying both *estA2-4/estA1* and *eltI* genes. *Stx1* and *Stx2* virulence gene determinants characterizing *EHEC* were not detected. All the 99 *EAEC* cases had the PCVD 432 gene while all the 37 cases of *EPEC* had the eae gene. All the 16 cases of *EIEC* and the 8 *Shigella* cases had the ipaH gene. Both organisms were genetically considered as one entity; however discrimination was done through identification by API20E system and serological agglutination.

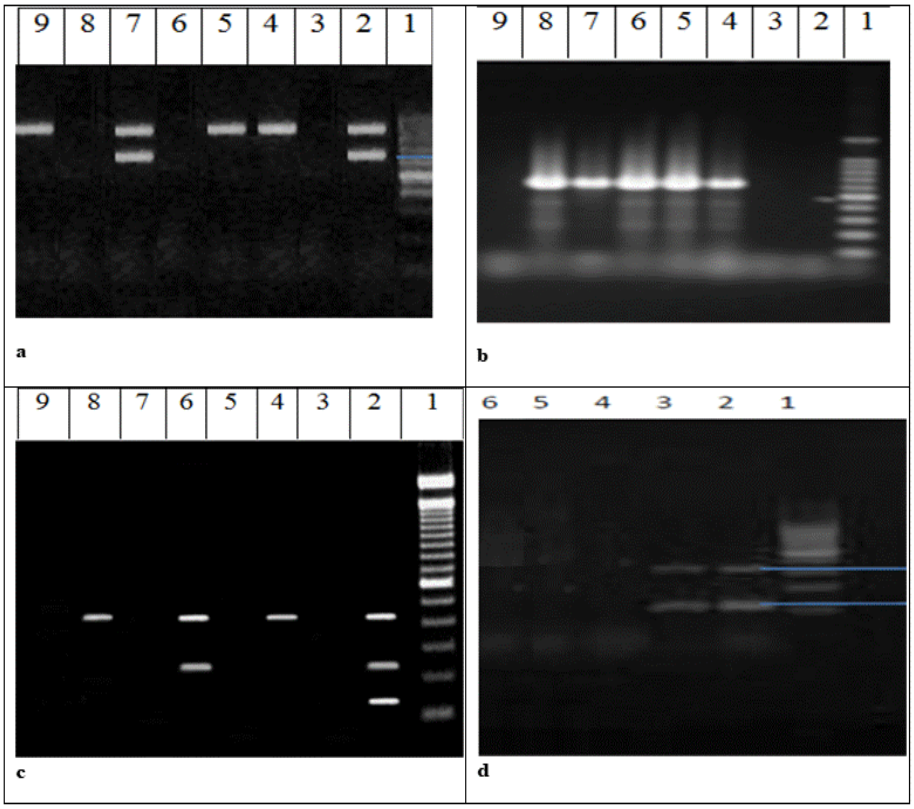

Fig. 1: PCR of diarrheagenic *E. coli*. a: Multiplex PCR of *EPEC* and *EIEC*. Lane 1: 100 bp size ladder; lane 2: Positive control (*eae* gene 890 bp and *ipaH* gene 619 bp); lane 3: negative control; lane 4, 5 and 9: three samples positive for *eae* gene; lane 7: one sample positive for *eae* and *ipaH* genes. **b: Uniplex PCR of *EAEC*.** Lane l: 100 bp size ladder; lane 2 and 3: 2 negative controls; lane 4: positive control; lane 5 to 8: 4 samples positive for *pCVD* 432gene; **c: Multiplex PCR of *ETEC*.** Lane 1: 100 bp size ladder; lane 2: positive control (*elt* I 402 bp, *estA I* 239 bp and *estA* 2-4 133 bp); lane 3 : negative control; lane 4 and 8: two samples positive for *elt* I; lane 6: one sample positive for *elt I* and *estA*. **d: Multiplex PCR of *STEC*.** Lane 1: 100 bp size ladder; lane 2 and 3: positive control (*Stx*1 gene 350 bp and *Stx*2 gene 110 bp); lane 4: negative control; lane 5 and 6: 2 samples negative for *EHEC*.

Campylobacter was identified in 14% (29/206) and classified into 21 *C. jejuni* and 8 *C. coli*. *Shigella* was detected in 3.8% (8/206) with *S. flexneri* group B type 1-6 in six cases, *S. dysentrae* group 1-7 in one case and *S. sonnei* in one case. *Salmonella* was detected in 1.4% (3/206) with Group C2 in two cases and Group C1 in one case. *Plesiomonas shigelloides*, *Aeromonas hydrophila* and *Hafnia alvei*; each was recovered once among total diarrheal cases (0.48%).

Comparing microscopic RBCs and pus cells in the stool samples among bacterial pathogens showed statistically significant difference only in *Shigella* and *EIEC* with a P- value of < 0.001.

Bacterial distribution among different age groups revealed significant recovery of *EAEC* and *ETEC* subtypes among children < 6 months of age with P-values 0.025 and 0.032, respectively. *Shigella* and *EIEC* showed significant recovery among 13-24 months age group with a P-value of 0.001. Significant correlation with water source was observed in 92% of *ETEC* cases who had an associated history of drinking municipal water with a P- value of 0.028. No significant correlation was found between the recovered bacterial pathogens and breast feeding or animal contact with P-values of 0.17 and 0.137, respectively.

The associated clinical findings with different bacterial pathogens were described in Table 2. Fever was significantly encountered in 84.8% of *EAEC* and in 96.6% of *Campylobacter* with P-values of 0.037 and 0.009, respectively. Vomiting was obviously encountered in 58.3% of *EIEC* and *Shigella* diarrheal cases having bloody stool (16 *EIEC and* 8 *shigella*) with a P-value of 0.009.

Table 2: The clinical presentation of patients compared to the bacterial pathogens

Organisms		Weight loss	Fever	Vomiting	Dehydration
EAEC (n =99)	Number	71	84	78	58
	% of cases	71.7%	84.8%	78.8%	58.6%
	P-value	0.733	0.037*	0.651	0.194
ETEC (n=51)	Number	37	45	37	25
	% of cases	52.7%	88.2%	52.7%	49%
	P-value	0.961	0.054	0.120	0.522
EPEC (n=37)	Number	27	28	30	22
	% of cases	73%	75.7%	81.1%	59.5%
	P-value	0.981	0.353	0.869	0.702
***Shigella* (8)** ***EIEC* (16)** (n=24)**	Number	13	20	14	16
	% of cases	54.2%	83.3%	58.3%	66.7%
	P-value	0.126	0.700	0.009*	0.155
Campylobacter (n =29)	Number	17	*28*	21	16
	% of cases	58.6%	96.6%	72.4%	55.2%
	P-value	0.088	0.009*	0.134	0.944
Salmonella (n =3)	Number	3	2	3	1
	% of cases	100%	66.6%	100%	33.3%
	P-value	-	-	-	-

* *P* value less than 0.05 is statistically significant.
**Twenty four cases with bloody stool divided into 16 with recovered *EIEC* and 8 with S*higella.*

Table 3 summarizes the results of antimicrobial susceptibility testing done to the recovered bacterial isolates; other than DEC.

Table 3: Antimicrobial susceptibility patterns of *bacterial isolates* by disk diffusion method

Organism	*Campylobacter*			*Shigella*			*Salmonella*			*Plesiomonas*			*Aeromonas*		
Number of isolates	29			8			3			1			1		
Antimicrobial agents	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Ampicillin 10 µg	4	3	22	1*	1	6	2	0	1	-	-	-	-	-	-
Amoxicillin/Clavulanate 30/10 µg	12	6	11	2**	1	5	2	0	1	0	0	1	0	0	1
Cephalothin 30 µg	0	2	27	0	0	8	0	0	3	0	1	0	0	1	0
Ceftriaxone 30 µg	10	10	9	6	2	0	2	0	1	1	0	0	1	0	0
Cefotaxime 30 µg	-	-	-	7	1	0	1	1	1	1	0	0	1	0	0
Cefotaxime/ Clavulanate 30/10 µg	-	-	-	7	1	0	1	1	1	1	0	0	1	0	0
Sulfamethoxazole 23.75 µg /trimethoprim 1.25µg	0	0	29	1	0	7	2	0	1	1	0	0	1	0	0
Gentamicin 10 µg	3	1	25	0	1	7	0	1	2	0	0	1	1	0	0
Ciprofloxacin 5 µg	0	0	29	8	0	0	1	0	2	1	0	0	1	0	0
Nalidixic acid 30 µg	0	0	29	8	0	0	1	0	2	-	-	-	-	-	-

S: Sensitive, **I**: Intermediate, **R**: Resistant

Viral agents were encountered in 42.7% (88/206) with a frequency of 67 *Rotavirus*, 11 *Norovirus*, 8 *Astrovirus* and 2 *Adenovirus*. *Giardia lamblia* and *Cryptosporidia* were detected with rates of 13.1% (27/206) and 12.1% (25/206), respectively.

The predominant pattern of mixed pathogens was the mixed bacterial and viral agents; it was encountered in 25.2% (52/206) of cases. Mixed bacterial and parasitic in 12.6% (26/206), mixed viral and parasitic in 0.97% (2/206), and mixed bacterial, viral & parasitic in 4.3% (9/206). No enteropathogen was detected in ١.45% (٣/206) of all diarrheal cases.

As described in Table 4, a significant difference in seasonality was observed with LT-producing *ETEC*, *Shigella*, *Rotavirus* and *Astrovirus*. *LT-ETEC* and Shigella were more recovered in warm season with a P-value of 0.005. *Rotavirus* and *Astrovirus* were more detected in cold with P-values of 0.000 and 0.005, respectively.

Table 4: Distribution of pathogens according to seasonality

Organism	**Warm season**	**Cold season**	**P-value**
	n (%)	n (%)	
EAEC	49 (49.49)	50 (50.51)	0.898
ETEC (ST)	19 (50)	19 (50)	0.895
ETEC (LT)	20 (71.43)	8 (28.57)	0.005*
EPEC	19 (51.35)	18 (48.65)	0.755
EIEC	11 (68.75)	5 (31.25)	0.147
Shigella	8 (100)	0 (0)	0.005*
Campylobacter spp	18 (62.07)	11 (37.93)	0.090
Salmonella spp	0 (0)	3 (100)	-
Rotavirus	20 (29.85)	47 (70.15)	0.000*
Norovirus	7 (63.64)	4 (36.36)	0.319
Astrovirus	0 (0)	8 (100)	0.005*
Adenovirus	1 (50)	1 (50)	-
Giardia lamblia	16 (59.26)	11 (40.74)	0.254
Cryptosporidium	18 (72)	7 (28)	0.014*

*P-value less than 0.05 is statistically significant.

DISCUSSION

Several studies reported a significantly higher prevalence of diarrhea in developing; than developed countries (P<0.016), because of the low living standards, bad health conditions and inadequate sanitation[3, 20]. The results of our study showed that diarrhea with at least one detected enteropathogen accounted for 98.5% of our 206 cases; a higher prevalence was reported by other studies (39-70%)[20-23] that could be due to variation in sample size and study population.

Regarding etiological agents, the highest contribution was for bacteria, followed by viruses and parasites. This agreed to another study which reported a higher recovery of bacteria, than viruses and protozoa[24]. Conversely, other studies reported the highest occurrence for viral agents [20, 21]. According to a systematic meta-analysis review, bacteria were more frequently detected than viruses among children in developing countries; while, the opposite in developed ones[3] which was probably owed to the seasonal variations and different social and health standards.

In the present study, no enteropathogen was detected in 1.45% of diarrheal cases, unlike other studies that experienced higher rates of 20.1% - 41.5%[20, 21, 25, 26]. This discrepancy might be due to: a) targeting only bacterial agents in some studies[26]; b) difficulty in detection of pathogens due to intermittent excretion in stool; c) diarrhea due to noninfectious causes[25].

In the present study, DEC represented the highest contribution among the isolated bacterial agents; similarly in the Middle East and North Africa[3, 23] and in other studies where DEC topped the list [24, 27].

On the contrary, DEC were less common in other studies with rates of 0.9% - 7.6%[20, 26] which could be due to higher age group children up to 12 years, variable sample sizes, and different methods of detection[20,21,25,28].

Some studies were designed as case-control, where DEC were isolated with high frequencies in both cases and controls, suggested of being colonizers; rather than pathogens[24]. This could be a limitation in our study which did not include a control group.

In the present study, the highest DEC subtype was *EAEC*, followed by *ETEC*, *EPEC*, and *EIEC*. That was consistent with another study in China, which reported DEC in a likely order of *EAEC*, *EPEC*, *ETEC*, and *EIEC*[20]. Fletcher et al.[3] observed a high prevalence of *EAEC*, mostly in the Middle East and North Africa; while, *EPEC* in Latin America. The present study observed more detection of ST-producing *ETEC* than LT-producing strains; which complied with one study in Egypt; however, confronted by another study, which reported higher *ETEC* isolates expressing LT than those expressing ST[29, 30].

EHEC and *EIEC* were usually isolated with the lowest frequencies worldwide[31]. Similar to many other studies, no *EHEC* was detected in the present study[20,23,24,26]. Unlikely, *EHEC* was reported in Gaza and China[25,32]. This variability could be attributed to the habit of eating undercooked meat in some countries[32].

In agreement to several studies, we encountered all DEC subtypes in the age group of less than two years[27, 33]; which would be related to immature local intestinal defense mechanisms of infants[31].

In accordance to several studies that reported a significantly higher rate of DEC in summer season[27, 33], we recorded significantly higher recovery of LT-producing *ETEC* in warm season. However, *EAEC*, ST-producing *ETEC* and *EPEC* were equitably distributed throughout the year with no distinct seasonality; might be due to moderate climate in Egypt most of the year.

In the present study, *Campylobacter* was the 2nd most commonly isolated bacteria after DEC with a prevalence of 14%, mostly *C. jejuni*, among the age group of 6- 12 months. Previously published data from Egypt reported *Campylobacter* with a prevalence range of 1-8% among < 5 years children[30, 34, 35].

Shigella was isolated in the present study with a prevalence of 3.8% close to previous studies in Egypt that reported prevalence of 4% and 1.2%[30, 34]; similarly, in India, China and Saudi Arabia with prevalence of 4%, 1.5% and 2%, respectively[20, 21, 23]. Unlikely, higher rates were reported by several studies with a prevalence range of 5.9% - 54.3%[25, 26, 28, 36]. That could be explained by the inclusion of children with higher age group > 5 years[25, 28]. *Salmonella* was isolated in the present study with a low rate of 1.4%; which was consistent with other studies in Egypt[30, 34]; due to more attention recently given by physicians to diagnosis and treatment of *Salmonella*[25, 37].

Clinically, fever showed statistically significant correlation with *Campylobacter* and *EAEC*, similarly was observed in other studies[24, 25]. Notably, our data showed a significant correlation between *ETEC* and water source; however, further studies of water sources are still required.

The present study showed a high frequency of antimicrobial resistance among *Shigella* (7 out of 8) against sulfamethoxazole and gentamycin, most probably due to selective pressure by their broad use therapeutically.

In the present study, viral agents were detected in 42.7% of diarrheal cases with the highest contribution for *Rotavirus*, followed by *Norovirus*, with rates of 32.5% and 5.3%, respectively; more significantly observed in cold than warm seasons which complied with that reported by other studies[38, 39]. Similar to some studies, we recorded the least contribution for *Astrovirus* and *Adenovirus* with rates of 3.8% and 0.97%, respectively[20, 21].

As for parasites, *Giardia lamblia* was detected with a rate of 13.1% of cases, in agreement to a study in India (10%)[23]. However, lower rates were reported by other studies[20, 21] which could be explained by geographical and health standard variations. According to Fletcher et al.[3] *Cryptosporidium* was more prevalent in Middle-East countries; complying with our study that recorded 12.1%. In the literature, the prevalence of *Cryptosporidium* in Egypt varied significantly from 0-47%[40].

More developed diagnostic techniques are needed for detection of mixed enteric pathogens; meanwhile, in such cases specifying the responsible pathogen for diarrhea would be confusing[20].

CONCLUSIONS

The present study concluded a high prevalence of diarrhea due to infectious causes among under five years children. Bacterial agents took the upper hand with a predominance of DEC; mostly *EAEC* and *EPEC*. Rotavirus and Giardia were frequently encountered.

List of abbreviations:
DEC: *Diarrheagenic E. coli*
EAEC: *Entero-aggregative E. coli*
EHEC: *Entero-haemorrhagic E. coli*
EIEC: *Entero-invasive E. coli*
ELISA: Enzyme-linked immunosorbent assay
EPEC: *Entero-pathogenic E. coli*
ETEC: *Entero-toxigenic E. coli*
PCR: Polymerase chain reaction
SD: Standard deviation

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- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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