Enteric Pathogens Associated with Childhood Diarrhea in Tripoli-Libya

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Abstract. Stool samples from children < 5 years of age with diarrhea ($N = 239$) were examined for enteric pathogens using a combination of culture, enzyme-immunoassay, and polymerase chain reaction methods. Pathogens were detected in 122 (51%) stool samples; single pathogens were detected in 37.2% and co-pathogens in 13.8% of samples. Norovirus, rotavirus, and diarrheagenic *Escherichia coli* (DEC) were the most frequently detected pathogens (15.5%, 13.4%, and 11.2%, respectively); *Salmonella*, adenovirus, and *Aeromonas* were detected less frequently (7.9%, 7.1%, and 4.2%). The most commonly detected DEC was enteroaggregative *E. coli* (5.4%). Resistance to ≥ 3 antimicrobials was observed in 60% (18/30) of the bacterial pathogens. *Salmonella* resistance to ciprofloxacin (63.1%) has become a concern. Enteric viral pathogens were the most significant causative agents of childhood diarrhea in Tripoli. Bacterial pathogens were also important contributors to pediatric diarrhea. The emergence of ciprofloxacin-resistant *Salmonella* represents a serious health problem that must be addressed by Libyan health authorities

INTRODUCTION

In developing countries, infectious diarrhea is associated with high rates of morbidity and mortality, mainly in childhood. In Libya, rotavirus and *Salmonella* have been documented as major causative agents of childhood diarrhea.¹ In the last few decades, several enteric pathogens including bacteria (e.g., *Campylobacter* spp., enterohemorrhagic *Escherichia coli*, and enteroadherent *E. coli*), viruses (e.g., norovirus, adenovirus, and astrovirus), and parasites (e.g., *Cryptosporidium* spp.) have been identified as important causes of diarrhea in humans, particularly in children.^{2–4} However, these pathogens either have not yet or have rarely been reported from pediatric diarrhea in Libya and other countries of the North Africa region.

Libya is located in North Africa situated between Egypt and Tunisia. The city of Tripoli is the capitol of Libya and has a population of around 1.5 million. The main objective of this work was to determine the prevalence of enteric pathogens among children with diarrhea in Tripoli.

MATERIALS AND METHODS

Stool samples from 239 children (102 females) with diarrhea, aged from a few days to 5 years, attending the Outpatient Clinics of Aljala Children's Hospital and Alkhadra Hospital in Tripoli were included in this study. Samples were collected between February and October 2008. After receiving informed consent from a parent or guardian, a clinical history for each patient was obtained. Results of the physical examination by medical doctors and clinical symptoms, including fever, vomiting, and dehydration were recorded in a standard proforma. We also recorded how children were fed (breast, artificial, or both).

Stool specimens from the children were examined for bacterial, viral, and parasitic agents. To isolate *Salmonella* spp.

(non-typhoidal), *Shigella* spp., and *Aeromonas* spp., stool samples were inoculated directly onto *Salmonella*-*Shigella* agar (SSA), MacConkey-lactose agar (MA), and blood agar supplemented with 15 mg/L ampicillin (ABA). Samples were also inoculated into selenite F broth (SFb) and alkaline peptone water (APW, pH 8). A loopful from SFb was inoculated onto SSA and a loopful from APW onto ABA. All media were incubated overnight at 37°C. Stool specimens were cultured for *Campylobacter* spp. using Skirrow's medium (containing Skirrow's selective supplement) and incubated at 42°C for 24–48 h in microaerophilic atmosphere of a candle jar. Suspected enteric pathogens from all media were identified biochemically using standard bacteriological methods^{5,6} and, whenever appropriate, the API 20E system (bioMérieux, Marcy l'Etoile, France). Isolates identified biochemically as *Salmonella* or *Shigella* were confirmed serologically. In addition, three lactose fermenting and any non-lactose fermenting colonies typical of *E. coli* were selected from MA plates and identified as previously mentioned. All microbiological media were purchased from Oxoid (Oxoid Ltd., Basingstoke, Hampshire, UK).

The disc diffusion method was used to determine the susceptibility of *Salmonella*, *Shigella*, and *Aeromonas* species isolates to different antimicrobial agents.⁷ *Escherichia coli* ATCC 25922 was used as a control organism.

Previously reported polymerase chain reaction (PCR) methods were used to screen isolates of *E. coli* for genes encoding virulence factors associated with diarrheagenic *E. coli* (DEC).^{8–11} These include genes located on the pCVD432 plasmid, indicative of EAEC, *eaeA* for enteropathogenic *E. coli* (EPEC) and EHEC, *ipaH* for enteroinvasive *E. coli* (EIEC), and the enterotoxigenic *E. coli* (ETEC) genes *estA* and *eltB* encoding for enterotoxins; heat-stable and heat-labile toxins, respectively. The PCR primer sequences used for detection of different genes associated with DEC are shown in Table 1.

Using commercial enzyme-immunoassays (EIAs) stool samples were examined for antigens of rotavirus (Premier Rotaclone, Meridian Bioscience, Inc., Cincinnati, OH), norovirus, adenovirus, and astrovirus (IDEIA, Oxoid Ltd.). In addition, second generation EIAs (*CRYPTOSPORIDIUM II*, *E. HISTOLYTICA II*, and *GIARDIA II*, TECHLAB, Blacksburg,

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TABLE 1
Polymerase chain reaction (PCR) primer sequences used for detection of virulence genes associated with diarrheagenic *Escherichia coli*

Target gene	Codes for:	Primer pair (5'-3')	PCR condition for 30 cycles	Amplicon size (bp)	Reference
pCVD432	EAEC*	CTGGCGAAAGACTGTATCAT and CAATGTATAGAAATCCGCTGTT	1 min 94°C, 1 min 55°C, 1 min 72°C†	630	8
eaeA	EPEC/EHEC*	AAACAGGTGAAACTGTTGCC and CTCTGCAGATTAACCCTCTGC	1 min 94°C, 1 min 52°C, 1 min 72°C†	454	9
ipaH	EIEC*	GTTCTTGACCGCCTTTCCGATACCGTC and GCCGGTCAGCCACCCTCTGAGAGTAC	1 min 94°C, 1 min 52°C, 1 min 72°C†	619	10
estA1	ETEC*	ATGAAAAAGCTAATGTTGGCA and TTAATAACATCCAGCACAGGCA	1 min 95°C, 30 s 58°C 1 min 72°C†	239	11
estA2-4	ETEC	AATTGCTACTATTCATGCTTTTCAGGAC and TCT TTT TCA CCT TTC GCT CAG G	1 min 95°C, 30 s 58°C 1 min 72°C‡	133	11
eltBI	ETEC	CATAATGAGTACTTCGATAGAGGAAC and GAAACCTGCTAATCTGTAAACCATCC	1 min 95°C, 30 s 58°C 1 min 72°C‡	402	11

* EAEC = enteroaggregative *E. coli*, EHEC = enteropathogenic *E. coli*/enterohemorrhagic *E. coli*; EIEC = enteroinvasive *E. coli*; ETEC = enterotoxigenic *E. coli*.

† A final extension step of 7 min at 72°C was performed.

‡ A final extension step of 10 min at 72°C was performed.

VA) were used to detect antigens of *Cryptosporidium*, *Entamoeba histolytica*, and *Giardia lamblia* in all specimens.

Epi Info, version 3.5.1 software (Centers for Disease Control and Prevention, Atlanta, GA) was used to analyze the data. *P* values were calculated using the χ^2 test; *P* values < 0.05 were considered statistically significant.

RESULTS

Enteric pathogens were detected in 122 (51%) stool samples examined; single pathogens in 37.2%, and multiple pathogens in 13.9% (Table 2). The most common pathogens detected were enteric viruses (82/239; 34.3%) followed by bacterial

pathogens (64/239; 26.8%); parasitic pathogens (10/239; 4.2%) were the minority of detected pathogens.

Overall, the most common individual pathogens detected were norovirus, rotavirus, and DEC. Among the DEC, pCVD432 (EAggEC) predominated at 5.4% (13/239) followed by eaeA (EPEC/EHEC) at 4.6% (11/239). No *est* and *eltB* genes (ETEC) were detected in this work. *Salmonella* species were detected in 7.9%; the majority of these were group C2. *Cryptosporidium* was the primary parasitic diarrheal pathogen detected (2.1%) (Table 2).

Total enteric pathogens were detected more frequently among diarrheic children ≤ 2 years of age (55.2%, 100/181; *P* < 0.03, odds ratio [OR] = 2.02) compared with children > 2 years of age (37.9%, 22/58). Although total enteric pathogens were detected at a higher rate in male (54%, 74/137) than in female (47.1%, 48/102) diarrheic children, the difference was not statistically significant (*P* > 0.05).

Of the 239 diarrheic children included in the study 202 (84.5%) had vomiting, 176 (73.6%) had fever, and 82 (34.3%) had dehydration. Vomiting, fever, and dehydration were significantly associated with children ≤ 2 years of age compared with children > 2 years of age (*P* < 0.02, OR = 2.52, *P* < 0.009, OR = 2.32, and *P* < 0.005, OR = 2.76, respectively). Table 3 shows clinical symptoms associated with enteric pathogens isolated from diarrheic children in Tripoli.

Rotavirus was significantly (*P* < 0.02, OR = 3.53) associated with dehydration (53.1%, 17/32) compared with norovirus (24.3%, 9/37). In addition, only dehydration was observed significantly associated with diarrheic children positive for single (34.4% [32/93], *P* < 0.0000001 OR = 29.11), multiple (39.4% [13/33], *P* < 0.0000001, OR = 36.08), and total enteric pathogens (35.7% [45/126], *P* < 0.0000001, OR = 30.83) compared with enteric pathogens-negative children (1.8% [2/113]).

The present investigation continued for 8 months (March to October) covering the seasons of spring, summer, and two-thirds of autumn (Supplementary Table 1). Single and total enteric pathogens and rotavirus were detected significantly more often (*P* < 0.05) during spring and autumn compared with summer. On the other hand, norovirus was detected significantly more (*P* < 0.05) in autumn compared with spring.

Of 181 diarrheic children included in this work ≤ 2 years of age, 34 (18.8%) were breastfed, 118 (65.2%) artificially fed, and 29 (16%) were on mixed feeding. Only multiple enteric

TABLE 2

Enteric pathogens isolated from 239 diarrheic children in Tripoli, Libya

Enteric pathogen (s)	No. (%) positive
Single	89 (37.2)
Multiple	33 (13.8)
Total	122 (51.0)
Diarrheagenic <i>Escherichia coli</i> *	27 (11.2)
pCVD432 (EAEC)	10 (4.1)
eaeA (EPEC/EHEC)	9 (3.8)
ipaH (EIEC)	4 (1.6)
pCDV432 + ipaH	2 (0.8)
eaeA + ipaH	1 (0.4)
pCVD432 + eaeA	1 (0.4)
<i>Salmonella</i> spp.	19 (7.9)
Salmonella group B	3 (1.3)
Salmonella group C1	1 (0.4)
Salmonella group C2	13 (5.4)
Salmonella group D1	2 (0.8)
<i>Shigella</i> spp.	1 (0.8)
<i>Campylobacter</i> spp.	7 (2.9)
<i>C. jejuni</i>	6 (2.5)
<i>C. coli</i>	1 (0.4)
<i>Aeromonas</i> spp.	10 (4.2)
Rotavirus	32 (13.4)
Norovirus	37 (15.5)
Adenovirus	17 (7.1)
Astrovirus	4 (1.7)
<i>Cryptosporidium</i> spp.	5 (2.1)
<i>Entamoeba histolytica</i>	2 (0.8)
<i>Giardia lamblia</i>	3 (1.3)
No pathogen detected	117 (49)

* *est* and *eltB* genes encoding for enterotoxigenic *E. coli* (ETEC) was not detected.

TABLE 3

Clinical symptoms associated with enteric pathogens isolated from diarrheic children in Tripoli, Libya

Agents	Cases detected	No. (%) positive		
		Vomiting	Fever	Dehydration*
Single	89	73 (82.8)	62 (69.9)	30 (33.7)
Multiple	33	30 (90.9)	24 (72.7)	13 (39.4)
Total	122	103 (84.4)	86 (70.4)	43 (35.2)
Diarrheagenic				
<i>Escherichia coli</i>	27	25 (92.6)	20 (74.1)	10 (37.0)
<i>Salmonella</i> spp.	19	15 (78.9)	15 (78.9)	4 (21.1)
<i>Shigella</i> spp.	1	1 (100)	1 (100)	0 (0.0)
<i>Campylobacter</i> spp.	7	3 (42.9)	4 (57.1)	0 (0.0)
<i>Aeromonas</i> spp.	10	10 (100)	6 (60)	5 (50)
Rotavirus†	32	32 (100)	24 (75)	17 (53.1)
Norovirus	37	34 (91.9)	23 (62.2)	9 (24.3)
Adenovirus	17	14 (82.4)	10 (58.8)	7 (41.2)
Astrovirus	4	3 (75)	4 (100)	2 (50)
<i>Cryptosporidium</i> spp.	5	2 (40)	4 (80)	2 (40)
<i>Entamoeba histolytica</i>	2	2 (100)	2 (100)	2 (100)
<i>Giardia lamblia</i>	3	2 (66.7)	3 (100)	1 (33.3)
No pathogen detected	117	95 (81.2)	87 (74.4)	2 (1.7)

* Dehydration was significantly associated with diarrheic children positive for single (33.7% [30/89], $P < 0.0000001$, odds ratio [OR] = 29.24), multiple (39.4% [13/33], $P < 0.0000001$, OR = 37.38), and total enteric pathogens (35.7% [43/122], $P < 0.0000001$, OR = 31.30) compared with children with no pathogen detected (1.7% [2/117]).

† Rotavirus was significantly ($P < 0.02$, OR = 3.53) associated with dehydration (53.1%, 17/32) compared with norovirus (24.3%, 9/37).

pathogens and *Salmonella* species were significantly ($P < 0.02$, OR = 8.43 and $P < 0.04$, OR = undefined, respectively) associated with artificial feeding (20.3% [24/118] and 11.8% [14/118], respectively) compared with breast feeding (2.9% [1/34] and 0.0% [0/34]).

Multiple-antibiotic resistance (resistance to ≥ 3 antimicrobials) was observed in 60% of the total enteric bacterial pathogens examined (Supplementary Table 2). Of the 19 *Salmonella* spp. isolated, 84.2% were resistant to nalidixic acid, 63.1% to ciprofloxacin, 78.9% to streptomycin, and 63.1% to tetracycline. Among the 10 isolated *Aeromonas* spp. 100%, 50%, 70%, and 30% were resistant to ampicillin, amoxicillin/clavulanic acid, cephalothin, and streptomycin, respectively; one *Aeromonas* isolate was resistant to imipenem.

DISCUSSION

Few studies have been conducted to determine the etiology of childhood diarrhea in Libya^{12–14}; however, the findings of this study confirm the importance of rotavirus and *Salmonella* as major causes of diarrhea in Libyan children. We found *Salmonella* in nearly 8% of the patients studied, which is consistent with previously published studies; rotavirus, however, in this investigation was found in only 13.4% of overall cases, a rate that is lower than the rates of 24–31% reported in the past.

Data regarding the role of norovirus, adenovirus, and astrovirus in pediatric diarrhea in North Africa and the Middle East are scarce. Sdiri-Loulizi and others¹⁵ in Tunisia used EIA to detect rotavirus, adenovirus, and astrovirus and PCR for norovirus detection from diarrheic children. They found rotavirus in 22.5%, norovirus in 17.4%, astrovirus in 4.1%, and adenovirus in 2.7% of samples examined. In this investigation norovirus and rotavirus were the predominant enteric viruses found among diarrheic pediatrics (15.5% and 13.4%, respectively), followed by adenovirus (7.1%) and astrovirus (1.7%). Predominance of norovirus and rotavirus infections in

children with diarrhea has also been reported from Egypt and Saudi Arabia.^{16,17} Norovirus, adenovirus, and astrovirus have never previously been reported from Libya.

Kamel and others,¹⁶ in Egypt, reported that all cases of severe dehydration in pediatric populations were associated with either rotavirus or norovirus monoinfections or mixed infections. A study from Tunisia reported that norovirus is equal to rotavirus infection in terms of necessity of hospitalization and severity of the clinical symptoms.¹⁸ In this study, only rotavirus was significantly associated ($P < 0.02$) with dehydrated children. However, our findings and those reported from Tunisia and Egypt indicate that rotavirus and norovirus are the leading causes of childhood diarrhea in the North Africa region. Several rotavirus vaccines are available that provide good protection to the pediatric population at risk.¹⁹ Recently, the Strategic Advisory Group of Experts (SAGE) on Immunization of the World Health Organization (WHO) recommended the inclusion of rotavirus vaccination of infants into all national immunization programs.²⁰

There is increased recognition of EAEC as an emerging enteric pathogen.²¹ The organism is associated with pediatric diarrhea, particularly persistent diarrhea, in developing countries.² The overall prevalence of EAEC among diarrheic children in Libya is not known, although a previous study examined 50 *E. coli* isolates (20 from diarrheic and 30 from healthy Libyan children) for their virulence traits.²² The investigators identified 9 EAEC (6 from diarrheic children) isolates. In this study, we found a prevalence rate of 5.4% (13/239) for EAEC among children with diarrhea. Recent studies from Brazil, India, Iran, and Kuwait have reported various detection rates for EAEC of 5.5%, 14.7%, 16.3%, and 2.6% from diarrheic children, respectively.^{23–26}

Campylobacter spp. are important enteric pathogens with *Campylobacter jejuni* usually responsible for the majority (80–90%) of enteric *Campylobacter* infections.²⁷ Previous reports from Libya suggest rates of 2–6% for *Campylobacter* spp. among children with diarrhea.^{13,14} In this investigation *Campylobacter* spp. were found in 2.9% (7/239) of patients, predominantly *C. jejuni* as determined by hippurate hydrolysis. Ghanim and others¹³ reported that Libyan children infected with *Campylobacter* spp. clinically presented with fever and vomiting. Although our numbers were small in this study, we also found almost 50% of *Campylobacter*-positive children with vomiting and fever; none were dehydrated.

Although the role of *Aeromonas* spp. in gastroenteritis is controversial, the literature indicated that some motile *Aeromonas* spp. may be emerging as food- and water-pathogens of importance.^{28,29} In developing countries prevalence of *Aeromonas* spp. in diarrheic children ranges between 4% and 22%, whereas in developed countries it is usually less than 3%.^{30–35} We detected these organisms in 4.2% of children with diarrhea. In addition, although there were only 10 cases identified as positive, 50% of the patients were dehydrated, and 100% and 60% presented with vomiting and fever, respectively. Studies from different Libyan cities have reported prevalence rates between 0% and 15% (12–14). Furthermore, Ghenghesh and others³⁶ in Tripoli reported that vomiting and fever were observed in more than one-third of children with *Aeromonas*-associated diarrhea.

Published reports from developing countries on the use of EIA assays for the detection of *Cryptosporidium*, *E. histolytica*, and *G. lamblia* in stools of diarrheic children are few.

Studies from Turkey and India reported infection rates of 4.9% and 7.6%, respectively, for *Cryptosporidium* in pediatric diarrheal cases using EIA.^{37,38} These rates are higher than the detection rate of 2.1% for *Cryptosporidium* observed in this study. However, a study from Thailand reported a very low prevalence rate (0.8%) of *Cryptosporidium* among preschool children with diarrhea.³⁹ We also found low prevalence rates of *G. lamblia* (1.3%) and *E. histolytica* (0.8%) among the studied patients.

Previous studies from Libya reported the occurrence of *G. lamblia* and *E. histolytica* between 1% and 18% and 4% and 46%, respectively, among diarrheic children using wet mount and microscopy techniques.^{12,40,41} *Entamoeba histolytica* is identical morphologically to the non-pathogenic *Entamoeba dispar*. The latter is the new species name for what was previously called “non-invasive” or “non-pathogenic” *E. histolytica*.⁴² Recently, Al-Harthi and Jamjoom⁴³ in Saudi Arabia examined 156 stool samples from diarrheic patients for *E. histolytica* by microscopy and *E. histolytica* II (EIA used in this work). They detected *E. histolytica* in 64.8% of samples by microscopic examination and in 2.6% by EIA. Therefore, the previously reported prevalence rates of *E. histolytica* from Libya, and other developing countries, diagnosed microscopically should be interpreted carefully.

Studies have shown that mortality rates can be reduced by several folds among infants breastfed for the first 4–6 months of life and its continuation during diarrheal illnesses.^{44–46} Ali and others¹² in their study on childhood diarrhea a decade ago reported that < 7% of diarrheic Libyan children < 2 years of age were breastfed. Although they detected enteropathogens at higher rates from non-breast fed compared with breastfed children, the difference was not statistically significant. In this study, a higher percentage of diarrheic children ≤ 2 years of age were breastfed, however a significant association of multiple enteric pathogens and *Salmonella* spp. ($P < 0.02$ and $P < 0.04$, respectively) was observed among artificially fed compared with breastfed diarrheic children.

In a one year study, Ghenghesh and others reported rotavirus, *Salmonella*, *Shigella*, and *Aeromonas* were detected more frequently during the autumn season however, they found no statistically significant differences in the isolation rates of different enteric pathogens during the four seasons of the year.¹⁴ Although the winter season was not included in this work, our findings are in line with their observation for rotavirus infection being detected significantly more in autumn and in spring than in summer. However, no significant difference in the detection rate of norovirus in autumn compared with summer. The lack of clear-cut seasonal variation in the detection rate of enteric pathogens observed in this and previous studies may be caused by the climate in Libya, which is characterized by a hot summer, warm autumn and spring, and mild winter.

Non-typhoid *Salmonella* are re-emerging as one of the most important etiological agents of infectious diseases in the world.⁴⁷ Multi-antibiotic resistance in non-typhoid *Salmonella* has been associated with enhanced virulence and excess mortality in patients compared with infection with sensitive strains.^{48,49} High rates of resistance to multiple antimicrobial agents (resistance to three or more classes of antibiotics) by enteric pathogenic were previously reported from Libya.^{12,50,51} Similarly, a high percentage of multiple-drug-resistance was identified among the bacterial pathogens examined in this work. Moreover, we found almost two-thirds of *Salmonella*

isolates were resistant to ciprofloxacin, a rate that was unexpected because previous studies from Libya reported fluoroquinolones susceptibility of all salmonellae isolated from diarrheic children.^{12,50} Although nalidixic acid and ciprofloxacin are not used routinely in the treatment of childhood infectious diarrhea in Tripoli hospitals (Dr. Suhila O. Almbrok, Al-Jala Children Hospital, Tripoli, personnel communication), this finding represents a serious health problem because fluoroquinolones are the drugs of choice in the treatment of salmonellosis and other infectious syndromes (e.g., urinary tract infections) in adults; in particular, a high rate of resistance to ampicillin and trimethoprim-sulphamethoxazole is observed among isolates recovered.^{52–54} Ciprofloxacin is licensed for use both in animals and humans in some developing countries and in Libya antimicrobials, including the fluoroquinolones, are available to the public without the need for medical prescription.^{12,55} High rates of multiple antimicrobial resistance among enteric bacterial pathogens will continue to increase unless proper measures are taken by the health authorities to combat this serious public health issue in Libya.

To our knowledge, this is the first study to identify norovirus, adenovirus, and astrovirus as enteric pathogens causing childhood diarrhea in Libya. The findings of this investigation indicate, in addition to *Salmonella* and rotavirus, the enteric pathogens norovirus, adenovirus, and EAEC are important causative agents of childhood diarrhea in Tripoli. On the other hand, *Cryptosporidium*, *Campylobacter*, and *Aeromonas* spp. appear to play a minor role in pediatric diarrheal disease in Tripoli. The emergence of ciprofloxacin-resistant *Salmonella* is a very serious health problem that should be addressed by public health authorities. More studies are necessary in other major cities of Libya and a dedicated national pathogen-specific surveillance system to identify various etiologies of pediatric diarrhea to determine the exact role of these enteric pathogens. Furthermore, introduction of a rotavirus vaccine into the vaccination program in Libya to protect the pediatric population is urgently needed.

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