Molecular Characterization of Diarrheagenic *Escherichia coli* from Libya

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Abstract. Diarrheagenic *Escherichia coli* (DEC) are important enteric pathogens that cause a wide variety of gastrointestinal diseases, particularly in children. *Escherichia coli* isolates cultured from 243 diarrheal stool samples obtained from Libyan children and 50 water samples were screened by polymerase chain reaction (PCR) for genes characteristic of enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC). The DEC were detected in 21 (8.6%) children with diarrhea; 10 (4.1%) cases were identified as EAEC, 3 (1.2%) as EPEC, and 8 (3.3%) were ETEC; EHEC, and EIEC were not detected. All DEC were grouped phylogenetically by PCR with the majority (> 70%) identified as phylogenetic groups A and B1. The EAEC isolates were also tested for eight genes associated with virulence using PCR. Multi-virulence (≥ 3 virulence factors) was found in 50% of EAEC isolates. Isolated EAEC possessed different virulence traits and belonged to different phylogenetic groups indicating their heterogeneity.

INTRODUCTION

Although part of the normal gut flora, *Escherichia coli* strains are also important intestinal pathogens. Diarrheagenic *E. coli* (DEC) cause a wide variety of gastrointestinal diseases, particularly among children in developing countries, resulting in significant morbidity and mortality. The DEC are differentiated into at least five pathotypes, according to their pathogenic mechanisms, that include enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) (also known as Shiga-toxin producing *E. coli* or verocytotoxigenic *E. coli*). Genes used to screen for different DEC pathotypes among *E. coli* isolated from fecal specimens include: pCVD432 encoding for EAEC; *eaeA* (mediates intimate attachment to epithelial cells), and *bfpA* (bundle-forming pilus) for EPEC; *stx1* and *stx2* for shiga-like toxin 1 (SLT-1), and shiga-like toxin 2 (SLT-2), respectively, of EHEC; *esta1* (STp) and *esta2* (STh) for heat-stable (ST) and eltB for heat-labile (LT) toxins of ETEC; and *ipaH* (invasion plasmid antigen) for EIEC.

The EAEC is a heterogeneous group of organisms possessing a broad range of virulence factors. The pathogenesis of EAEC infection is not well understood. For this reason, additional virulence genes have been used to screen EAEC-positive *E. coli* that include: *aggR*, a transcriptional activator; *aggA*, fimbriae AAfI; *aafA*, fimbriae AAF II; *agg3A*, fimbriae AAF III; *asta*, aggregative stable toxin 1 (EAEC 1); *pet*, plasmid-encoded heat-labile toxin; *aap*, anti-aggregation protein; and *pic*, protein involved in colonization.

High isolation rates of DEC, particularly EAEC, among diarrheic children have been reported from some developing countries. Vargas and others determined the distribution of enteropathogens, including bacteria, viruses, and parasites, causing diarrhea among children < 5 years of age in Ifakara, Tanzania. They reported that DEC (35.7%) were the most isolated enteropathogens with predominance of EAEC (20.6%), ETEC (9.3%), and EPEC (5.3%). Presterl and others found EAEC in 38% and ETEC in 4.7% of 150 children with diarrhea in Gabon. However, they did not detect EHEC and EIEC. Orlandi and others in Brazil reported a lower detection rate for EAEC (5.5%). The EAEC is increasingly recognized as an emerging enteric pathogen associated with children's diarrhea, particularly persistent diarrhea, in developing countries.

The EPEC pathotype is subdivided into typical EPEC (tEPEC) that harbors both *eae* and *bfpA* genes, and atypical EPEC (aEPEC) that lacks the *eaeA* gene. Although rare in industrialized countries, tEPEC strains are the leading cause of infantile diarrhea in developing countries. On the other hand, ETEC is an established agent of diarrhea in developing and developed countries and cause diarrhea through the action of either the LT and/or ST enterotoxin. There are two distinct classes of ST that differ in structure and mechanism of action, STa and STb. Two subtypes of STa designated STp (ST porcine) and STh (ST human) exist; both variants can be found in human ETEC strains. In addition, ETEC isolates express surface proteins termed colonization factors (CFs) that promote their colonization of the epithelium. Recently, Nada and others in Egypt, designed and validated a multiplex polymerase chain reaction (PCR) assay for the identification of ETEC and associated structural genes of CF antigen (CFA)/I, CS1 to CS8, CS12 to CS15, CS17 to CS22, and PCFO71. They reported the predominance of CFA/I, CS21, and CS6 (a sub-unit of CFA/IV) among the ETEC strains examined.

Studies that describe the distribution of the various alleles of these genes in children with DEC from the North Africa countries are few. There are no reports of DEC from Libyan cities and there is no information regarding their frequency in water sources. Recently, we have found DEC among the leading enteric pathogens, in addition to rotavirus and norovirus, associated with childhood diarrhea in Tripoli, Libya, with the predominance of EAEC. The aims of this work were to determine the frequency of DEC among children with diarrhea and from Zliten and Alkhomes city water sources; furthermore, the phylogenetic grouping and virulence-encoding
genes of the isolated DEC were determined by PCR and DNA sequence analyses.

MATERIALS AND METHODS

Bacterial strains. Escherichia coli isolates were recovered from 243 diarrheal stool samples of Libyan children a few days of age up to 12 years of age (>90% were <3 years of age). Stool samples were collected at Al-Shafa Private Clinic and outpatient clinics of the Central Hospitals in Zliten and Alkhomes cities between April 2000 and March 2001 and the summer of 2007. After receiving informed consent from a parent or guardian, a clinical history for each patient was obtained. Histories were obtained through physical examination by medical doctors. Clinical symptoms, including fever, vomiting, abdominal pain, and dehydration were recorded in cooperation by medical doctors. Clinical symptoms, including fever, vomiting, abdominal pain, and dehydration were recorded in cooperation by medical doctors.

Stool specimens from enrolled children were collected using wide-mouthed sterile plastic containers and transported immediately to the microbiology laboratory for analysis within two hours of collection.

Stool specimens were directly streaked onto MacConkey agar (Difco, Detroit, MI) for isolation of E. coli. After overnight incubation at 37°C, three lactose-fermenting colonies and a representative non-lactose fermenting colony of a different morphological type were picked and identified by the API 20E System (bioMerieux, Marcy l’Etoile, France).

Fifty water samples (including tap water and drinking water from wells and reservoirs) were collected randomly, during the summer of 2007, from different areas of Zliten and Alkhomes. They were examined for the presence of E. coli using a previously reported technique. Water samples were collected aseptically in sterile glass containers (100 mL) containing 0.1 mL sodium thiosulphate (1.8% w/v) and examined within 6 hours from collection. Identification of E. coli from water samples was carried out as described previously. All E. coli isolates from stool and water samples were cryopreserved at −70°C until further use.

Genotyping methods. Identification of DEC and screening of virulence factors. Previously reported PCR methods1−9 were used to screen isolates of E. coli for genes associated with DEC (Table 1). Furthermore, PCR techniques were used to screen all EAEC-positive E. coli for virulence genes.12,19 Primer sequences, the amplification program, and expected amplicon sizes are shown in Table 1. Whole-cell lysates, obtained by boiling20 or QIAamp DNA Mini Kit (Qiagen, Valencia, CA), from each isolate were used as DNA template. Amplification was carried out in a 50 μL reaction mixture containing 3 μM MgCl2, 400 μM each dNTP, 10 μL 5X Green GoTaq Flexi buffer (Promega, Madison, WI), 2.5 U GoTaq Flexi DNA polymerase (Promega), 1 μL each primer and 5 μL whole-cell lysate. Reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA). The ETEx-positive E. coli isolates were examined for coli surface (CS) antigens by PCR as previously reported.5 All of the primers used in the study were purchased from Sigma-Genosys (The Woodlands, TX).

Table 1

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer pair (5′−3′)</th>
<th>Anneling temperature of PCR condition for 30 cycles</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
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<td>pCVD432</td>
<td>CTGGCAGAGATTGATCAT and CAAATGTAAGAAATCCGCTT</td>
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<td>aggR</td>
<td>CTTAATCATCAATCGATGA and ATGAGTAATCCCTGATT</td>
<td>1 min 52°C* 308</td>
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<tr>
<td>aggA</td>
<td>TCTAATCTCTCTAGGG and AATTAATGCGGCTG</td>
<td>1 min 60°C* 450</td>
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<tr>
<td>aapA</td>
<td>AGTATATATTGAGGTIGAC and TATTATATTGACACAAGTCG</td>
<td>1 min 55°C* 518</td>
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<tr>
<td>aggB</td>
<td>GATCATGAGCGCATCCTTACAG and CCCTTCTGAGCTCAG</td>
<td>1 min 50°C* 462</td>
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<td>pic</td>
<td>CTTTTCTGCCATCTTGGGT and GTAAACACTCCCTTGAAGT</td>
<td>1 min 52°C* 232</td>
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<td>pet</td>
<td>GCCCTAGCATTTACACCGACGC and CGGAAATTCGGCAGC</td>
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<td>astA</td>
<td>CCACTCCACAGATATCTCGGA and GGGTCGGTGTGACG</td>
<td>1 min 55°C* 111</td>
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<td>stx1</td>
<td>CCACTCCTGGTAGTCTCCAG and CCTCCCTACCTGTAATA</td>
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<td>stx2</td>
<td>ATGCGTTCGCTCAGCTCTACTGTTG and CTGCGTGTAACGTGAA</td>
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<td>eaeA</td>
<td>AATACGATGAAAATCTTGCC and CTCGGCAGATTACCCCTGCC</td>
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<td>bfpA</td>
<td>AATTAGCTGATGCTGCACGTTG and GCCCGTTATCCACACTTGT</td>
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<tr>
<td>ipaH</td>
<td>GTTCCTTCTGACAGCGCCTTGCAGTACCCCTGACT</td>
<td>1 min 52°C* 619</td>
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<td>estA1 (STp)</td>
<td>ATGAAAAGCTAATGGTGC and TATATATATCAGCAGCAGCA</td>
<td>30 s 58°C* 239</td>
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<tr>
<td>estA2-4 (STh)</td>
<td>ATGGTACTTATATCATGCTGACGAC and TCTTCTACCAATGTGAGAAGGC</td>
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<td>eltB (LT)</td>
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*The amplification conditions used were 30 cycles of 94°C for 1 min; annealing temperature as shown in table above; elongation temperature of 72°C for 1 min. One final cycle was added in which the extension step was increased to 10 min.

†The amplification conditions used were 30 cycles of 95°C for 1 min; annealing temperature as shown in table above; elongation temperature of 72°C for 1 min. One final cycle was added in which the extension step was increased to 10 min.
Distribution of diarrheagenic Escherichia coli from diarrheic Libyan children according to gender, age, type of feeding, and source of drinking water

<table>
<thead>
<tr>
<th>DEC</th>
<th>Gender</th>
<th>Type of feeding</th>
<th>Source of drinking water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F (N = 97)</td>
<td>M (N = 146)</td>
<td>6 (N = 80)</td>
</tr>
<tr>
<td>EAEC</td>
<td>8 (8.2)†</td>
<td>2 (1.4)</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>EPEC</td>
<td>0 (0.0)</td>
<td>3 (2.1)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>ETEC</td>
<td>3 (3.1)</td>
<td>5 (3.4)</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>Total DEC</td>
<td>11 (11.3)</td>
<td>10 (6.9)</td>
<td>5 (6.3)</td>
</tr>
</tbody>
</table>

*Only children aged ≥2 years positive for diarrheagenic Escherichia coli (DEC) were included.
†Enterohemorrhagic E. coli (EAEC) was significantly associated with female compared with male diarrheic children (P < 0.02 OR = 6.47).
‡DEC was significantly associated with non-breastfed compared with breastfed diarrheic children (P < 0.05 OR = undefined).

RESULTS

The DEC was found in 8.6% of diarrheic stool samples examined from Libyan children. Only EPEC (1.2%), ETEC (5.3%), and EAEC (4.1%) were detected. EHEC and EIEC were not detected. Vomiting was observed in 90.5%, fever in 95.2%, abdominal pain in 42.9%, dehydration in 76.2%, and diarrhea duration of more than 48 h in 76.2% of Libyan children with DEC.

Table 2 shows the distribution of DEC isolated from Libyan children according to gender, age, type of feeding practices, and source of drinking water. The EAEC was significantly associated with female Libyan children with diarrhea (P < 0.02 odds ratio [OR] = 6.47). Furthermore, DEC was significantly associated with diarrheic children who were not breastfed (P < 0.05 OR = undefined). With the exception of one diarrheic child, none of the children included in this study had traveled abroad 30 days before stool collection. Of the 50 water samples examined for DEC only one (2%) was positive, and contained an EPEC pathotype.

Table 3 shows the distribution of virulence factors in DEC isolated from children with diarrhea in Libya. Greater than 90% of EAEC carried the aggR gene. Three or more virulence genes (multivirulent isolates) were found in 50% of EAEC. Typical EPEC (eae + ve and bfpA + ve) were found in one of three of EPEC. All ETEC had genes coding for STp. No LT genes were detected in Libyan ETEC. The CS antigens were found in three ETEC isolates (37.5%, 1 CS6 and 2 CS13).

Phylogenetic group A was detected in 28.6% (6 of 21), group B1 in 42.9% (9 of 21), group B2 in 14.3% (3 of 21), and group D in 14.3% (3 of 21) of total DEC. Of the ten EAEC isolates three were identified as belonging to group A, three to group B1, one to group B2, and three to group D. Of the eight ETEC isolates two were identified as belonging to group A, four to group B1, and two to group B2. Of the three EPEC isolates, one belonged to group A, and two to group B1.

DISCUSSION

The EAEC predominates in this study at 4.1% (48% of all the DEC isolated) with the remainder being EPEC and ETEC. Similarly, Rahouma and others26 reported that EAEC (5.4%) is the main pathotype of DEC in diarrheic Libyan children in Tripoli. Several studies from developing countries reported high detection rates (20–38%), whereas other investigators reported low detection rates (5–6%) for EAEC from children with diarrhea.

In this study EPEC was the least frequently detected DEC at 1.2% (3 of 243, one of the three strains being tEPEC) of diarrheic children. Data regarding the detection of tEPEC or aEPEC in the Mediterranean North Africa region are rare. Al-Gallas and others35 found EPEC in 3.2% (6 of 188, 2 tEPEC) of children with diarrhea in Tunisia. Dow and others,36 in Libya, reported six out of nine EPEC strains from children with and without diarrhea being tEPEC.

Variation in toxin types can provide important insight into the origin and spread of specific ETEC clones. Bölün and others37 tested 46 STI (Sta) only or STI (Sta)/LT E. coli strains from Egyptian children with diarrhea previously identified by enzyme-linked immunosorben assay (ELISA) for the prevalence of STp and STh by PCR. They found STp and STh genotypes in 37% (17 of 46) and in 65% (29 of 46), respectively. On the other hand, Steinsland and others38 in Guinea-Bissau, examined the prevalence of ETEC in young children by PCR detection, reported that only ETEC strains producing STh alone or in combination with LT, but not STp-producing strains, were associated with diarrhea. Identification of ETEC from diarrheic children using PCR or ELISA has not been reported previously from Libya. We detected ETEC in 3.3% (8 of 243) of children with diarrhea.
All (100%) ETEC-positive strains were ST subtype STp. The detection of subtype STp among Libyan children with diarrhea indicate that this subtype may play a role in children's diarrhea in Zliten and Alkhomes from where the stool samples were obtained. Detection of specific toxin types may have important implications for development of vaccines targeting ETEC. Additional studies are needed on ETEC in Libya to confirm our findings. Others reported similar findings among diarrheic children in Guatemala, South America.35

Several pathogens of E. coli such as EHEC and EPEC have captured the attention of the public because of high profile outbreaks. No EHEC or EIEC were found in this study. Other workers reported similar findings.31 The variation in the detection rates of the different DEC pathotypes, reported in present and previously mentioned studies can be attributed to several factors that include geographical locations, social status, dietary behavior, housing, and quality of sanitation.37

In this investigation, fever, dehydration, and vomiting were observed in >76% of DEC-positive children. Typical EAEC illness is manifested by a watery, mucoid, secretory diarrhea with low-grade fever and little to no vomiting.1 However, severe symptoms associated with EAEC have been reported. Cobeljic and others58 reported an outbreak caused by EAEC in the nursery of a hospital in Belgrade, Serbia. Of the 19 affected infants 16 (84%) required intravenous hydration.

Three-fourths (76%) of the children with DEC-associated diarrhea in this study were ≥6 months of age. Other investigations in North and Sub-Saharan Africa have reported similar findings.33,39 Gonzalez and others40 in Venezuela, focused on EAEC, a specific DEC, and found that they were significantly associated with 0 to 2 months of age but not with older infants. Infections caused by EAEC have generally been reported to be unique because of the relatively long duration of symptoms.1 We found that most of the DEC-infected children had diarrheal episodes for >2 days. The high rates of these clinical symptoms among affected children supports the etiological importance of DEC in this region where diarrhea is endemic. Our findings and of those investigators reported previously supports the need for proper measures to be taken by the health authorities to reduce the burden of illness caused by these organisms.

No DEC was isolated from breastfed children. Breast feeding has been observed to protect the infant from the morbidity and mortality of diarrhea in the first few months of life and when given exclusively it offers the greatest protection.41,42

Parents of >57% of DEC-positive Libyan children reported having untreated water in their homes. However, such data could not be relied on because most of the parents reported that on certain occasions, during shortages of water, particularly in summer; water was used from other sources in which treatment conditions were unknown. In addition, the findings of the present investigation indicate that drinking and other types of water sources may not be the major source of the DEC isolated from the study population; only 1 of 50 water samples examined was positive for DEC. Some investigators suggested that meat and vegetables may be involved in transmission of E. coli pathotypes in the community.33 Studies are needed in Libya to determine what types of foods could be the source of DEC isolated from children with diarrhea.

In this research the three predominant genes detected in EAEC were aggR (90%), astA (70%), and aap (50%). Elias and others44 reported aggR in 81% and astA in 48% of EAEC from diarrheic Brazilian children. Several workers reported relatively similar results.15,45 Recently, Harrington and others46 suggested the term “typical EAEC” to refer to strains expressing the aggR regulon. Results of this study show an abundance of typical rather than atypical EAEC among diarrheic Libyan children, as reported elsewhere.47,48

Previous studies have reported that astA (EAST1) is not restricted to EAEC isolates and is significantly associated with diarrhea.49,50 Furthermore it has been observed that, in addition to toxin EAST1, pet (Pet) seems to be common in the E. coli population and not restricted to EAEC.21 In addition, Okeke and others59 reported data that suggest that the aafA (AAF/II) in EAEC may be a marker for other pathogenic strains of E. coli. The findings of this work are in accordance with the previously mentioned studies as far as astA is considered but not pet and aafA. The pet gene was found in only one EAEC isolate and aafA (AAF/II) was not detected in this investigation. Similar to our findings, Spencer and others61 examined 22 EAEC strains isolated from four outbreaks of diarrheal disease in England and found two isolates expressed the AAF/II genotype. Recently, a study from Tunisia reported astA in 100% of EAEC strains from diarrheic children, adults and controls.33

It has been hypothesized that the combination of different virulence genes increases strain virulence.47 We found three or more virulence genes (multivirulent isolates) in 50% of EAEC isolates. Saranuyta and others60 reported multivirulence in >30% of EAEC from diarrheic children in Mongolia. A study from Brazil examined EAEC from diarrheic and control children for 11 virulence markers and found multivirulence in >74% of EAEC strains.44 Differences in the reported rates of virulence genes among EAEC from different regions may be caused by differences in geography, source of fecal samples examined (e.g., from diarrheic children or adults), number of EAEC strains examined, and methods used (phenotypic or genotypic).

Around 50% of ETEC strains worldwide known CFs can be detected.52 Recently, an ETEC colonization factor was identified in 49% of ETEC isolates from Egypt.53 In this study CFs were identified in 37.5% (3 of 8) of the ETEC strains examined. Previous studies from Egypt reported closely similar findings with the predominance of CFA/IV, especially CS6 in ETEC strains.54,55 Two of these three CFs in ETEC strains from Libya were CS13. The CS13 (previously referred to as PCFO9) is another human ETEC-fimbria more related to other animals than to human ETEC-fimbriae.56 Recently, Navarro and others57 examined 23 E. coli strains (11 from Egypt, 10 from Mexico, and 2 from Bangladesh) from children with diarrhea for the CFs. The CS13 was the predominant CF being detected in 72.7% of E. coli from Egypt, 50% of E. coli from Bangladesh, and not detected in E. coli from Mexico. This is the first time CS13 is being reported in E. coli from Libya.

We identified the majority (>70%) of the isolated DEC as phylogenetic groups A and B1. Rügèles and others33 determined the phylogenetic groups among different E. coli pathotypes isolated from diarrheic children and from foods. They reported that EAEC strains belonged to groups A and B1, and EPEC and ETEC to groups B2 and D. In accordance with their findings we found most of EAEC belong to groups A and B1. However, contrary to their observation most of our
ETEC and all EPEC belong to groups A and B1. This could be because none of our DEC was isolated from foods.

In conclusion, EAEC is the predominant DEC isolated from the examined Libyan children with diarrhea. The isolated EAEC possessed different virulence traits and belonged to different phylogenetic groups indicating their heterogeneity. More studies are needed from different regions of the country to determine the exact role of these pathogens in children with diarrhea in Libya.

Received May 24, 2011. Accepted for publication November 18, 2011.

Acknowledgments: We acknowledge the excellent laboratory work provided by Hakam Abd El Fattah and Salem Awad.

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