Etiology of Acute Diarrhea in Tunisian Children with Emphasis on Diarrheagenic *Escherichia coli*: Prevalence and Identification of *E. coli* Virulence Markers

*Imen BEN SALEM-BEN NEJMA [1,2], Mouna HASSINE ZAAFRANE [1,3], Fredj HASSINE [4], Khira SDIRI-LOULIZI [3], Moncef BEN SAID [5], Mahjoub AOUNI [1], Ridha MZOUGHI [2]

1. *Laboratory of Infectious Diseases and Biological Agents, Faculty of Pharmacy, University of Monastir, TU-5000 Monastir, Tunisia*
2. *Laboratory of Hygiene, 5 Rue de Kairouan 4000 Sousse, Tunisia*
3. *National Reference Center for Enteric Viruses, Laboratory of Virology, CHU of Dijon, 2 Rue Angélique Ducoudray, University of Bourgogne, 21000 Dijon, France*
4. *Polyclinique CNSS Sousse- Khzema Sousse 4000-Tunisia*
5. *Laboratory of Parasitology-Farhat Hached Hospital, Sousse 4000, Tunisia*

*Corresponding Author: Email: imen.ben-salem@laposte.net

Abstract

Background: Diarrheal diseases can be caused by viral, bacterial and parasitic infections. This paper provides a preliminary image of diarrhea with regards to etiology and epidemiologic factors in Tunisian children less than five years of age.

Methods: Overall, 124 diarrhoeal stools were collected from patients suffering from acute diarrhea and 54 stool samples from healthy children. All stools were examined for the presence of enteric pathogens.

Results: In diarrheagenic children, 107 pathogenic bacteria were isolated (12 *Salmonella spp.* (9.7%) and 95 diarrheagenic *Escherichia coli* strains (76.6%): 29 enteroaggregative *E.coli* (EAEC) (23.4%), 15 enteroinvasive *E.coli* (EIEC) (12.1%), 17 enteropathogenic *E.coli* (EPEC) (13.7%), 26 enterotoxigenic *E.coli* (ETEC) (21%) and 2 enterohemoragic *E.coli* (EHEC) (1.6%). However, in the control group, 23 pathogenic *E.coli* strains were isolated (42.6%): 8 EAEC (14.8%), 12 EIEC (22.2%) and 3 EPEC (5.5%). Among diarrheagenic *E.coli* (DEC), only ETEC strains were significantly recovered from diarrheagenic children than from healthy controls ($P < 0.0003$). Group A rotavirus was identified in 33.9% (n=42) of diarrheagenic children and in 11.1% among the control group (n=6). Concerning norovirus, 8.9% (n=11) of the samples collected from diarrheagenic children and 9.2% (n=5) from the control group were positive. The prevalence of rotaviruses and *Salmonella spp* were also significantly higher in patients with diarrhea than in controls ($P = 0.002$ and $P < 0.019$, respectively). Finally, enteropathogenic parasites (*Entamoeba coli* and *cryptosporidium Oocystes*) were isolated from 4.8% and 9.2% of diarrheagenic and control children, respectively.

Conclusion: These results provide baseline data about the relative importance of different enteropathogens in Tunisian children.

Keywords: Enteric pathogens, *Escherichia coli*, Diarrhea, Children, Diagnosis, Tunisia

Introduction

Acute diarrhea is still a relevant health problem, particularly in early infancy, in both developing and developed countries. Furthermore, acute gastroenteritis is the major cause of morbidity and mortality worldwide (1). Diarrheal diseases cause over two million deaths every year, most of them affecting children less than 5 years of age living in less developed countries (www. who. int., 2005).
A wide range of enteropathogens cause diarrhea in children such as viruses, bacteria and parasites (2, 3, 4). The data on rotavirus and norovirus gastroenteritis published by the United States Centers for Disease Control and Prevention, showed that rotavirus infections were estimated to cause 25 million clinical visits, 2 million hospitalizations and 453,000 deaths worldwide each year in children younger than 5 years (5). However, it was estimated that norovirus caused 900,000 clinical visits and 64,000 hospitalizations among children in developed countries and up to 200,000 deaths of children younger than 5 years in developing countries each year (6).
Among the bacterial pathogens, *Salmonella, Shigella* and some *Escherichia coli* (*E.coli*) strains can cause severe and life-threatening diarrhea. Diarrheagenic *E.coli* (DEC) strains can be currently classified into five major categories on the basis of distinct epidemiological and clinical features, specific virulence determinants, and association with certain serotypes (7) : (i) enteroaggregative *E.coli* (EAEC), (ii) enteroinvasive *E.coli* (EIEC), (iii) enterohemorrhagic *E.coli* (EHEC), (iv) enteropathogenic *E.coli* (EPEC), and (v) enterotoxigenic *E.coli* (ETEC). Other additional categories have been also described as DEC strains (7); however their clinical significance, epidemiology and pathogenesis have not been well understood yet.
In North Africa, several studies about the role of DEC in diarrhea have been carried out especially in Egypt. However, little is known about the role of DEC in diarrheal diseases in the Maghreb area. During the last two decades, only one study has been conducted in Tunis (North of Tunisia), and highlighted an important rate of ETEC in children

and adults with or without diarrhea, suggesting an endemic status in our country (8).
The aim of this prospective study was to determine the etiology of infantile diarrhea including viruses, bacteria and parasites and to examine the virulence markers of pathogenic *E.coli* strains.

Materials and Methods

Subjects and fecal specimens
From December 2008 to November 2009, 124 stool samples were collected from non-hospitalized children (median age: 21 months; range: 3 month to 60 months) who consulted the CNSS polyclinic and suffered from acute diarrhea and gastro-enteric symptoms. A control group consisting of 54 healthy children representing the same age group (median age: 20 months; range: 4 month to 60 months) was also tested for the presence of enteric pathogens. Children with chronic diarrhea were excluded from this study.
Diarrhea was characterized by watery stools with or without blood and mucus. Children patients had no history of travelling outside Tunisia and did not take antibiotics in the week preceding the sampling. Stools were classified as bloody when blood was visible upon examination of the samples macroscopically. Other clinical symptoms, including fever, vomiting, nausea, abdominal pain or dehydration were obtained through questionnaires reported by the physicians.
The samples were immediately transported to the laboratory. Specimens which were not analyzed for more than four hours were rejected. Patients and healthy persons, who mostly were on a low income, were informed by doctors about the study and agreed to participate and to inform doctors of any information needed. This study did not require approval by the ethic committee.

Bacteriological Methods
Phenotypic procedures
All fecal specimens were cultured on sorbitol MacConkey agar (Oxoid) and were incubated for

24 hours at 37°C. Colonies suspected to be *E.coli* (3-5 colonies from each culture plate) were selected. These colonies were further studied by other characteristic: enzymes specific for *E.coli* strains (Tryptophanase that cleaves Tryptophan into pyruvate, indol, and ammonia; by using reagents (Kovac's and DMCA). Identification was confirmed with the Api 20E system (bioMérieux, Marcy-l'étoile, France).

Samples were subcultred on Hektoen agar (Oxoid) and on other media (SS agar (Oxoid)) for the identification of *Salmonella* and *Shigella* spp. followed by an overnight incubation at 37°C. Colonies morphologically resembling *Shigella* and *Salmonella* spp. were further identified based on appropriate biochemical reactions using the API 20E system and the results were confirmed by slide agglutination testing using commercially available antisera (Biorad, France).

Molecular procedure for the identification of diarrheagenic E. coli

For DNA extraction, all *E.coli* strains were grown in enrichment broth. Genomic DNA was prepared from each isolate by boiling (9) and used as target for polymerase chain reaction (PCR).

Thus, the DNA templates were subjected to multiplex and monoplex PCRs with specific primers for the detection of the following virulence markers: *bfp*A (BFP1, BFP2) structural gene for the bundle-forming pilus of EPEC (10), *eae* (*eae*1, *eae*2) (attaching and effacing lesions of EPEC (10, 11), shiga toxins and their variants *stx*, *stx*1 (STX1f, STX1r) *and* *stx*2 (STX2f, STX2r) of EHEC (12), *elt* (heat-labile enterotoxin) (LTL, LTR) and/or *est* (heat-stable enterotoxin) (AL65, AL125) (enterotoxins of ETEC) (13, 14), *ipa*H (*ipa*III, *ipa*IV) invasion-adhesion locus of the invasion plasmid found in EIEC (15) and *agg*R (*aggRks*1, *aggRkas*2) transcriptional activator of *AAF* I and *AAF* II of EAEC (16). The sequences of primers selected for use in the amplification method matched the sequences of the corresponding genes of EPEC, EHEC, ETEC, EIEC and EAEC.

The minimum criteria for determining diarrheagenic *E.coli* were defined as follows: the presence of *est* and/or *elt* for ETEC strains, the presence of *bfp*A and *eae* for typical EPEC strains whereas the only presence of *eae* gene confirmed the detection of atypical EPEC strains, the presence of shiga toxins genes and their variants (*stx*) for STEC strains, the presence of *ipa*H for EIEC and the presence of *agg*R for EAEC. Differentiation between EIEC and *Shigella spp* for strains possessing *ipa*H gene was also achieved by agglutination with *Shigella* specific antisera (Bio-Rad laboratories).

To further investigate the toxin type in diarrheagenic EHEC strains, we conducted two other monoplex PCRs. The first monoplex PCR was performed to detect shiga toxins 1 gene (*stx*1) (STX_1f, STX_1r) and the second monoplex PCR to detect all shiga toxins 2 gene variants (*stx*2) (STX_2f, STX_2r)) (Table 1).

All reactions were performed in a final volume of 34 μl containing 5μl of template DNA, 0.2 mM dNTPs, 2 Mm MgCl2, 5.0 units of Taq polymerase, 50 ng of each primer and deionized water to make up the volume. All assays used the same cycling parameters. Primers and cycling parameters used in multiplex and monoplex PCRs were summarized in (Table 1, 2, 3).

A negative control lacking the DNA template was included in each experiment to exclude the possibility of reagent contamination. Multiplex PCR assays were performed with each reference strain of diarrheagenic *E. coli* from pure culture. We used non-pathogenic *Escherichia coli* strain HB 101 as a negative control. Positive controls were as follows: enterotoxigenic *Escherichia coli* ETEC H10407 for the *elt* gene, Jep5683 for the *est* gene, EDL933 (O157:H7) for the *stx* gene, EPEC2348/69 (O127:H6) for the *eae* and *bfp*A genes, EIEC 11741 for the *ipa*H gene and finally, EAEC 17-2 for the *agg*R gene. These strains were kindly provided by Professor Ridha Ben Aissa (Institut Pasteur Tunis).

Next, in order to test the reproducibility of these results and equally the reproducibility of mPCR method, the mPCR assays were repeated at least twice with all tested strains whereby the DNA template of strain that was freshly prepared for each repeat. Finally, the specificity of the mPCR

assay was also tested with each control reference *E. coli* strain listed above.

Combining the molecular results of all of these primers permitted an easier detection of the five categories of diarrheagenic *E. coli* and also the *E.coli* strains that expressed unusual virulence factors (intermediate *E. coli* pathotypes).

The amplified DNA products were electrophoresed in 2% or 1% (for the multiplex PCR I) agarose gels containing ethidium bromide (Agarose LE; Roche Applied Science, Penzberg, Germany) and were then visualized under UV light. A 100-pb DNA ladder was used as a molecular mass marker.

Virological methods

The stool samples were routinely screened for the presence of VP6 group A rotavirus antigen by enzyme immunoassay using a commercial ELISA kit (Rotavirus ELISA kit, Premier™ Rotaclone®). All rotavirus positive samples were confirmed by RT-PCR and genetically characterized. The viral RNA was extracted as previously described (17). Rotavirus G and P genotyping was performed using semi-nested type specific multiplex RT-PCRs that

could detect seven G-types and six P-types, as previously described (17).

Human noroviruses were detected by RT-PCR using several sets of primers in separate reactions. Primer set JV12 and JV13 (Vinjé and Koopmans, 1996) was used to amplify a fragment of the RNA polymerase genes. Primer set G1SKF and G1SKR and primer set G2SKF and G2SKR (Kojima et al., 2002) were used to detect a fragment of the capsid genes of genogroup I (GGI) and genogroup II (GGII) noroviruses, respectively. RT-PCRs were performed using a Qiagen One Step RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

All PCR products were examined by gel electrophoresis in 2% agarose gels containing 10 µg/ml ethidium bromide, and amplicons were viewed with UV light.

The P and G types were determined by the size of the amplicons and the genotyping of noroviruses was performed by direct sequencing of the PCR products with the same primers used for amplification by using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit and a 3100 DNA sequencing system (both from Applera Corporation, Forster City, CA).

Table 1: Primers used in multiplex and monoplex PCRs

Designation	Primer sequence (5'-3')	Target gene	Amplicon size (bp)
AL65	5'-TTAATAGCACCCGGTACAAGCAGG-3'	est	147
AL125 5'-	5'-CCTGACTCTTCAAAAGAGAAAATTAC-3'	est	
LT_L	5'-TCTCTATGTGCATACGGAGC–3'	elt	322
LT_R	5'-CCATACTGATTGCCGCAAT-3'	elt	322
eae1	5'CTGAACGGCGATTACGCGAA 3'	eae	917
eae2	5'CCAGACGATACGATCCAG3'	eae	917
VTcom-u	5'gACCgAAATAATTTATATgTg3'	stx	518
VTcom-d	5'TgATgATggCAATTCAgTAT3'	stx	518
ipaIII	5'gTTCCTTgACCgCCTTTCCgATACCgTC3'	ipaH	916
ipaIV	5'gCCggTCAgCCACCCTCTgAgAgTAC3'	IpaH	916
aggRks1	5'gTATACACAAAAgAAggAAgC3'	aggR	254
aggRkas2	5'ACAgAATCgTCAgCATCAgC3'	aggR	254
BF1	5'AATggTgCTTgCgCTTgCTgC3'	bfpA	326
BF2	5'gCCgCTTTATCCAACCTggTA3'	bfpA	326
STX1f	5'ATAAATCgCCATTCgTTgACTAC3'	stx_1	180
STX1r	5'AgAACgCCCACTgAgATCATC3'	stx_1	180
STX2f	5'ggCACTgTCTgAAACTgCTCC3'	stx_2	225
STX2r	5'TCgCCAgTTATCTgACATTCTg3'	stx_2	225

Table 2: Cycling parameters used in multiplex and monoplex PCRs

	Primers	PCR programme
Multiplex PCR I	STX eae ipaH	Enzyme activation 5min at 94°C 40 Cycles Denaturation: 30s at 94°C Primer annealing30s 62°C DNA extension: 1min at 72°C Final elongation 5min at 72°C
Multiplex PCR II	bfpA aggR	Enzyme activation 5min at 94°C 40 Cycles Denaturation: 30s at 94°C Primer annealing30s 62°C DNA extension: 1min at 72°C Final elongation 5min at 72°C
Multiplex PCR III	Est Elt	Enzyme activation 5min at 94°C 40 Cycles Denaturation: 30s at 94°C Primer annealing30s 62°C DNA extension: 1min at 72°C Final elongation 5min at 72°C
Monoplex PCR I	STX1	Enzyme activation 5min at 94°C 40 Cycles Denaturation: 30s at 94°C Primer annealing30s 62°C DNA extension: 1min at 72°C Final elongation 5min at 72°C
Monoplex PCR II	STX2	Enzyme activation 5min at 94°C 40 Cycles Denaturation: 30s at 94°C Primer annealing30s 62°C DNA extension: 1min at 72°C Final elongation 5min at 72°C

Sequence analysis was conducted using Fasta software, version 3, available from the European Bioinformatics Institute.

Parasitological methods

Parasitological examination was based on direct examination of fresh stool followed by a concentration method and completed by a staining method: MIF coloration (Merthiolate-iode-formol).

Statistical analyses

The chi-square test was used to determine the statistical significance of the data. A *P* value of <0.05 was considered as significant.

Results

Bacterial causes of gastroenteritis

Among the 124 diarrheic children that have visited the CNSS polyclinic during the study period,

107 enteropathogenic bacteria (86.3%) were iso-lated. These pathogens were classified into two groups: 12 strains of *Salmonella* spp. (9.7%) and 95 strains of diarrheagenic *E. coli* (76.6%). For the healthy group, 23 diarrheagenic *E. coli* (42.6%) from 54 children were isolated. No *Shigella* strains were isolated from any of the children tested (diarrheic and healthy children). Rates of detection of bacterial pathogens in patients and healthy groups are shown in Table 4.

Pathotype identification

Table 2 recapitulates the different mPCRs used in order to detect the five different categories of diarrheagenic *E.coli*. PCR products were derived from the pure culture of reference strains of non pathogenic *E.coli*, EHEC EDL933, EPEC 2348/69, ETEC 10407, ETEC Jep 5683, EIEC 11741 and EAEC 17-2, respectively.

Therefore, when the multiplex PCR was positive for EHEC screened from stool samples, two spe-cific monoplex PCRs were run (separately with primers specific for the shiga toxins1 (*stx*1 gene), and shiga toxins 2 variants (*stx*2 gene) to deter-mine whether the strain possessed the *stx*1 and/or *stx*2.

These multiplex PCRs showed positive results for diarrheagenic *E.coli* strains and negative results for non diarrheagenic *E.coli* strains (Table 4, Fig. 1). The prevalence of diarrheagenic *E. coli* in both groups was respectively 76.6% and 42.6%. No significant differences in the prevalence of isola-tion of diarrheagenic *E. coli* were noted in both diarrheic and healthy groups.

Among the group with diarrhea the PCR assays detected 29 (23.4%) EAEC isolates (*agg*R positive gene), 15 (12.1%) EIEC isolates (*ipa*H positive gene), 17 (13.7%) EPEC isolates among which 11.3% were typical and 2.4% were atypical EPEC strains, 26 (21%) ETEC isolates (*elt* and/or *est* positive gene) and 2 (1.6%) EHEC isolates (*stx* positive gene) (Table 4, Fig. 1). The prevalence of EAEC, EIEC and typical EPEC strains in the healthy group was 14.8% (8 strains), 22.2% (12 strains), and 5.5% (3 strains) respectively. No EHEC, ETEC and atypical EPEC strains were

isolated from any of the healthy children tested (Table 4).

Concerning the ETEC isolated strains, 10 of 26 (8.1%) possessed both heat-labile toxin gene and heat-stable toxin gene, 5 of 26 (4%) had only the heat-labile toxin gene and 11 of 26 (8.9%) pre-sented only the heat-stable toxin gene (Table 4).

Interestingly, we detected in patients a collection of diarrheagenic *E.coli* (n=6) (4.8%) that expressed unusual virulence factor profiles apparently repre-senting intermediate pathotypes: 2 ETEC/EPEC strains (*est*+, *elt*+, *eae*+), 2 ETEC/EIEC strains (*est*+, *ipa*H+), 1 ETEC/EAEC strain (*est*+, *agg*R+), and 1 ETEC/EPEC strain (*est*+, *bfp*A+, *eae*+). These intermediate pathotypes were absent in the healthy control group (Table 4).

Regarding the two monoplex PCRs that aimed to a further investigation of shiga toxin type in EHEC strains, we noted that the two EHEC strains presented only the shiga toxins 1 target gene.

Twelve *Salmonella spp.* (9.7%) were isolated from the patient group and none from the healthy group (Table 4) while no *Shigella spp.* were isolated from any of the children tested (patient and healthy groups).
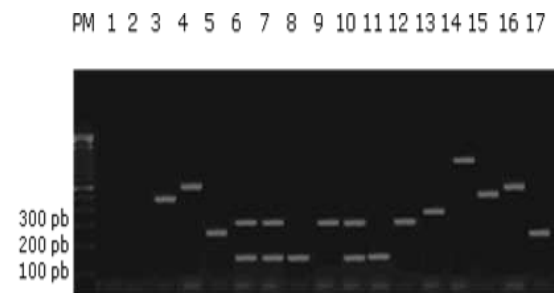

Fig.1: Agarose gel electrophoresis of multiplex PCRs (mPCR 1, 2, and 3) amplification of laboratory strains isolated from diarrheagenic children (Only positive results of each multiplex PCR were present in this Figure) **PM**; **Lanes: 1,** negative control: mixture control; 2, non pathogenic *E.coli* **HB101**; **3,** Strain 103 (EHEC); **4,** Strain 113 (EIEC) ; **5,** Strain 102 (EAEC); **6,** Strain 119 (ETEC); **7,** Strain 42 (ETEC); **8,** Strain 21 (ETEC); **9,** Strain 51 (ETEC); **10,** Strain 5 (ETEC); **11,** Strain 22 (ETEC); **12,** Strain 88 (ETEC); **13,** Strain 103 (EPEC); **14,** Strain 103 (EPEC); **15,** Strain 104 (EHEC); **16,** Strain 30 (EIEC); **17,** Strain 35 (EAEC)

Viral causes of gastroenteritis

Among the GEA-group (n=124), 53 (42.7%) presented a viral infection. Group A rotaviruses and noroviruses were identified as causative agents in 42 (33.9%) and 11 (8.9%) of the fecal samples, respectively. From the healthy group, we isolated 6 (11.1%) rotaviruses and 5 (9.2%) noroviruses. Mixed infections with the two viruses were detected in 2 stool samples (3.7%).

In diarrheal children, rotavirus genotype combination G2P [4] was predominant with 12.9% (n=16). This was followed by G3P [8] 10.5% (n=13), G1P [8] 6.4% (n=8), and G4P [8] 2.4% (n=3). Among two P [8] rotavirus strains (1.6%), the G type remained nontypeable. In the healthy group, we isolated only genotype G1P [8]. The genotyping of noroviruses revealed that, in diarrheal patients, we detected only genogroup II (GGII) 8.1% (n=10) with GGII.b/II.3 being the predominant genotype. As for children in the control group, the genotype GGI.b/I.6 of genogroup I (GGI) was the most common genotype isolated. It has to be noted that no mixed infections with GGI and GGII strains were observed.

Parasitologic detection

Parasitologic study showed that only 6 (4.8%) of 124 diarrheal samples were positive for parasites (*Entamoeba coli* (n=2), *Cryptosporidium Oocystes* (n=2), *Giardia lamblia* (n=1) and *Blastocystis hominis* (n=1)) Mixed infections with ≤ 4 different organisms isolated from a single stool sample were observed in the majority of the specimen. A variety of combinations was detected in the diarrheagenic group especially with ETEC pathotype where the most frequent combination was ETEC and other *E.coli* pathotypes (n=14). ETEC and rotavirus (n=7) were also observed.

Seasonal distribution of enteropathogens

We did not observe any particular temporal distribution of different enteropathogens. In fact, the highest prevalence of bacterial agents was observed in autumn (n=36) and summer (n=34), but bacterial infection can be also detected in winter. Viral infections were detected in autumn and in winter showing a decrease in the frequency of their isolation in summer and spring. However, the six cases of parasitic infections were detected only in autumn.

Enteropathogen identification according to sex and age groups

A higher rate of infection among male patients was found for all enteropathogens tested. Infections with enteropathogens were identified in 54.8% of males (n=68) and 45.2% of females (n=56) suffering from gastroenteritis. This one-year surveillance also showed that 50% of cases with gastroenteritis were found in 12-35 months age group (n=35 of male and n=27 of female children).

Table 3: International reference of *E. coli* strains used as control for PCRs amplifications

Strain	International Designation	Positive gene(s)
ETEC	H10407	*elt*
ETEC	Jep5683	est
E.coli strain (negative control)	HB 101	No virulence gene
EHEC	EDL933 (O157:H7)	*stx*
EPEC	EPEC2348/69 (O127:H6	*eae* and *bfp*A
EIEC	EIEC 11741	*ipa*H
EAEC17-2	EAEC17-2	*agg*R

Table 4: Distribution of enteropathogens identified from stool samples of 178 children: 124 patients and 54 controls

Microorganism			Total no. of samples (%) positive from children: (n=178)			Pathogenicity
			diarrheic children (124) No. of cases (%)	healthy children (54) No. of cases (%)	*P*	index[a]
Bacterial (total)			107 (86.3)	23 (42.6)	NS	
Escherichia coli Diarrheagenic (as identified in multiplex and monoplex PCRs)			95 (76.6)	23 (42.6)	NS	1.8
ETEC	All		26/124 (21)	0/54 (0)	<0.0003	-
	est		11/124 (8.9)	0/54 (0)		-
	elt		5/124 (4)	0/54 (0)		-
	est/elt		10/124 (8.1)	0/54 (0)		-
EAEC	aggR		29/124 (23.4)	8/54 (14.8)	NS	1.58
EIEC	ipaH		15/124 (12.1)	12/54 (22.2)	NS	0.54
EHEC	All		2/124 (1.6)	0/54 (0)		-
	stx		2/124 (1.6)	0/54 (0)		-
	stx1		2/124 (1.6)	0/54 (0)		-
	stx2		0/124 (0)	0/54 (0)		-
EPEC	All		17/124 (13.7)	3/54 (5.5)	NS	2.49
Typical EPEC	bfpA, eae		14/124 (11.3)	3/54 (5.5)		-
Atypical EPEC	eae		3/124 (2.4)	0/54 (0)		-
Intermediate *E. coli* strains			6/124 (4.8)	0/54 (0)	NS	-
ETEC/ EIEC	est/ipaH		2/124 (1.6)	0/54 (0)		-
ETEC/ EPEC	est/bfpA		1/124 (0.8)	0/54 (0)		-
ETEC/ EAEC	est/aggR		1/124 (0.8)	0/54 (0)		-
ETEC/ EPEC	est/elt/eae		2/124 (1.6)	0/54 (0)		-
Salmonella spp.			12/124 (9.7)	0/54 (0)	<0.019	-
Shigella spp.			0/124 (0)	0/54 (0)		-
Viral			124 (100)	54 (100)		
Enteropathogenic viruses			53 (42.7)	13 (24.1)	0.018	1.8
Rotavirus			42/124 (33.9)	6/54 (11.1)	0.002	3
	Typage G/P					
		G1P[8]	8/124 (6.4)	6/54 (11.1)	NS	-
		G3 P[8]	13/124 (10.5)	0/54 (0)	NS	-
		G4 P[8]	3/124 (2.4)	0/54 (0)	NS	-
		G2 P[4]	16/124 (12.9)	0/54 (0)	NS	-
		* NTP[8]	2/124 (1.6)	0/54 (0)	NS	-
Norovirus			11/124 (8.9)	5/54 (9.2)	NS	0.96
GGII.4			1/124 (0.8)	0/54 (0)		-
GGII.b/II.3			10/124 (8.1)	0/54 (0)		-
GGIb/I.6			0/124 (0)	4/54 (7.4)		-
GGIIb/II.13			0/124 (0)	1/54 (1.85)		-
Viral Co-infections			0/124	2/54 (3.7)		-
G1P[8] & GGIIb/II.3			0/124 (0)	1/54 (1.85)		-
G1P[8] & GGIIb/II.13			0/124 (0)	1/54 (1.85)		-
Parasitic			124 (100)	54 (100)		
Entamoeba coli			2 (1.6)	0 (0)		-
Oocystes of cryptosporidies			2 (1.6)	0 (0)		-
Giardia lamblia			1 (0.8)	2 (3.7)		-
Blastocystis hominis			1 (0.8)	0 (0)		-
Dientamoeba fragilis			0 (0)	2 (3.7)		-
Endolimax nanus			0 (0)	1 (1.8)		-
Total			6 (4.8)	5 (9.2)	NS	-

[a] The pathogenicity index was calculated as the percentage of case patients shedding a particular pathogen or shedding *Escherichia coli* carrying a target gene, divided by the respective percent of control patients with positive test results for that same pathogen or factor; *NS= not significant; ETEC= enterotoxigenic *E.coli* ; EAEC= enteroaggregative *E.coli* ; EIEC= enteroinvasive *E.coli* ; EHEC= enterohemorrhagic *E.coli* ; EPEC= enteropathogenic *E.col,* NT*= Nontypeable

Discussion

This study, which covered a one-year period, is the first one in our region and the second in Tunisia that has estimated the prevalence of enteric pathogens associated with acute gastroenteritis in outpatient children in Sousse, Tunisia.

Diarrhea in developing countries is caused by an increasingly long list of viral, bacterial, and parasitic pathogens with rotavirus, diarrheagenic *E. coli*, *Shigella* spp. and *Salmonella* spp. (18) being the most prevalent.

In the present study, ETEC were the most common cause of diarrhea in outpatient children followed by rotaviruses and *Salmonella* (Table 4). These microorganisms were significantly recovered from children with diarrhea than from healthy controls ($P < 0.0003$, $P = 0.002$ and $P < 0.019$, respectively). However, the prevalence of diarrheagenic *E.coli* was not significantly higher in the patient group than in controls (76.6% versus 42.6%) ($P > 0.05$).

ETEC and EAEC were the most prevalent pathogens isolated from children with diarrhea. In fact, ETEC pathotype is recognized as one of the main important diarrheagenic *E.coli* in developing countries. High rates of ETEC have been previously reported in North Africa especially in Egypt where a strong association with diarrhea has been shown (19). However, many other studies have not reported any significant association of ETEC with diarrhea; in fact ETEC strains were found among healthy children (8, 20, 21). These discrepancies have been explained by the geographical and temporal variation of ETEC rates in childhood (22).

Among the 26 ETEC isolates, the percentage of strains possessing *est* gene only, *elt* gene only and both *elt* and *est* genes were respectively 42.3% (n=11), 19.2% (n=5), and 38.5% (n=10); thus they were different from those previously reported in north Africa (19, 8). In fact Al-Gallas et al. have reported a percentage of 22% of ETEC strains in patients (16% of strains possess *est* gene only, 3.7% possess *elt* gene only and 2% possess both *elt* and *est* genes) and a percentage of 35% in controls (32.5% of strains possess *est* gene only and 2.6% possess *elt* gene only) (8). This wide variation in the frequency of toxin phenotypic subgroups among ETEC isolates was also noted in most developing world. Knowledge of the distribution of ETEC toxin phenotypic subgroups in a population may be useful to assess endemic disease incidence, as LT-ETEC is thought to be less likely to cause disease than ST or ST and LT ETEC (22). Thus, in this study, the high rate of ETEC strains harboring *est* gene (with or without *elt* gene) (80.8%) (n=21) may explain why ETEC strains are isolated only in patients with diarrhea but not in healthy controls (Table 4). Other virulence factors (adhesive factors (CFA) in ETEC strains) that were not characterized in this study have been reported to play an effective role in the pathogenesis of ETEC diarrhea and therefore should be explored (7, 19, 22). Co-infections with other pathogens in children with diarrhea are frequently observed with this pathotype. Indeed, 14 co-infections with other *E.coli* pathotypes, 7 co-infections with rotaviruses and one infection with norovirus have been detected.

In the present study, the identification of EAEC strains was only based on the detection of *Agg*R gene by PCR. This test has been shown to lack sensitivity in comparison to the hep-2 cell adherence test considered as the "gold standard" (7) but the latter requires specialized facilities and is time consuming. Yet, the present study increased the knowledge of the role of EAEC in acute diarrhea in children in the region of Sousse. In fact, EAEC was the most common pathotype among DEC isolated from children (30.5%) (n=29). However, in accord with many previous reports from different geographic areas, among the 95 DEC strains no significant differences between patients and controls ($P > 0.05$) were found. This lack of association between both groups reflects the high frequency of carrier state among the studied child population (14.8%) (n=8), as it has been reported previously in many less developed countries (23, 24), suggesting their role as enteropathogenic bacteria with an endemic status. Moreover, we have found a high percentage of EAEC in wastewater as well as in entrance and in exit

points even after bacteriological wastewater assessment. These environmental findings could explain the important prevalence of EAEC strains in patients and in controls (25). In this way, as it is the first study to report a high rate of EAEC isolates from Tunisia, a further characterization of these strains is required for a better understanding of their role in diarrheal diseases in this country. Co-infections especially with rotaviruses were frequently detected (n=8).

Besides ETEC and EAEC, enteropathogenic *E.coli* (EPEC) is the leading cause of infantile diarrhea (26). EPEC infection is primarily a disease of infants younger than 2 years of age in developing world. EPEC classification is currently based on the presence of specific genes. The *eae* (intimin) and the *bfp*A (bundle-forming-pilus) genes have both been used for the identification of EPEC and for the subdivision of this group of bacteria into typical (*eae*+, *bfp*A+) and atypical (*eae*+, *bfp*A-) strains. For many years typical EPEC strains have been considered to be the leading cause of infantile diarrhea in developing countries. However current data suggest that atypical EPEC strains are emerging enteropathogens that have been detected worldwide either in developing or in developed countries. As reviewed by Levine and Edelman (27), numerous case-control studies in many countries have found out that EPEC is more frequently isolated from children with diarrhea than from healthy controls (7). In the present study, the prevalence of isolation of EPEC strains from children with diarrhea was not significantly higher than that from the controls (13.7% versus 5.5%) ($P > 0.05$). 14 EPEC strains (11.3%) isolated possess both *eae* gene and also structural gene encoding the bundle-forming pilus *bfp* and so are classified as typical EPEC strains whereas only three strains (2.4%) were atypical EPEC strains. The prevalence of atypical EPEC strains is still low compared with previous reports in Tunisia and elsewhere (8).

EIEC strains were shown to be capable of causing diarrhea in children. This pathotype can be difficult to distinguish from *Shigella* spp. and from other *E. coli* strains, including non-pathogenic strains. The *ipa*H PCR was reported to be effec-

tive in identifying EIEC strains from other *E. coli* pathotypes but other techniques like DNA probes could be used in conjunction with molecular method to characterize better EIEC strains (28). In Sousse, 15 EIEC (12.1%) in children with diarrhea were isolated. But, interestingly, the prevalence of this pathotype in children older than 1 year of age was significantly lower than from children younger than 1 year of age ($P < 0.05$). To sum up, it has been reported that the prevalence of EIEC pathotype vary in different geographical areas (23, 26, 29, 30).

In contrast to the relatively high prevalence of EIEC in this study in both diarrheagenic and control group, EHEC strains were detected at a low prevalence in diarrheagenic children (1.6%) and were not isolated in control group.

This result assert with the low prevalence of EHEC infection in many developing countries (23, 31-33). Interestingly, 6 intermediate *E.coli* strains (4.8%) that presented specific virulence genes of more than one specific *E. coli* pathotype were also found. These intermediate *E.coli* strains that expressed unusual virulence factor profiles can apparently be explained by the extraordinary plasticity of the *E. coli* genome. These intermediate pathotypes were also described in a previous report in Tunisia (8).

For rotaviruses, all fecal positive specimens contained only one type of rotavirus strain. The predominant single G/P combination was G2P [4] (12.9%), followed by G3P [8] (10.5%), G1P [8] (6.4%), G4P [8] (2.4%). This variability in the genotype profiles has been shown in previous studies to be dependent on a given place and a period of time (34-36).

For noroviruses, strains classified as GGII.b/II.3 were almost the only ones detected in diarrheagenic children while, in the control group, GGI.b/I.6 was the predominant circulating genotype. Another study in Tunisia (37) demonstrated the predominance of genogroup II and showed that recombinant GGII.b strains were the second most frequently detected noroviruses in Tunisian children. The natural emergent recombinant GGII.b strains were detected for the first time in France in August 2000 (38) and then spread

throughout France and all over Europe in the following seasons, with outbreaks and sporadic cases reported in Spain, Sweden, and Hungary (39-41). They were also reported in other parts of the world, such as in Japan in the 2003-2004 seasons (42) and India in the 2005-2007 seasons (43). This study has limitations on account of analysis restricted to a small size of samples and a short period of collection, it is indicative of the notable role of noroviruses in causing gastroenteritis and the need for longitudinal studies on surveillance of norovirus diseases and strains in Tunisia. Earlier reports have described GGII.b strains as naturally occurring recombinant norovirus strains with different capsid types (44). This requires more work towards a complete characterization of RNA polymerase and capsid genes for a better understanding of the mechanism involved in the recombination and pathogenicity of noroviruses in Tunisia.

In this study, mixed infections with more than one pathogen were frequently detected with the majority of *E.coli* pathotype, particularly bacterial co-infections. Viral co-infections were also observed (n=7).

Regarding the epidemiology of enteric infections, all detected enteropathogens occurred most frequently in children aged between one and three years (n=62) (data not shown). This distribution can be explained by the interruption of breast-feeding after the age of one year and the exposure of these children to environmental contamination (food and water contamination) (25). Therefore, improvement in the quality of the water distribution network might help reduce the burden on the healthcare system by reducing the number of infected children.

The peak of viral infections occurred during the winter months, while bacterial infections were often predominant during the summer or warm months (data not shown). These tendencies agree with the findings of other studies (45- 48).

Conclusion

An understanding of the relative contribution of enteric pathogens in gastroenteritis is essential for the implementation of appropriate public health measures controlling these diseases.

The findings of this study should urge us to implement management guidelines for *E.coli* associated diarrhea in Tunisia. In particular, laboratory diagnostic methods have to take in account ETEC and EAEC as potential pathogens that should be correctly identified.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

Acknowledgments

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