Etiology of acute diarrhea in children and adults in Tunis, Tunisia, with emphasis on diarrheagenic Escherichia coli: prevalence, phenotyping, and molecular epidemiology.

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Etiology of Acute Diarrhea in Children and Adults in Tunis, Tunisia, with Emphasis on Diarrheagenic *Escherichia coli*: Prevalence, Phenotyping, and Molecular Epidemiology

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Abstract. A total of 271 stool specimens were collected from children (diarrheagenic, n = 115 and control, n = 54) and adults (diarrheagenic, n = 73 and control, n = 29) from Tunis, Tunisia, and processed to detect bacterial enteropathogens, parasites, and viruses. Diarrheagenic *Escherichia coli* (DEC) were identified by their virulence genes (polymerase chain reaction) and adherence patterns (tissue culture assays). The most frequently isolated enteric pathogens from diarrheagenic children were enterotoxigenic *E. coli* (ETEC, 32.3%), enteroaggregative *E. coli* (EAEC, 11.3%), enteroinvasive *E. coli* (EIEC, (11.3%), adenovirus (10.4%), enterohemorrhagic *E. coli* (EHEC, 10.4%), and *Salmonella* spp. (9.5%). For children in the control group, ETEC (37%), EAEC (15%), EHEC (11.1%), and typical enteropathogenic *E. coli* (EPEC, 11.1%) were the most common enteric pathogens. In adults in the diarrheagenic group, *Salmonella* spp. (34.2%), ETEC (12.3%), adenovirus (7%), and *Shigella* spp. (4%) were the most common enteric pathogens. In adults in the control group, ETEC (31%) was the most common enteric pathogen. Multiple pathogens were recovered from 22% of the diarrheagenic children and 7% of the diarrheagenic adults. *Escherichia coli* strains showed high resistance rates to tetracycline, streptomycin, and β-lactams. The most frequent combinations were ETEC-rotavirus and ETEC-adenovirus. Pulsed-field gel electrophoresis for DEC indicated a large number of DEC clones (five major clones) persistent in the community reservoir for a considerable period of time that caused diarrhea in the population. This suggests the confluence of small epidemics by clonally related DEC strains circulating in this region.

INTRODUCTION

Diarrhea, especially acute diarrhea, remains a major public health problem. In developing countries, an estimated 12 or more diarrheal episodes per child per year occur within the first five years of life. Annually, approximately 4.6 million pediatric deaths, approximately 25–30% of all deaths among children less than five years of age, can be attributed to acute diarrhea.[1,2] Acute diarrhea also contributes considerably to morbidity and medical expenses in developed countries.[3] Adult residents of developing countries are less likely to have sporadic diarrhea, and when it occurs, it is unlikely to be life-threatening. Therefore, diarrheal pathogens in adults in developing countries have been the subject of few investigations, and little is known about the etiologic epidemiology of pathogens other than epidemic *Vibrio cholerae* and *Shigella*.

The causes of acute diarrhea include a wide range of viruses, bacteria, and parasites.[4] Among the bacterial pathogens, *Escherichia coli* plays an important role. *Escherichia coli* is the predominant non-pathogenic facultatively anaerobic member of the human intestinal microflora. However, some strains of *E. coli* can cause severe and life-threatening diarrhea. Diarrheagenic strains of *E. coli* can be divided into five main categories on the basis of distinct epidemiologic and clinical features, specific virulence determinants and association with certain serotypes: enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC).[5] Diffuse-adherent *E. coli*, also known as diarrhea-associated hemolytic *E. coli*, and cytolethal distending toxin-producing *E. coli* have also been described as diarrheagenic.[5] However, their epidemiology and diarrheagenic potential are not yet clear.[6]

This is the first study of its kind to be carried out in Tunisia; the last similar study was conducted 20 years ago and was limited to children with acute diarrhea.[7] The aims of the present study were 1) to determine the prevalence of enteropathogens, including bacteria, viruses, and parasites, that cause acute diarrhea among children and adults in Tunis, Tunisia; and 2) to investigate the presence and frequency of diarrheagenic *E. coli* (DEC) in children and adults with acute diarrhea by examining their virulence markers and cell-adherent patterns. The isolates were further examined for their O antigens and susceptibility to common antibiotics. In addition, we used pulsed-field gel electrophoresis (PFGE) to determine clonal relatedness among *E. coli* isolates in Tunisia.

MATERIALS AND METHODS

Definition. Diarrhea was defined as at least three loose stools in a 24-hour period accompanied by at least one of the following symptoms: nausea, vomiting, abdominal cramps, or fever > 38°C. Acute diarrhea was defined as diarrhea that lasted ≤ 14 days at the time of presentation.

Samples and subjects. The study was conducted between November 2001 and November 2004 in Tunis, the capital of Tunisia. Nine different pediatric and health centers provided stool samples. These centers were Children-Care Center of Mellassine, Children's Hospital (Hôpital d'Enfant), Al-Rabta Hospital, and the Community Centers of Ben Arous, which composed of six centers in the Ben Arous region: Rades, Fouchana, Mohammadia, Naassan, Hammam Lemf, and Ezzahra. All enrolled patients (children and adults) were outpatients visiting the clinical health with acute diarrhea as reported by the physicians. The diarrhea was characterized by watery stools with or without blood and mucus. The patients (children < 1–15 years of age, n = 115 and adults > 18 years of age, n = 73) had not taken antibiotics in the week preceding

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sampling and had no history of traveling outside Tunisia in the last month preceding sampling. Stools were classified as bloody when blood was visible upon examination of the samples macroscopically or reported as present by the submitting physician. Other clinical symptoms, including fever, vomiting, abdominal pain, or dehydration (reduced consciousness, sunken eyes, dryness of mucous membranes, thirst, skin turgor), were reported by the physicians. A control group of apparently healthy persons (children, n = 54 and adults, n = 29) without a history of diarrhea and who did not take any antibiotics for at least one month was selected at the same clinical centers in the same period. This group had similar ages and were from the same area of residence as the patients. These persons came to the health centers for non-diarrheal illnesses. All persons were from urban, middle-income families. All stool samples were sent to our laboratory (Laboratoire de Contrôle des Eaux et Denrées Alimentaires-Institut Pasteur de Tunis). Specimens that were not refrigerated and did not contain transport medium for more than four hours were rejected. A total of 271 persons who met the criteria described above were included in the study. All the subjects (patients and healthy persons) were informed by the doctors about the study and they agreed to participate by giving stool samples and informed the doctors about any needed information. This study did not require approval or review by our institutional review board.

Culture and identification of bacterial isolates. All specimens were processed by routine microbiologic and biochemical tests to identify different enteropathogenic bacteria. For *E. coli*, all stool samples were inoculated onto MacConkey agar (Oxoid, Basingstoke, United Kingdom) and sorbitol MacConkey agar (Oxoid) containing cefixime (0.05 mg/L) and tellurite (2.5 mg/L) and 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 biochemical tests specific for *E. coli* strains. Identification was confirmed with the Api 20E system (bioMérieux, Marcy-l'Etoile, France). Stools were subcultured onto Hecktoen agar (Oxoid) for identification of *Salmonella* spp. For selective enrichment of *Salmonella* spp., stools were inoculated into selenite broth, incubated for 24 hours at 37°C, inoculated onto Hecktoen agar, and incubated for 24 hours at 37°C. *Shigella* spp. were isolated by subculturing onto Hecktoen agar and incubating for 24 hours at 37°C. For *Campylobacter* spp., stools were inoculated onto modified charcoal cefoperazone deoxycholate agar (Oxoid) and incubated for 48 hours at 42°C under microaerophilic conditions. Identification was confirmed with the ApI Camp system (bioMérieux). *Yersinia enterocoliticus* was isolated by inoculation of samples onto cefsuldine-irgasan-novobiocin (CIN) agar (Oxoid) and incubation for 24 hours at 32°C. Identification was confirmed with the ApI 20E system (bioMérieux). In addition, glucose broth was inoculated with samples, incubated for one week at 4°C, and subcultured onto CIN agar. For detection of *Aeromonas* spp., blood agar plates containing 10 μg of ampicillin/mL were inoculated and incubated for 24–48 hours at 37°C. Identification was confirmed with the ApI 20NE system (bioMérieux). For selective enrichment of *Vibrio* spp., stools were inoculated onto peptone water containing 3% NaCl, pH 8, incubated for 24 hours at 37°C, inoculated onto thiosulfate-citrate-bile-saccharose agar (Oxoid), and incubated for 24 hours at 37°C. Identification was confirmed with the ApI 20NE system (bioMérieux). All bacterial strains were stored at −80°C in brain heart infusion broth containing 20% glycerol and subcultured before testing

Investigation of stool samples for parasites was performed by direct examination of stools in cosin solution for *Entamoeba histolytica*, *Giardia lamblia*, and other parasites. Stool samples also examined for rotavirus and adenovirus using an enzyme-linked immunosorbent assay kit.

DNA extraction. All isolated *E. coli* strains (353 strains) were grown on Luria-Bertani broth (Sigma, St. Louis, MO) overnight at 37°C. Genomic DNA was extracted by a boiling method as described previously[8] and used as the target for polymerase chain reaction (PCR) assays.

Detection of diarrheagenic *E. coli*. The 353 *E. coli* strains isolated from 132 diarrheal cases (96 children and 36 adults) and 60 healthy persons (48 children 12 adults) were evaluated by PCR for identification of nine virulence genes of distinct DEC groups. Attaching and effacing gene (*eae* 790 basepairs [bp]) and bundle-forming pilus gene (*bfpA* 324 bp) were identified for EPEC.[9,10] Attaching and effacing gene (*eae* 790 bp), shiga toxin gene (*stx* 900 bp) and enterohemolysin gene (*ehxA* 321 bp) were identified for EHEC.[9,11,12] To detect and identify the *stx* gene, we used the Lin-all system PCR, followed by digestion with *Hinc* II to further identify *stx1* and *stx2*.[11] Invasion plasmid antigen (*ipaH* 424 bp) was identified for EIEC.[10] The heat-stable enterotoxin I gene (*astA* 111 bp) and aggregative adherence fimbria (*aaf/I* 432 bp) were identified in EAEC.[13] The heat-labile enterotoxin gene (*elt* 708 bp) was identified in ETEC.[10] Details of each primer sequence, final amplification reaction, and the conditions for PCR were obtained from the relevant references. The heat-stable enterotoxin (*sta*) gene was amplified using primers with the following sequences: forward (5′-TTAATAGCACGCGGTAAAGCAGG-3′) and reverse (5′-CACCCGGTACAAGGCAGGATT-3′). These amplified a 180-bp fragment and were designed on the basis of GenBank data (accession no. M29255). Amplification of the *sta* gene was conducted in a final amplification reaction of 50 μL containing 10 mM Tris-HCl, pH 8, 50 mM KCl, 2 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate (Amersham, Piscataway, NJ), 2.5 units of *Taq* polymerase (Amersham), and 25 pmol of each primer. The reaction mixture was subjected to 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 90 seconds, and extension at 72°C for 90 seconds. The amplified DNA products were resolved by electrophoresis on agarose gels and visualized by ultraviolet transillumination after staining with ethidium bromide. The PCRs were performed in an automated thermocycler (Perkin-Elmer, Waltham, MA)

Reference strains. Reference strains used in PCR in this study included EHEC EDL933(O157:H7) for the *stx* and *ehxA* genes (shiga toxin gene and enterohemolysin gene, respectively), EPEC 2348/69(O127:H6) for the *eae* and *bfpA* genes (attaching and effacing gene and bundle-forming pilus gene, respectively), ETEC H10407 for the *elt* gene (heat-labile enterotoxin), ETEC Jep5683 for the *sta* gene (heat-stable enterotoxin), EIEC 11741 for the *ipaH* gene (invasion plasmid antigen), and EAEC 17-2 for the *astA and aaf-I* genes (heat-stable enterotoxin I and aggregative adherence fimbria, respectively). The non-pathogenic *E. coli* strain HB 101 was used as a negative control.

Vero-cell and Hep-2 cell adherence assay. The DEC strains were subjected to a Vero cell and HEP-2 cell adherence test

by a method described previously,[8] except that neutralization with an anti-Stx antibody was included in the Vero cell adherence assay.

***Escherchia coli* antimicrobial drug susceptibility.** Antimicrobial drug susceptibility testing was done for bacterial strains using standard methods (disc diffusion method) using Mueller-Hinton agar and interpreted according to the guidelines of the Antibiogram Committee of the Société Française de Microbiologie.[14] *Escherichia coli* strains were examined for susceptibility to cephalothin (30 μg), ticarcillin (75 μg), cefotaxime (30 μg), chloramphenicol (30 μg), amoxicillin (25 μg), cefoxitin (30 μg), amoxicillin plus clavulanic acid (20 and 10 μg), trimethoprim-sulfamethoxazole (1.25 and 23.7 μg), gentamicin (10 μg), nalidixic acid (30 μg), sulfonamide (200 μg), tetracycline (30 μg), kanamycin (30 IU), ciprofloxacin (5 μg), streptomycin (10 IU), and ofloxacin (5 μg). *Salmonella cholerea suis* strain ATCC14028 was used as a control for each test. Characterization of strains as susceptible, intermediately resistant, or resistant was determined with OSIRIS software version 3× (Bio-Rad Laboratories. Hercules, CA).

Serotyping. Serogrouping and serotyping were performed by the slide agglutination method to identify somatic O antigen and flagellar H antigens. The O:H serotypes of *E. coli* strains were determined by agglutination tests with 12 different sera contained in a commercially available O:H serotype kit (Bio-Rad Laboratories) at the National Center for Enteropathogenic Bacteria (*Salmonella*, *Shigella*, and *Vibrio* spp.) at Pasteur Institute in Tunis, Tunisia. *Salmonella* strains were serotyped according to the Kauffmann-White scheme with antiserum (Bio-Rad Laboratories). *Shigella* strains and *Vibrio* strains were serotyped with antiserum (Bio-Rad Laboratories).

Pulsed-field gel electrophoresis. Molecular relationships between *E. coli* strains were assessed by studying PFGE patterns of genomic DNA. Three hundred fifty-three *E. coli* strains (170 DEC strains and 183 non-pathogenic *E. coli* strains) and one reference strain (EDL933 O157:H7) were grown overnight at 37°C in 5 mL of brain heart infusion broth. Cells were harvested and washed with 0.85% NaCl by centrifugation at 12,000 × *g* for 10 minutes at 4°C. The optical density of the culture was subsequently adjusted to 0.8 at 620 nm with 100 mM Tris-HCl, pH 7.5, 100 mM EDTA, 150 mM NaCl. This suspension was mixed with an equal volume of 2% low-melting-point agarose (Amersham Bioscience, Little Chalfont, United Kingdom) at 50°C. The bacterial agarose suspension was introduced into a plug mold (Bio-Rad Laboratories) and allowed to solidify at 4°C for 20 minutes. The plugs were incubated overnight at 37°C in lysis buffer (6 mM Tris-HCl, pH 7.5, 100 mM EDTA, 1 M NaCl, 0.5% Brij 58, 0.2% sodium deoxycholate, and 0.5% sodium-N-lauryl sarcosine) and washed in buffer (50 mM EDTA, pH 8, 50 mM Tris-HCl, 1% sodium-N-lauryl sarcosine, and 200 μg/mL of proteinase K) for 24 hours at 55°C. The plugs were then washed three times (one hour per wash) in Tris-EDTA buffer at room temperature and sliced into 2-mm pieces. The DNA in each plug was digested with 30 units of *Xba* I (Amersham Biosciences) for 18 hours at 37°C. The PFGE was performed on a 1% agarose gel (Bio-Rad Laboratories) by using a CHEF DR III apparatus (Bio-Rad Laboratories) in 0.5× Tris-borate-EDTA buffer at 14°C under the following conditions: 6 V/cm at a field angle of 120°; block 1, 12 hours, with an initial switching time of 5 seconds to a final switching time of 15 seconds; block 2, 12 hours, with an initial switching time of 30 seconds to a final switching time of 50 seconds. The gel was stained with ethidium bromide and photographed. A lambda DNA ladder with a size range of 48.5 kb to 1 mb (Amersham, Biosciences) was used as a size marker. Strain EHEC EDL933 (O157:H7) was used as a control in each test.

Numerical analysis of PFGE profiles for isolated *E. coli* strains. The PFGE patterns of *E. coli* strains digested with *Xba* I were analyzed visually. In addition, a numerical analysis after conversion, normalization, and analysis of similarity in band patterns was performed using MVPS3.31 software (Gel-Pro Analyzer version 3.1, Media Cybernetics, Atlanta, GA). Similarities between profiles were calculating using the Dice coefficient, with a maximum position tolerance of 1%. The PFGE patterns obtained were clustered by unweighted pair group method with arithmetic mean.

Statistical analysis. Statistical analysis was done with SPSS version 11.5 software (SPSS Inc., Chicago, IL) using Fisher's exact test. P values < 0.05 were considered significant.

RESULTS

Enteropathogens isolated from patients and healthy subjects. From November 2001 through November 2004, a total of 271 persons were enrolled in this study. They were divided into two groups: 169 children (115 patients and 54 controls) and 102 adults (73 patients and 29 controls). Most patients had watery diarrhea; only 9% (17 of 188) had bloody diarrhea. Watery diarrhea was observed in 13 (11.3%) of 115 diarrheagenic children and in 4 (5.5%) of 73 diarrheagenic adults. Bloody diarrhea was associated with DEC in children (EIEC n = 10, ETEC n = 3) and adults (ETEC n = 4). Dehydration was present in 46 (24.5%) of 188 patients: 33 (28.7%) of 115 diarrheagenic children and 13 (18%) of 73 diarrheagenic adults.

All 271 stool samples were tested for bacteria, parasites, and viruses. Parasitologic studies showed that only 7 (2.6%) of 271 samples were positive for parasites. *Giardia lamblia* was the only pathogenic parasite identified and was isolated from diarrheagenic children (Table 1). A virus was found in 33 (12.2%) of 271 samples. Adenovirus was the most common virus in the diarrheagenic group (Table 1).

A wide range of bacterial pathogens was detected. Bacterial were isolated from 236 (87%) of 271 stool samples (children: 115 patients and 54 controls and adults: 66 patients and 12 controls). The rates of detection of bacterial pathogens in patients and controls are shown in Table 1. In diarrheagenic adults, *Salmonella* spp. was the most common pathogen (34.2%, 25 of 73 cases), followed by ETEC (12.3%, 9 of 73) and *Shigella* spp. *Salmonella* spp. were significantly associated with diarrhea ($P < 0.005$). Conversely, the difference in the incidence of ETEC and *Shigella* spp. between patients and controls was not statistically significant. In diarrheagenic children, many enteropathogens were frequently isolated, However, typical EPEC was the only one significantly associated with acute diarrhea (Table 1). *Campylobacter* spp. and *Aeromonas* spp. showed an incidence < 2%. *Vibrio* spp. were only found in children in the control group. No *Yersinia* spp. or typhoidal *Salmonella* were isolated.

Age distribution data in diarrheagenic children showed that bacterial infection was most the common etiology in different age groups. In diarrheagenic children less than one year of age (n = 28), ETEC was the main cause of diarrhea (n = 7), followed by rotavirus (n = 2). In children between one and

TABLE 1
Enteropathogens isolated from diarrheagenic and control groups Tunis, November 2001–November 2004)*

Enteropathogens	Children (n = 169)			Adults (n = 102)		
	Diarrheagenic (115 patients) No. of cases (%)	Control (54 persons) No. of cases (%)	*P*	Diarrheagenic (73 patients) No. of cases (%)	Control (29 persons) No. of cases (%)	*P*
Bacterial (236 cases)	115 (100)	54 (100)		66 (90.4)	12 (41.4)	
Escherichia coli	15 (13)	4 (7.4)		24 (33)	2 (7)	
Diarrheagenic	81 (70.4)	44 (81.5)	NS	12 (16.4)	10 (34.5)	NS
E. coli						
ETEC	37 (32.3)	20 (37)	NS	9 (12.3)	9 (31)	NS
EAEC	13 (11.3)	8 (15)	NS	1 (1.4)	1 (1.4)	NS
EIEC	13 (11.3)	2 (3.7)	NS	1 (1.4)	0	
EHEC	12 (10.4)	6 (11.1)	NS	0	0	
Typical EPEC	2 (1.7)	6 (11.1)	< 0.03	0	0	
Atypical EPEC	4 (3.5)	2 (3.7)	NS	1 (1.4)	0	
Salmonella spp.	11 (9.5)	1 (2)	NS	25 (34.2)	0	< 0.005
Enteritidis	3 (2.6)	0		3 (4)	0	
anatum	4 (3.5)	0		7 (9.6)	0	
corvallis	1 (1)	0		1 (1.4)	0	
Infantis	1 (1)	0		1 (1.4)	0	
agona	1 (1)	0		2 (2.7)	0	
altona	1 (1)	0		0	0	
heidelberg	0	0		1 (1.4)	0	
amsterdam	0	0		2 (2.7)	0	
cerro	0	0		2 (2.7)	0	
hadar	0	0		1 (1.4)	0	
kentucky	0	0		3 (4)	0	
zanzibar	0	0		1 (1.4)	0	
montevideo	0	0		1 (1.4)	0	
arizona	0	1 (2)		0	0	
Shigella spp.	4 (3.5)	1 (2)	NS	3 (4)	0	NS
boydii	3 (2.6)	0		1 (1.4)	0	
flexeniri	1 (1)	1 (2)		0	0	
sonnei	0	0		2 (2.7)	0	
Campylobacter spp.	2 (1.7)	1 (2)	NS	1 (1.4)	0	NS
coli	2 (1.7)	1 (2)		1 (1.4)	0	
Aeromonas spp.	2 (1.7)	1 (2)	NS	0	0	
sobria	1 (1)	0		0	0	
hydrophila	1 (1)	1 (2)		0	0	
Vibrio spp.	0	2 (3.7)		0	0	
vulnificus	0	1 (2)		0	0	
cholerae non-01	0	1 (2)		0	0	
Yersinia spp.	0	0		0	0	
Viruses (33 cases)	19 (16.5)	6 (11.1)	NS	6 (8.2)	2 (7)	NS
Adenovirus	12 (10.4)	3 (5.5)		5 (7)	2 (7)	
Rotavirus	7 (6.1)	3 (5.5)		1 (1.4)	0	
Protozoa (7 cases)	2 (1.7)	4 (7.4)	NS	1 (1.4)	0	NS
Giardia lamblia	2 (1.7)	0		0	0	
Entamoeba coli	0	2 (3.7)		1 (1.4)	0	
Endolimax nana	0	2 (3.7)		0	0	

* NS = not significant; ETEC = enterotoxigenic *E. coli*; EAEC = enteroaggregative *E. coli*; EIEC = enteroinvasive *E. coli*; EHEC = enterohemorrhagic *E. coli*; EPEC = enteropathogenic *E. coli.*

five years of age (n = 37), ETEC (n = 17) was the most common cause of diarrhea, followed by EHEC (n = 10), EAEC (n = 9), EIEC (n = 6), adenovirus (n = 4), and rotavirus (n = 3).

Mixed infections with ≤ 4 different organisms isolated from a single specimen were seen in 27 (14.4%) of 188 diarrheagenic patients. Mixed infections were seen in 22 (19%) of 115 diarrheagenic children. A variety of different combinations were observed in this group but the most frequent combination was ETEC and adenovirus (n = 8). Among diarrheagenic adults, mixed infections were found in 5 (7%) of 73 persons: ETEC plus *C. coli* (n = 1), EAEC plus *S. sonnei* (n = 1), *Salmonella* spp. plus adenovirus (n = 1), ETEC plus EPEC plus *S. boydii* (n = 1), and *Salmonella* spp. plus adenovirus plus *Entamoeba coli* (n = 1).

Season and diarrhea. The seasonality of enteropathogen infection was also determined and is shown in Table 2. Large numbers of children with acute diarrhea were observed between May and December with a peak in autumn (September, October, and November). Infection with ETEC and EAEC reflected this distribution with 22% (for each) of the infections with these bacteria. Adult acute diarrhea reached its peak in July and August (summer) (Table 2). The highest incidence of bacterial agents and viruses were observed during the summer (90%, 35 of 39 and 10.2%, 4 of 39; respectively). *Salmonella* spp. had the greatest effect on the epidemiology of acute diarrhea in adult group (34.2% in the summer). No etiologic agent was isolated during the months of January, February, and March (Table 2).

Prevalence of diarrheagenic *E. coli*. A total of 353 *E. coli*

TABLE 2

Seasonal distribution of the most frequent enteropathogenic agents in children and adults groups (diarrheal and control groups) and the diarrheal incidence rate, Tunis, 2001–2004*

	Winter†		Spring				Summer				Autumn			
	Children No. of cases (%)		Children No. of cases (%)		Adults No. of cases (%)		Children No. of cases (%)		Adults No. of cases (%)		Children No. of cases (%)		Adults No. of cases (%)	
Enteropathogens	Patients	Control	Patients	Control	Patients	Control	Patients	Control	Patients	Control	Patients	Control	Patients	Control
Diarrheal incidence rate	23 (20)	24 (44.4)	25 (22)	6 (11.1)	1 (1.4)	–	24 (21)	16 (31)	39 (53.4)	9 (31)	51 (44.3)	14 (26)	8 (11)	3 (10.3)
Bacteria	23 (100)	21 (87.5)	20 (80)	2 (33.3)	–	–	19 (79.2)	16 (100)	35 (90)	9 (100)	40 (78.4)	11 (78.6)	6 (75)	(33.3)
ETEC	11 (48)	4 (17)	12 (48)	–	–	–	3 (12.5)	10 (62.5)	7 (18)	8 (89)	11 (22)	6 (43)	2 (25)	1 (33.3)
EAEC	–	4 (17)	–	2 (33.3)	–	–	2 (8.3)	2 (12.5)	1 (2.6)	1 (11)	11 (22)	–	–	–
EIEC	6 (26)	2 (8.3)	–	–	–	–	1 (4.2)	–	1 (2.6)	–	8 (16)	–	–	–
EHEC	4 (17.4)	6 (25)	8 (32)	–	–	–	–	–	–	–	–	–	–	–
EPEC	–	3 (12.5)	–	–	–	–	–	3 (19)0	1 (2.6)	–	6 (12)	2 (14.3)	–	–
Salmonella	2 (8.7)	–	–	–	–	–	8 (33.3)	–	24 (61.5)	–	1 (2)	1 (7.1)	1 (12.5)	–
Shigella	–	1 (4.2)	–	–	–	–	3 (12.5)	–	1 (2.6)	–	1 (2)	–	2 (25)	–
Campylobacter	–	–	–	–	–	–	–	–	–	–	2 (4)	1 (7.1)	1 (12.5)	–
Aeromonas	–	1 (4.2)	–	–	–	–	2 (8.3)	1 (6.2)	–	–	–	–	–	–
Vibrio	–	–	–	–	–	–	–	–	–	–	–	1 (7.1)	–	–
Viruses	–	3 (12.5)	4 (16)	2 (33.3)	–	–	4 (16.6)	–	4 (10.2)	–	11 (21.6)	1 (7.1)	2 (25)	2 (66.6)
Adenovirus	–	–	3 (12)	2 (33.3)	–	–	3 (12.5)	–	3 (7.7)	–	6 (12)	1 (7.1)	0	2 (66.6)
Rotavirus	–	3 (12.5)	1 (4)	–	–	–	1 (4.2)	–	1 (2.6)	–	5 (10)	–	2 (25)	0
Protozoa	–	–	1 (4)	2 (33.3)	1	–	1 (4.2)		–	–	–	2 (14.3)	–	–
Giardia lamblia	–	–	1 (4)	–	–	–	1 (4.2)	–	–	–	–	0	–	–
Entamoeba coli	–	–	–	1 (17)	1	–	–	–	–	–	–	1 (7.1)	–	–
Endolimaw nana	–	–	–	1 (17)	–	–	–	–	–	–	–	1 (7.1)	–	–

* For definitions of abbreviations, see Table 1.

† There were no specific enteropathogens in patients and controls during the winter.

strains (isolated from 192 of 271 persons) were evaluated by PCR to detect nine virulence genes and classify the five DEC categories (ETEC, EPEC, EHEC, EIEC, and EAEC). The incidence of each virulence gene among *E. coli* strains in the diarrheal and control groups is shown in Table 3. One hundred seventy *E. coli* strains were classified as DEC, 93 from cases (81 children and 12 adults) 54 from controls (44 children and 10 adults). The most prevalent category in the diarrheagenic persons was ETEC, followed by EAEC, EIEC, EHEC, and EPEC. The most prevalent category in the controls was ETEC, followed by EPEC, EAEC, EHEC, and EIEC.

ETEC with the *sta* gene was significantly associated with diarrheal disease ($P < 0.0182$), especially in diarrheagenic children ($P = 0.004$) (Table 3). Strains with the *elt*-gene and strains with the *sta* and *elt* genes were weakly associated with diarrhea in adults and children.

EAEC are defined as strains that do not secrete *elt* or *sta* gene products and that adhere to Hep-2 cell in an aggregative adherence (AA) pattern.[5] The PCR showed that 39 (15.3%) of 254 strains in the diarrheal groups had the *staA* gene, of which 19 strains were EAEC, 11 strains were ETEC, 6 strains were EIEC, 2 strains were ETEC/EIEC, and 1 strain was ETEC/EPEC; 15% of 99 strains in the control groups had the *sta*A gene, of which 5 strains were ETEC and 1 strain was ETEC/EPEC. The *aaf/I* gene was not detected (Table 3). The incidence of strains carrying both *sta* and *astA* genes (or in association with other genes) were higher in the diarrheal group (2.4%, 10 of 254) than in the control group (5%, 5 of 99). However, this difference was not statistically significant ($P < 0.887$) (Table 4).

TABLE 4
Combination of virulence genes identified in diarrheagenic *Escherichia coli* (DEC) categories*

DEC category (No. of strains)	Gene combination	No. of strains: Diarrheal (n = 254)	No. of strains: Control (n = 99)
ETEC/EPEC (10)	*sta eae*	1	0
	sta bfpA	1	6
	sta bfpA astA	1	1
ETEC/EIEC (5)	*sta ipaH*	2	0
	elt ipaH	1	0
	sta ipaH astA	1	0
	elt st ipaH astA	1	0
ETEC/EHEC (1)	*sta stx*	1	0
ETEC (18)	*sta astA*	4	4
	elt astA	4	1
	sta elt	0	2
	elt sta astA	3	0
EHEC (2)	*stx eae ehxA*	1	1
EIEC (6)	*ipaH astA*	6	0

* For definitions of abbreviations, see Table 1.

We examined EAEC strains for the AA pattern in 19 strains from diarrheal groups (18 from children and 1 from an adult) and 9 strains from control groups (8 from children and 1 from an adult). Almost all strains showed the typical AA pattern with honeycomb formation. Thus, the *astA* gene was had a role in the production of diarrhea only in EAEC strains, and not in strains with other virulence genes.

EIEC (*ipaH* gene) was detected in only 2 (2.6%) of 77 strains from children in the control group. This gene showed an association with acute diarrhea in 13 (7%) of 188 children

TABLE 3
Distribution of diarrheagenic *Escherichia coli* categories among strains isolated*

DEC	No. of strains (%): Diarrheal (n = 254)	No. of strains (%): Control (n = 99)	*P*	No. of strains (%) in each group: Children: Diarrheal (n = 188)	Children: Control (n = 77)	Children: *P*	Adults: Diarrheal (n = 66)	Adults: Control (n = 22)	Adults: *P*	Serotypes (No. of strains)
ETEC (total)	50 (19.7)	36 (36.4)	< 0.0182	41 (22)	27 (35)	0.036	9 (13.6)	9 (41)	0.012	
st	35 (14)	29 (29.3)	0.001	30 (16)	25 (32.5)	0.004	5 (7.6)	4 (18)	NS	NT (8), O111:B4 (6), O86:B7 (18), O128:B12 (11), O126:B16 (5), O127:B8 (16)
elt	11 (4.3)	5 (5)	NS	7 (3.7)	2 (2.6)	NS	4 (6)	3 (13.6)	NS	NT (5), O111:B4 (2), O86:B8 (9).
sta/elt	4 (1.6)	2 (2)	NS	4 (2)	0	NS	0	2 (9)	NS	NT (5), O111:B4 (1).
EAEC (total)	19 (7.5)	9 (9)	NS	18 (9.6)	8 (10.4)	NS	1 (1.5)	1 (4.5)	NS	
astA	19 (7.5)	9 (9)	NS	18 (9.6)	8 (10.4)	NS	1 (1.5)	1 (4.5)	NS	NT (8), O125:B15 (8), O55:B5 (12).
aaf/I	0	0	0	0	0	0	0	0	0	
EHEC (total)	12 (5)	9 (9)	NS	12 (6.3)	9 (11.7)	NS	0	0	0	
stx	11 (4.3)	8 (8)	NS	11 (6)	8 (10.4)	NS	0	0	0	NT (19).
stx/eae/ehxA	1 (0.4)	1 (1)	NS	1 (0.5)	1 (1.3)	NS	0	0	0	NT (1), O157:H7 (1).
EPEC (total)	7 (3)	12 (12.1)	0.001	6 (3.2)	12 (15.6)	< 0.001	1 (1.5)	0	NS	
eae/bfpA	2 (0.8)	9 (9)	< 0.001	2 (1)	9 (11.7)	< 0.001	0	0	0	NT (4), O128:B12 (6), O126:B16 (1).
typical EPEC										
eae	5 (2)	3 (3)	NS	4 (2)	3 (4)	NS	1 (1.5)	0	N	NT (1), O55:B5 (7).
atypical EPEC										
EIEC (total)	14 (5.5)	2 (2)	NS	13 (7)	2 (2.6)	NS	1 (1.5)	0	NS	
ipaH	14 (5.5)	2 (2)	NS	13 (7)	2 (2.6)	NS	1 (1.5)	0	NS	NT (3), O128:B12 (6), O126:B16 (7)
Total	102 (40)	68 (68.7)	< 0.008	90	58	< 0.04	12	10	NS	

*DEC = diarrheagenic *E. coli*; NT = non-typeable. For definitions of other abbreviations, see Table 1.

in this group. However, this association was not statistically significant ($P = 0.253$). Only one strain of this type was isolated from an adult patient (Table 2).

All 21 EHEC strains were detected in children (12 strains from diarrheagenic cases and 9 strains from control cases). The 21 EHEC strains were isolated from 18 stool specimens (6 from controls and 12 from diarrheagenic children). EHEC strains were also recovered from non-bloody stools. All EHEC strains were positive for the *stx* gene (*stx1* and *stx2*). Digestion with *Hinc* II confirmed the presence of the PCR fragments (705 bp and 158 bp) for s*tx1* and the fragments (555 bp and 262 bp) for s*tx2* in all EHEC strains. Two of these strains were also positive for the *eae* and *ehxA* genes.

EHEC strains showed a degree of cytotoxicity. The verotoxin titer was expressed as the reciprocal of the highest filtrate dilution that caused 50% cell detachment after incubation for 48 hours. The breakpoint for a positive result was a titer of 4. The EHEC EDL933 strain, which was used as a control, had a titer > 64. Strains were classified into two categories: moderately cytotoxic (titer = 4–32 in 3 strains from diarrheagenic children) and highly cytotoxic (titer ≥ 64 in 9 strains from diarrheagenic children).

EPEC strains showed a higher prevalence in diarrheagenic children (3.2%, 6 of 188) than in the control group (15.6%, 12 of 77) ($P < 0.001$). Among the 19 EPEC strains isolated, only 1 was found in the diarrheagenic adult group. Eleven strains were typical EPEC (*eae*+, *bfpA*+), among which six strains (4 from diarrheagenic children and 2 from children in the control group) showed the classic localized adherence pattern in Hep-2 cells. Eight strains were atypical EPEC (*eae*+) (Table 3).

As shown in Table 4, three different combinations of DEC strains were identified. The most prevalent combination was ETEC and EPEC strains, especially in the control groups.

Age distribution of diarrheagenic *E. coli*. The age distribution of patients with DEC is shown in Figure 1. Diarrheagenic *E. coli* strains were observed in children of all ages in the diarrheagenic group, with an increasing trend in children 6–12 years of age and those 1–2 years of age. The prevalence of ETEC was highest in diarrheagenic adults.

Antimicrobial drug susceptibility testing of *E. coli* strains. Of 353 *E. coli* strains, 223 (63.2%) of 353 were resistant to one or more antibiotics. High levels of resistance were observed for tetracycline (28.6%, n = 101), streptomycin (24%, n = 85), ticarcillin (20.4%, n = 72) amoxicillin (20.4%, n = 72), and sulfonamide (20%, n = 70). The incidence of antimicrobial drug resistance to these five antibiotics varied during the study period. There was a significant increase in the level of resistance to all five antibiotics from November 2001 to November 2002, followed by a decrease in incidence from November 2002 to November 2003. However, in the last year of study period (November 2003 to November 2004), resistance to antibiotics was observed only for tetracycline (2.5%, n = 8). Other antibiotics to which some *E. coli* strains were resistant were nalidixic acid (n = 48 strains), trimethoprim-sulfamethoxazole (n = 48), chloramphenicol (n = 38), amoxicillin plus clavulanic acid (n = 32), cephalotin (n = 30), kanamycin (n = 22), ofloxacin (n = 18), ciprofloxacin (n = 18), cefoxitin (n = 13), gentamicin (n = 13), and cefotaxime (n = 7).

Serotypes of *E. coli* strains. Wide arrays of O-typeable strains were observed among 201 (57%) the 353 *E. coli* strains: 116 (68.2%) of 170 diarrheagenic *E. coli* and 73 (40%) of 183 of non-diarrheagenic *E. coli* were typeable for O antigens. In addition, 40% (68 of 170), 12% (12 of 170), 0.6% (1 of 170), 8.2% (14 of 170), and 7.6% (13 of 170) of ETEC, EAEC, EHEC, EPEC, and EIEC, respectively, were O-antigen typeable. The *E. coli* strains were serotyped as follows: O86:B7 (n = 53), O128:B12 (n = 41), O127:B8 (n = 31), and O55:B5 (n = 31), O111:B4 (n = 16), O126:B16 (n = 16), O125:B15 (n = 12) and O157:H7 (n = 1). There were four serotypes that were exclusively associated with a single pathotype: O111:B4 (ETEC), O86:B7 (ETEC), O125:B15 (EAEC), and O157:H7 (EHEC)

Chromosomal DNA analysis by PFGE for *E. coli* strains. To determine clonal relatedness among *E. coli* strains, we analyzed their *Xba* I patterns by PFGE. We analyzed 353 *E. coli* strains and one reference strain (EHEC EDL 933 O157:H7), which was used as a control. Pulsed-field gel electrophoresis produced 11–22 fragments ranging in size from approximately 50 kb to 600 kb. DNA degradation was observed for 6 *E. coli* strains because of nuclease activity. Dendogram analysis of the PFGE patterns (Figure 2) showed that the strains were distributed among 20 main clusters. The four largest clusters were *X01*, *X02*, *X03*, and *X05*. Thirty-nine unique profiles were observed among 39 *E. coli* strains.

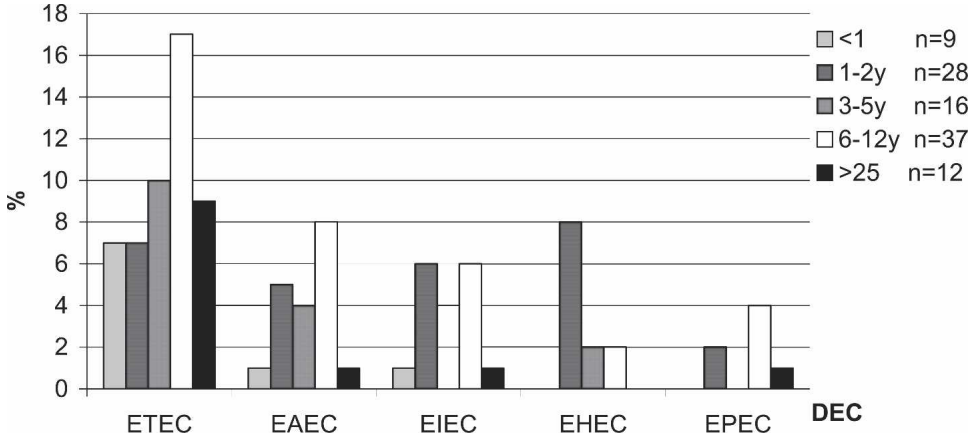

FIGURE 1. Distribution of diarrheagenic *Escherichia coli* (DEC) in the study groups, Tunis, Tunisia. y = years; ETEC = enterotoxigenic *E. coli*; EAEC = enteroaggregative *E. coli*; EIEC = enteroinvasive *E. coli*; EHEC = enterohemorrhagic *E. coli*; EPEC = enteropathogenic *E. coli*.

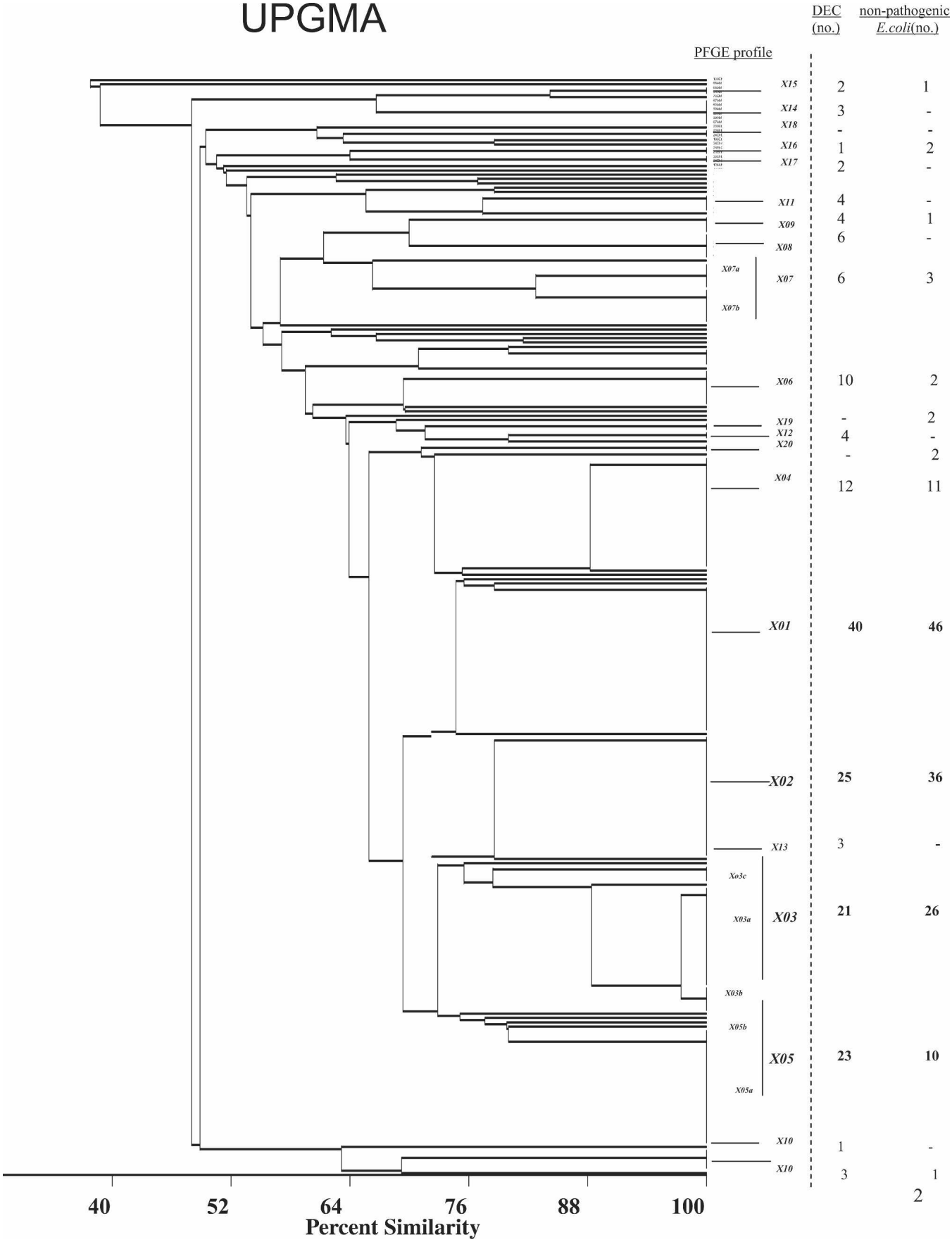

FIGURE 2. Dendrogram showing percentage similarity calculated by the Dice similarity index of pulsed-field gel electrophoresis (PFGE) restriction endonuclease digestion profiles for 353 *Escherichia coli* strains (170 diarrheagenic *E. coli* [DEC] and 183 non-pathogenic *E. coli*). UPGMA = unweighted pair group method with arithmetic mean.

A total of 86 (24.4%) of 353 *E. coli* strains belonged to *X01*, the largest major cluster. The PFGE patterns of *E. coli* strains in this cluster were 100% genetically similar. Most *E. coli* strains in this cluster were obtained from children (33 with diarrhea and 31 from controls), and 22 *E. coli* strains were obtained from adults (11 with diarrhea and 11 from controls). Three different DEC categories were included in this cluster: ETEC, EPEC, and EAEC. The most prevalence DEC category was ETEC with the *sta* genetic marker.

The second major cluster, *X02*, contained 61 *E. coli* strains with identical profiles (100%). In this cluster, four different DEC categories were observed (ETEC, EPEC, EIEC, and EAEC) in association with non-pathogenic *E. coli*. Fourty-seven *E. coli* strains in this cluster were obtained from children (24 with diarrhea and 23 from controls) and 14 *E. coli* strains were obtained from diarrheagenic adults.

In the third major cluster, cluster *X03* (47 *E. coli* strains obtained from children, 19 with diarrhea and 17 controls, and from adults, 9 with diarrhea and 2 from controls), three closely related sub-clusters were identified with a genetic similarity of approximately 88%. The first sub-cluster (*X03a*) contained 28 *E. coli* strains isolated from children and adult groups with identical profiles (100%). Two DEC categories were included in this sub-cluster: ETEC and EHEC. The second sub-cluster (*X03b*) contained 18 *E. coli* strains (100% identical profiles). ETEC was the only DEC category includes in this sub-cluster. The third sub-cluster (*X03c*) included only one strain (ETEC).

Two main sub-clusters were identified in the fourth major cluster (*X05*) for 33 *E. coli* strains, 22 from children (16 with diarrhea and 6 from controls) and 11 from adults (10 with diarrhea and 1 from a control). The first sub-cluster (*X05a*) included *E. coli* strains representing 2 DEC categories (EAEC and EHEC) and 10 strains with no genetic markers isolated from diarrheagenic children. The genetic similarity in this sub-cluster was 100%. The second sub-cluster contained the only O157:H7 isolate in this study with 80% similarity. The predominant genotype in cluster *X05* was *astA*. The percentage of genetic similarity between the 20 main clusters was 64%. No association was observed between clones and genetic markers or serotypes.

DISCUSSION

The most important outcome of the present study is the description of etiologic agents of diarrhea in children and adults in Tunis, Tunisia, with special emphasis on diarrheagenic *E. coli*. This study of 169 Tunisian children < 1–15 years of age and 102 Tunisian adults used modern diagnostic techniques and provided an accurate description of the contribution of different pathogens to the overall burden of acute diarrheal disease. In addition, it evaluated the potential associations between diarrheagenic *E. coli* and acute diarrhea.

The association of diarrheal illness with seasons is well-established.[15] The largest number of acute diarrheal cases in children was found from May to December. The largest number of acute diarrheal cases in adults were found during the summer. The high incidence of acute diarrhea in children during the summer (June, July, and August) and in November was due to vacations from school, which resulted in increased exposure to environmental pathogens and contaminated food.

Microbiologic investigations showed that ETEC (32.2%) was the most commonly found pathogen in Tunisian diarrheagenic children, followed by EAEC (11.3%), EIEC (11.3%), adenovirus (10.4%), EHEC (10.4%), and *Salmonella* spp. (9.6%). However, the difference in the isolation rates of these enteric pathogens between diarrheagenic children and healthy children was not statistically significant. Typical EPEC was not frequently isolated from the diarrheagenic group; however, this finding was statistically significant. This finding is similar to reports from Iran, Poland, South Africa, the United Kingdom, and Australia, in which atypical EPEC outnumber typical EPEC strains as a cause of acute diarrhea, although EPEC was not frequently isolated.[16] Our findings do not minimize the importance of DEC in Tunis as an etiology of acute diarrhea because DEC was frequently found to be the only enteropathogen isolated from diarrheagenic children without mixed infections with other enteric pathogens. In those cases, there was an activation of virulence genes of DEC and expression of toxins leading to diarrhea.[5] However, in controls these virulence genes were not activated and toxins were not expressed.[5] This was confirmed by the results of tissue culture assays because toxins were not detected at titers high enough in strains isolated from healthy children to cause diarrhea (compared with reference strains used).

Our data indicate that ETEC, followed by rotavirus, EAEC, and EIEC, are the most frequently isolated enteropathogens from diarrheagenic children less than one year of age. In children one to five years of age, ETEC was the most common pathogen, followed by EHEC, EAEC, EIEC, adenovirus, and rotavirus. These results differ from those obtained 20 years ago in Tunisia, in which *Campylobacter* spp. and adenovirus were significantly associated with diarrheagenic children.[7]

Adults rarely visit a health center when they have diarrhea, unless they perceive the diarrhea as being serious. This study suggests that although many enteric pathogens were isolated from diarrheagenic adults, only *Salmonella* spp. were significantly associated with diarrheagenic adults in Tunisia. In our study, mixed infections were detected in 22% of diarrheagenic children and 7% of diarrheagenic adults. However, mixed infection were less frequent than monoinfections, a finding that has been previously confirmed.[17]

No information is available about the current status of DEC as a diarrheagenic agent in Tunisia because it has not been routinely assayed in stool samples. In this study, special attention was paid to investigating the presence of selected virulence factors of ETEC, EPEC, EIEC, EHEC, and EAEC in stool samples of patients with acute diarrhea and stool samples of those without diarrhea. All classes of DEC were found in children with diarrhea. The difference in the number of DEC strains or non-pathogenic *E. coli* strains between patients and controls was statistically significant ($P < 0.008$). However, this finding was not observed in adults. The results observed with ETEC, in which shiga toxin–producing strains showed a stronger association with acute diarrhea than strains producing labile toxin and strains producing shiga toxin and labile toxin, are consistent with those of other studies.[18,19]

We detected infections with more than one pathogen, particularly coinfection with adenovirus, in children with diarrhea (Table 4). Unlike previous results,[20] the prevalence of ETEC in the persons with diarrhea in our study was higher in

children more than two years of age than in those less than two years of age (Figure 1).

According to the consensus definition of the Second International Symposium on EPEC,[21] typical EPEC strains produce the A/E lesion without *stx* genes and have the EAF plasmid. Atypical EPEC strains lacks the EAF plasmid, which been shown to contain a 14-gene bundle-forming pilus operon that encodes a type IV pilus (bundle-forming pilus).[22] Some atypical EPEC strains express the intimin-mediated localized adherence–like pattern,[23] but all of our atypical EPEC strains were non-adherent. We found that typical EPEC strains were more prevalent in the diarrheagenic group, but this result was not statistically significant. However, typical EPEC strains were significantly associated with diarrhea in children. Our result are in contrast to those of previous reports from developing countries and other industrialized countries,[5,24] and underscore the emergence of atypical EPEC strains worldwide.

The most notable feature of the epidemiology of disease caused by EPEC is the striking age distribution of the patients. Infection with EPEC is primarily a disease of infants less than two years of age.[25] Within the diarrheal group, the highest prevalence (20%) was in children 6–12 years of age, followed by children less than two years of age (15%). Thus, EPEC is not a common cause of childhood acute diarrhea. A similar observation was previous reported.[26]

EAEC was found in 13 (11.3%) of 155 children with diarrhea and was the second most common pathogen among DEC categories. However, this finding was not statistically significant (Table 3). The highest prevalence of EAEC was in children 6–12 years of age, followed by children 1–2 years of age. The *aaf/I* gene was not found in any EAEC strains. However, the EAST1 toxin was found in EAEC, ETEC, EPEC, and EIEC strains. It has been shown that EAST1 is not restricted to EAEC isolates and a study showed an association of this gene with diarrhea.[27] In our study, the EAEC strains producing EAST1 were associated with children acute diarrhea, which suggests the pathogenic role of this toxin.

Our study increased understanding the role of EIEC in acute diarrhea in children in Tunis. We isolated 15 EIEC strains, 2 from controls and 13 from patients. The higher prevalence of EIEC in children with diarrhea was found in persons 1–2 and 6–12 years of age. Notably, EIEC was not found in children 3–5 years of age. Coinfection with other pathogen in children with diarrhea was observed only in one case (Table 4). This reflects the role of EIEC as a pathogen responsible for acute diarrhea in children in our study. However, this finding is in contrast to the results of other investigators, who reported that EIEC was not associated with acute diarrhea.[22]

We isolated 12 and 9 EHEC strains from patients and controls, respectively. Most strains in this study were *stx* positive. The only EHEC O157:H7 strain isolated was from the control group.[28]Although most patients infected with EHEC stop excreting the organism shortly after acute illness has passed, a substantial proportion may continue to carry the organism for several months. This may partially explain the presence of these pathogens in control children. Coinfection was not observed with EHEC. The highest prevalence of EHEC was in children 1–2 years of age.

In our study, EHEC strains were recovered from non-bloody stools. This emphasizes that non-bloody diarrhea does not rule out EHEC infection. According to another study,[29] screening visibly bloody stools would have identified all patients with severe EHEC-associated disease, but would have missed most EHEC-positive patients.

All classes of DEC except EHEC were found in adults with diarrhea. The observed differences between recovery rates in the diarrheal and the control groups for ETEC showed a significant association with diarrhea ($P = 0.012$). Strains producing heat-stable toxin or heat-labile toxin showed a strong association with acute diarrhea. Strains producing heat-stable and heat-labile toxins were not found. Low prevalence of EIEC, typical EPEC, atypical EPEC, and EAEC was detected. EHEC strains were not found in the diarrheal or control groups. Coinfections in diarrheagenic adults were rare (Table 4).

Bloody diarrhea was not common and was associated with EIEC and ETEC strains isolated from diarrheagenic children and adults. Patients had bloody diarrhea with ETEC, a finding is similar to that in a study in Gabon in which diarrhea caused by ETEC was accompanied by bloody diarrhea.[30] Although we were meticulous in looking for all possible pathogens, another pathogen may not have been detected because ETEC do not cause damage to the mucosa.

Our data show that the prevalence of resistance to most drugs tested in *E. coli* strains is high in Tunis. However, the trend in *E. coli* strains is toward decreasing antibiotic resistance, in contrast to the results of previous studies in developing countries.[31,32] There was no significant difference in antibiotic resistance in *E. coli* strains isolated from diarrheagenic groups compared with strains from control groups. Resistance to more than one antibiotic was detected in 65.2% of all *E. coli* strains. The most commonly observed resistance was to tetracycline, streptomycin, amoxicillin, ticarcillin, and sulfonamide. The decrease in the prevalence of antibiotic resistance during the last year of the study may indicate the predominance of sensitive bacterial populations.

There was a wide diversity of serotypes among the 353 *E. coli* strains. Approximately 10.6% of ETEC, 4.7% of EAEC, 11.7% of EHEC, 3% of EPEC, and 2% of EIEC were not typeable, and 40% of non-diarrheagenic *E. coli* strains were typeable. These findings indicate that a significant number of EHEC strains and non-diarrheagenic *E. coli* strains would have been misidentified by serotype-based diagnosis. The unrestricted nature of serotypes among different pathotypes was also observed.

Typing by PFGE has been shown to be superior to multilocus enzyme electrophoresis and ribotyping in discriminating isolates of *E. coli* and has been used in epidemiologic investigations.[33,34] Because there was no information concerning the distribution of fingerprints among *E. coli* strains in Tunisia, we compared DEC strains and non-pathogenic *E. coli* strains by PFGE to determine if clones were frequently found in the general population in Tunisia. *Xba* I was the only restriction endonuclease used because it has been shown to be more discriminative for studying *E. coli*.[35] The PFGE results for 353 *E. coli* strains indicated a clonal relationship among the isolates and suggested that four major distinct patterns (*X01*, *X02*, *X03*, and *X05*) were circulating in the study area from November 2001 to November 2004. These genotypes are stable and persist over a considerable period.

Clonal diversity among *E. coli* is high. Such genetic heterogeneity among *E. coli* strains belonging to the same serotypes has been recorded previously.[36,37] Serotype heterogeneity

within genetically derived clusters should not be a source of great concern in evaluating the validity of genotyping methods. Beltran and others[38] speculated that this pattern might be the result of horizontal transfer of genes for the O and H antigens forming the basis of serotype classification.

Our study showed that typical EPEC were present in Tunisian children. Infections with typical EPEC occurred throughout the year. *Salmonella* spp. were also associated with diarrheagenic adults in Tunisia. However, other pathotypes of DEC should not be neglected because they were frequently isolated in our study from diarrheagenic cases and controls. We used molecular diagnostic methods to re-evaluate the prevalence of different diarrheagenic *E. coli* in Tunisian subjects with or without diarrhea. Application of PFGE typing indicates that large numbers of stable DEC clones are present in Tunisia and may cause diarrhea. This finding could be important in further studies of the epidemiology of DEC in Tunisia.

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