Gene encoding virulence markers among *Escherichia coli* isolates from diarrhoeic stool samples and river sources in rural Venda communities of South Africa

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Abstract

River water sources and diarrhoeic stools of residents in the Venda Region, Limpopo Province of South Africa were analysed for the prevalence of *Escherichia coli* (*E. coli*) and the presence of virulence genes among the isolates. A control group of 100 non-diarrhoeic stool samples was included. *Escherichia coli* was isolated and identified by standard cultural and biochemical methods. Pathogenicity of environmental and human isolates was determined by amplification of genes associated with virulence of *E. coli,* using specific primers.

Of a total of 228 water and river sediment samples screened, *E. coli* was recovered from 200 (87.7%), and 135 (67.5%) of these had one or more genes associated with pathogenicity. The highest frequency of isolation of pathogenic strains was found in Ritavi River water and sediment (80.6%), followed by Lotanyanda River (76.9%), and the least (45.8%) in Nzhelele River 2. *Escherichia coli* was recovered from all of the 252 diarrhoeic stools tested (100%), and 119 (47.28%) of these had one or more genes associated with pathogenicity. The frequency of isolation of potential pathogenic *E. coli* from humans was highly significant ($t = 6.3$; $pd \bullet 0.01$) in comparison to water isolates. Cytotoxic necrotizing Factor 1 (*cnf1*) and cytotoxic necrotising Factor 2 (*cnf2*) coding for necrotoxigenic *E. coli* (NEC); bundle-forming pilus (*bfpA*) and enteropathogenic attachment and effacement (*eaeA*) coding for enteropathogenic *E. coli* (EPEC), occurred in 35% and 34% respectively of river isolates. Heat-stable (*ST*) and heat-labile (*LT*) toxin genes coding for enterotoxigenic (ETEC) and Shiga-like toxin 1 (*Stx1*) and Shiga-like toxin 2 (*Stx2*) coding for Shiga-like toxin-producing *E. coli* (STEC) were not encountered in the river isolates. Isolates from stool samples had 21.8% and 12.6% of EPEC and NEC strains respectively; while enterotoxigenic (ETEC), Shiga-like toxin-producing (STEC) and enteroaggregative *E. coli* (EAEC) had a prevalence of 5%, 5.8% and 5.8% respectively. One human isolate possessed *stx2* and *eaeA* indicating *E. coli* O157: H7. No genes associated with pathogenicity were observed in human non-diarrhoeic stool isolates. Results have revealed a possibility of a recycling of pathogenic *E. coli* strains, particularly the EPEC and NEC strains, between the water sources and the local population.

Keywords: *Escherichia coli*, virulence markers, water, stool, Venda, South Africa

Introduction

Over 500 x 10^6 cases of acute diarrhoea have been reported to occur yearly in children aged less than 5 years across the globe (Snyder and Merson, 1982). Diarrhoeal diseases are responsible for a huge proportion of morbidity and mortality in developing countries, particularly among children (Snyder and Merson, 1982; DuPont, 1995a). Apart from protozoans such as *Giardia lamblia*, *Entamoeba histolytica, Cryptosporidium parvum*, *Isospora belli*, and viruses such as Rotavirus, and Norwalk-like virus, implicated in cases of diarrhoea, frequently isolated bacterial diarrhoeagenic agents include *Escherichia coli*, *Campylobacter jejuni/coli, Salmonella*, *Shigella,* and *Aeromonas* species (Obi et al., 1995, 1997; Lainson and Silva, 1999; Coker et al., 2002; Oyofo et al., 2002). *Escherichia coli* is, however, the focus of this study. Although a normal flora of animals and humans, some *E. coli* strains are pathogenic and may account for life-threatening infections. Such infections include urinary tract infections (Falagas and Gorbach, 1995), haemolytic colitis, neonatal meningitis, nosocomial septicaemia, haemolytic uremic syndrome and surgical site infections (Klein et al., 1986; Thielman and Guerrant, 1999). Diarrhoeal diseases due to the virulent strains have been extensively reported and account for a substantial degree of morbidity and mortality in different age groups (El-Sheikh and El-Assouli, 2001; Galane and Le Roux, 2001). Virulent strains of *E. coli* include enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and enterotoxigenic *E. coli* (ETEC) (Giron et al., 1991; Falbo et al., 1992; Blanco et al., 1992; Chan et al., 1994; Levine, 1987). The clinico-epidemiological patterns of the virulent strains vary, and association with travellers' diarrhoea, extra-intestinal infections, acute, chronic or persistent diarrhoea are known (Falbo et al., 1992; Chan et al., 1994). Indices of pathogenicity among *E. coli* strains include pili, k-antigen, haemolysin, adhesive factor, enterotoxins, cytotoxins, effacement factors and cytotoxic necrotic factors (Galane and Le Roux, 2001).

Animals, humans and the environment including water sources serve as natural habitats of virulent strains of *E. coli* (DuPont, 1995; Nataro et al., 1998; Griffin, 1999; Kuhnert et al., 2000; Stephan and Schumacher, 2001). Infection with pathogenic *E. coli* strains is closely linked to poor sanitation and personal hygiene. In developing countries infection could be due to the consumption of contaminated water from wells, rivers, and other surface waters (Grasso et al., 2000; Welch et al., 2000; Tumwine et al., 2002). In many rural areas of South Africa, clean potable water and sanitation are

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TABLE 1
Primers and cycling conditions used in the amplification of specific genes fragment for *cnf1*, *cnf2*, *stx1*, *stx2*, *ST*, and *LT* responsible for toxin production; and *bfpA*, EAEC, *eaeA* genes responsible for adhesion properties of *E. coli*

Pathogenic factor	Primers	Primer sequences (5'-3')	Product size (base pairs)	Cycling conditions	Reference
Cytotoxic necrotising Factor 1 (*cnf1*)	CNF 1a CNF 1b	GCTCAACGAGACTATGCTCTG ACGCTGCTAAGTACCTCCTGG	278 bp	95°C for 30s, 5 cycles of 72°C for 1 min; 20 cycles of 95°C for 30s, 63°C for 30s, 72°C for 30s; 72°C for 5 min final extension.	Falbo et al., 1992
Cytotoxic necrotising Factor 2 (*cnf2*)	CNF 2a CNF 2b	GTGAGGCTCAACGAGATTATGCACTG CCACGCTTCTTCTTCAGTTGTTCCTC	839 bp	5 cycles of 95°C for 30s, 72°C for 1 min; 20 cycles of 95°C for 30s, 63°C for 30s, 72°C for 30s; 72°C for 5 min final extension.	Pass et al., 2000
Shiga-like toxin 1 (*stx1*)	Stx 1a Stx 1b	CAGTTAATTTGGTGGCGAAG CTGCTAATAGTTCTGCGAATC	894 bp	95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 final extension.	China et al., 1996
Shiga-like toxin 2 (*stx2*)	Stx 2a Stx 2b	CCTCGGTATCCTATTCCCGG GGATGCATCTCTGGTCATTG	478 bp	95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 final extension.	China et al., 1996
Heat-stable toxin (*ST*)	StaP a StaP b	TTAATAGCACCCGGTACAAGCAGG CTTGACTCTTCAAAAGAGAAAATTAC	147 bp	95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 final extension.	Matar et al., 2002
Heat-labile toxin (*LT*)	LT a LT b	TCTCATTGTGCATACGGAGC CCATACTGATTGCCGCAAT	320 bp	95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 final extension.	Matar et al., 2002
Bundle-forming pilus (*bfpA*)	Bfp a Bfp b	ATTGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	326 bp	95°C for 5 min followed by 30 cycles of 94°C for 30 sec, 56°C for 1min, 72°C for 2 min; final incubation for 10 min at 72°C	Yatsuyanagi et al., 2002
Enteroaggregative gene (*eaeC*)	EAgg a EAgg b	CTGGCGAAAGACTGAATCAT CAATGTATAGAAATCCGCTGTT	630 bp	95°C for 5 min, 30 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 1 min, and final extension of 72° for 10 min	Schmidt et al., 1995
Enteropathogenic attachment and effacement (*eaeA*)	Eae a Eae b	AGGCTTCGTCACATGTG CCATCGTCACCAGAGGA	579 bp	95°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and final extension for 10 min at 72°C	China et al., 1996

lacking. Village communities depend on untreated water from rivers, wells, fountains and other surface waters for drinking, laundry, and recreational purposes. (Von Shirnding et al., 1993; Pegram et al., 1998). A previous report on the microbial quality of river water sources in Venda rural communities in Limpopo, South Africa, showed that the water sources were unsafe for human consumption, and *E. coli* was the predominant potential pathogen isolated. (Obi et al., 2002). Values reported for faecal coliforms, total coliforms, heterotrophic plate counts, enterococci and somatic coliphages (Obi et al., 2002) exceeded minimum acceptable values prescribed in the guidelines of the Department of Water Affairs and Forestry (DWAF, 1996).

In this study, stool samples and river water sources in the Venda Region, Limpopo Province of South Africa were screened for the prevalence of *E. coli* and the presence of virulence factors associated with the organism using the polymerase chain reaction in an endeavour to elucidate the molecular epidemiology of virulent strains.

Materials and methods

Sources of samples and study sites

Five hundred millilitres of river water and 500 g of river sediments were collected from six rivers in the Venda region of South Africa. The rivers were: Nzhelele 1, Nzhelele 2, Elim, Ritavi, Lotanyanda and Xikari. A total of 228 river samples were collected, and 252 diarrhoeic stool samples were obtained on a weekly basis from outpatients attending the Elim and Siloam Hospitals in the same region. One hundred non-diarrhoeic stools were included as controls. Stool samples were collected from individuals who make use of one or more of the rivers as their water sources. Water and stool samples were collected between June 2001 and September 2002, and processing was done within 6 h. Informed consent was obtained before the collection of stool samples. Diarrhoea in this study was defined as the passage of watery stools at least thrice daily.

Isolation and identification of *E. coli*

Isolation of *E. coli* from stool samples was done as previously described (Maier et al., 2000). Briefly, stool samples were inoculated unto nutrient broth (Merck, South Africa), and incubated at 37°C for 24 h, followed by streaking on eosin methylene blue agar (Merck, South Africa), and incubated again at 37°C for 24 h. Five millilitres river water and 10 g river sediment were used as inocula for the isolation of *E. coli* from river water and river sediments. Dark blue colonies or colonies showing metallic sheen were confirmed using biochemical tests as described (Edward and Ewing, 1972; Forbes et al., 1998).

Amplification of pathogenic gene sequences

Bacterial DNA was isolated as described by Reischl et al. (2002). Briefly, an overnight bacterial culture was suspended in sterile distilled water and heated at 94°C for 13 min; this was followed by centrifugation at 14 000 $r \cdot min^{-1}$ for 15 min to pellet the cell debris. The supernatant was used as template for amplification reactions. Gene regions coding for the following pathogenic properties were amplified for each bacterial isolate: Bundle-forming pilus (*bfpA*), cytotoxic necrotising Factors 1 and 2 (*cnf1, cnf2*), heat-labile toxin (*LT*), heat-stable toxin (*ST*), Shiga-like Toxin 1 and 2 (*stx1*, *stx2*), enteropathogenic attachment and effacement (*eaeA*), and enteroaggregative (EAEC) gene , using specific primers. For each gene of interest the master mix consisted of 50mM KCl, 1mM Tris HCl, 1.5mM $MgCl_2$, 100mM of dNTPs and 1U of Taq polymerase. Five microlitres of DNA template was added to 45 mℓ of the master mix and amplified accordingly. Specific primers and amplification conditions for the different pathogenic gene coding regions were employed as previously described (Falbo et al., 1992; Schmidt et al., 1995; Pass et al., 2000; China et al., 1996; Matar et al., 2002; Yatsuyanagi et al., 2002;). Details are shown in Table 1. Amplified gene products were verified by gel electrophoresis (2% agarose) at 80V for 90 min and visualised under ultraviolet light. Isolates were classified according to the genes detected as follows: *cnf1* and *cnf2* for necrotoxigenic; *ST* and *LT* for enterotoxigenic; *stx1* and *stx2* for Shiga toxin producing; *bfpA* and *eaeA* for enteropathogenic, and EAE*C* for enteroaggregative *E. coli*. Reference strains representing the amplified genes, BM2-1 (*cnf1*), B20A (*cnf2*), C600-933J (*stx1*), C600-933W (*stx2*), and previously obtained amplicons of *ST*, *LT*, *bfpA*, *eaeA* and EAEC were obtained from the Department of Medical Microbiology, Medical University of Southern Africa, South Africa.

Results

Frequency of isolation of pathogenic *E. coli* from water sources

Of a total of 228 river water and river sediment samples collected, *E. coli* was recovered from 200 (87.7%), and 135 (67.5%) of these had one or more of the genes responsible for pathogenicity of *E. coli*. The highest frequency of isolation of pathogenic strains was found in Ritavi River surface and sediment (80.6%), followed by Lotanyanda River (76.9%), and the least number of isolates was found in Nzhelele River 2 (45.8%)(Table 2).

TABLE 2
Frequency of isolation of *E. coli* and pathogenic *E. coli* strains from six rivers and their sediments, and from diarroeal stools from in-patients of two hospitals, in the Limpopo Province of South Africa

Sampling sites (n = number of samples)	Number of (%) *E. coli* isolates	Number of pathogenic *E. coli* isolates (%)
Rivers (n=Surface water + sediments)		
Nzhelele 1 (n=29)	24 (82.7%)	16 (66.7%)
Nzhelele 2 (n=26)	24 (92.3%)	11 (45.8%)
Elim bridge (n=31)	26 (83.9%)	14 (53.8%)
Ritavi (n=34)	31 (91.1%)	25 (80.6%)
Xikari (n=78)	69 (88.4%)	49 (71.0%)
Lotanyanda (n=30)	26 (86.7%)	20 (76.9%)
Total (n=228)	**200 (87.7%)**	**135 (67.5%)**
***Hospitals* (diarrhoeic stools)**		
Siloam Hospital (n=124)	124 (100%)	58 (46.8%)
Elim Hospital (n=128)	128 (100%)	61 (47.7%)
Total	**252 (100%)**	**119 (47.2%)**
***Controls* (non-diarrhoeic stools, n = 100)**	**100 (100%)**	**00 (0.0%)**

TABLE 3
Frequency of occurrence of pathogenic coding genes of *E. coli* isolated from river water and river sediments, and diarrhoeic stools. Pathogenic *E. coli* strains and corresponding coding genes are indicated.

Sample source	Frequency of occurrence of pathogenic coding genes							
	Cnf1	***Cnf2***	***ST***	***LT***	***Stx1***	***Stx2***	***BfpA***	***EaeA***	**EAEC**
Water and sediments (n=135)	22 (16.3%)	26 (19.3%)	8 (4.0%)	8 (4.0%)	3 (2.2%)	3 (2.2%)	35 (26%)	11 (8.1%)	19 (14.1%)
E. coli strain	NEC 48 (35.6%)		ETEC 16 (11.8%)		STEC 6 (4.4%)		EPEC 46 (34.1%)		19 (14.1%)
Diarrhoeal stools (n=119)	4 (3.5%)	21 (17.6%)	9 (7.6%)	7 (5.9%)	11 (9.2%)	8 (6.7%)	27 (22.7%)	13 (10.9%)	19 (16%)
E. coli strain	NEC 25 (21%)		ETEC 16 (13.4%)		STEC 19 (16%)		EPEC 40 (33.6%)		EAEC 19(16%)

Key:
Cnf1 = cytotoxic necrotising Factor 1
Cnf2 = cytotoxic necrotising Factor 2
ST = heat stable toxin
LT = heat labile toxin
Stx1 = Shiga-like Toxin 1
Stx2 = Shiga-like Toxin 2
BfpA = bundle-forming pilus
EaeA = enteropathogenic attachment and effacement
EAEC = enteroaggregative
NEC = Necrotoxigenic *E. coli*
EPEC = Enteropathogenic *E. coli*
ETEC = Enterotoxigenic *E. coli*
STEC = Shiga-like toxin-producing *E. coli*
EAEC = Enteroaggregative *E .coli*

Frequency of isolation of pathogenic *E. coli* from humans

E. coli was recovered from all of the 252 (100%) diarrhoeic stool specimens screened. However, 119 (47.28%) of these had genes associated with pathogenicity. Among the 124 isolates obtained from Siloam Hospital, 58 (46.8%) were pathogenic, while 61 (47.7%) were pathogenic strains among the 128 isolates obtained from Elim Hospital. *E. coli* was isolated from all the control non-diarrhoeic stools samples (100%). However, no pathogenic strains were identified (Table 2). The frequency of isolation of potential pathogenic *E. coli* from humans was highly significant (t = 6.3; pd•0.01) in comparison to river water sources.

Frequency of occurrence of pathogenic coding genes of *E. coli* isolates

The *bfpA* gene coding for enteropathogenic *E. coli* (EPEC) was most frequently detected (26%) among *E. coli* isolates from river water sources and their sediments; followed by *cnf2* (19.3%), and *cnf1* (16.3%), both coding for necrotoxigenic *E. coli* (NEC). *ST*, *LT*, and *stx1*,*stx2*, and EAEC coding for enterotoxigenic *E. coli* (ETEC), Shiga-like toxin-producing strains (STEC), and enteroaggregative *E. coli* (EAEC) occurred with a frequency of 4%, 4%, 2%, 2% and 14.1% respectively. Overall genes coding for NEC were most frequent (35.5%), followed by genes coding for EPEC (34.1%). Among human *E. coli* isolates *BfpA* occurred most frequently (22.7%), followed by *cnf2* (17.6%), and the least was *cnf1* (3.5%). Genes coding for EPEC were most observed (33.6%), followed by genes coding for NEC (21%)(Table 3). One human isolate contained the stx2 and *eaeA* genes. No other isolate had more than one gene coding for pathogencity. No genes associated with pathogenicity were identified in the isolates obtained from non-diarrhoeal stools. A representative gel electrophoresis profile of amplified products of the investigated pathogenic coding genes is shown in Fig. 1.

Discussion

In the absence of treated potable water, many rural communities in South Africa depend on rivers and other water bodies for domestic and recreational purposes. Unfortunately, these water sources are not protected, and are open to microbial contamination from humans, animals and the environment (Nevondo and Cloete, 1999; Lehloesa and Muyima, 2000). River water sources in the Limpopo Province have been shown to be heavily contaminated with *E. coli* (Obi et al., 2002). Moreover, strains of *E. coli* have been implicated in a variety of clinical infections. The presence of pathogenic *E. coli* in water sources is always of public health concern.

In this study, rivers used for drinking and washing purposes in the Venda Region of the Limpopo Province were found to be contaminated with pathogenic strains of *E. coli*. The detection of necrotoxigenic and enteropathogenic strains means that these water sources present a health hazard to their users. Among the water isolates NEC (35.6%) and EPEC (34.1%) were most frequently encountered. EPEC has been shown to be a major cause of diarrhoea in young children (Kuhnert et al., 2000), while NEC is responsible for diarrhoea in cattle (Orden et al., 1999; DebRoy et al., 2001). NEC isolated from animals and humans can belong to the same serogroups and produce or carry genes coding for fimbrial and afimbrial adhesins (Mainil et al., 1999). The isolation of NEC from some of the rivers investigated may confirm the faecal contamination of these water sources by grazing cattle, and raises the question of possible zoonotic infections. We observed 21% NEC isolates from diarrhoeal stools, and none from the control

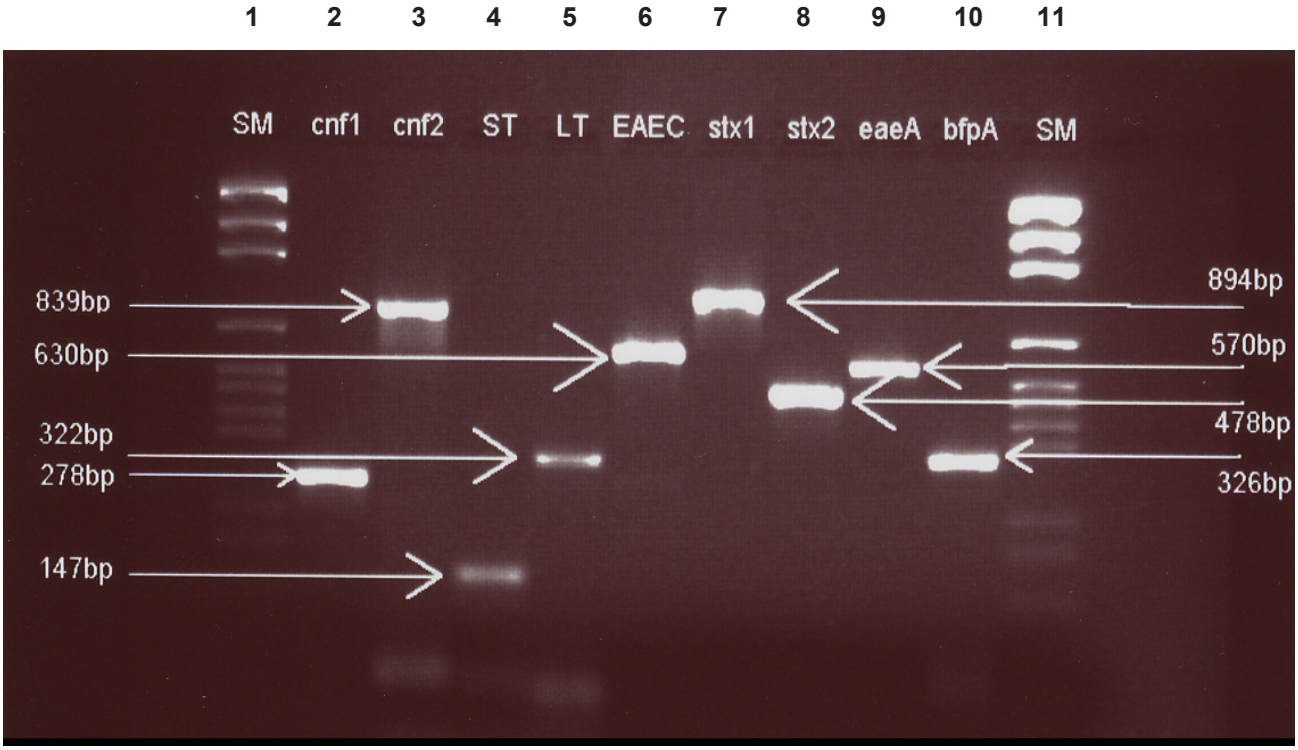

Figure 1

A representative gel electrophoresis profile of different virulence genes of isolated E. coli. *Lanes 1 and 11, molecular weight marker (GIBCO BRL 1000kb ladder); Lane 2,* cnf1*; Lane 3,* cnf2*; Lane 4,* ST; *Lane 5,* LT; *Lane 6, EAEC; Lane 7,* Stx1*; Lane 8,* Stx2*; Lane 9,* eaeA;, *Lane 10,* bfpA.

group. The prevalence of EPEC observed in this study among diarrhoeagenic human isolates was marginally higher (33.6%) compared to the report of Galane and Le Roux (2001), who reported a prevalence rate of 26.5% among South African children with diarrhoea. In this study, a marginally higher proportion of atypical EPEC (32.5%) against 28.2% reported by Galane and Le Roux (2001) was noted. In our findings, NEC (21%), EPEC (33.6%) were also the predominant isolates from clinical (stool) samples. ETEC (13.4%), STEC (16%) and EAEC (16%) occurred more frequently in stool isolates than in water isolates. One human isolate had *stx2* and *eaeA* genes indicating a pathogenicity island typical of *E. coli* O157:H7, known to cause diarrhoea in infants, haemorrhagic colitis, and haemolytic uremic syndrome, a severe clinical manifestation of infections with Shiga-like toxin-producing *E. coli* (Blank et al., 2003; Tozzi et al., 2003). The predominance of NEC and EPEC in both the human and water isolates indicates a possibility of a recycling of pathogenic *E. coli* strains between the water sources and the local population. This could explain an endemic situation of diarrhoeal cases due to *E. coli*. EPEC and ETEC have been identified as leading pathogens in developing countries, particularly in children (Georges et al., 1984). Finally, this study has revealed the presence of virulence markers in *E. coli* isolates from river water sources and stool samples of patients with diarrhoea and also emphasises the importance of safe water supply, good hygiene and sanitation practices in rural communities.

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