Characterization of enterotoxigenic *Escherichia coli* from diarrhoeal patients in Bangladesh using phenotyping and genetic profiling

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A total of 99 isolates out of 370 colonization factor (CF)-positive, well-characterized enterotoxigenic *Escherichia coli* (ETEC) strains belonging to 13 different CF types isolated from diarrhoeal patients admitted to the hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh, were tested. The isolates were selected at random based on expression of the major CFs prevailing in Dhaka, Bangladesh, from 1996 to 1998. These isolates were characterized by O-antigenic serotyping, randomly amplified polymorphic DNA (RAPD) analysis and biochemical fingerprinting using the PhenePlate (PhP) system. The 99 ETEC isolates belonged to 10 O serogroups, the predominant ones being O6 ($n=28$), O115 ($n=20$) and O128 ($n=20$). Most isolates of serogroup O6 (CS1 + CS3, 11/14; CS2 + CS3, 5/8) belonged to the same PhP/RAPD type (H/f), whereas other isolates of serogroup O6 ($n=12$) belonged to different PhP/RAPD types (Si/f and F/c). Eleven serogroup O128 (CFA/I) isolates belonged to the same PhP/RAPD type (E/b), whereas the other O128 isolates formed different PhP/RAPD types. Fifteen (75%) serogroup O115 isolates (together with fourteen isolates from serogroups O25, O114, O142 and O159) demonstrated two closely related common groups by PhP typing (A and A1) and belonged to the same PhP/RAPD type (A/a). Three major clonal groups were identified among the ETEC strains in this study, largely based on O-antigenic type, CF expression pattern and toxin profile.

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INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) has been reported to be a major cause of diarrhoea in humans throughout the world (Sack, 1975; Albert *et al.*, 1995; Gaastra & Svennerholm, 1996), especially in developing countries (Black *et al.*, 1984). It is a cause of morbidity and mortality in children up to 5 years of age in developing countries (Black, 1993). It has also been found to be the most common cause of traveller's diarrhoea in all countries where surveys have been conducted (Black, 1990). ETEC strains express well-defined enterotoxins, heat-stable toxin (ST) and/or heat-labile toxin (LT). The bacteria first colonize and multiply in the small intestine and produce these toxins. Colonization is usually associated with the presence of surface adhesins known as colonization factors (CFs) that mediate attachment to the small intestine.

Over 22 different CFs are known to be associated with adherence of ETEC to the gut mucosa; these include the *E. coli* surface antigens CS1–CS6 (Viboud *et al.*, 1993; Gaastra & Svennerholm, 1996). ETEC strains express somatic (O) antigen on the cell surface by which O serogrouping of *E. coli* is determined (Ørskov & Ørskov, 1992). Epidemiological investigations show that ETEC strains belong to a wide range of O-antigenic serovars (Wolf, 1997). The predominant CFs of ETEC are frequently associated with a wide range of O serotypes, and such combinations can be found regardless of location and time (Wolf, 1997). Molecular typing methods have shown that ETEC strains expressing the same serotype and other phenotypic traits usually have reduced genetic diversity (Ochman & Selander, 1984; Caugant *et al.*, 1985). However, in several cases, genetically unrelated clones may display the same phenotype or strains may differ antigenically within the same clonal group (Ochman & Selander, 1984; Caugant *et al.*, 1985). Therefore, classification based on phenotypic traits, although invaluable for clinical and epidemiological analyses, may not reflect the genetic relatedness of bacterial strains. Thus, bacteriological

Abbreviations: CF, colonization factor; CFA, colonization factor antigen; ETEC, enterotoxigenic *Escherichia coli*; LT, heat-labile toxin; RAPD, random amplification of polymorphic DNA; ST, heat-stable toxin.

traits such as biochemical phenotype, serotype and virulence factors may act as indicators of clonality in clinical isolates.

Random amplification of polymorphic DNA (RAPD) is a well-known molecular technique applied to the study of genetic diversity of bacteria due to its simplicity, sensitivity, flexibility and relatively low cost (Welsh & McClelland, 1990; Williams *et al.*, 1990). The aim of this study was to investigate relatedness among ETEC strains using various phenotypic and genetic traits.

METHODS

Bacterial strains. Ninety-nine ETEC strains (Table 1) were selected randomly from a collection ($n=662$) gathered during a prospective study of CF antigens (CFA) and enterotoxin (ST and LT) types of ETEC isolated from diarrhoeal patients (Qadri *et al.*, 2000). The strains were stored at -70°C in Luria broth (Difco) containing 25% glycerol. At the start of the study, the expression of virulence

markers was confirmed by GM1 ganglioside ELISAs for the detection of ST and LT (Qadri *et al.*, 2000). Enterotoxin-positive *E. coli* colonies from CFA agar plates were tested for 13 different CFs using monoclonal antibody-based dot-blot assays (Qadri *et al.*, 2000).

Serotyping. ETEC strains were serogrouped with commercially available antisera (Denka Seiken) for specific somatic (O) antigens. Strains were subcultured on blood agar and serological reactions were performed using a slide agglutination test as described by Guth *et al.* (1989). Both live and heated (100°C for 30 min) bacterial suspensions were used for slide agglutination.

Biochemical fingerprinting. A rapid, semi-automated and computerized typing method for *E. coli*, based on measurements of the kinetics of 12 biochemical reactions, known as the PhenePlate (PhP) system (PhP-RE plates; PhPlate), was used for typing. A single colony from each sample was used in these assays. Similarities between the strains were calculated as a correlation coefficient (Kühn, 1985) and clustered according to the unweighted pair group method using arithmetic means (UPGMA) (Sneath & Sokal, 1973). The reproducibility of the system was evaluated in duplicate by assaying 20 known isolates. The level of identity between isolates was defined as the mean of correlation coefficients obtained between these duplicate assays minus 2 SD. PhP types consisting of more than one isolate were named common (C) types, whereas those consisting of only one isolate were termed single (Si) types. An identity level of 0.975 was set, based on reproducibility of the system (Kühn, 1985; Kühn *et al.*, 1997). Strains showing >0.975 similarity were regarded as identical and assigned the same PhP type.

DNA extraction. Chromosomal DNA of *E. coli* isolates was extracted and purified by the method of Maniatis *et al.* (1989), with minor modifications. Briefly, cells were harvested from overnight Luria broth cultures by a 3 min centrifugation at 14 000 r.p.m. (model 5415 D, 24-position standard rotor for 1.5 and 2.0 ml tubes; Eppendorf), washed in PBS, resuspended in 250 μl TES [10 mM Tris/HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl] and treated with 10% SDS (25 μl) at 65°C for 10 min. Following proteinase K treatment [25 μl (5 mg ml^{-1}) solution] at 45°C for 5 h, DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) using centrifugation at 14 000 r.p.m. for 8 min. DNA was purified by ethanol precipitation, dried and dissolved in TE buffer. RNase treatment [3 μl (10 mg ml^{-1}) solution] was performed at 37°C for 1–2 h, and the DNA purified as above and stored at -20°C .

RAPD fingerprinting. Common PhP types containing three or more isolates were employed for RAPD analysis. This fingerprinting was carried out using primers 1281 (5'-AACGCGCAAC-3') and 1283 (5'-GCGATCCCCA-3'). PCRs (25 μl final volume) containing 2.5 μl $10\times$ PCR buffer (Invitrogen), 20 ng genomic DNA, 4 μl 25 mM MgCl_2 (Perkin Elmer), 20 pmoles primer, 2 U AmpliTaq DNA polymerase (Invitrogen) and 2.5 μl 2.5 mM dNTPs were performed under a drop of mineral oil in an automated thermal cycler (Biometa). The cycling program involved 45 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min. PCR products were separated by electrophoresis in 1% agarose gel containing ethidium bromide (0.5 mg ml^{-1}). A 1 kb DNA ladder (New England Biolabs) was used as a marker in all gels. DNA was visualized with a UV transilluminator and gel images were digitized with an ID Gel documentation system (Bio-Rad). Isolates with 90% of the amplified bands in common were regarded as identical and assigned to a common RAPD type, denoted by lower-case letters. Isolates with three or more different bands in their RAPD profiles were regarded as unrelated RAPD types (Tenover *et al.*, 1995).

Table 1. Phenotypic characteristics and RAPD patterns of 99 ETEC isolates from Bangladesh

ND, Not determined; n , number of isolates.

Serogroup (n)	Phenotypic characteristic		RAPD type
	CF (toxin type/ n)	PhP type	
O6 (28)	CS1 + CS3 (ST/12)	H,H1,H3,Si	f
	CS1 + CS3 (STLT/2)	H1,H2	f
	CS2 + CS3 (ST/7)	H,H2,Si	f
	CS2 + CS3 (STLT/1)	H3	f
	CS4 + CS6 (ST/4)	F	c
	CS14 (STLT/2)	F1	c
O8 (1)	CS21 (ST/1)	G	ND
O15 (3)	CS14 (STLT/3)	C	e
O25 (7)	CFA/I (ST/1)	A	a
	CS4 + CS6 (STLT/3)	Si	ND
	CS5 + CS6 (STLT/2)	A	a
	CS8 (STLT/1)	Si	ND
O114 (7)	CS2 + CS3 (ST/1)	H	ND
	CS7 (LT/6)	A,A1	a
O115 (20)	CS3 (ST/2)	D2	ND
	CS5 + CS6 (ST/9)	A,A1	a
	CS6 (ST/1)	Si	ND
	CS6 (STLT/6)	A,A1	a
	CS21 (ST/2)	G	ND
O126 (1)	CS8 (STLT/1)	Si	ND
O128 (20)	CFAI (ST/11)	E	b
	CFA/I (ST/1)	E1	ND
	CS14 (ST/2)	I	ND
	CS17 (ST/5)	D,D1	d
	CS21 (ST/1)	Si	ND
O142 (3)	CFA/I (ST/3)	A	a
O159 (9)	CFA/I (ST/2)	A1	a
	CS12 (STLT/7)	B,B1	b

Fig. 1. UPGMA dendrogram derived from clustering of the PhP typing data of 99 ETEC strains belonging to 10 O serogroups, isolated from a prospective study of diarrhoea in Bangladesh. ID, identity.

RESULTS AND DISCUSSION

The 99 ETEC strains used in this study were classified into 10 O serogroups and 16 PhP types (Table 1). Different PhP types were denoted by capital letters, whilst non-identical PhP types belonging to the same cluster were denoted by the same capital letters followed by different numbers (e.g. H1, H2, H3). The predominant PhP common types that contained three or more isolates were included for RAPD

typing. A total of six common RAPD types were observed among the selected strains tested (Table 1).

The predominant serogroups found were O6 ($n=28$), O115 ($n=20$) and O128 ($n=20$) (Table 1). Results of PhP and RAPD typing indicated that most isolates of serogroup O6 ($n=16$) belonged to the same PhP/RAPD type (i.e. H, H1/f) and expressed CS1+CS3 or CS2+CS3 and ST. A few O6 serotype isolates produced closely related PhP subtypes (H2

or H3) or Si type, and expressed ST/LT (Table 1). Six O6 serogroup isolates belonged to another PhP/RAPD type (F,F1/c) and expressed CS4 + CS6 and ST (*n*=4) or CS14 and ST/LT (*n*=2). Most isolates (*n*=15) of the second predominant serotype (O115) expressed CS5 + CS6 and ST (*n*=9) or CS6 and ST/LT (*n*=6) and formed a common PhP group (A,A1) with several other serogroups (Figs 1 and 2). A third common PhP type (E) was found (serogroup O128 isolates, *n*=11; PhP/RAPD type E/b). Five of the remaining serogroup O128 isolates formed a separate PhP/RAPD type (D,D1/d). Interestingly, two PhP types (E and B,B1) displayed RAPD type b (Fig. 2).

Infection due to ETEC continues to be a major cause of childhood diarrhoea in developing countries, especially in children under 2 years of age. A strong association of ETEC with diarrhoea (18 %) is found in Bangladesh, which is

similar to many other developing countries (Echeverria *et al.*, 1989; Kim *et al.*, 1989; Black, 1993). In a previous study (Qadri *et al.*, 2000), the prevalence of toxin types and CFs of ETEC was reported. Unlike CFs and toxins, which are proteins, the O antigens are carbohydrates and are part of the lipopolysaccharide. The O serogroup has previously been used to differentiate between pathogenic and commensal *E. coli* (Wolf, 1997). However, in recent years, classification of pathogenic *E. coli* has involved enterotoxins and CF expression more than O serotype. However, O serogrouping provides worthwhile epidemiological information about the variety of *E. coli* strains geographically (Wolf, 1997; Pacheco *et al.*, 1998). Furthermore, polyphasic strategies, combining conventional serotyping with other phenotypic and/or molecular techniques has been shown to be useful in determining the clonal nature of ETEC strains (Selander *et al.*, 1987; Pacheco *et al.*, 1998). Such studies

Fig. 2. UPGMA dendrogram derived from clustering of PhP typing data (major clusters) and their RAPD types of prevalent O serogroups of ETEC strains isolated from a prospective study of diarrhoea in Bangladesh. ID, identity.

have shown that a few ETEC serogroups are responsible for a majority of the infections in each geographical area (Mitsuda *et al.*, 1998; Pacheco *et al.*, 1998, 2001). The results of the present study not only showed the presence of three dominant serogroups among the CF-positive ETEC isolated from diarrhoeal patients in Bangladesh, but also indicated that the majority of the isolates (69 %) belonged to three common groups. In addition, a clear relationship was found between O serogroup, PhP type and RAPD type, CF antigens and toxin production.

Serogroup O6 has traditionally been recognized as prevalent worldwide among ETEC strains. Most of the serogroup O6 isolates in this study expressed CS1 + CS3 or CS2 + CS3 and enterotoxin, and were related to each other according to the typing methods used; thus, they may have a common clonal origin. However, one strain of serogroup O114 appeared to belong to this clonal group, as it expressed CS2 + CS3 and ST, and displayed PhP type H. Similar properties have been found in ETEC of serogroup O6 collected from different geographical locations (Pacheco *et al.*, 1998). The remaining six isolates of serogroup O6 in this study produced a second common group (F, F1/c) and expressed CS4 + CS6 or CS14. These two common groups (H, H1–H3/f and F, F1/c) of serogroup O6 may arise from two different lineages.

Most isolates of the third dominant serogroup, O115 ($n=15$; CS5 + CS6 and CS6), along with other minor serogroups, O114 ($n=6$; CS7), O25 ($n=3$; CS5 + CS6 and CFA/1), O142 ($n=3$; CFA/1) and O159 ($n=2$; CFA/1), constituted a large common group showing a similar PhP/RAPD type (A, A1/a; Table 1). These isolates expressed one or more of the different enterotoxins (ST and LT) and CFs according to serotype specificity, in accordance with previous studies (Pacheco *et al.*, 1997, 1998; Wolf, 1997). Other than these three major clusters, which comprised the predominant serogroups associated with ETEC diarrhoea in Bangladesh, seven serogroup O159 isolates produced a minor common group B, B1/b expressing CS12 and ST/LT.

Our results suggest that three major groups of ETEC strains (PhP/RAPD types A, A1/a, E/b and H/f) were predominantly associated with diarrhoea in Dhaka, Bangladesh, during the period 1996–1998. In the present study, the presence of polymorphic bands allowed us to detect different RAPD types among selected ETEC strains. The use of RAPD and PhP typing to investigate the diversity of ETEC strains isolated from Bangladesh has shown that strains of the same serotype that expressed different CFs and enterotoxin may be genetically and phenotypically distinct (type H/f and F/c for serotype O6; type E/b and D/d for serotype O128). However, some isolates of different O serotypes seemed to be closely related according to PhP/RAPD typing (A, A1/a).

Our results have shown that there were three major clonal groups belonging to serogroups O6, O128 and O115, with several other minor serogroups, that were associated with ETEC diarrhoea in Bangladesh. Clonal relatedness, such as that identified in this study, may be an important

consideration in the development and evaluation of vaccines directed at protection against ETEC infection.

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REFERENCES

- Albert, M. J., Faruque, S. M., Faruque, A. S. G., Neogi, P. K. B., Ansaruzzaman, M., Bhuiyan, N. A., Alam, K. & Akbar, M. S. (1995). Controlled study *Escherichia coli* diarrheal infection in Bangladeshi children. *J Clin Microbiol* **33**, 973–977.
- Black, R. E. (1990). Epidemiology of travelers' diarrhea and relative importance of various pathogens. *Rev Infect Dis* **12** (Suppl. 1), S73–S79.
- Black, R. E. (1993). Epidemiology of diarrhoeal disease: implications for control by vaccines. *Vaccine* **11**, 100–106.
- Black, R. E., Brown, K. H. & Becker, S. (1984). Effect of diarrhea associated with specific enteropathogens on the growth of children in rural Bangladesh. *Pediatrics* **73**, 799–805.
- Caugant, D. A., Levin, B. R., Ørskov, I., Ørskov, F., Eden, C. S. & Selander, R. K. (1985). Genetic diversity in relation to serotype in *Escherichia coli*. *Infect Immun* **49**, 407–413.
- Echeverria, P., Taylor, D. N., Lexsomboon, U., Bhaibulaya, M., Blacklow, N. R., Tamura, K. & Sakazaki, R. (1989). Case-control study of endemic diarrhoeal disease in Thai children. *J Infect Dis* **159**, 543–548.
- Gaastera, W. & Svennerholm, A.-M. (1996). Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). *Trends Microbiol* **4**, 444–452.
- Guth, B. E. C., Silva, R. M., Toledo, M. R. F., Lima, T. M. O. & Trabulsi, L. (1989). Virulence factors and biochemical characteristics of serotypes of *Escherichia coli* serogroup O29. *J Clin Microbiol* **27**, 2161–2164.
- Kim, K.-H., Suh, I.-S., Kim, J. M., Kim, C. W. & Cho, Y.-J. (1989). Etiology of childhood diarrhea in Korea. *J Clin Microbiol* **27**, 1192–1196.
- Kühn, I. (1985). Biochemical fingerprinting of *Escherichia coli*: a simple method for epidemiological investigations. *J Microbiol Methods* **3**, 159–170.
- Kühn, I., Albert, M. J., Ansaruzzaman, M., Bhuiyan, N. A., Alabi, S. A., Islam, M. S., Neogi, P. K., Huys, G., Janssen, P. and other authors (1997). Characterization of *Aeromonas* spp. isolated from humans with diarrhea, from healthy controls, and from surface water in Bangladesh. *J Clin Microbiol* **35**, 369–373.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mitsuda, T., Muto, T., Yamada, M., Kobayashi, N., Toba, M., Aihara, Y., Ito, A. & Yokota, S. (1998). Epidemiological study of a food-borne outbreak of enterotoxigenic *Escherichia coli* O25:NM by pulsed-field gel electrophoresis and randomly amplified polymorphic DNA analysis. *J Clin Microbiol* **36**, 652–656.

- Ochman, H. & Selander, R. K. (1984). Evidence for clonal population structure in *Escherichia coli*. *Proc Natl Acad Sci U S A* **81**, 198–201.
- Ørskov, F. & Ørskov, I. (1992). *Escherichia coli* serotyping and disease in man and animals. *Can J Microbiol* **38**, 699–704.
- Pacheco, A. B. F., Guth, B. E. C., Soares, K. C. C., Nishimura, L., de Almeida, D. F. & Ferreira, L. C. S. (1997). Random amplification of polymorphic DNA reveals serotype specific clonal clusters among enterotoxigenic *E. coli* strains isolated from humans. *J Clin Microbiol* **35**, 1521–1525.
- Pacheco, A. B. F., Soares, K. C., de Almeida, D. F., Viboud, G. I., Binsztein, N. & Ferreira, L. C. S. (1998). Clonal nature of enterotoxigenic *E. coli* serotype O6:H16 revealed by randomly amplified polymorphic DNA analysis. *J Clin Microbiol* **36**, 2099–2102.
- Pacheco, A. B. F., Ferreira, L. C. S., Pichel, M. G., Almeida, D. F., Binsztein, N. & Viboud, G. I. (2001). Beyond serotypes and virulence-associated factors: detection of genetic diversity among O153:H45 CFA/I heat-stable enterotoxigenic *E. coli* strains. *J Clin Microbiol* **39**, 4500–4505.
- Qadri, F., Das, S. K., Faruque, A. S. G., Fuchs, G. J., Albert, M. J., Sack, R. B. & Svennerholm, A.-M. (2000). Prevalence of toxin types and colonization factors in enterotoxigenic *Escherichia coli* isolated during a 2-year period from diarrheal patients in Bangladesh. *J Clin Microbiol* **38**, 27–31.
- Sack, R. B. (1975). Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. *Annu Rev Microbiol* **29**, 333–353.
- Selander, R. K., Caugant, D. A. & Whittam, T. S. (1987). Genetic structure and variation in natural populations of *E. coli*. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 1625–1648. Edited by F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter & H. E. Umberger. Washington, DC: American Society for Microbiology.
- Sneath, P. H. A. & Sokal, R. R. (1973). *Numerical Taxonomy: the Principles and Practice of Numerical Classification*. San Francisco: W. H. Freeman.
- Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.
- Viboud, G. I., Binsztein, N. & Svennerholm, A.-M. (1993). Characterization of monoclonal antibodies against putative colonization factors of enterotoxigenic *Escherichia coli* and their use in an epidemiological study. *J Clin Microbiol* **31**, 558–564.
- Welsh, J. & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**, 7213–7218.
- Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* **18**, 6531–6535.
- Wolf, M. K. (1997). Occurrence, distribution and association of O and H serogroup, colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli*. *Clin Microbiol Rev* **10**, 569–584.