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Antibiotic Resistance Pattern of Heat-Labile Enterotoxin (LT) Producing *Escherichia coli* Isolated from Children with Diarrhoea in Bangladesh: Clonal Relationships among Isolates with Different Resistant Phenotypes

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ABSTRACT

Fifty-six heat-labile, enterotoxin-producing (LT^+) *Escherichia coli* isolated from 33 children less than 5 years of age with diarrhoea were analysed for resistance to antibiotics, plasmid contents, and clonal relationships among isolates by ribosomal RNA (rRNA) fingerprinting (ribotyping). Fifty-five (98.2%) of the LT^+ isolates were resistant either to tetracycline alone (48.2%) or to tetracycline and one or more other antibiotic, i.e. ampicillin, streptomycin, chloramphenicol, trimethoprim-sulfamethoxazole, or nalidixic acid. Most of the isolates harboured one or more plasmid but antibiotic resistance patterns did not always correlate with particular plasmid patterns. Ribotyping of the isolates using the restriction endonuclease *Eco*RI revealed a total of 7 different ribotypes, and ribotypes were shared by *E. coli* isolates with different antibiotic resistant phenotypes. The results indicate that in Bangladesh at least 7 different clones of LT^+ *E. coli* acquired resistance to one or more different antibiotics in various combinations. However, a similar drug resistance pattern was not mediated by the same set of plasmids in all strains. The mechanism for the emergence and spread of antibiotic resistance among *E. coli* should be investigated further in Bangladesh, where LT^+ *E. coli* is an important agent of early childhood diarrhoea.

Key words: LT-producing *E. coli*; Plasmids; Antibiotic resistance; Ribotyping; Diarrhoea.

INTRODUCTION

Enterotoxin-producing *E. coli* are a major cause of diarrhoea in infants and young children in developing countries (1-3). These *E. coli* can produce either heat-labile enterotoxin (LT) or heat-stable enterotoxin (ST) or both, which contribute to the development of diarrhoea (1). In Bangladesh, many infants and young children develop diarrhoea due to infections with enterotoxigenic *E. coli* (ETEC) (4-6), and a significant number of these patients excrete heat-labile toxin-producing (LT^+) *E. coli* in their stools (4). The genetic determinants for LT are carried on large plasmids (7-9), and plasmid-borne LT genes have been cloned (10,11). However, there is at least one report indicating that LT synthesis is occasionally linked to phage conversion (12) and also that an LT-like toxin produced by an *E. coli* strain SA53 was not plasmid-mediated (13). Plasmids are also known to carry genes for antibiotic resistance, sequestering of ions, utilisation of substrates, and mobilisation of plasmid themselves between strains, species, and genera (14). Analysis of plasmid profiles has been shown to be a powerful tool in epidemiological studies of infectious diseases caused by various microorganisms (14,15) and has now become a part of a routine system for surveillance of nosocomial infections in many countries (16).

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Conjugal transfer of plasmids may cause transfer of genetic determinants for antibiotic resistance to recipient strains. To determine the origin and spread of antibiotic resistance genes and relatedness between resistant and susceptible strains, it is necessary to identify different clones of bacteria based on stable genetic markers. Recent developments in DNA analysis techniques have introduced several typing methods dependent on genotypes rather than phenotypes. One of these techniques is the use of ribosomal RNA (rRNA) gene probes to study restriction fragment length polymorphisms (RFLPs) of rRNA genes in different strains of bacteria. The probing of chromosomal DNA with *E. coli* rRNA probe provides a widely applicable system to investigate the molecular epidemiology as well as evolutionary relatedness between clones of bacteria (17). In the present study, LT^+ *E. coli* isolates have been analysed for antibiotic resistance, plasmid contents, and clonal relationships among the isolates.

MATERIALS AND METHODS

Bacterial isolates. Fifty-six heat-labile, toxin-producing (LT^+) *E. coli* isolated from the stools of 33 children less than 5 years of age with diarrhoea were obtained from two previous studies (4,5) under the diarrhoea surveillance programme of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). More elaborate descriptions of the surveillance system have been published previously (4,6). These isolates were positive for heat-labile enterotoxin (LT) both by Y1 adrenal cell assay (18) and DNA probe assay (4,5), and a single colony of each confirmed positive isolate was grown in trypticase soy broth with 0.3% yeast extract (TSBY; Gibco Laboratories, Detroit, Michigan, USA), containing 15% glycerol and was stored at −70°C until required for this study.

Antimicrobial susceptibility tests. The antimicrobial susceptibility tests were done by the method described by Bauer *et al.* (19) using commercially available disks (BBL Microbiology Systems, Cockeysville, MD, USA) at the following antibiotic concentrations (μg/disc): ampicillin, 10; chloramphenicol, 30; streptomycin, 10; tetracycline, 30; trimethoprim-sulfamethoxazole, 1.25 and 23.75 respectively; kanamycin, 30; gentamicin, 10; and nalidixic acid, 30.

Isolation and analysis of plasmid DNA. Plasmids were prepared by the alkaline lysis method described by Birnboim and Doly (20) from a 1.5 ml overnight culture of the bacteria in Luria-Bertini (LB) broth. The extracted plasmid DNA was electrophoresed in 0.7% agarose gels at room temperature at 30 mA (90 volts) for 3-4 h. Molecular size markers, either plasmid DNAs of known molecular weight or supercoiled DNA ladder (Bethesda Research Laboratories, Gaithersburg, MD, USA) were also run in an adjacent lane of each gel. Gels were stained in 0.5 μg/ml aqueous ethidium bromide for 20-30 min and were photographed on type 57 Polaroid films using short wave ultraviolet light.

Preparation of Southern blots. Southern blots were prepared from agarose gels containing intact plasmids, high molecular weight chromosomal DNA, and restriction endonuclease digested chromosomal DNA. High molecular weight chromosomal DNA was isolated from strains grown overnight in TSBY at 37°C with aeration, as described previously (17). Approximately 5 μg of purified whole cell DNA was digested with the appropriate restriction endonucleases as instructed by the manufacturer (Bethesda Research Laboratories) using 5 units of enzyme per μg of DNA. The digested DNAs were electrophoresed in 0.8% agarose gels in Tris-borate-EDTA buffer, using a standard method (21), and transferred onto nylon membranes (Hybond-N, Amersham International plc., UK) by Southern blotting (22).

Hybridisation of Southern blots. Southern blots derived from plasmid preparations and from high molecular weight chromosomal DNA were hybridised with a DNA probe for LT, and those derived from restriction endonuclease digested chromosomal DNA were hybridised with a gene probe for rRNA. The ribosomal RNA gene probe was a 7.5-kb *Bam*HI fragment of pKK3535 (23) and the LT probe was a 1-kb *Bam*HI fragment of pCVD404 (4). The probes were purified and labelled by random priming (24) using [α-^{32}P]-deoxycytidine triphosphate (3000 Ci/mmol, Amersham) as described previously (4). Southern blots were hybridised, washed under stringent conditions and autoradiographed as described by Stull *et al.* (17).

RESULTS

Analysis of the antibiotic resistance pattern showed that 55 of the 56 LT^+ isolates (98.2%) were resistant to tetracycline; of these, 27 isolates (48.2%) were resistant to tetracycline alone. The remaining 28 isolates were resistant to tetracycline and one or more other antibiotic (Table), including ampicillin (23.2%), streptomycin (42.8%), chloramphenicol (8.9%), nalidixic acid (7.1%), and trimethoprim-sulfamethoxazole (3.5%). None of the isolates were resistant to gentamicin, and one isolate was sensitive to all the antibiotics used (Table).

Plasmid analysis of the 56 LT^+ *E. coli* isolates showed 10 different patterns (Fig. 1a). Isolates from the same patient always showed similar plasmid profiles. Southern-blot hybridisation of plasmid DNA using an LT gene probe (Fig. 1b) showed that the probe hybridised with more than one plasmid band in some isolates, and with the sheared chromosomal DNA band in all the LT isolates. High molecular weight chromosomal DNA obtained from these isolates also hybridised with the probe (data not shown).

Plasmid profile produced by the different isolates did not always correlate with the drug resistance patterns. Isolates with similar plasmid profiles but obtained from different patients showed different antibiotic resistance patterns, and all isolates from the same patient showed similar antibiotic resistance patterns.

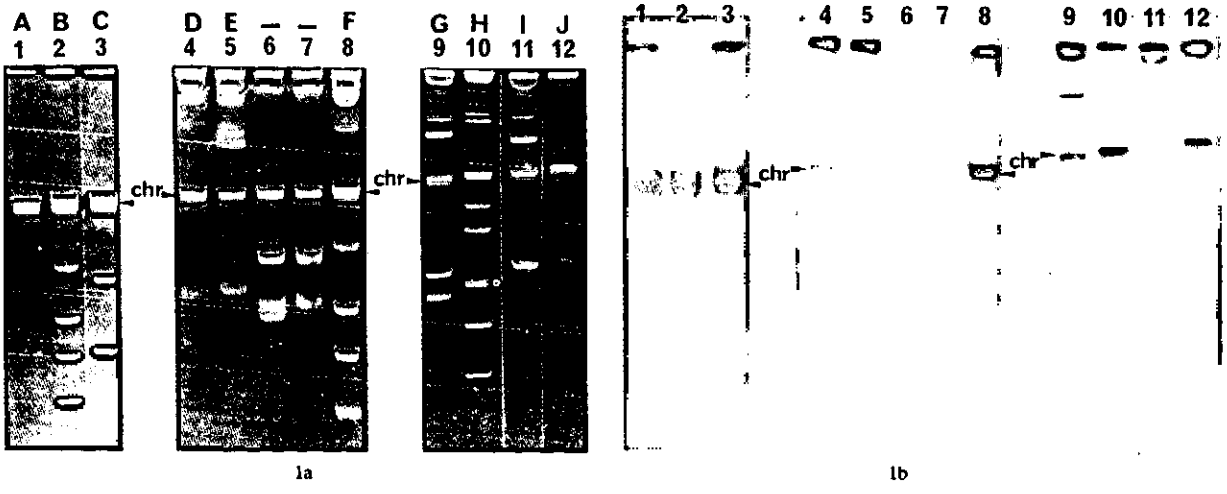
DISCUSSION

The objective of this investigation was to study the antibiotic resistance pattern and the possible origin and spread of antibiotic resistance genes among LT$^+$ *E. coli* isolates in Bangladesh.

Table. Antibiotic resistance patterns and ribotype distribution of 56 heat-labile toxin producing *Escherichia coli* isolated from 33 children up to 5 years old with diarrhoea.

Antibiotic[a] resistance pattern	No. of isolates belonging to different ribotypes							Total no. of isolates (%)		Total no. of patients (%)		Plasmid[b] pattern
	I	II	III	IV	V	VI	VII					
Tc	5	2	5	1	8	3	3	27	(48.2)	15	(45.4)	C,D
Tc,S	4	5	3	0	0	2	1	15	(26.7)	9	(27.2)	I,J
Tc,Am	0	0	0	2	0	2	0	4	(7.1)	2	(6.0)	B,E
Tc,S,Am,C	1	0	2	0	1	1	0	5	(8.9)	4	(12.1)	F
Tc,S,Am,N	0	0	0	0	0	2	0	2	(3.5)	1	(3.0)	H
Tc,S,Am,N,SxT	0	0	0	0	0	2	0	2	(3.5)	1	(3.0)	G
All sensitive	0	0	0	0	0	0	1	1	(1.7)	1	(3.0)	A
Total	10	7	10	3	9	12	5	56	(100%)	33	(100%)	

[a]Isolates were resistant to: Am = Ampicillin C = Chloramphenicol, Tc = Tetracycline S = Streptomycin N = Nalidixic acid, and SxT = Trimethoprim-sulfamethoxazole. All isolates were susceptible to Gentamicin.
[b]Of the Tc^R isolates, 9 produced plasmid pattern C, and 18 isolates produced pattern D; of the Tc^RS^R isolates 9 and 6 isolates produced plasmid pattern I and J respectively, and 2 each of the Tc^RAm^R isolates produced pattern B and pattern E.



1a 1b

Fig. 1. Plasmid DNA extracted from *E. coli* isolates were electrophoresed in 0.8% agarose gels and Southern blots derived from the gels were hybridized with a radioactively labelled probe for heat-labile toxin. A photograph of an ethidium bromide stained gel (1a) and the corresponding autoradiograph (1b) are shown. Lanes 1 through 5: plasmid patterns A through E, Lanes 8 through 12: plasmid patterns F through J, lanes 6 and 7: plasmid isolated from two non-toxigenic *E. coli* strains.

Ribotyping of all 56 LT$^+$ isolates was carried out using the enzymes *Hin*dIII, *Eco*RI, and *Bam*HI. Optimum differentiation of the strains was obtained with *Eco*RI and a total of 7 different ribotypes (Fig. 2) were detected among the 56 *E. coli* isolates. Ribotypes were shared by isolates with different antibiotic resistance patterns (Table).

Since resistance to antimicrobial agents is often mediated through plasmids which are transferable genetic elements (12), it is possible that susceptible strains of LT$^+$ *E. coli* acquired resistance to various antibiotics by spontaneous transformation due to conjugal transfer of plasmids from appropriate donors in

the environment. This assumption is supported by the rRNA gene restriction patterns (ribotypes) of the LT$^+$ *E. coli* isolates, since ribotypes were shared by strains with different antibiotic resistance patterns and also a strain susceptible to all the antibiotics tested in the study (Table).

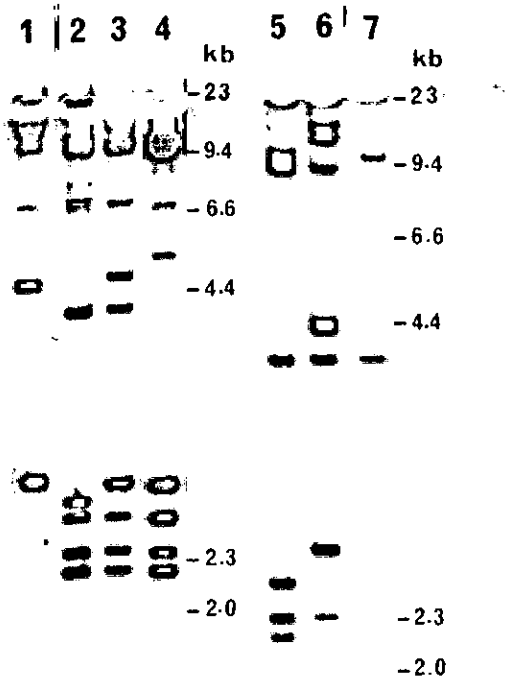


Fig 2. Southern hybridisation analysis of genomic DNA from *E. coli* isolates digested with *Eco*RI and probed with a 7.5 kb *Bam*HI fragment of the *E. coli* rRNA clone pKK3535. Lanes 1 through 7 show the patterns corresponding to 7 different ribotypes of *E. coli*. Numbers indicating molecular sizes of bands correspond to *Hind* III fragments of bacteriophage lambda DNA used as molecular size markers.

Plasmid analysis of LT-producing *E. coli* isolates showed 10 different plasmid profiles in isolates obtained from 33 patients. Identical drug resistance patterns did not in all cases correlate to an identical plasmid pattern. This indicates that the genetic determinants for drug-resistance were probably distributed among plasmids of various sizes. Plasmid profile and drug resistance have been used to identify and differentiate strains in epidemiological studies (14,15). Plasmid profiles have also been shown to correlate with drug resistance patterns in such studies (15). However, results of the present study show that strains with a similar plasmid profile are not necessarily identical, nor are strains with similar drug resistance patterns.

Southern-blot hybridisation of plasmid DNA (Fig. 1) showed that in all LT$^+$ strains the LT gene probe hybridised with the sheared chromosomal DNA, and in some strains the probe hybridised with more than one of the plasmid bands. The multiple hybridisation bands in some strains could be due to different forms (relaxed circle, supercoiled, and nicked circle or linear) of the LT$^+$ plasmid (Fig. 1). Hybridisation with chromosomal DNA indicated the chromosome comprised a sequence which shared some homology with the LT gene sequence. Whether the sequence was cryptic or encoded the production of LT was not determined. However, there are reports that genetic determinants for LT production can occasionally be chromosomally located (12,13).

Most of the LT$^+$ isolates (98.2%) were resistant to tetracycline and some of these strains were resistant to one or more other antibiotics in addition to tetracycline. These results suggest a high frequency of tetracycline resistance among LT$^+$ *E. coli*, and also that different clones of tetracycline-resistant LT$^+$ *E. coli* may have independently acquired the genetic determinants for resistance to other antibiotics, possibly by multiple conjugations with antibiotic-resistant microbial flora. This process may have been enhanced due to widespread and unrestricted use of antibiotics in the community, which favoured the resistant strains to predominate (25). In support of this assumption, the resistance of almost 100% of LT$^+$ strains to tetracycline could be correlated to the widespread use of tetracycline in Bangladesh for treatment of cholera (25,26) which resembles, to some extent, the watery diarrhoea caused by LT$^+$ *E. coli*. This also emphasises the need for controlled use of antimicrobial agents. The mechanism for the spread of antibiotic resistance should be seriously considered in a population like this where *E. coli* strains feature prominently in early childhood diarrhoea (4).

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