JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1988, p. 1477–1481
0095-1137/88/081477-05$02.00/0

Vol. 26, No. 8

Clonal Origin, Restricted Natural Distribution, and Conservation of Virulence Factors in Isolates of Enterotoxigenic *Escherichia coli* Serogroup O126

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Received 7 March 1988/Accepted 29 April 1988

Enterotoxigenic *Escherichia coli* serogroup O126 isolates have been isolated in Hong Kong since 1982 from sporadic cases of infantile diarrhea and from one outbreak in a neonatal ward. A 64-megadalton plasmid encoding colonization factor antigen I and heat-stable enterotoxin was identified in all 23 isolates. Enterotoxigenic *E. coli* strains producing heat-stable enterotoxin from different regions of Southeast Asia were collected and compared by biotyping, antibiotic resistance patterns, and plasmid profiles. Restriction endonuclease digestion of plasmids and subsequent Southern blot analysis with the heat-stable enterotoxin gene probe of representative strains showed a unique plasmid was harbored by all heat-stable enterotoxin-producing O126 strains tested. These results are consistent with conservative inheritance of enterotoxin plasmids within enterotoxigenic *E. coli* strains over a 2-year period in Hong Kong.

Enterotoxigenic *Escherichia coli* (ETEC) is primarily a water-borne pathogen associated with diarrhea of children and travellers (16). In an urban center such as Hong Kong, where there is a good water supply, it is a rare cause of diarrhea. In a study of 2,246 episodes of diarrhea among hospitalized patients aged 5 years or younger from May 1983 to October 1985, only 21 were attributed to ETEC infections (18). Consistent with the low endemicity of the infection, these enteric pathogens have not been isolated from non-diarrheal control subjects. All ETEC isolates except one were clonally diverse and were associated with sporadic cases. In a separate prospective study of 375 infants from birth to 2 years of age conducted during the same time period in an industrial suburban area of Hong Kong, only two strains of ETEC were isolated.

In contrast to the pattern described above, one ETEC strain persisted in hospitalized patients throughout the 2-year period of the study. It caused sporadic cases and an outbreak of diarrhea in the neonatal ward of a maternity hospital in September 1982 (18). Isolates of this strain were serogroup O126 and harbored the same or similar 64-megadalton (MDa) plasmid bearing the gene encoding heat-stable enterotoxin (ST-H). These are referred to as ST-H-O126 isolates; the clonal relationship between them was further indicated by their having the same biotype and pattern of antibiotic resistance. Additionally, these isolates were positive for the colonization factor antigen I (CFA/I) as indicated by the mannose-resistant hemagglutination test.

Studies on ST-H-O126 isolates show that they persisted in Hong Kong since 1982, causing an outbreak in a neonatal ward and sporadic cases in hospitalized patients. The distribution of this organism is geographically confined to Hong Kong and Guangzhou. It has not been detected in the tested isolates from other Southeast Asian countries and Japan. In this study, we further characterize the enterotoxin-bearing plasmid from these isolates to better understand its persistence in an urban center.

(This work was done in partial fulfillment of the Ph.D. degree at the University of Hong Kong by W.C.Y.)

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MATERIALS AND METHODS

Strains. A total of 23 ETEC (ST-H-O126) strains were collected from infants and children suffering from diarrhea in Queen Mary Hospital between May 1983 and October 1985 and one *E. coli* outbreak (15 strains) in another maternity hospital in Hong Kong in September 1982 (18). In another prospective study of 375 infants conducted during the same period in an industrial suburban area, one strain producing ST-H (134097) and one strain producing a heat-labile enterotoxin were isolated in two community clinics in 1984 by using DNA filter assays (12, 13, 17). One ST-H-O126 strain from Guangzhou, People's Republic of China, and three ST-H strains from Thailand collected in the same period were serogrouped in Hong Kong as described by Edwards and Ewing (7) and with the infant mouse assay (4) and the DNA filter assay (12, 13, 17). Four ST-H strains from Osaka, Japan, were also included in this study (17). The probes were prepared from bacterial strains harboring recombinant plasmids EWD299 (heat-labile toxin), pRIT 10036 (ST-P), and pSLM004 (ST-H) supplied by S. Falkow of Stanford University. M421C1 (ST-H) was provided by S. Moseley of the Childrens' Orthopedic Hospital and Medical Center, Seattle, Wash. *E. coli* K-12 strain JP995, used as a recipient in conjugation experiments, was given to us by J. Ling of the Chinese University of Hong Kong.

Plasmid curing. Six selected ST-H-O126 strains, including four sporadic isolates and one outbreak isolate (85652) in Hong Kong, and the isolate from Guangzhou were grown in L broth containing different concentrations of acridine orange (25 to 200 μg/ml) at 37°C for 24 h (2). Broth cultures showing slight turbidity after overnight incubation were subcultured on MacConkey plates. For each strain, 100 isolated colonies from subculture plates were picked for biotyping, antibiotic susceptibility testing, serogrouping, and CFA/I and ST-H screening. Cured derivatives negative for ST-H production were subjected to DNA analysis.

Plasmid conjugation. ST-H strains selected for plasmid curing were also subjected to conjugation experiments. Resistance factor (R-factor) transfer from these strains to *E. coli* K-12 strain JP995 was done in broth culture by standard methods for plasmid conjugation (3). Mueller-Hinton agar

plates containing rifampin (25 μg/ml), in combination with either streptomycin (10 μg/ml), chloramphenicol (10 μg/ml), tetracycline (10 μg/ml), or sulfamethoxazole (100 μg/ml), were used to screen for transconjugants carrying resistance factors transferred from ST-H strains. These transconjugants were biotyped, serogrouped, and screened for CFA/I and ST-H toxin, and their DNA was analyzed.

Detection of CFA/I and CFA/II. Bacterial growth of ETEC obtained from 18-h Casamino Acids-yeast extract-salts agar cultures grown at 37°C was subsequently subjected to hemagglutination with human group A, bovine, guinea pig, and chicken erythrocytes suspended in phosphate-buffered saline containing 1% mannose to detect CFA/I and CFA/II (8, 9).

Antibiotic resistance pattern. The disk diffusion method (1) was used for testing antibiotic resistance. Chloramphenicol, ampicillin, streptomycin, tetracycline, and sulfamethoxazole were tested.

Biotyping. Sugar fermentation tests were performed as described by Edwards and Ewing for identification of members of the family *Enterobacteriaceae* (7). Carbohydrates used for fermentation tests included adonitol, cellobiose, dulcitol, raffinose, L-rhamnose, salicin, sucrose, and D-xylose.

DNA analysis. Plasmid DNA was extracted by the method of Kado and Liu (10), electrophoresed in horizontal agarose gels, transferred to nitrocellulose by the method of Southern (15), and hybridized with ^{32}P-labeled ST-H probe as described previously (17). *E. coli* plasmids of known molecular mass, including 40R660 (25.9 MDa), 40R646 (37.8 MDa), 34R193 (31.7 MDa), 40R268 (64 MDa), 40R448 (77.6 MDa), and 28R823 (143.7 MDa), were electrophoresed in parallel with plasmid DNAs from the test strains. Plasmid DNAs from seven ST-H-O126 strains and one ST-H strain (134097) from Hong Kong, one ST-H-0126 strain from Guangzhou, and one ST-H strain (P179) from Thailand were further digested by endonucleases *Hin*dIII and *Hpa*II (Anglian Biotechnology, Ltd.), respectively, as described by Maniatis et al. (11), electrophoresed in 1% agarose horizontal gels, transferred to nitrocellulose by the method of Southern (15), and hybridized with ^{32}P-labeled ST-H probe as described earlier. A lambda phage DNA marker (*Hin*dIII digest) was electrophoresed in parallel with endonuclease-digested DNAs of ST-H strains.

RESULTS

Twenty-four ETEC ST-H strains isolated in Hong Kong, one from Guangzhou, three from Thailand, and four from Japan were characterized in this study. All but one Hong Kong strain were serogroup O126 and possessed CFA/I (Table 1). These strains harbored multiple plasmids, among which a 64-MDa plasmid carried the ST-H gene. The isolate from a Hong Kong community clinic (134097) differed in carriage of ST-H enterotoxin gene on a larger plasmid and the lack of both O126 serological specificity and CFA/I. The isolate from Guangzhou was similar to the 23 ST-H-O126 hospital strains from Hong Kong. None of the strains isolated from Thailand or Japan was identical to ST-H-O126 strains. The three strains from Thailand were not serogroup O126. Strain P204 possessed CFA/I, but its ST-H gene was encoded in a smaller plasmid. A 64-MDa plasmid harboring the ST-H gene was present in strain P179, but this strain lacked CFA/I. ST-H strains isolated from Japan did not harbor a 64-MDa plasmid encoding ST-H. They also lacked the O126 antigen, and only T130 possessed CFA/I. The ST-H plasmids from strains in Hong Kong, Guangzhou, and Thailand harbor multiple plasmids (Fig. 1). The ST-H gene is encoded by a plasmid of 64 MDa in all specimens except 134097, which has a larger 73-MDa plasmid encoding ST-H.

TABLE 1. Characteristics of ETEC ST-H strains

Source and strain no.	Serogroup	CFA type	Approx plasmid size (MDa)[a]
Hong Kong Hospital			
15 outbreak strains	O126	I	70, **64**, 51, 33
87099	O126	I	70, **64**
87175	O126	I	71, **64**, 27
83550	O126	I	70, **64**, 59, 29
92059	O126	I	70, **64**
12876	O126	I	**64**
104785	O126	I	68, **64**
112547	O126	I	71, **64**, 55
107108	O126	I	70, **64**, 56
Hong Kong community clinic			
134907			**73**, 69
Guangzhou			
G33	O126	I	70, **64**
Thailand			
P36			**71**
P179			**64**
P204		I	70, **60**, 54
Japan[b]			
T103			**50**
T122			**76**, 46, 42
T130		I	69, **56**, 52
T132			**70**

[a] Numbers in boldface type indicate plasmids carrying ST-H genes.
[b] ETEC strains of traveler's diarrhea from Osaka, Japan (18).

Further evidence for the clonal relationship of the 23 ST-H-O126 strains in Hong Kong and 1 isolate from Guangzhou is shown in Table 2. These 24 strains had identical sugar fermentation patterns and antibiograms. The other eight ST-H strains which lacked O126 displayed variable biotypes and antibiograms. During the course of this study, nontoxigenic strains of serogroup O126 were collected from hospitalized infants with diarrhea, and characteristics of these six strains are shown in Table 2. The heterogeneous patterns of sugar fermentation and antibiograms of these strains further support the unique epidemiological features of the ST-H-O126 strain in Hong Kong.

To determine whether the 64-MDa plasmid encoding ST-H also encodes other characteristics of these isolates, we treated representative strains with acridine orange. Among the resulting colonies antibiotic resistance and toxigenicity segregated independently. The colonization factor (CFA/I) and toxigenicity (ST-H) cosegregated. Southern blots of cured strains probed with ST-H (Fig. 2) showed disappearance of the plasmid, together with the loss of ST-H and CFA/I in subsequent tests. In all but one case, the organisms contained two plasmids, of which one was lost after curing. Multiple plasmids were present in strain 85652. After curing, one plasmid had a lower mobility but was not lost. Presumably a portion of the plasmid encoding ST-H in strain 85652 had been lost. In addition, among all 100 colonies from each tested strain after plasmid curing, the serogroup and biotype of ST-H-O126 were resistant to acridine orange treatment (data not shown).

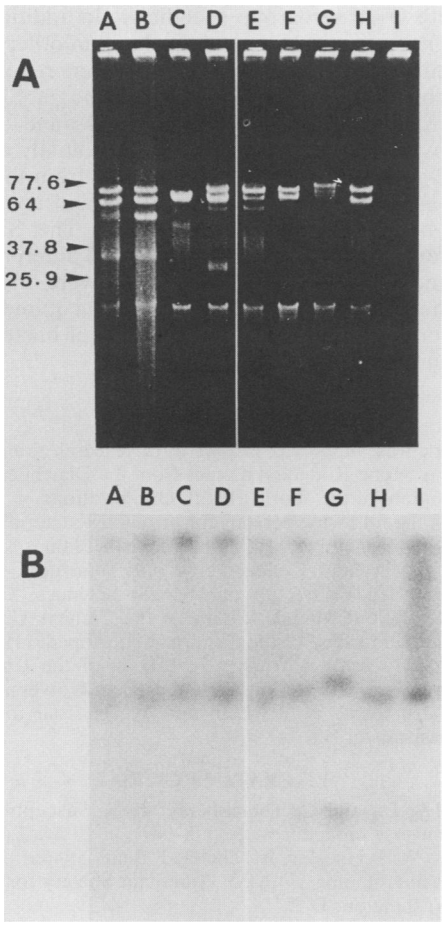

FIG. 1. Identification of ST-H plasmid in isolates from different geographical regions. (A) Ethidium bromide visualization of plasmids; (B) autoradiogram of plasmids probed with ST-H. Lanes: A and B, ST-H-O126 strains (outbreak cases); C through F, ST-H-O126 strains (sporadic cases); G, ST-H strain 134097 (community clinic); H, ST-H-O126 strain G33 (Guangzhou); I, ST-H strain P179 (Thailand).

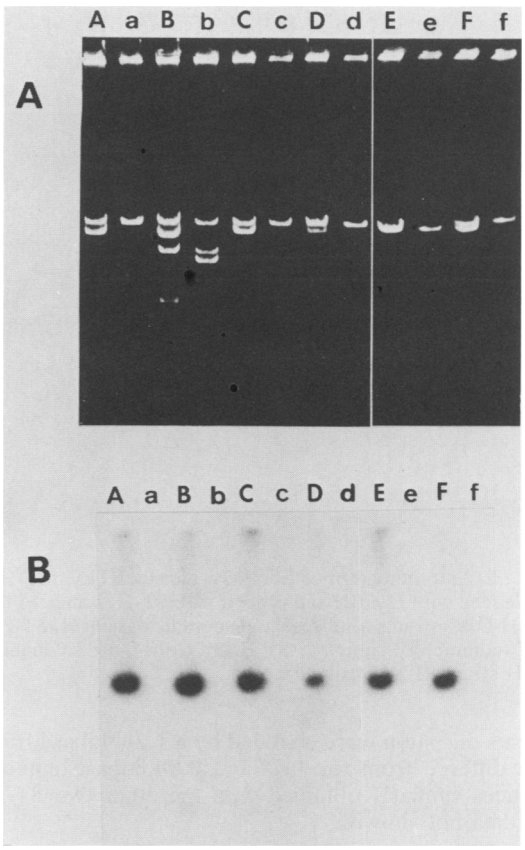

FIG. 2. Gel electrophoresis and Southern blot analysis of ST-H-O126 plasmids after curing with acridine orange. (A) Ethidium bromide visualization of plasmids; (B) autoradiogram of plasmids probed with ST-H probe. Lanes: A through F, strains G33, 85652, 104785, 92059, 12876, and 87099, respectively; a through f, cured derivatives of respective strains.

Conjugation experiments of the above strains with *E. coli* K-12 recipients revealed that cotransfer of toxin plasmid with R factor did not occur in four transconjugants carrying the antibiotic resistance plasmids. Among the six tested strains, conjugation was only successful with strain 85652. Transconjugants resistant to tetracycline were only detected at a transfer frequency of 10^{-7}. By DNA analysis the tetracycline resistance gene was found to be encoded by a 33-MDa plasmid (data not shown).

The 64-MDa plasmid which simultaneously bears the genes for ST-H and the CFA/I in ST-H-O126 strains apparently evolved separately and is indigenous to Hong Kong. A comparison of the Southern blots indicates that the ST-H and the CFA/I in ST-H-O126 strains apparently evolved separately and is indigenous to Hong Kong. A comparison of the Southern blots of ST-H ETEC isolated from other countries in Southeast Asia revealed a clonal diversity of these organisms (Table 1). The plasmids from representative isolates were purified and digested with *Hin*dIII, followed by gel electrophoresis and Southern blotting. Fragments carrying the toxin (ST-H) sequences were located with the radiolabeled ST-H probe as described previously. Only isolates of ST-H-O126 showed an identical 4.2-kilobase fragment carrying the toxin sequences after *Hin*dIII digestion (Fig. 3). The enterotoxin sequences identified from the two other ST-H isolates were 2.9 and 3.3 kilobases. These results were confirmed by using another restriction enzyme, *Hpa*II. The enterotoxin sequences obtained from ST-H-O126 strains

TABLE 2. Characteristics of ETEC ST-H and enteropathogenic *E. coli* serogroup O126

ST-H production	Serogroup O126	No. of isolates fermenting carbohydrate/total								No. of strains susceptible/total				
		Adonitol	Cellobiose	Dulcitol	Raffinose	L-Rhamnose	Salicin	Sucrose	D-Xylose	Ampicillin	Streptomycin	Chloramphenicol	Tetracycline	Sulfamethoxazole
+	+	0/24	0/24	0/24	24/24	24/24	24/24	24/24	24/24	24/24	0/24	0/24	0/24	0/24
+	−	0/8	0/8	5/8	6/8	7/8	5/8	6/8	7/8	7/8	6/8	7/8	5/8	7/8
−	+	0/6	1/6	6/6	3/6	6/6	5/6	4/6	6/6	4/6	3/6	5/6	4/6	2/6

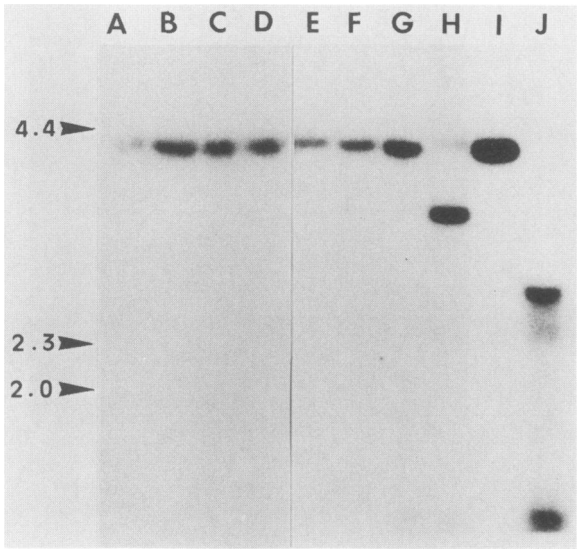

FIG. 3. Autoradiogram of Southern blot of ETEC (ST-H) plasmids cleaved with *Hin*dIII and probed with ST-H. Lanes: A through G, ST-H-O126 strains (outbreak and sporadic cases); H, ST-H strain 134097 (community clinic); I, ST-H-O126 strain G33 (Guangzhou); J, ST-H strain P179 (Thailand).

after this digestion were encoded by a 1.2-kilobase fragment which differed from the 0.7- and 0.8-kilobase enterotoxin sequences similarly obtained with the other two ST-H isolates (data not shown).

DISCUSSION

It was evident that ST-H-O126 strains isolated from hospitalized diarrheal patients and those from an outbreak in a maternity hospital are clonally related. These organisms persisted in Hong Kong, at least since 1982, causing severe diarrhea in infants and young children territory-wide. This strain appears indigenous to Hong Kong and Guangzhou, and there is no evidence yet for its spread beyond these confines.

The organisms were found to be simultaneously positive for the ST-H enterotoxin and CFA/I. The latter characteristic is a pathogenic attribute of some ETEC. Both ST-H and CFA/I are encoded on a plasmid of 64 MDa, and these pathogenic attributes segregate coordinately after treatment of the organism with acridine orange. This is similar to the finding of genes encoding both ST-H and CFA/I on the same plasmid by previous workers (5). The organism also exhibited the same pattern of antibiotic resistance and sugar fermentation. The O126 serogroup, on the other hand, was probably chromosomally determined and consequently was not cured by treatment with acridine orange, although plasmid-encoded lipopolysaccharide O polysaccharide expression by EPEC has recently been reported (14). The identity of the enterotoxin plasmid harbored by different ST-H-O126 isolates was further established by endonuclease digestion followed by Southern blotting. As described previously, the prevalence of ETEC infection in Hong Kong is substantially lower than that reported from its neighboring countries (18). Also unlike the case in its neighbors, asymptomatic carriage of ETEC in Hong Kong is rare (17). The number of isolates available for studying natural distribution of this plasmid was limited. Nonetheless, it has not yet been found to occur in any other of the tested ST-H ETEC or in enteropathogenic *E. coli* with O126 serogroup specificity. In addition, ST-H-O126 strains were not commonly isolated in other countries (5, 6), and detailed studies have not been reported. Our results show that the enterotoxin plasmid of ST-H-O126 isolated from the different sporadic cases and a hospital outbreak were identical and has been essentially conserved over the 2-year study period. The isolates from the hospital outbreak have the same plasmid profile and are therefore likely to be of the same clonal origin. The ST-H-O126 isolates from sporadic cases, however, had different plasmid profiles, and it is not certain whether they were originally the same clone of bacteria which has since acquired different plasmids or were different related strains of bacteria which have acquired the same 64-MDa plasmid.

ACKNOWLEDGMENTS

This work was supported in part by a research grant from the University of Hong Kong and a grant from the Diarrhoeal Diseases Control Program of the World Health Organization.

We thank the following persons for providing bacterial strains: S. Falkow of Stanford University, Stanford, Calif.; James Kaper of the University of Maryland, College Park; Y. K. Yan and G. Y. Guo of the Sun Yat Sen University of Medical Sciences, Guangzhou, People's Republic of China; J. Ling of the Chinese University of Hong Kong; S. Moseley of the Children's Orthopedic Hospital and Medical Center, Seattle, Wash.; T. Takeda of the University of Tokyo, Japan; and T. Pramote of Khon Kaen University of Thailand. We also thank W. P. Lam and W. H. Lau for excellent technical assistance.

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