Genetic relationships and virulence factors among classical enteropathogenic *Escherichia coli* serogroup O126 strains

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Summary. Thirty-nine *Escherichia coli* strains of the enteropathogenic (EPEC) serogroup O126 isolated from sporadic and outbreak cases of infantile diarrhoea between 1982 and 1988 were studied. These strains consisted of four serotypes showing close genetic relationships between their virulence markers, outer-membrane protein and lipopolysaccharide profiles, and electrophoretic types by multilocus enzyme electrophoresis. None of these strains exhibited localised adherence to HEp-2 cells or the attaching-effacing properties of classical type I EPEC. Of the 39 strains, 31 were of serotype O126:H12 and enterotoxigenic; one strain was serotype O126:H10 and enteroaggregative. The remaining six strains of serotype O126:H21 and one strain of serotype O126:H8 harboured no known virulence factors for diarrhoeagenic *E. coli*.

Introduction

The principal evidence for enteropathogenicity in *Escherichia coli* has been derived from epidemiological studies of different outbreaks. Certain strains of *E. coli* have been implicated as causative agents of several clinically distinct diarrhoeal syndromes.^{1,2} Five recognised classes of *E. coli*—enteropathogenic (EPEC), enteroinvasive (EIEC), enterohaemorrhagic (EHEC), enteroaggregative (EAaggEC) and enterotoxigenic (ETEC)—can cause diarrhoeal diseases in man. Each class has a characteristic phenotype and corresponding genotype which allows differentiation from non-pathogenic strains. Among the pathogenic attributes of EPEC is the capacity to produce distinctive microscopic lesions in the intestinal mucosa, known as attachment and effacement, and it is associated with a chromosomal locus, termed *eae*.³ A second genetic locus, found in most EPEC strains, is the EPEC attachment factor (EAF). This is located on a plasmid that regulates expression of *eae* and also codes the ability of bacteria to adhere to cultured epithelial cells in a distinctive pattern known as localised adherence.⁴ As in shigellosis, EIEC invade and multiply within the mucosal cells lining the large bowel, causing inflammation and ulceration. It has been shown that the invasive property of EIEC is borne on a plasmid.⁵ For EHEC, two types of cytotoxin have been recognised and implicated as possible causes of haemorrhagic

colitis.⁶ ETEC elaborate a heat-labile enterotoxin (LT) resembling cholera toxin (CT), a heat-stable enterotoxin (ST), or both. Genes encoding production of LT and ST occur on transmissible plasmids.⁷ *E. coli* with aggregative adherence patterns to intestinal mucosa are known as EAaggEC; they are associated epidemiologically with persistent diarrhoea in infants, especially in developing countries.⁸

Earlier studies have indicated that *E. coli* possess a clonal population structure consisting of groups of bacteria with highly uniform properties; therefore, the bacteria within each group can be considered to have descended from a single ancestral cell, and can be referred to as clones.⁹ A successful taxonomic analysis of *E. coli* population structures would yield valuable insight into microbial ecology and pathogenicity. Because of the potential instability of many microbial traits, clonal analysis of pathogenic *E. coli* by new molecular techniques has proved to be more discriminating than conventional methods for epidemiological typing.¹⁰

E. coli strains of the O126 serogroup are implicated in human enteric disease and the serogroup is recognised as one of the classical EPEC O groups.^{1,2} The aim of this study was to assess the overall genetic relationships and virulence along O126 strains obtained from cases of infantile diarrhoea collected over a 6-year period by means of outer-membrane protein (OMP) and lipopolysaccharide (LPS) profiles, virulence markers, enzyme polymorphisms detected by multilocus enzyme electrophoresis and other phenotypic traits.

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Materials and methods

Strains

Thirty-nine isolates of *E. coli* serogroup O126 were collected from infants and children suffering from diarrhoea in Hong Kong and Guangzhou, People's Republic of China, between 1982 and 1988, as described previously.^{11,12} These included 15 strains isolated from an outbreak at a maternity hospital in Hong Kong during 1982. Selected DNA probes were investigated for their ability to hybridise with the individual test strains by colony and Southern blotting techniques as described previously.^{11,12} The DNA probes were derived from the following plasmids (the corresponding virulence-associated property is given in parentheses): pEWD299 (LT, heat-labile toxin); pSLM004 (ST-H, heat-stable enterotoxin of ETEC, human subtype); pRIT10036 (ST-P, heat-stable enterotoxin of ETEC, porcine subtype); pCVD419 (adhesion-associated plasmid of EHEC); pCVD432 (AggA, adhesion-associated plasmid of EAggEC); pSLM852 (DA, associated with diffused-adherence ability of *E. coli*); pJPN16 (EAF, associated with the plasmid-encoded localised-adherence phenotype of EPEC); pCVD434 (*eae* gene, associated with the attaching-effacing ability of *E. coli*); pJN37-19 (Shiga-like toxin type I of EHEC); pNN110-18 (Shiga-like toxin type II of EHEC); and pRM17 (enteroinvasive capacity of EIEC).^{11,13-16}

DNA analysis

Plasmid DNA was extracted by the method of Kado and Liu.¹⁷ Electrophoresis was performed in horizontal agarose gels, and after transfer to nitrocellulose by the method of Southern, hybridisation was performed with ³²P-labelled DNA probes as described previously.^{11,12}

Serogrouping and serotyping

Serogrouping of enteropathogenic *E. coli* (EPEC) strains was performed with Burroughs Wellcome *E. coli* antisera by standard procedures.^{18,19} EPEC was screened by slide agglutination, initially with polyvalent O antisera then with monovalent O antisera, of *E. coli* colonies. The serogrouping results were confirmed by tube agglutination of heat-inactivated *E. coli* suspensions with monovalent O antisera. Serotyping with H antisera was performed by Y. Takeda of the University of Tokyo and T. Tsukamoto of Osaka Prefectural Institute of Public Health, Osaka.

Outer-membrane protein analysis

OMPs were prepared by the method of Achtman *et al.*²⁰ The OMPs, extracted from 100 ml of a mid-exponential phase bacterial culture, were suspended in 50 µl of electrophoresis sample buffer containing 0.0625 M Tris, pH 6.8, SDS 2% v/v, glycerol 10% v/v,

bromophenol blue 0.002% v/v and mercaptoethanol 5% v/v. Each sample was boiled for 10 min before SDS gel electrophoresis. A polyacrylamide gel was prepared with a resolving gel 10% w/v overlaid by a stacking gel 2.5% w/v. Resolving gel buffer was 3 M Tris, pH 8.8, and stacking gel buffer was 0.5 M Tris, pH 6.8. The tank running buffer contained 0.25 M Tris, pH 8.3, 1.92 M glycine and SDS 1% w/v. Samples were loaded and electrophoresis was performed at a constant current of 20 mA (stacking gel) and 30 mA (resolving gel) for 4 h. The gel was stained with Coomassie Blue 0.3% w/v overnight at room temperature, destained with a solution of methanol:acetic acid:water (3:1:6). A protein mol.-wt marker (Rainbow[®]) was electrophoresed in parallel with the samples.

Lipopolysaccharide analysis

Whole-cell lysates were prepared according to the method of Hitchcock *et al.*²¹ A polyacrylamide gel was prepared with resolving gel 12.5% w/v overlaid by a stacking gel 4% w/v. For each sample, 60 µl of extract was subjected to SDS-PAGE with Tris-glycine SDS 0.1% w/v buffer, pH 8.3 at 35 mA for 3 h followed by silver staining.²¹

Enzyme electrophoresis

Cultures were grown overnight at 37°C in 100 ml of nutrient broth. Cells were harvested by centrifugation at 15000 g for 10 min, suspended in buffer containing 10 mM Tris, 1 mM EDTA, 0.5 mM NADP, pH 6.8, and sonicated (20 cycles, 15 s on, 20 s off; MSE Soniprep 150 cell disrupter) with cooling in an ice bath. Unbroken cells were removed by centrifugation at 30000 g for 20 min and 10 µl of the supernate was subjected to horizontal starch gel electrophoresis at 40 V for 4 h; selective protein staining was performed as described previously.²² The following nine enzymes were assayed: malate dehydrogenase, 6-phosphogluconate dehydrogenase, adenylate kinase, β-galactosidase, phosphoglucose isomerase, isocitrate dehydrogenase, glucose 6-phosphate dehydrogenase, mannose phosphate isomerase and leucine aminopeptidase. For each enzyme, distinctive mobility variants were designated as electromorphs and numbered in order of decreasing rate of anodal migration. Distinctive profiles of electromorphs, corresponding to unique multilocus genotypes, were designated as electrophoretic types (ETs).

Haemagglutination tests

Colonisation factor antigens I and II (CFA/I and CFA/II) were detected by the mannose resistant haemagglutination test (MRHA) with human group A, bovine, guinea-pig and chicken erythrocytes.^{23,24} Cultures of *E. coli* were grown overnight at 37°C on CFA agar casamino acids 1% w/v, yeast

Table. Characteristics of *E. coli* serogroup O126

Number of isolates	H-type	Strain no.	ET	Iso-enzyme score*	LPS pattern	OMP pattern	Toxin	CFA/I	HEP-2 cell adhesion	DNA probe hybridisation
2	H12	A1-A2	1	113112212	1	1	ST	+	NA	ST-H
3	H12	B1-B3	2	113102212	1	1	ST	+	NA	ST-H
26	H12	C1-C26	3	112112212	1	1	ST	+	NA	ST-H
5	H21	D1-D5	4	121213213	2	2	...	—	NA	...
1	H21	E1	5	111211111	2	2	...	—	NA	...
1	H8	F1	1	113112212	2	3	...	—	NA	...
1	H10	G1	6	121213213	3	4	...	—	AA	AggA

LPS, Lipopolysaccharide; OMP, outer-membrane proteins; NA, not adherent; AA, aggregative adherence; ET, electrophoretic type; ST, heat-stable enterotoxin; CFA, colonisation factor antigen; ST-H, heat-stable enterotoxin of ETEC, human subtype; AggA, adhesion-associated plasmid of EAggEC.

* Listed in order: malate dehydrogenase, 6-phosphogluconate dehydrogenase, adenylate kinase, β -galactosidase, leucine aminopeptidase, phosphoglucose isomerase, isocitrate dehydrogenase, glucose 6-phosphate dehydrogenase and mannose phosphate isomerase.

Fig. 1. Identification of ST-H plasmid in representative isolates of *E. coli* serogroup O126: **a**, ethidium bromide visualisation of plasmids; **b**, autoradiogram of plasmids probed with ST-H. Lanes: **A**, O126: H8 (strain F1); **B, D–G**, O126: H21 (strains D1, D4, E1); **C**, O126: H10 (strain G1); **H**, *E. coli* K12; **I–N**, O126: H12 (strains A1, C5, B2, B3, C1, C20).

extract 0.15% w/v, MgSO_4 0.005% w/v, MnCl_2 0.0005% w/v in agar 2% w/v. Erythrocytes were washed several times with phosphate-buffered saline (PBS), pH 7.2, and the washed cells were diluted 1 in 4

with PBS to test for haemagglutination and 1 in 4 with mannose 1% w/v in PBS to test for MRHA. *E. coli* cultures from CFA agar were mixed with a drop of the appropriate species of blood (c. 0.02 ml) on a glass

Fig. 2. SDS-PAGE of OMPs from representative isolates of *E. coli* O126. Clonal designations of strains according to the table are shown at the bottom. Lanes: A, O126:H8 (strain F1); B–D, O126:H21 (strains D1, D4, E1); E, O126:H10 (strain G1); F–L, O126:H12 (strains A1, C5, C8, B2, B3, C1, C20).

slide at room temperature. After observation for 1 min, the slides showing less than maximum haem-agglutination were placed on ice and observed for at least 2 min with intermittent mixing by rotation of the slide.

Detection of bacterial adhesion to cell culture

The pattern of bacterial adherence to HEp-2 epithelial cells was assayed as reported previously.^{16, 25, 26} The fluorescent actin staining (FAS) assay, which corresponds to the ability of bacteria to produce attachment-effacement lesions in the intestine, was performed with 3- and 6-h incubation periods as described previously.^{27, 28, 29}

Assay for enterotoxin

Cytotoxin production was assayed in Vero cells by the method of Marques *et al.*³ Biken and infant mouse assays were used to assay LT and ST of ETEC.^{31, 32}

Results

Characteristics of O126 strains

All the isolates in this study were obtained from infants with diarrhoea from whom no other con-

ventional intestinal pathogen was isolated.^{11, 12} The most frequency serotypes present were O126:H12 (31 isolates) and O126:H21 (six isolates) (table). Isolates of H8 and H10 were found once only during the 6-year study period. DNA hybridisation showed that only the 31 O126:H12 strains harboured the ST-H toxin gene and produced positive results with the infant mouse assay. A common plasmid of identical molecular size (64 kDa) was detected in these strains (fig. 1). These strains also gave positive results for CFA/I by MRHA. Only one strain (O126:H10) was classified as EAggEC by the characteristic pattern of adherence to HEp-2 cells and by the ability to hybridise with the corresponding DNA probe. Assays for enterotoxins and adhesion to HEp-2 cells correlated with DNA hybridisation for all probes used in this study. None of the strains possessed the virulence-associated properties of classical type I EPEC, EHEC or EIEC.

Genetic relationships in the O126 serogroup

To determine whether *E. coli* O126 strains associated with infantile diarrhoea were clonally related, the associated virulence and genetic relationships of O126 strains, which had been isolated over a 6-year period from Hong Kong and the People's Republic of China were examined. These strains were grouped into four

Fig. 3. SDS-PAGE of whole-cell lysates of representative isolates of *E. coli* O126 stained by LPS silver staining. Clonal designations of strains according to the table are shown at the bottom. Lanes: A, O126:H21 (strain D1); B, O126:H10 (strain G1); C, O126:H12 (strain A1).

serotypes, with distinct virulence genes, ETs, OMP and LPS patterns (table). Each serotype possessed a unique OMP pattern. All isolates had major OMPs of

mol. wt from 21–42 kDa (fig. 2). Four major OMP patterns were identified. On LPS analysis, O126:H12 and O126:H10 strains exhibited patterns 1 and 3, whereas O126:H21 and O126:H8 strains exhibited an identical LPS pattern, type 2 (fig. 3). In all 39 isolates, seven of the nine enzymes assayed were polymorphic. Comparisons of the multilocus genotypes of isolates revealed six ETs and the electrophoretic variation in isocitrate dehydrogenase is shown (fig. 4). Different serotypes of O126, except for the only O126:H8 strain, showed distinct ETs. The O126:H8 strain exhibited an ET identical to that of two isolates of O126:H12. All the 15 O126:H12 strains (nos. C3–C17) isolated from the same outbreak in 1982 exhibited ET 3. As regards virulence traits, ST-H and CFA/I were found associated only with O126:H12, and enteroaggregative activity was associated with O126:H10. Six strains of O126:H21 and one strain of O126:H8 had none of the known virulence factors associated with diarrhoeagenic *E. coli*. All *E. coli* O126 isolates, except O126:H12 which harboured a plasmid of uniform size (64 kDa) coding for bacterial virulence, possessed diverse plasmid DNA profiles (fig. 1).

Discussion

The *E. coli* O126 strains examined were isolated at different times from various epidemiological sources. Some clonal isolates exhibited uniform properties. Correlation of virulence factors, serotypes, ETs, migration patterns of major OMPs and LPS was observed among the strains collected over a 6-year period. We propose that there is no causal association among these properties, rather that they are independent, conserved characteristics of clonal groups. It is clear from this study that there is a clonal association

Fig. 4. Electrophoretic variation in isocitrate dehydrogenase of representative isolates of *E. coli* serogroup O126. Lanes: A–F, O126:H12 (strains A1, C5, B2, B3, C1, C20); G, O126:H8 (strain F1); H–J, O126:H21 (strains D1, D4, E1); K, O126:H10 (strain G1).

of *E. coli* O126 with H serotypes. It has been reported with other pathogenic *E. coli* that clonality is strongly associated with H, rather than with O serotypes.³³

Plasmid analysis was unsuitable for recognising clonal groups, with the exception of O126:H12 strains, since most bacteria possess too many different plasmids. However, OMP analysis is a powerful tool which has been found to correlate well with other phenotypic characters for studying the clonal nature of EPEC.^{33,34} Minor differences in OMP patterns 2 and 3 suggest that O126:H21 and O126:H8 are subclones. This is supported by the identical LPS patterns of these two serotypes. The multilocus electrophoresis technique attempts to determine overall genetic relatedness among isolates by examination of a small fraction of the structural gene loci of the genome. Except for only a few strains of ET 1 present in both O126:H12 and O126:H8, each serotype consisted of strains with unique ETs.

These findings suggest that characterisation of phenotypic attributes is essential for understanding the relationships among pathogenic *E. coli*, even of the one O serogroup. In this collection of O126 strains, both ETEC and EAggEC were found along with two groups lacking known virulence factors. None of the strains belonged to the classic EPEC O126:H2 subtype, or harboured the EAF or *eae* virulence factors which are associated mainly with type I EPEC, as reported recently.²⁹ Different pathogenic mechanisms may be associated with clonal groups in *E. coli* O126 strains. In recent years, the identification of virulence factors has enabled the subdivision of classical EPEC strains into classes causing diarrhoea by different mechanisms.¹ This study confirms previous findings that strains belonging to the same EPEC serogroup are a heterologous group.³⁵ The existence of invasive and toxigenic *E. coli* strains in the same serogroup has been reported for serogroup O167.³⁶

LPS composition may contribute to the virulence of EPEC by favouring selection of strains for the establishment, maintenance, or infectivity of the clone.³³ This may account for the persistence of

O126:H12 (with type 1 LPS pattern) in this 6-year study period. A previous study showed that the enterotoxin (ENT) plasmid associated with O126:H12 is identical¹² and the present findings on the serotype, ETs, OMP and LPS patterns of this clone further confirm the identity of the bacterial host harbouring this ENT plasmid. Maintenance of this plasmid within a cell line for at least 6 years suggests that it confers a selective advantage. In this study, the number of isolates available for the examination of the genetic relationships and pathogenic mechanisms of *E. coli* O126 was limited. However, *E. coli* O126 was not detected among 100 control subjects during the same period.¹¹ Since toxigenic strains of O126 were not commonly isolated in neighbouring countries,³⁷ O126:H12 may represent a recent clone of ETEC from the EPEC serotype O126:H12 which has acquired the ENT plasmid in the locality. Indeed, a predominant ETEC strain that produced only ST and belonged to serotype O126:H12 was reported recently from Thailand.³⁸ The latter strain may be identical to the isolates in this study, and may reflect the spread of this clone in Southeast Asia. The finding that O126:H10, categorised as EAggEC, is noteworthy in the light of the recent report by Scotland *et al.* that the EPEC strains of serotype O126:H27, obtained from patients with diarrhoea in the UK, were in fact EAggEC.³⁹

Further study of *E. coli* as a diarrhoeal agent depends on increased understanding of the pathogenesis of the disease. This study presents the results of comprehensive screening of virulence factors in diarrhoeagenic *E. coli*. Plasmid-borne pathogenic attributes such as enterotoxins, CFA or enteroaggregation were detected in the majority of the strains. However, none of the *E. coli* strains of serotypes O126:H21 and O126:H8 possessed any of the pathogenic attributes investigated.

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