Production of Cytolethal Distending Toxin and Other Virulence Characteristics of *Escherichia coli* Strains of Serogroup O86

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Genetic and phenotypic virulence markers of different categories of diarrhoeagenic Escherichia coli *were investigated in 106 strains of enteropathogenic* E. coli *(EPEC) serogroup O86. The most frequent serotype found was O86:H34 (86%). Strains of this serotype and the non motile ones behaved as EPEC i.e., carried eae, bfpA and EAF DNA sequences and presented localised adherence to HeLa cells. Serotypes O86:H2, O86:H6, O86:H10, O86:H18, O86:H27 and O86:H non determined, belonged to other categories. The majority of the strains of serotype O86:H34 and non motile strains produced cytolethal-distending toxin (CDT). The ribotyping analysis showed a correlation among ribotypes, virulence markers and serotypes, thus suggesting that CDT production might be a property associated with a universal clone represented by the O86:H34 serotype.*

Key words: enteropathogenic *Escherichia coli* - serogroup O86 - cytolethal distending toxin - virulence markers - diarrhoea

Escherichia coli strains associated with diarrhoeal disease were first classified on the basis of their somatic (O) and flagelar (H) antigens. In 1987, the World Health Organization (WHO) recognized as enteropathogenic *E. coli* (EPEC) the following serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158 (WHO 1987). With the introduction of tissue culture assays and DNA probes to study the virulence of *E. coli* strains associated with diarrhoea, it has been possible to demonstrate the existence of at least six established or putative categories of diarrhoeagenic *E. coli*: enteropathogenic *E. coli* (EPEC), enterohemorragic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and difuselly adhering *E. coli* (DAEC). Recently, a new category termed atypical EPEC has been proposed (Nataro & Kaper 1998). In addition to the defining characteristics, strains of some serogroups might present other virulence properties that could contribute to their pathogenesis.

Studies carried out by several authors in the last few years, have shown that the EPEC serogroups are very heterogeneous. In general, they include more than one of the seven diarrhoeagenic *E. coli* categories (with the exception of EIEC) and each category have specific H antigens, corresponding to defined *E. coli* serotypes (Campos et al. 1994, Rodrigues et al. 1996, Gonçalves et al. 1997, Valle et al. 1997).

The first *E. coli* strain of serogroup O86 (*E. coli* E990) was identified by Taylor and Charter (1952) as the cause of an outbreak of acute diarrhoea in children committed to day care in London. Since then, strains of this serogroup, mainly of serotype O86:H34, have been isolated in outbreaks and sporadic cases of diarrhoea in many parts of the world (Toledo et al. 1983, Gomes et al. 1989, Scotland et al. 1996, Cravioto et al. 1996).

Cytolethal Distending Toxin (CDT) is a heatlabile factor which induces a progressive distension and death of various cell lines cultivated in vitro. CDT production has been detected in the supernatants of some *E. coli*, *Shigella* spp., *Campylobacter* spp., *Haemophilus ducrey* and *Actinobacillus actinomycetemcomitans* strains (Johnson & Lior 1987, 1988a,b, Cope et al. 1997, Mayer et al. 1999). However, so far, CDT produc-

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tion has not been associated to any particular category of diarrhoeagenic *E. coli*, except for a few strains of EPEC serogroups including the O86:H34 serotype (Johnson & Lior 1988a, Guth et al. 1994).

The purpose of this study was to determine the virulence characteristics of serogroup O86 strains isolated in Brazil and in other countries and to investigate the relationship between serotypes and ribotypes in this serogroup.

MATERIALS AND METHODS

Bacterial strains and serotyping - The study employed 90 *E. coli* strains which were isolated from faeces of children with diarrhoea at Instituto Adolfo Lutz, São Paulo, Brazil, between 1977 and 1991. For comparative purposes we included 14 *E. coli* strains from other countries, kindly provided by Dr Fleming Schultz, from Department of Gastrointestinal Infections, Denmark and *E. coli* strains from Chile and Centers for Disease Control (CDC), Atlanta, Ga, included in Dr Luiz R Trabulsi's collection.

The original strains kept at room temperature were grown in Triptic Soy Broth (TSB) and streaked out onto nutrient agar for serogroup confirmation and determination of H antigens (Edwards 1986).

Adherence to HeLa cells - Adherence was assayed in the presence of 1% D-mannose (Cravioto et al. 1979). Briefly monolayers were examined after 3 h of incubation, and poorly adherent and non-adherent strains were submitted to a period of assay of 6 h *E. coli* strains E2348/69, O42, and C1845 were used as control of localized, aggregative and diffuse adherence, respectively (Valle et al. 1997).

Hybridisation with DNA probes - All strains were submitted to colony hybridisation assays (Maas 1983), using specific DNA probes for different categories of diarrhoeagenic *E. coli*: *eae* (*E. coli* attaching and effacing gene encoding intimin), EAF (EPEC adherence factor plasmid), *bfpA* (bundle forming pilus), *daaC* (associated with diffuse adherence), EAEC (associated with aggregative adherence), INV (*E. coli* invasiveness), Stx-1 and 2 (Shiga-toxin types 1 and 2), LT-I and II (heat-labile enterotoxin types I and II), ST-I p and h (heat-stable enterotoxin type I, from *E. coli* of porcine and human origin, respectively) (Rodrigues et al. 1996). The CDT probe used was the 1,375 bp- *Acc* I fragment of pCVD448 derived from *E. coli* E6468/62 (O86:H34) (Scott & Kaper 1994). Probes were isotopically labelled and used in colony hybridisation assays as described elsewhere (Rigby et al. 1977).

Citotoxicity assays - CDT production was investigated in CDT probe positive and negative strains by testing in HeLa cells as described previouly (Johnson & Lior 1988a). Briefly, strains were grown statically in 2 ml of Brain Heart Infusion broth (BHI) at 37°C, for 48 h. The cells were pelleted and the supernatant fraction was retained and filtered. To prepare the sonicate, the pellet was resuspended in 1 ml of Eagle's Minimal Essential Medium, modified with Earle's salts (MEM) and the suspension was sonicated as described by Pickett et al. (1994). Supernatants and sonicates were diluted 1:4 and 1:10 respectively for the assays and morphological changes were monitored every 24 h for 5 days. Strain 0741-4 (O86:H34) (Guth et al. 1994) was used as positive control. Sonicates presenting activity similar to that of the CDT control were submitted to neutralisation assays with rabbit anti-CDT serum, kindly provided by Dr H Lior. Equal volumes of sonicate diluted 1:5 and antisera (imunne and non-imunne) diluted 1:128 in MEM, were mixed and incubated at 37°C. After 2 h the mixture was submitted to citotoxicity assays.

Ribotyping - The genomic DNA of 33 strains carrying different H antigens and virulence markers, was extracted by the method described by Brenner et al. (1982). Approximately 2 µg of DNA were digested with *Bgl*I (Sigma) and eletrophoresed with a marker (*Haemophilus aegyptius* strain, 320/86), digested with *Eco*RI (Dalla-Costa et al. 1998). The DNA was transferred onto a nylon membrane (Magnagraph, USA) in a vacuum blotting system (Vacum Gene XL, Pharmacia), according to the manufacture's instructions. A cDNA probe prepared by reverse transcriptation of 16S plus 23S rRNA (Boerhinger, Germany) and labelled using the digoxigenin DNA labelling and detection Kit (Boerheinger) as described (Popovic et al. 1993).

RESULTS

The 106 strains tested, belonged to six distinct serotypes. Seven strains were non motile (O86:H) and the H antigen of one strain could not be determined (O86:H?) (Table I).

The data in Fig. 1 showed that O86:H34 was the predominant serotype in São Paulo, between 1977 and 1982.

None of the strains studied reacted with the probes for *daaC*, INV, LT-I and II, ST-Ip and h, and Stx 1 and 2. Table II shows the results obtained with the other probes and the adherence patterns presented by different strains. Presence of EPEC sequences (*eae*, EAF and *bfpA*) was detected in most strains of serotype O86:H34 (72 of 78 Brazilian strains and all 6 strains of other countries). Moreover, these probe positive strains produced localised adherence (LA) in HeLa cells. Four of 7 O86:H- strains had these same properties. All 10

TABLE I

Sources and flagellar antigens (H types) of 106 *Escherichia coli* strains

Origin	Period of isolation	No. of strains	No. of the H type							
			H?	H-	H2	H6	H10	H18	H27	H34
São Paulo (Brazil)	1977-1991	90	1	7	-	1	2	1	-	78
Denmark	1966-1989	9	-	-	5	-	-	1	1	2
Guine Bissau	1990	3	-	-	2	-	-	-	-	1
India	1986	2	-	-	1	-	-	-	-	1
CDC (Atlanta)	Unknow	1	-	-	-	-	-	-	-	1
Chile	Unknow	1	-	-	-	-	-	-	-	1
Total		106	1	7	8	1	2	2	1	84

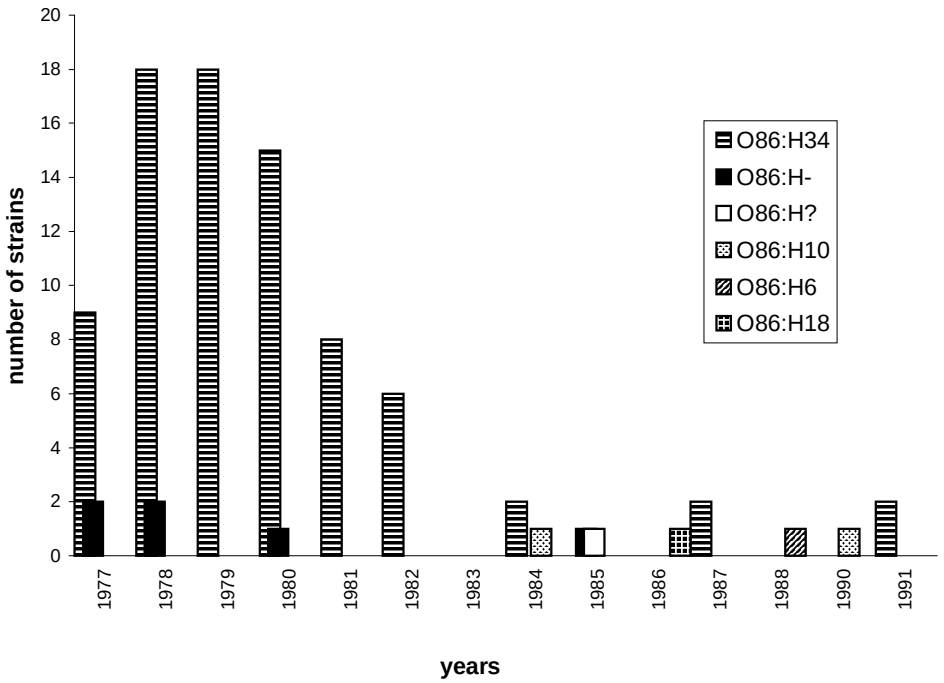

Fig. 1: distribution of *Escherichia coli* strains belonging to six serotypes of Enteropathogenic *E. coli* serogroup O86, São Paulo, 1977-1991.

strains of serotypes O86:H18 and O86:H2 produced the aggregative adherence (AA). Strains of the remaining serotypes were *eae* positive; (O86:H6, O86:H?) or lacked any virulence property searched for; (O86:H27, O86:H10).

Most of the strains (79 of 85) of serotypes O86:H34 and O86:H- isolated in São Paulo and all strains of serotype O86:H34 from other countries had the CDT sequence and produced a progressive distention on HeLa cells after 48 h of incubation. The distending activity of the sonicates was completely neutralized by rabbit antiserum to CDT.

Among the *E. coli* O86 strains isolated in São Paulo we observed five ribotypes (RT) named RT-A to RT-E. The O86:H34 and the virulent O86:H- strains, including the prototype strain E990 (O86:H-), belonged to RT-A. The O86:H- with no virulence sequences belonged to RT-B. The serotypes O86:H10, O86:H18 and O86:H6 belonged to RT-C, RT-D and RT-E, respectively. The O86 *E. coli* strains isolated in other countries belonged to four ribotypes, RT-I to RT-IV. The O86:H2, O86:H18, O86:H34 and O86:H27 strains belonged to RT-I, RT-II, RT-III and RT-IV, respectively (Fig. 2).

TABLE II
Virulence properties of 106 *Escherichia coli* O86 strains

Serotype	Origin	Virulence DNA sequences	CDT Probe	Adherence patterns
		eae, *bfpA*, EAF (72)	+(68)	LA[a] (72)
H34 (78)	Brazil	*eae* (4)	+(4)	I[b] (4)
		bfpA, EAF (2)	+(2)	LA[a] (2)
H34 (6)	Denmark, India, G. Bissau, Chile, CDC	*eae*, *bfpA*, EAF (6)	+(6)	LA[a] (6)
H27 (1)	Denmark	None (1)	-	NA (1)
H18 (1)	Brazil	EAEC (1)	-	AA[a] (1)
H18 (1)	Denmark	EAEC (1)	-	AA[a] (1)
H10 (2)	Brazil	None (2)	-	NA (2)
H6 (1)	Brazil	*eae* (1)	-	NA (1)
H2 (8)	Denmark, G. Bissau, India	EAEC (6)	-	AA[b] (6)
		None (2)	-	AA[b] (2)
H-(7)	Brazil	*eae*, *bfpA*, EAF (4)	+(4)	LA[a] (4)
		eae (1)	+(1)	NA (1)
		None (2)	-	NA (2)
H? (1)	Brazil	*eae* (1)	-	NA (1)
E990 (O86:H-)	Prototype strain	*eae* (1)	+(1)	I[b] (1)

a: adhesion test of 3 h; *b*: adhesion test of 6 h; LA: localized adherence pattern; AA: aggregative adherence pattern; I: indefinite adherence pattern; NA: not adherent; ( ) no. of strains

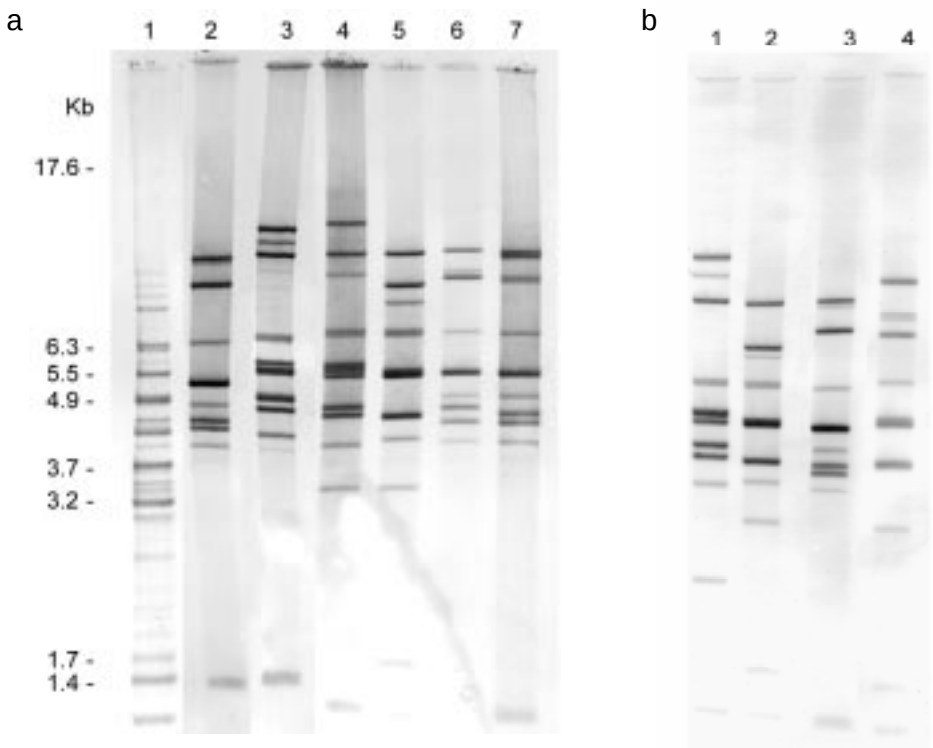

Fig. 2: ribotypes and serotypes of *Escherichia coli* strains, serogroup O86. a: isolated in Brazil. Slot 1-Marker; Slot 2-RT-A, O86:H34 and O86:H-(with virulence sequences); Slot 3-RT-B, O86:H-(with no virulence sequences); Slot 4-RT-C, O86:H10; Slot 5-RT-D, O86:H18; Slot 6-RT-E, O86:H6; Slot 7-RT-A, E990, O86:H-(with virulence sequence); b: isolated in other countries Slot 1-RT-I, O86:H2; Slot 2-RT-II, O86:H18; Slot 3-RT-III, O86:H34; Slot 4-RT-IV, O86:H27

DISCUSSION

In this study, we examined a diverse collection of *E. coli* O86 strains isolated from human diarrhoea, in different countries. We observed that the most frequent serotype of serogroup O86, found in São Paulo (O86:H34) (Toledo et al. 1983, Gomes et al. 1989) is not the most frequent in other countries (Scotland et al. 1996, Giamanco et al. 1996). Therefore, geographic variation in the predominance of the EPEC serotypes may occur. The serotype O86:H34 was predominant in São Paulo between 1977 and 1991 and since then, it has been isolated no more frequently than the other less common serotypes of this serogroup, found in São Paulo. This kind of distribution suggested the ocurrence of an outbreak, which started before or at the beginning of 1977 and disappeared in 1982, or that this serotype was endemic until 1990, when its frequency started to fall (Fig. 1).

Typical EPEC strains, i.e., strains carrying EAF, *bfpA*, *eae* and presenting LA in the 3 h adherence assay, were found in 82 (77.4%) of the strains studied, which were comprised in the serotypes O86:H34 and O86:H-. The strains of serotypes O86:H18 and 6 of 8 O86:H2 strains were categorised as EAEC, since they presented the aggregative adherence pattern and hibridised with the EAEC probe. Lack of hybridisation with the EAF probe in four O86:H34 strains and with the EAEC probe in two O86:H2 strains, is probably attributed to genetic alterations that often occur in E. coli stored for long periods (Rodrigues et al. 1996, Gonçalves et al. 1997). Strains of serotypes O86:H6 and O86:H? showed the characteristics of atypical EPEC (i.e., *eae* only). No sequences associated with intestinal infections were found in the strains of serotypes O86:H27 and O86:H10 (Table II). The data described above, agreed with the findings reported by other authors (Scotland et al. 1996, Cravioto et al. 1996, Giamanco et al. 1996).

Of particular interest in this study was the demonstration that besides having all the virulence characteristics of typical EPEC, most of the strains of serotypes O86:H34 and the virulent O86:H- strains produced CDT, and it could act as an additional virulence factor of diarrhoea. Although O86:H34 and O86:H- may be considered as distinct serotypes, it is likely that the virulent H- strains were derived from O86:H34 strains, because they have identical virulence characteristics and were isolated in the same period of time (Fig. 1, Table II). Until now, among the EPEC serotypes, frequent production of CDT seemed to be an exclusive characteristic of the serotype O86:H34. In fact, in a survey of CDT production by EPEC serogroups O55, O86, O119, O126, O127 and O142, Guth et al. (1994) found that only O86:H34 strains produced CDT. CDT producing strains have been reported among *E. coli* strains isolated in Canada and India, although in one of these studies serotyping was not performed (Bouzari et al. 1990) and in the other one, a single CDT positive strain of serotype O86:H34 was found (Johnson & Lior 1988a). A significant percentage of CDT producing strains belonging to the same serotype has not been reported so far. The role of CDT in the pathogenicity of *E. coli* strains has not been studied. It is possible that CDT production alone, has no implication for pathogenesis but its production by *E. coli* strains in combination with other virulence factors, such as the ability to adhere to the intestinal mucosa, may be significant. Albert et al. (1996) did not find an important association between CDT positive *E. coli* strains and diarrhoea but it has been shown in a recent study that this toxin has diarrhoeagenic properties in an animal model (Okuda et al. 1997).

We found an association between O:H serotypes and ribotypes among the studied strains. This relationship allowed the identification of non motile strains (O86:H-) by comparing their fingerprints with those of the motile strains, i.e., RT-B (O86:H-) and RT-I (O86:H2), the prototype strain E990 (O86:H-), and RT-A and RT-III (O86:H34) (Fig. 2). This finding confirmed that this kind of flagellar variation occurred with relatively high frequency, a phenomenon that has also been observed in other EPEC serogroups (Rodrigues et al. 1996, Gonçalves et al. 1997). Despite of that, the ribotype presented by the O86:H34 strains isolated in Brazil (RT- A) was the same of the O86:H34 strains isolated in other countries (RT- III) (Fig. 2), suggesting that CDT production could be associated with a clone distributed all over the world represented by strains belonging to the serotype O86:H34.

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