IDENTIFICATION AND TYPING OF BACTERIA

Serotyping and categorisation of *Escherichia coli* strains isolated between 1958 and 1992 from diarrhoeal diseases in Asia

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A total of 3065 strains of *Escherichia coli* isolated between 1958 and 1992 from patients with diarrhoea in different countries were examined for virulence factors by hybridisation with biotinylated DNA probes for genes that coded for production of heat-labile and heat-stable enterotoxins, enteroinvasiveness, production of verotoxins and attaching-and-effacing factor and were serotyped. Of the 3065 strains, 1998 were placed into one of four pathogenic categories by their virulence factors: 1057 enterotoxigenic *E. coli* (ETEC) comprising 30 O-groups, 73 serovars and 137 untypable strains; 132 enteroinvasive *E. coli* (EIEC) comprising 11 O-groups and 13 serovars; 64 verotoxin-producing *E. coli* (VTEC) comprising 11 O-groups, 17 serovars and 13 untypable strains; and 745 enteropathogenic *E. coli* (EPEC) comprising 34 O-groups, 92 serovars and 91 untypable strains. The remaining 1067 strains did not hybridise with any of the DNA probes used. About half the number of O-groups recognised were not restricted to a single pathogenic category, although the combinations of O- and H-antigens were different in each category.

Introduction

Following recognition as a human diarrhoeagenic pathogen and the establishment of a serotyping system, *Escherichia coli* serovars associated with diarrhoeal diseases have gained increasing interest in all parts of the world. There are numerous reports of the serotypes of *E. coli* strains isolated from patients with diarrhoea. Initially the classification of enteropathogenic *E. coli* isolates relied essentially on historical and epidemiological findings, because the mechanism by which *E. coli* produced diarrhoeal diseases was not known. Now four principal categories of pathogenic *E. coli* are recognised in diarrhoeal diseases according to the virulence factors implicated: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), verotoxin (Shiga-like toxin)-producing or enterohaemorrhagic *E. coli* (VETC or EHEC) and enteropathogenic *E. coli* (EPEC).

Since 1958, serotyping of *E. coli* isolates from patients with diarrhoea in Japan has been carried out routinely at the Enterobacteriology Laboratory of the National Institute of Health, Tokyo. The results obtained up to 1974 were reported by Sakazaki *et al.* [1, 2]. From 1980, in addition to serotyping, various tests to demonstrate the virulence potential of each of the four categories of *E. coli* have been introduced into the laboratory routine as they became available. In this report, the results of serotyping and of assays of virulence factors on 3065 strains of *E. coli* collected up to 1992 are described.

Materials and methods

E. coli strains

The 3065 isolates of *E. coli* examined were received originally for serotyping at the National Institute of Health, Tokyo between 1958 and 1992. All were submitted as isolates from patients with diarrhoea in Japan, Southeast Asia, India and the Middle East. However, epidemiological information such as patient age and scale of infection was not available in most cases. After an initial examination for purity, and

Received 2 Sept. 1995; revised version accepted 15 March 1996.

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biochemical characterisation, all the strains were stored in rubber-stoppered bottles on semi-solid medium containing Bacto-Casitone (Difco) 1%, yeast extract 0.3%, NaCl 0.5% and agar 0.3% and were kept at room temperature in the dark.

Serotyping

All strains were serotyped with 176 O-antisera representing 173 recognised O-antigens of *E. coli*, three O-antigens from Ewing (OX2, OX4 and OX13) to which antigen numbers have not been assigned and 57 *E. coli* H-antisera. The agglutination methods used were based on those described by Ewing [3].

Virulence factor assay

Although 650 strains in this collection examined before 1985 were tested for enterotoxin production with Y1 adrenal cells and infant mice and for invasiveness with HEp-2 cells, the remaining 2415 strains were assayed for genes coding for the production of both heat-labile and heat-stable enterotoxins (LT and ST), enteroinvasiveness, production of verotoxins (VT1 and VT2), and *E. coli* attachment-and-effacing factor (EAE) with biotinylated DNA probes as described previously [4–6]. The following specific gene probes were used: LT, a 850-bp *HincII* fragment from plasmid pEWD 299 [7]; ST1a, a 157-bp *EcoRI*-*Bam*HI fragment from pDAS 101 [8]; ST1b, a 215-bp *Bam*HI-*Pst*I fragment from pDAS 100 [8]; EIEC, a 17-kbp *EcoRI* fragment from pRM 17 [9]; VT1, a 1142-bp *Bam*HI fragment from pJN 37-19 [10]; VT2, a 842-bp *Pst*I-*Sma*I fragment from pNN 110-8 [10]; and EAE, a 1-kbp *Sal*I-*Kpn*I fragment from pCVD 434 [5]. The DNA probe fragments were labelled with biotin-7-dATP (BRL).

Pathogenic categorisation of strains

E. coli strains with demonstrable virulence factors were categorised according to the following criteria: ETEC for strains that hybridised with the LT or ST probe or both; EIEC for strains that hybridised with the probe for EIEC; VTEC for strains that hybridised with the VT1 or VT2 probe or both; and EPEC for strains, regardless of their serovar, that hybridised with the EAE probe but not the VT probes.

More recently, two additional categories of pathogenic *E. coli*, enteroaggregative *E. coli* (EAggEC) and diffuse adherent *E. coli* (DAEC), have been identified. However, these two categories were not considered in this study, as their significance as causes of diarrhoeal disease is still controversial.

Results

Of 3065 strains of *E. coli* examined, virulence factors were demonstrated in 1998. These were divided into

the following groups: 1057 ETEC, 132 EIEC, 64 VTEC and 745 EPEC. The serovars of 1757 of these 1998 strains were identified, but those of the remaining 241 strains could not be determined either because they were in the R-form or because they did not react with any of the antisera. The serovars of the strains in each pathogenic category are shown in Tables 1–4.

ETEC (Table 1)

Most of the ETEC strains were from outside Japan. The 1057 ETEC strains consisted of 920 serotypable and 137 untypable strains. The 920 typable strains comprised 30 O-groups and 73 serovars. O-groups most frequently recognised included O6, O8, O15, O25, O27, O63, O73, O78, O114, O119, O126, O127, O128, O146, O148, O153, O159, O161, O167, O168 and O169. Of these O-groups, O6 which included two serovars, O6:K15:H16 and O6:K15:NM, was the predominant O-serogroup representing 292 (31.7%) of the 920 ETEC typable strains. Significant differences in the geographic distribution of strains with O-groups recognised frequently were not observed.

EIEC (Table 2)

The 132 strains that hybridised with the probe for EIEC represented 11 O-groups. Most of EIEC strains were non-motile; motile strains were found in only nine instances. Of the 11 O-groups, O28, O124, O136 and O144 were predominant. None of these 11 O-groups occurred among the other pathogenic categories, ETEC, VTEC and EPEC. Forty-four of the strains were late fermenters of lactose and did not produce gas from glucose.

VTEC (Table 3)

The serovars of 51 of the 64 strains that hybridised with VT1 or VT2 probes or both were determined. The 51 strains comprised 11 O-groups and 17 serovars. Of the 11 O-groups, O157 was the most frequent followed by O103, O111, and O26. Many of the VTEC strains, but not all, also hybridised with the EAE probe.

EPEC (Table 4)

EPEC strains are usually characterised by the production of attaching-and-effacing factor (EAE), causing lesions in the enterocyte brush border membrane, as well as by the production of fimbrial adherence factor (EAF). However, the EAE factor is also produced by VTEC strains and the adherence factor EAF occurs only in some EPEC serovars (class 1 EPEC). Therefore, in the present study the EPEC category was defined as strains that hybridised with the EAE probe but not with any of LT, ST and VT probes.

Of the 745 strains included in the EPEC category, 654 were identified to serovar level. The 654 strains

Table 1. Serovars of 1057 strains of enterotoxigenic *E. coli*

O-group			H-antigen	Enterotoxin			Number of strains
				LT	ST	LT+ST	
6	16(152)	NM(140)		112	15	165	292
8	2(8)	9(21)	32(4)	16	0	46	62
11	27(6)	33(2)	NM(1)	9	0	0	9
15	10(6)	11(13)	NM(3)	18	0	4	22
20	11(2)	NM(2)		0	2	2	4
25	7(6)	42(3)	NM(20)	8	0	21	29
27	7(15)	20(16)	NM(5)	0	30	6	36
63	6(5)	12(4)	NM(6)	15	0	0	15
73	18(9)	34(9)	NM(5)	23	0	0	23
78	11(11)	12(15)	NM(8)	20	9	5	34
82	8(8)	NM(1)		0	0	9	9
114	NM(18)			18	0	0	18
115	8(2)	33(3)	40(1)	0	0	11	11
119	6(15)	NM(2)		9	0	8	17
126	12(25)	NM(10)		0	29	6	35
127	9(19)	NM(2)		3	18	0	21
128	7(2)	12(43)	21(3)	6	15	32	53
146	7(3)	21(3)	NM(10)	1	6	9	16
148	28(52)	NM(6)		0	58	0	58
153	7(4)	10(5)	NM(8)	0	17	0	17
158	NM(5)			1	0	4	5
159	21(8)	34(10)	NM(22)	0	40	0	40
161	NM(15)			15	0	0	15
162	NM(5)			5	0	0	5
163	NM(5)			0	5	0	5
165	NM(2)			2	0	0	2
166	15(3)	NM(7)		7	1	2	10
167	5(8)	NM(9)		7	0	10	17
168	23(4)	41(9)	NM(10)	10	2	11	23
169	10(1)	41(5)	NM(11)	2	15	0	17
Subtotal				307	262	351	920
Unknown or undetermined serovars				45	31	61	137
Total				352	293	412	1057

Figures in parentheses indicate the number of strains.

NM, non-motile strain.

Table 2. Serovars of 132 strains of enteroinvasive *E. coli*

O-group	H-antigen	Number of strains
7	NM(3)	3
28	NM(26)	26
29	NM(8)	8
124	30(2)	17
136	NM(29)	29
143	NM(8)	8
144	25(7)	17
152	NM(6)	6
164	NM(6)	6
171	NM(5)	5
173	NM(7)	7
Total		132

Figures in parentheses indicate the number of strains.

NM, non-motile strain.

comprised 34 O-groups and 92 serovars. The O-groups found most frequently were: O18, O26, O44, O55, O86, O103, O111, O114, O119, O125, O126, O127, O-128, O142, O153, O159, O160, O165, O166 and O167. Five strains of serogroup O157, a well-known VTEC O-group, gave positive reactions in tests for sorbitol fermentation and β -glucuronidase activity, unlike the VTEC O157 strains.

Discussion

In the study, 1067 of 3065 *E. coli* strains did not hybridise with any of the virulence factor gene probes used, although some of the strains belonged to the same O-groups or serovars as those of the ETEC, EPEC or VTEC category. However, the *E. coli* strains studied were collected over a period of 30 years. It is considered likely, therefore, that some had lost genes for virulence factors during storage and subculture.

The allocation of strains to the EPEC and VTEC categories in this study must also be considered. As well as EAE and verotoxin production, adherence factors are important for virulence potential in these *E. coli* strains. However, the adherence factor in EPEC and VTEC is not limited to a single category. Indeed, EAF, which is an adherence factor in EPEC strains, is present only in certain EPEC O-groups, termed class 1 [11]. It is interesting that enteroaggregative strains of *E. coli* (EAggEC) have been implicated epidemiologically as one of the aetiological agents of diarrhoea [12–14]. Even in EPEC serogroups, it has been suggested that EAggEC strains are probably more important than those which are EAF-positive [15]. On the other hand, it has also been proposed that some *E. coli* strains showing 'diffuse adherence' (DAEC) also

Table 3. Serovars of 64 strains of verotoxin-producing *E. coli*

O-group	H-antigen		Verotoxin			eae	Number of strains
			VT1	VT2	VT1 + VT2		
1	1(1)		0	1	0	0	1
26	11(4)	NM(2)	2	3	1	6	6
55	7(1)	10(3)	0	4	0	3	4
103	2(4)		0	4	0	3	4
111	8(5)	30(1)	6	2	0	8	8
113	21(5)	NM(2)	0	4	1	1	5
128	8(3)	NM(2)	2	3	0	4	5
157	7(8)	NM(5)	1	7	5	13	13
163	NM(1)		0	1	0	0	1
165	NM(1)		0	0	1	1	1
172	NM(3)		0	3	0	3	3
Subtotal			11	32	8	42	51
Unknown or undetermined serovars			3	7	3	10	13
Total			14	39	11	52	64

Figures in parentheses indicate the number of strains.

Table 4. Serovars of 745 strains of enteropathogenic *E. coli*

O-group	H-antigen					Number of strains
1	7(6)					6
18	14(3)	NM(12)				15
26	2(2)	8(3)	12(1)	21(3)	NM(10)	19
44	18(6)	28(2)	33(2)	NM(7)		17
55	5(3)	6(12)	33(4)	34(4)	NM(36)	59
63	6(5)	NM(4)				9
86	21(2)	27(24)	34(7)	47(2)	NM(2)	37
103	19(1)	NM(13)				14
111	2(2)	5(1)	12(7)	21(8)	NM(28)	46
114	6(3)	10(3)	32(7)	NM(13)		26
119	6(65)	21(3)	NM(18)			86
125	19(3)	21(13)	NM(8)			24
126	21(8)	27(3)	NM(4)			15
127	10(2)	21(4)	NM(18)			24
128	2(4)	41(5)	NM(8)			17
142	6(25)	33(11)	NM(19)			55
146	1(2)	NM(6)				8
151	10(6)	NM(2)				8
153	11(2)	NM(15)				17
157	39(3)	NM(2)				5
158	23(3)	42(1)				4
159	19(5)	38(6)	NM(9)			20
160	7(1)	33(4)	48(2)	NM(9)		16
162	NM(5)					5
163	6(1)	45(5)	NM(3)			9
165	7(5)	NM(15)				20
166	4(6)	6(2)	NM(10)			18
167	9(5)	33(3)	45(3)	NM(21)		32
168	10(1)	NM(5)				6
169	34(1)	45(1)	NM(1)			3
171	NM(4)					4
OX2	9(2)					2
OX4	8(4)					4
OX13	8(4)					4
Subtotal						654
Unknown or undetermined serovars						91
Total						745

Figures in parentheses indicate the number of strains.

OX2, OX4 and OX13 are O-groups designated by Ewing.

NM, non-motile strain.

cause diarrhoea. However, the aetiological and epidemiological significance of DAEC remains controversial [16–21]. Similarly, the EAE gene occurs not only in EPEC, but also in VTEC strains. VTEC strains in which the gene coding for verotoxin production has been lost may thus be misidentified as EPEC strains.

Many of the serovars listed in the Tables, particularly those that occurred frequently, resemble those reported by other investigators [3, 12, 22–36]. For example, the principal serogroups of strains isolated from human diarrhoeal diseases by Ørskov and Ørskov [22] were found in this collection, although some differences of serovars were observed. A high proportion of ETEC

isolates from Southeast Asia and the Middle East belong to a limited number of serogroups [23, 24, 29]. Similar results of serogroup distribution among ETEC isolates from Spain were reported [31, 32]. In his review of adhesion and virulence of EPEC, Law [37] described EPEC serogroups and serovars traditionally associated with infantile diarrhoea. Most of these serogroups and serovars were also predominant in our EPEC collection. Scotland *et al.* [34] and Echeverria *et al.* [25] reported serogroups of EPEC found recently in diarrhoeal cases in Britain and Thailand, respectively. All of these serogroups were present in the EPEC strains in our collection. Although there is some variation, these findings suggest that the serovars of *E. coli* that cause diarrhoea may be more or less the same throughout the world. As has already been reported by others [22, 34, 38, 39], the occurrence of distinct categories of pathogenicity within a single O-group was observed frequently in this study. Of the 59 O-groups listed here, 24 occurred in more than one pathogenic category, although the combination of O- and H-antigens differed with the category. In this respect, five strains of O-serogroup 157 in the EPEC category are of interest as Schmidt *et al.* [40] and Scotland *et al.* [41] also reported non-toxigenic O157 strains. These non-toxigenic strains of O157 not only possessed H-antigens other than H7 but also fermented sorbitol and produced β -glucuronidase, unlike VTEC O157 strains. These findings suggest that phenotypic characterisation, including H-antigen determination, may be important for understanding the relationships among the different pathogenic categories.

E. coli remains an important cause of infantile diarrhoea in developing countries. In industrial countries, although outbreaks caused by such *E. coli* occur occasionally, they constitute an important finding in travellers returning from warmer countries with diarrhoea. However, diarrhoeal disease caused by infection with ETEC and EPEC strains in adults and children is self-limiting. The full identification of strains of *E. coli* that cause diarrhoea includes serotyping based on both O- and H-antigens as well as determination of virulence factors by various methods such as tissue cell culture assays and hybridisation with gene probes or PCR. These procedures are complex and time-consuming and, for this reason, are generally restricted to reference laboratories even in developed countries. However, EIEC strains produce dysentery-like symptoms and there is often person-to-person transmission of infection. In the absence of antisera for the EIEC serovars, antisera for *Shigella* may be used for presumptive identification because the O-antigens of EIEC serovars are closely related to those of *Shigella* serovars. Furthermore, H-antigen determination is not necessary for EIEC strains as most of them are non-motile. On the other hand, outbreaks of haemorrhagic colitis due to VTEC infection are a serious problem in industrial

countries. Although various serovars are included in the VTEC category most outbreaks of haemorrhagic colitis are caused by O-group 157. In view of its importance, a selective isolation agar was developed for VTEC O157 strains [42] but as it does not detect all VTEC serovars, further identification of isolates from cases of haemorrhagic colitis may be necessary. Thus, the identification of EIEC and VTEC O157 strains can be achieved readily in routine laboratories with a small number of diagnostic antisera and only a few biochemical tests.

We thank Dr P. Echeverria, US Medical Institute of Research, Bangkok, for providing plasmids encoding virulence genes of *E. coli*.

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