

Original Article

The Relationship between O-Antigens and Pathogenic Genes of Diarrhea-Associated *Escherichia coli*

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SUMMARY: To evaluate the serogrouping-based diagnosis of diarrheagenic *Escherichia coli*, a total of 1,130 strains of *E. coli* isolated in several countries were studied. The strains were regarded as enterovirulent on the basis of their O-antigens determined using a commercially available kit containing 43 antisera, and the presence of diarrhea-associated genes (*eae*, *stx*, *aggR*, *est*, *elt*, *ipaH*) was evaluated by PCR. Two hundred sixty-three strains of 1,130 (23.3%) were identified as diarrheagenic based on the presence of at least one pathogenic gene. The probability that *E. coli* identified as diarrheagenic on the basis of serogrouping actually possessed some pathogenic gene was highest for serogroup O119 (78.4%); other serogroups with a positive rate for pathogenic genes higher than 60% were O111 and O126. No target genes were detected among the strains belonging to serogroups O1, O29, O112ac, O143, O158 and O168. Our results suggest that, in practice, serogrouping is useful for the identification of diarrheagenic *E. coli* in a very limited number of serogroups.

INTRODUCTION

Escherichia coli is a member of human intestinal flora and usually remains harmlessly in the intestine. However, the possibility of enteric infection with *E. coli* was already recognized in the early 1900s, and antigenic determination of *E. coli* associated with diarrhea was reported by Bray in 1945 (1). The term enteropathogenic *E. coli* (EPEC) was proposed by Neter et al. to refer to certain specific serotypes (2), and by the mid-1950s, serotyping-based EPEC had been incriminated as an important cause of diarrhea (3). Thereafter, enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC) in 1971 (4,5), enterohemorrhagic *E. coli* (EHEC) in 1982 (6), and enteroadherent *E. coli* in 1985 (7) were also reported and accepted as diarrheagenic *E. coli* (DEC). Since the pathogenic factors of DEC have been clarified, they are now identified by detecting these specific factors. The basic characteristics of EPEC are attaching and effacing (A/E) histopathology (8) and the absence of Shiga toxin (Stx). All factors necessary to produce A/E lesions are encoded in the 35.5-kb chromosomal region known as the locus of enterocyte effacement (LEE) (9). EIEC is defined as Sereny test-positive *E. coli* (9). The pathogenicity-based identification of EPEC and EIEC are too laborious, therefore, identification of these *E. coli* usually depends on PCR to detect the pathogenic genes in the research fields. However, in clinical laboratories, the traditional serotyping- or serogrouping-based method has been widely used. In 1987, Levine proposed 10 specific O-antigens (10) as the most important serogroups of EPEC (O26, O55, O86a, O111, O119, O125, O126, O127a, O128,

O142); these are referred to as class I classical EPEC. He also proposed an additional 4 serogroups as less important (O18, O44, O112ac, O114, class II classical EPEC) and another 9 O-antigens (O28ac, O29, O124, O136, O143, O144, O152, O164, O167) as the serogroups of EIEC (10).

In Japan, a kit of antisera to 43 O-antigens (including the 23 identified by Levine) called "pathogenic *E. coli* immune sera" is commercially available without specifying each category of DEC and is widely used in clinical laboratories to determine DEC. Therefore, it is important to clarify the relationship between O-serogroups and pathogenic factors. Previous studies have determined the reliability of serogrouping-based diagnosis in a limited number of strains or serogroups (11-13). Giammanco et al. (11) studied the prevalence of pathogenic genes in strains belonging to 12 EPEC serogroups in Italy, and found that 75% of the strains identified as EPEC by commercial antisera may possess potential virulence properties. However, only 17.5% of *E. coli* strains isolated in Japan that agglutinated with one of the 43 O-serogroups included in the kit were recognized to have pathogenic properties (14). Therefore, there is now a need to evaluate the reliability of serogrouping-based diagnosis of diarrheagenic *E. coli* in a wide number of strains isolated from diverse areas. The purpose of the present study was to clarify the positive rate of pathogenic genes in *E. coli* strains isolated in different countries and belonging to 42 specific O-serogroups determined by using antisera of Japanese preparation.

MATERIALS AND METHODS

Bacterial strains: The strains to be examined were restricted to *E. coli* with one of the 42 O-antigens shown in Table 1 (since most of *E. coli* O157 strains were intentionally collected as Shiga toxin-producing *E. coli* (STEC), serogroup O157 was omitted in this study). The organisms were collected

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Table 1. Antisera against *E. coli* O-antigens group

polyvalent sera	O-antigens						
1 (7 serogroups)	1	26	86a	111	119	127a	128
2 (6 serogroups)	44	55	125	126	146	166	
3 (6 serogroups)	18	114	142	151	157	158	
4 (6 serogroups)	6	27	78	148	159	168	
5 (5 serogroups)	20	25	63	153	167		
6 (4 serogroups)	8	15	115	169			
7 (5 serogroups)	28ac	112ac	124	136	144		
8 (4 serogroups)	29	143	152	164			

Table 2. Countries and years of isolation for the strains included in this study

Countries	District	Year of isolate	Origin	No. of isolates
Japan	Hyogo	1996	diarrheal patients	15
	Shizuoka	2000	diarrheal patients	72
	Nagasaki	1994-2000	diarrheal patients	129
	Okinawa	2001	diarrheal patients	3
	Oita	2003	diarrheal patients	137
Lao PDR	Vientiane	1996-2002	diarrheal patients	326
	Vientiane	1996	healthy children	50
	Khammouan	1996	healthy villagers	64
Vietnam	Hanoi	1984-1987	diarrheal patients	43
	Hanoi	2002	diarrheal patients	37
Indonesia	Surabaya	1996-2000	pediatric diarrhea	187
Dominican Rep.	Santo Domingo	1996	diarrheal patients	61
Kenya	Nairobi	1981	diarrheal patients	6

in epidemiological studies conducted in a variety of countries between 1981 and 2003. The strains included in this study were selected to ensure that they were isolated from different people that were not linked temporally. Most of the strains were isolated from sporadic cases of infection and several small outbreaks; only one strain from each outbreak was included in this study. A total of 1,130 strains, 356 from Japan, 440 from the Lao People's Democratic Republic (Lao PDR), 61 from the Dominican Republic, 187 from Indonesia, 80 from Vietnam and 6 from Kenya, were examined. Of the 1,130, 1,016 and 114 isolates were collected from diarrheal and non-diarrheal stools, respectively (Table 2).

Determination of O-antigens: *E. coli* strain proliferated on a nutrient agar plate was suspended in normal saline solution and autoclaved for 15 min, then examined by slide agglutination using commercially available antisera in a kit identified as "pathogenic *E. coli* immune sera" (Denka Seiken Co., Ltd., Tokyo, Japan) (Table 1).

Terminology: In the present paper, we tentatively defined the various types of DEC as follows: "EPEC" or "true EPEC" indicates *E. coli* having the intimin gene (*eae*) and not

having the Stx gene (*stx*); "Class I classical EPEC" indicates *E. coli* having the O-antigen O26, O55, O86a, O111, O119, O125, O126, O127, O128 or O142; "Class II classical EPEC" indicates *E. coli* having O-antigen O18, O44, O112a or O114; "ETEC" means *E. coli* having the heat-stable toxin gene (*est*) and/or the heat-labile toxin gene (*elt*); "EIEC" means *E. coli* having the invasive plasmid antigen gene (*ipaH*); and "serogrouping-based EIEC" means *E. coli* belonging to the O28ac, O29, O124, O136, O143, O144, O152, O164 or O167 serogroup. "STEC" indicates *E. coli* having the *stx* gene, and "Enterotoxigenic *E. coli* (EAEC)" indicates *E. coli* having the *aggR* (transcriptional activator of AAF/I and AAF/II) gene.

Detection of pathogenic genes: The target genes were the 6 genes associated with diarrheagenic *E. coli*: *eae*, *stx*, *aggR*, *est*, *elt* and *ipaH*. DNAs were extracted from the organisms as described by Yokoyama (15). The genes were detected by multiplex PCR as reported by Toma et al. (16), but the primer set VTcom-u/VTcom-d was changed to MK-1/MK-2 for detecting the *stx* family as reported by Phantouamath et al. (17). The strains with positive multiplex PCR were confirmed by PCR using one of the primer sets for the single gene. Briefly, the PCR mixture consisted of a total volume of 30 μ l containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.75 U of *Taq* DNA polymerase (Toyobo, Osaka, Japan), 0.2 mM deoxynucleoside triphosphate, a 0.25- μ M concentration of primer, and 3 μ l of the DNA template. The PCR program consisted of 25 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1 min. The amplified PCR product was electrophoresed on a 2.5% agarose gel and visualized by staining with ethidium bromide.

RESULTS

Pathogenic gene positive rate: Figure 1 shows the PCR amplification products of the target genes studied for representative strains. The results concerning the presence of pathogenic genes in each O-serogroup are presented in Table 3. From a total of 1,130 *E. coli* strains that agglutinated with one of the 42 anti-O sera, 84 (7.4%) belonging to 19

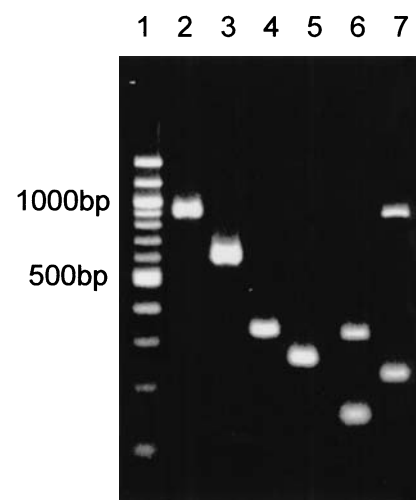


Fig. 1. PCR analysis of pathogenic genes in representative strains of different DEC categories. Lanes: 1, 100-bp ladder (New England Biolabs); 2, EPEC strain O111-29 (*eae*, 881 bp); 3, EIEC strain O28ac-1 (*ipaH*, 619 bp); 4, ETEC strain O167-2 (*elt*, 322 bp); 5, EAEC strain O111-28 (*aggR*, 254 bp); 6, ETEC strain O151-6 (*elt*, 322 bp and *est*, 147 bp) and 7, STEC strain O111-22 (*eae*, 881 bp and *stx*, 224 bp).

Table 3. Strains positive for pathogenic genes in each O-serogroup

Serogroup category	O-serogroup	No. examined	No. of					% any gene positive
			EPEC	STEC	ETEC	EIEC	EAEC	
Class I Classical EPEC <i>n</i> = 383	O119	51	38	0	0	0	2	78.4
	O111	42	11	6	0	0	14	73.8
	O126	66	1	0	28	0	15	66.7
	O86a	22	1	0	1	0	8	45.5
	O55	31	12	0	0	0	1	41.9
	O128	40	3	0	7	0	5	37.5
	O26	49	3	9	1	0	1	28.6
	O142	22	1	0	1	0	0	9.1
	O127a	27	0	0	0	0	2	7.4
	O125	33	1	0	0	0	0	3.0
Class II Classical EPEC <i>n</i> = 136	O44	45	0	0	6	0	8	31.1
	O114	21	1	0	0	0	1	9.5
	O18	65	0	0	1	0	2	4.6
	O112ac	5	0	0	0	0	0	0.0
EIEC serogroups <i>n</i> = 107	O136	4	0	0	1	1	0	50.0
	O144	15	1	0	1	0	3	33.3
	O28ac	22	0	0	1	4	1	27.3
	O152	12	1	0	2	0	0	25.0
	O164	16	1	0	0	1	2	25.0
	O124	5	1	0	0	0	0	20.0
	O167	5	0	0	1	0	0	20.0
	O29	17	0	0	0	0	0	0.0
	O143	11	0	0	0	0	0	0.0
	O-antigens other than Class I, II and EIEC <i>n</i> = 504	O78	7	0	0	0	0	4
O27		10	1	0	4	0	0	50.0
O63		5	2	0	0	0	0	40.0
O169		23	0	0	8	0	0	34.8
O159		12	0	0	3	0	1	33.3
O151		10	0	0	1	0	1	20.0
O25		24	0	0	3	0	1	16.7
O153		37	3	0	1	0	1	13.5
O115		18	1	0	1	0	0	11.1
O15		27	0	0	0	0	3	11.1
O148		11	0	0	1	0	0	9.1
O146		24	0	0	0	2	0	8.3
O6		62	0	0	5	0	0	8.1
O20		17	1	0	0	0	0	5.9
O8		21	0	0	1	0	0	4.8
O166		51	0	0	0	0	1	2.0
O158	16	0	0	0	0	0	0.0	
O1	124	0	0	0	0	0	0.0	
O168	5	0	0	0	0	0	0.0	
Total		1,130	84	15	79	8	77	23.3

serogroups carried the *eae* gene without having the *stx* gene (EPEC). EPEC was most frequently seen in *E. coli* O119; specifically, 38 of 51 *E. coli* O119 strains were found to belong to EPEC (74.5%). *E. coli* O63, O55, O111, O124 also included EPEC at rates of 40, 38.7, 26.2 and 20.0%, respectively. The frequency of EPEC in the other serogroups was lower than 10%. Seventy-nine strains from 22 serogroups were found to possess ETEC-pathogenic genes, and the rate of ETEC strains was higher in serogroups O126 (28 strains of 66, 42.4%), O27 (4 strains of 10, 40%) and O169 (8 strains of 23, 34.8%) than in other serogroups. Among the 79 ETEC strains, 59 carried *est*, 7 carried *elt*, and 13 carried both genes. EIEC was found in 3 EIEC- serogroups: O28ac (4 strains of 22), O136 (1 strain of 4) and O164 (1 strain of 16). Two strains belonging to O146, a non-EIEC serogroup, were also positive for *ipaH*. STEC was found only in O26 and O111 at rates of 18.4% (9 of 49) and 14.3% (6 of 42), respectively.

All STEC strains were also positive for the *eae* gene. EAEC was found in 21 serogroups. The serogroups that showed the highest positive rates for *aggR* were O78 (4 of 7, 57.1%), O86a (8 of 22, 36.4%), O111 (14 of 42, 33.3%) and O126 (15 of 66, 22.7%).

The serogroups of *E. coli* that showed the highest positive rate for any of the 6 pathogenic genes were O119 (78.4%), O111 (73.8%), O126 (66.7%) and O78 (57.1%). In the other serogroups, positive rates were lower than 50%.

Class I and Class II classical EPEC: There were 383 cases of class I classical EPEC. Among them, 71 strains carried the *eae* gene without the *stx* gene; that is, 18.5% of class I classical EPEC was regarded as true EPEC. The EPEC serogroups with the highest probability of a correct diagnosis of *eae*-positive/*stx*-negative strains were O119, O55 and O111. In the other 7 class I serogroups, true EPEC was found in less than 10% of strains. In the class II serogroups, only 1 of 136

strains examined was identified as true EPEC (Table 3).

EIEC serogroups: One hundred and seven strains were found to belong to the 9 serogroups for EIEC proposed by Levine (10). Only 6 (5.6%) carried the *ipaH* gene and were regarded as EIEC.

***E. coli* in diarrheal and non-diarrheal stools:** Among the strains examined, there were 114 from non-diarrheal stools and 326 from diarrheal stools in the Lao PDR samples, therefore, the detection rates of pathogenic genes in both groups were compared. Fifty-one of the 326 diarrheal strains (15.6%) and 11 of the 114 non-diarrheal strains (9.6%) carried a pathogenic gene. This difference was not statistically significant ($P = 0.11132$), as determined by chi-square test.

DISCUSSION

Studies on serogrouping-based diagnosis of DEC have so far been confined to only a few serogroups. In view of the wide use of serogrouping-based diagnosis of DEC, there is now a need for the evaluation of the 43 O-antisera used in clinical laboratories. In the present study, we studied 1,130 *E. coli* strains as putative DEC which were agglutinated with one of the 42 antisera commercially available in Japan. However, only 263 of them (23.3%) were found to carry any of the 6 pathogenic genes examined. The Stx encoding prophage and toxin-encoding plasmids have been shown to be unstable (18) and they could be lost during storage on agar stabs; we therefore can not rule out the possibility that some of the strains became negative for pathogenic genes during storage. However, our results were comparable with those of previous reports. During an epidemiological study performed in Japan, only 40 strains of 229 (17.5%) that had been identified as enterovirulent on the basis of their O-antigens were recognized to be diarrheagenic (14). Scotland et al. (19) studied the production of heat-labile or heat-stable enterotoxins for strains of some serogroups, finding that 3% of O44 strains and 22% of O128 were ETEC, while we found that 13.3% of O44 and 17.5% of O128 were ETEC.

In 383 *E. coli* strains that agglutinated with one of 10 antisera specified for class I classical EPEC as proposed by Levine (10), only 71 of 383 (18.5%) were defined as true EPEC (*eae+stx-*). Giammanco et al. (11) found that 75% of strains isolated in Italy between 1987 and 1992 identified as EPEC by commercial antisera may possess virulence properties. In other countries, the detection of virulence factors was lower in EPEC-serogroup strains (13,20,21). The observation that many strains belonging to EPEC serogroups are actually devoid of any known virulence factor have also led some investigators to consider O-serogrouping an outmoded method for identifying EPEC (22). However, 74.5% of serogroup O119 strains were identified as EPEC in the present study, suggesting a strong association between O119 and EPEC. A total of 110 *E. coli* O119 strains from different countries were examined by Gonçalves et al. (23), who also reported a high percentage of strains belonging to EPEC. Interestingly, we found 2 EAEC strains in O119 samples. These strains were isolated in 2000 and 2002 in Indonesia and Laos. EAEC is an emerging cause of diarrhea in both developed and developing countries and is increasingly reported to be associated with classical EPEC O-serogroups (24).

STEC has been found in many serogroups (25), and the most common serogroups associated with human disease other than O157 were O26, O103, O111 and O113 (26). In

the present study, 15 STEC strains belonged to O26 and O111, however, strains of other DEC categories were also found in these serogroups.

The detection rate of pathogenic genes in diarrheal and non-diarrheal stool was not statistically different. Therefore, the detection rate of 23.3% for pathogenic genes obtained in this study was not influenced by *E. coli* samples from non-diarrheal stools. It is important to know to which serogroups DEC belongs. In the present study there were 84 true EPEC strains, of which 71 (84.5%) belonged to class I serogroups. In the case of EIEC, 6 of 8 EIEC (75%) belonged to one of the EIEC-serogroups proposed by Levine (10). These results suggest that, for EPEC and EIEC, the sensitivity of the serogrouping method is not very bad and the specificity is very poor. The serogroups of ETEC have been well described by Wolf (27), who examined 78 serogroups detected in 954 ETEC isolates, concluding that O6, O78, O8, O128 and O153 accounted for over half of the tested ETEC. It is important to know what percentage of strains within a certain serogroup are ETEC. In the present study, the serogroups that had a positive rate of ETEC higher than 40% were O126 and O27. A study on virulence factors among 39 strains belonging to serogroup O126 reported by Yam et al. also found an association of this serogroup with ETEC (28). However, in the present study, 15 of 66 *E. coli* O126 were EAEC. This serogroup has been reported to be a common EAEC serogroup in childhood diarrhea in England (29), Japan (30) and Israel (31). Although some serogroups such as O78 possessed a positive rate for *aggR* higher than 50%, serogrouping-based diagnosis for EAEC is difficult because they belong to a large variety of serogroups.

The pathogenic gene carrying rate was very different in the serogroups studied. Regarding *E. coli* O119, 78.4% were pathogenic including 38 EPEC and 2 EAEC strains. In the case of *E. coli* O111, 73.8% were pathogenic including 11 EPEC, 6 STEC and 14 EAEC strains. Campos et al. have reported that serogroup O111 is associated with these three pathotypes (32). Thus, even in the serogroups with a high positive rate, different DEC categories were observed.

In conclusion, our results show that: (i) some serogroups such as O1 are rarely associated with virulent strains; (ii) it is recommended to perform tests for the detection of virulence properties regardless of serogrouping results; and (iii) serogrouping-based diagnosis of DEC should be restricted to those serogroups that are most likely to be associated with virulent strains (such as O119, O111, O126, etc.).

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