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Characterization of Enteropathogenic and Enteroaggregative *Escherichia coli* Isolated from Diarrheal Outbreaks

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Virulence characteristics of diarrheal outbreak-associated *Escherichia coli* O55:NM, O126:NM, and O111:NM were examined. The *E. coli* O55:NM strains were atypical enteropathogenic *E. coli* (EPEC), while the *E. coli* O126:NM and O111:NM strains should be classified as enteroaggregative *E. coli* (EAggEC). The contributions of EPEC and EAggEC to the human disease burden in Japan might be significantly greater than is currently appreciated.

There are six categories of *Escherichia coli* that cause diarrhea: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli*, enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli*, and diffusely adherent *E. coli* (21). EPEC causes characteristic attaching-and-effacing lesions (A/E), which can be observed by intestinal biopsy in both human patient (19) and animal (29) models. A/E is characterized by loss of microvilli, intimate adherence of bacteria between epithelial cell membranes (27, 30), and cytoskeletal changes such as actin polymerization directly beneath the adherent bacteria (15). Generally, EPEC causes infantile diarrhea in developing countries and sporadic diarrhea in developed countries (21). EAggEC, on the other hand, is an enteric pathogen defined by its distinctive aggregative or "stacked-brick" pattern of adherence to cultured human epithelial cells (22). EAggEC associates mainly with persistent diarrhea in developing countries (21). Only two reports in Japan have described diarrheal outbreaks caused by EAggEC or EPEC. Itoh et al. (11) reported the isolation of EAggEC from the stools of patients with severe diarrhea in elementary and junior high schools. Makino et al. (18) reported the isolation of EPEC from a mass outbreak. In this paper, we describe three cases of diarrheal outbreaks in Japan caused by *E. coli* belonging to the traditional EPEC serotype.

Chromosomal DNA-embedded agarose plugs for pulsed-field gel electrophoresis (PFGE) analysis were prepared by using the CHEF Bacterial DNA Plug Kit (Bio-Rad, Hercules, Calif.) and were digested with *Xba*I (Nippon gene; Osaka, Japan) at a concentration of 30 U/plug for 4 h at 37°C. The plugs were applied to a 1% PFC Grade Agarose (Bio-Rad) gel. Electrophoresis was performed in 0.5× Tris-Borate EDTA buffer at 14°C using a CHEF DR-II PFGE apparatus (Bio-Rad) under the following conditions: voltage, 6 V/cm; block 1, 11 h, with initial switching time of 4 s to final switching time of 8 s; block 2, 9 h, with initial switching time of 8 s to final switching time of 50 s. The HEp-2 cell assay was performed following the method described by Craviotto et al. (4), with modifications involving 3 or 6 h of incubation (15). The *E. coli* isolates were examined for the presence of the following virulence genes by PCR: *stx1* (Shiga toxin) and *stx2* (16), *eaeA* (*E. coli* attaching and effacing) (12), *bfpA* (bundle-forming pilus) (9), *perA* (EPEC plasmid-encoded regulatory region) (8), *astA* (EAggEC heat-stable enterotoxin) (28), *aggR* (transcriptional activator for EAggEC aggregative adherence fimbria I expression) (20), and *pet* (EAggEC plasmid-encoded heat-labile toxin) (6) using the primers listed in Table 1. EPEC E2348/69 and EAggEC 17-2 were kindly provided by James B. Kaper, and EAggEC 042 was kindly provided by James P. Nataro, University of Maryland School of Medicine, Baltimore, Md.

The diarrheal patients were junior high school students in case 1, adults who attended a party in case 2, and infants of a day care center in case 3. The only diarrheagenic bacterial pathogens isolated from the patients were three *E. coli* O126:NM isolates from four of nine patients in case 1, nine *E. coli* O111:NM isolates from 9 of 21 patients in case 2, and four *E. coli* O55:NM isolates from four of four patients in case 3. As shown in Fig. 1, *E. coli* strains isolated within the same case showed identical PFGE patterns, suggesting that the strains originated from the common infectious sources in the respective cases. These results indicated that these *E. coli* strains were the causative agents of the diarrheal outbreak cases. As shown in Fig. 2, *E. coli* O55:NM possessed *eaeA* and showed a localized HEp-2 cell adherence pattern only in the 6-h assays but was negative for *bfpA* and *perA*, indicating that *E. coli* O55:NM is an atypical EPEC. The *E. coli* O55:NM isolate was negative for *aggR*, *astA*, and *pet*. On the other hand, both *E. coli* O126:NM and O111:NM strains were negative for the *eaeA*, *bfpA,* and *perA* genes but positive for the *aggR* and *astA* genes and showed an aggregative HEp-2 cell adherence pattern, indicating that they should be classified as EAggEC, displaying features of the traditional EPEC serotypes. The *E. coli* O126:NM was *pet* positive, while *E. coli* O111:NM was negative for this gene. All three *E. coli* isolates were negative for both *stx1* and *stx2* genes.

At the Second International Symposium on EPEC (13), a consensus on the basic characteristics of EPEC infection was

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TABLE 1. PCR primers used in this study

Designation	Location	Sequence (5′ to 3′)	Target gene	Amplicon size (bp)	Reference
V1	213–230	AGT-TAA-TGT-GGT-GGC-GAA			
V5	1013–1029	GAC-TCT-TCC-ATC-TGC-CG	*stx1*	817	16
V3	289–306	TTC-GGT-ATC-CTA-TTC-CCG			
V4	745–762	TCT-CTG-GTC-ATT-GTA-TTA	*stx2*	474	16
EA-1	1846–1865	AAA-CAG-GTG-AAA-CTG-TTG-CC			
EA-2	2280–2299	CTC-TGC-AGA-TTA-ACC-TCT-GC	*eaeA*	454	This study
EP-1	2773–2793	AAT-GGT-GCT-TGC-GCT-TGC-TGC			
EP-2	3076–3096	GCC-GCT-TTA-TCC-AAC-CTG-GTA	*bfpA*	324	9
PerAS	522–541	TGT-CAT-CCT-TAG-TGC-TTC-AT			
PerAAS	856–875	GGC-AAT-GTT-CCT-TGT-GTA-AT	*perA*	354	This study
EAST-1S	63–82	GCC-ATC-AAC-ACA-GTA-TAT-CC			
EAST-1AS	149–168	GAG-TGA-CGG-CTT-TGT-AGT-CC	*astA*	106	This study
AggRks1	100–120	GTA-TAC-ACA-AAA-GAA-GGA-AGC			
AggRkas2	353–334	ACA-GAA-TCG-TCA-GCA-TCA-GC	*aggR*	254	26
PetS	557–576	TCA-TTT-CCA-GCA-CTT-CCT-GT			
PetAS	979–998	CTC-CGA-CAG-TAT-TTG-CTC-GT	*pet*	442	This study

reached, identifying them as the presence of A/E histopathology and the absence of Shiga toxin. The A/E phenotype is closely related to the localized adherence phenomenon displayed by EPEC (14). DNA probes and PCR primers have been developed and used for the evaluation of the three major characteristics of EPEC: A/E (12), the presence of a ca. 60-MDa plasmid designated EPEC adherence factor plasmid (EAF) (23), and lack of Shiga toxin (16). Some EPEC strains possess EAF-encoding bundle-forming pilus (BFP) (5). Typical EPEC strains possess the *eaeA* for A/E and the EAF or *bfpA*, while atypical EPEC strains possess the *eaeA* gene only, and there is some controversy over whether atypical EPEC strains are true diarrheagenic pathogens (13). On the other hand, EAggEC infection is diagnosed definitively by isolation from the stools of patients of *E. coli* showing the aggregative HEp-2 cell adherence pattern (21). EAggEC strains possess the *aggA* gene that encodes the aggregative adherent fimbria I (AAF/I) protein (24), the *aggR* gene for transcriptional activation of AAF/I expression (20), and the *astA* gene that encodes the enteroaggregative *E. coli* heat-stable enterotoxin I protein (28). In this study, we examined *E. coli* isolated from patients with diarrhea from three outbreak cases. Serotyping of *E. coli* isolates showed the pathogenic strains to be O55:NM, O111:NM, and O126:NM, representing traditional EPEC serotypes. Based on phenotypic and genotypic tests, the O55:NM strain was identified as an atypical EPEC; it showed localized adher-

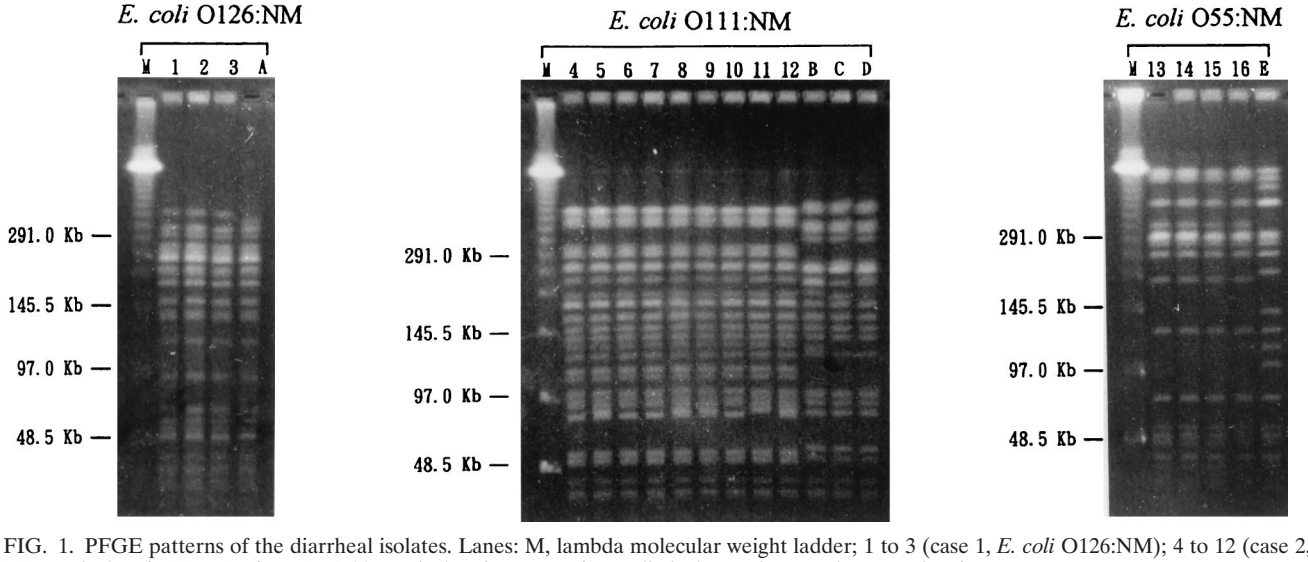

FIG. 1. PFGE patterns of the diarrheal isolates. Lanes: M, lambda molecular weight ladder; 1 to 3 (case 1, *E. coli* O126:NM); 4 to 12 (case 2, O111:NM isolates); 13 to 16 (case 3, O55:NM isolates); A to E (sporadic isolates of respective organisms).

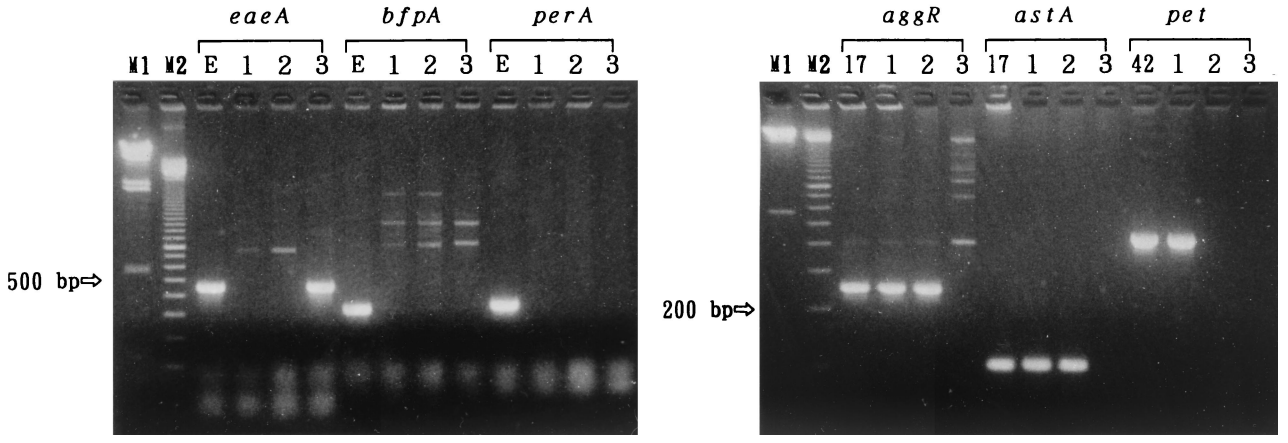

FIG. 2. Agarose gel electrophoresis of the PCR products of *E. coli* virulence genes showing virulence traits of the *E. coli* isolates from three outbreak cases. Lanes: M1, lambda/*Hin*dIII molecular weight marker; M2, 100-bp-ladder molecular weight marker; 1, *E. coli* O126:NM EC-152 from case 1; 2, *E. coli* O111:NM EC-560 from case 2; 3, *E. coli* O55:NM EC-1045 from case 3; E, EPEC E2348/69; 17, EAggEC 17-2; 42, EAggEC 042.

ence and possessed the *eaeA* without the EAF. However, *E. coli* isolates from the other two cases were identified as EAggEC, because they showed aggregative adherence and possessed *astA* and *aggR* but not *eaeA* or EAF. In general, EPEC infection is primarily a disease of infants younger than two years old (17), and EAggEC is associated with persistent diarrhea (1). Several outbreaks of diarrhea due to EPEC have been reported in the United States, the United Kingdom, Finland, and other developed countries. These outbreaks frequently occur in day care centers (2, 25) and occasionally occur in pediatric wards (10). However, reports of outbreaks due to atypical EPEC are infrequent. Recently, Hedberg et al. (10) reported an outbreak caused by an atypical EPEC among adults who ate at a gourmet buffet in the United States. This atypical EPEC strain was unique, because its serotype was O39:NM, which did not belong to any of the traditional EPEC serotypes, and it was positive for *astA* along with *eaeA* but negative for the EAF. Our present data, along with Hedberg's observation, suggest that atypical EPEC is a diarrheic pathogen. Furthermore, the number of reports describing outbreaks due to EAggEC is increasing (3, 7). In Japan, there are only two reports describing outbreaks of diarrhea involving EPEC and EAggEC (11, 18). Based on our cases and the two reports just mentioned, the contribution of EPEC and EAggEC to the human disease burden in Japan might be significantly greater than is currently appreciated.

REFERENCES

1. **Bahn, M. K., P. Raj, M. M. Levine, J. B. Kaper, N. Bhandari, R. Srivastava, R. Kumar, and S. Sazawal.** 1989. Enteroaggregative *Escherichia coli* associated with persistent diarrhea in a cohort of rural children in India. J. Infect. Dis. **159:**1061–1064.
2. **Bower, J. R., B. L. Congeni, T. G. Cleary, R. T. Stone, A. Wanger, B. E. Murray, J. J. Mathewson, and L. K. Pickering.** 1989. *Escherichia coli* O114: nonmotile as a pathogen in an outbreak of severe diarrhea associated with a day care center. J. Infect. Dis. **160:**243–247.
3. **Cobeljic, M., B. Miljkovic-Selimovic, D. Paunovic-Todosijevic, Z. Velickovic, Z. Lepsanovic, D. Savic, R. Ilic, S. Konstantinovic, B. Jovanovic, and V. Kostic.** 1996. Enteroaggregative *Escherichia coli* associated with an outbreak of diarrhoea in a neonatal nursery ward. Epidemiol. Infect. **117:**11–16.
4. **Craviotto, A., R. J. Gross, S. M. Scotland, and B. Rowe.** 1979. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes. Curr. Microbiol. **3:**95–99.
5. **Donnenberg, M. S., J. A. Girron, J. P. Nataro, and J. B. Kaper.** 1992. A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. Mol. Microbiol. **6:**3427–3437.
6. **Eslava, C., F. Navarro-Garcia, J. R. Czeczulin, I. R. Henderson, A. Craviotto, and J. P. Nataro.** 1998. *Pet*, as autotransporter enterotoxin from enteroaggregative *Escherichia coli*. Infect. Immun. **66:**3155–3163.
7. **Giron, J. A., F. Qadri, K. J. Jarvis, J. B. Kaper, and M. J. Albert.** 1995. Monoclonal antibodies specific for the bundle-forming pilus of enteropathogenic *Escherichia coli*. Infect. Immun. **63:**4949–4952.
8. **Gomez-Duarte, O. G., and J. B. Kaper.** 1995. A plasmid-encoded regulatory region activates chromosomal *eaeA* expression in enteropathogenic *Escherichia coli*. Infect. Immun. **63:**1767–1776.
9. **Gunzberg, S. T., N. G. Tornieporth, and L. W. Riley.** 1995. Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. J. Clin. Microbiol. **33:**1375–1377.
10. **Hedberg, C. W., S. J. Savarino, J. M. Besser, C. J. Paulus, V. M. Thelen, L. J. Myers, D. N. Cameron, T. J. Barrett, J. B. Kaper, and M. T. Osterholm.** 1997. An outbreak of foodborne illness caused by *Escherichia coli* O39:NM: an agent that does not fit into the existing scheme for classifying diarrheagenic *E. coli.* J. Infect. Dis. **176:**1625–1628.
11. **Itoh, Y., I. Nagano, M. Kunishima, and T. Ezaki.** 1997. Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable:H10 associated with a massive outbreak of gastrointestinal illness. J. Clin. Microbiol. **35:** 2546–2550.
12. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87:**7839–7843.
13. **Kaper, J. B.** 1996. Defining EPEC. Rev. Microbiol. Sao Paulo **27:**130–133.
14. **Knutton, S., A. D. Phillips, H. R. Smith, R. J. Gross, R. Shaw, P. Watson, and E. Price.** 1991. Screening for enteropathogenic *Escherichia coli* in infants with diarrhea by the fluorescent-actin staining test. Infect. Immun. **59:**365–371.
15. **Knutton, S., P. H. Williams, and A. S. McNeish.** 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic *Escherichia coli*. Infect. Immun. **57:**1290–1298.
16. **Kobayashi, K.** 1991. Detection of enterohemorrhagic *Escherichia coli* using PCR. Rinsyo to Biseibutsu. **18:**507–513. (In Japanese.)
17. **Levine, M. M., and R. Edelman.** 1984. Enteropathogenic *Escherichia coli* of classic serotypes associated with infant diarrhea: epidemiology and pathogenesis. Epidemiol. Rev. **6:**31–51.
18. **Makino, S., H. Asakura, T. Shirahata, T. Ikeda, K. Takeshi, K. Arai, M. Nagasawa, T. Abe, and T. Sadamoto.** 1999. Molecular epidemiological study of a mass outbreak caused by enteropathogenic *Escherichia coli* O157:H45. Microbiol. Immunol. **43:**381–384.
19. **Moon, H. W., S. C. Whipp, R. A. Argenzio, M. M. Levine, and R. A. Giannella.** 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. **41:** 1340–1351.
20. **Nataro, J. P., D. Yikang, D. Yingkang, and K. Walker.** 1994. *aggR*, transcriptional activator of aggregative adherence fimbria I expression in enteroaggregative *Escherichia coli*. J. Bacteriol. **176:**4691–4699.
21. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. **11:**142–201.
22. **Nataro, J. P., J. B. Kaper, R. Robins-Browne, V. Prado, P. Vial, and M. M.**

Levine. 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. Pediatr. Infect. Dis. J. **6:**829–831.
23. **Nataro, J. P., M. M. Baldini, J. B. Kaper, R. E. Black, N. Bravo, and M. M. Levine.** 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. J. Infect. Dis. **152:**560–565.
24. **Nataro, J. P., Y. Deng, D. R. Maneval, A. L. German, W. C. Martin, and M. M. Levine.** 1992. Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect. Immun. **60:**2297–2304.
25. **Paulozzi, L. J., K. E. Johnson, L. M. Kamahere, C. R. Clausen, L. W. Riley, and S. D. Helgerson.** 1986. Diarrhea associated with adherent enteropathogenic *Escherichia coli* in an infant and toddler center, Seattle, Washington. Pediatrics **77:**296–300.
26. **Ratchtrachenchai, O. A., S. Subpasu, and K. Ito.** 1997. Investigation on enteroaggregative *Escherichia coli* infection by multiplex PCR. Bull. Dept. Med. Sci. **39:**211–220.
27. **Rothbaum, R., A. J. McAdams, R. Giannella, and J. C. Partin.** 1982. A clinicopathological study of enterocyte-adherent *Escherichia coli*: a cause of protracted diarrhea in infants. Gastroenterology **83:**441–454.
28. **Savarino, S. J., A. Fasano, J. Watson, B. M. Martin, M. M. Levine, S. Guandalini, and P. Guerry.** 1993. Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of *E. coli* heat-stable toxin. Proc. Natl. Acad. Sci. USA **90:**3093–3097.
29. **Taylor, C. J., A. Hart, R. M. Batt, C. McDougall, and L. McLean.** 1986. Ultrastructural and biochemical changes in human jejunal mucosa associated with enteropathogenic *Escherichia coli* (O111) infection. J. Pediatr. Gastroenterol. Nutr. **5:**70–73.
30. **Ulshen, M. H., and J. L. Rallo.** 1980. Pathogenesis of *Escherichia coli* gastroenteritis in man—another mechanism. N. Engl. J. Med. **302:**99–101.