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NOTE

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Identification of the ETT2 locus in human diarrheagenic *Escherichia coli* by multiplex PCR

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Abstract An *Escherichia coli* type III secretion system 2 (ETT2) locus was discovered in enterohemorrhagic *E. coli* O157:H7. To determine presence or absence of the ETT2 locus in diarrheagenic *E. coli*, a multiplex polymerase chain reaction (PCR) encoding Shiga toxins (stx_1 and stx_2), intimin (*eaeA*), and ETT2 (*etrA*) was developed for rapid detection. The ETT2 locus was identified not only in Shiga toxin-producing *E. coli* (STEC) but also in various non-STEC.

Key words ETT2 · Enterohemorrhagic *Escherichia coli* · Diarrheagenic *Escherichia coli* · Multiplex PCR

The genome sequence of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 has been found to contain an additional type III secretion system (TTSS), the *E. coli* type III secretion system 2 (ETT2).[1] A characteristic of the TTSS encoded by a pathogenicity island known as the locus of enterocyte effacement (LEE) region, or ETT1, is its ability to deliver effector proteins into the cytoplasm of a target eukaryotic cell through a translocation apparatus that connects the bacterial and eukaryotic cells; the ETT1 is responsible for the development of attaching and effacing lesions.[2] The ETT2 locus consists of the *epr*, *epa*, and *eiv* genes, similar to the sequence of a pathogenicity island in *Salmonella enterica* serovar Typhimurium.[3–5]

To determine the presence or absence of the ETT2 locus in human diarrheagenic *E. coli* strains, which include various serotypes, target genes selected for multiplex polymerase chain reaction (PCR) were *eaeA*, encoding the protein intimin (which is involved in LEE regions), stx_1 and stx_2 for Shiga toxin, and *etrA* (which is involved in the ETT2 locus). In particular, *etrA*, the target gene in the ETT2 locus, has been identified between the *epr* and *epa* genes as an ETT2 regulator; it has a profound effect on gene transcription in the LEE region.[6]

The PCR assay was tested with a laboratory collection of 83 human diarrheagenic *E. coli* isolates and 34 *E. coli* isolates from healthy individuals, obtained from Kobe University Hospital, Nishi-Kobe Medical Center, and the Kobe Institute of Health, all in Japan. These strains were previously examined for O-serotypes and for the production of Shiga toxins. The strains were incubated at 37°C for 18 to 24h in 2ml Heart infusion broth medium (Eiken Chemical Co. Ltd., Tokyo, Japan). The culture was the incubated at 100°C for 10min, followed by centrifugation at 13000rpm for 5min prior to removal of the template for PCR. The *E. coli* O157:H7 strain (ATCC 35150) was used as a positive control in this study, and the *E. coli* K-12 strain JM109 was the negative control. For our protocol, 5µl of DNA was added to 45µl of reaction mixture containing final concentrations of 1× PCR reaction buffer (500mM Tris-HCl, 100mM KCl, 50mM $(NH_4)_2SO_4$, 20mM $MgCl_2$; pH 8.3), 0.2mM deoxynucleotide triphosphate mix (Roche Diagnostics, Penzberg, Germany), and 0.4U of FastStart Taq polymerase (Roche). The four primer sets (Table 1) were added at final concentrations of 0.05µM for *etrA*, 1µM for *eaeA*, and 0.5µM for stx_1 and stx_2. The concentration of each primer gave satisfactory amplification of each gene. The PCR consisted of an intial denaturation step at 94°C for 4min, amplified by 30 cycles of denaturation at 94°C for 30s, annealing at 50°C for 1min, and extension at 72°C for 3min, ending with a final extension at 72°C for 10min, in a TaKaRa PCR Thermal Cycler Personal (Takara Bio, Ohtsu, Japan). DNA fragments were resolved by gel

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Table 1. PCR primers used in this study

Gene target	Primer sequence (5′-3′)	Product size (bp)	Reference no.
etrA	CTTCTTCCTAACGAAACTATCATTA TGACATATCAACTTTCTCTTACGC	913	7
eaeA	GCTTAGTGCTGGTTTAGGAT TCGCCGTTCAGAGATCGC	488	8
stx_1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614	9
stx_2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	9

Table 2. Conservation of virulence factors in diarrheagenic *E. coli*

	Serotype (no. of isolates)	*etrA*	*eaeA*	stx_1	stx_2
STEC	O157:H7 (17)	+	+	+	+
	O157:H7 (7), O157:H-(3)	+	+	−	+
	O26 (5), O157:H7 (1), O157:H-(1)	+	+	+	−
	O26 (2)	+	−	+	−
	O177 (1)	−	−	+	+
non-STEC	O26 (1)	+	+	−	−
	O26 (1)	−	+	−	−
	O1 (6), O8 (1), O44 (1), O114 (1), O119 (1), O126 (1), O114 (2), O158 (1), O166 (1), O168 (1), O169 (1)	+	−	−	−
	O1 (8), O6 (5), O15 (1), O18 (5), O25 (3), O27 (1), O114 (1), O128 (2), O164 (1)	−	−	−	−

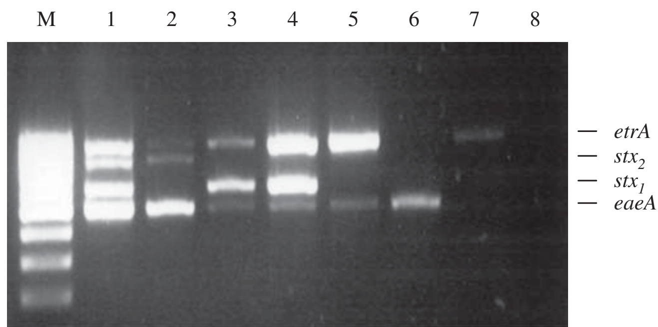

Fig. 1. Agarose gel electrophoresis of DNA fragments generated by multiplex polymerase chain reaction (PCR) with serotyping strains. *Lanes*, *M*, DNA size markers (ϕ × 174-*Hae*III digest); *1*, ATCC35150(O157:H7); *2*, E-232(O157:H7); *3*, E-179(O157:H7); *4*, E-321(O26); *5*, E-354(O157:H7); *6*, E-355(O26); *7*, E-5(O1), *8*, JM109 (negative control)

electrophoresis on a 2% agarose gel (1 × Tris-acetate-ethylenediamine tetraacetic acid [EDTA] buffer), stained with ethidium bromide, and visualized with UV illumination.

Figure 1 shows that the four virulence genes were clearly detected. PCR products were obtained in the expected sizes for *eaeA* (488 bp, lanes 1 to 6), stx_1 (614 bp, lanes 1, 3, and 4), stx_2 (779 bp, lanes 1 and 2), and *etrA* (913 bp, lanes 1 to 5, and 7), while no PCR product was detected in the negative control (lane 8). Eighty-three strains isolated from diarrheic patients were characterized by multiplex PCR. These strains were identified with the aid of those obtained by single PCR for each gene and phenotype (Stx1 and Stx2). The detect of virualence factors in diarrheagenic *E. coli* are shown in Table 2. *etrA* was the most prevalent in the samples (54 of 83; 65%), compared to *eaeA* (36 of 83; 43%). Shiga toxin-producing *E. coli* (STEC) strains were characterized as stx_1 (27 of 83; 33%), stx_2 (28 of 83; 34%), and all possessed *etrA,* except for O177. A small proportion of non-STEC strains did not possess *eaeA*, and *etrA* was contained in many non-STEC strains (18 of 46; 39%), especially O1. Furthermore, a few of the 34 strains from healthy individuals showed the presence of *etrA* (2 of 34; 6%) but none showed the presence of *eaeA*, stx_1, or stx_2. These results indicate that, using multiplex PCR, the ETT2 locus can be identified in various *E. coli*, both STEC snd non-STEC. The ETT2 locus contains 19 open reading frames (ORFs) highly homologous with SPI-1 (*Salmonella enterica* serovar Typhimurium pathogenicity island 1).[3] SPI-1 comprises the *inv-spa* complex, which specifies a TTSS involved in the export of antigens that promote cell entry.[4] The *epr-epa-eiv* genes of the ETT locus were not found in K-12 MG1655.[5] These findings indicate that the ETT2 locus is important as a virulence factor in diarrheagenic *E. coli*. This suggests that the presence of ETT2 is not only associated with EHEC/STEC but also with other diarrheagenic *E. coli*.

References

1. Perna NT, Plunkett G 3rd, Burland V, Mau B, Glasner JD, Rose DJ, et al. Genome sequence of enterohemorrhagic *Escherichia coli* O157:H7. Nature 2001;409:529–33.

2. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli.* Clin Microbiol Rev 1998;11:142–201.
3. Makino S, Tobe T, Asakura H, Watarai M, Ikeda T, Takeshi K, et al. Distribution of the secondary type III secretion system locus found in enterohemorrhagic *Escherichia coli* O157:H7 isolates among Shiga toxin-producing *E. coli* strains. J Clin Microbiol 2003;41:2341–7.
4. Galan JE. Molecular genetic bases of *Salmonella* entry into host cells. Mol Microbiol 1996;20:263–71.
5. Ren CP, Chaudhuri RR, Fivian A, Bailey CM, Antonio M, Barnes WM, et al. The ETT2 gene cluster, encoding a second type III secretion system from *Escherichia coli*, is present in the majority of strains but has undergone widespread mutational attrition. J Bacteriol 2004;186:3547–60.
6. Zhang L, Chaudhuri RR, Constantinidou C, Hobman JL, Patel MD, Jones AC, et al. Regulators encoded in the *Escherichia coli* type III secretion system 2 gene cluster influence expression of genes within the locus for enterocyte effacement in enterohemorrhagic *E. coli* O157:H7. Infect Immun 2004;72:7282–93.
7. Hartleib S, Prager R, Hedenstrom I, Lofdahl S, Tschape H. Prevalence of the new, SPI1-like, pathogenicity island ETT2 among *Escherichia coli.* Int J Med Microbiol 2003:292:487–93.
8. Sueyoshi M, Fukui H, Tanaka S, Nakazawa M, Ito K. A new adherent form of an attaching and effacing *Escherichia coli* (*eaeA*+, *bfp*−) to the intestinal epithelial cells of chicks. J Vet Med Sci 1996;58:1145–7.
9. Gannon VP, King RK, Kim JY, Thomas EJ. Rapid and sensitive method for detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. Appl Environ Microbiol 1992;58:3809–15.