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Prevalence and Characteristics of Shiga Toxin-Producing *Escherichia coli* from Healthy Cattle in Japan

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The prevalence of Shiga toxin-producing *Escherichia coli* (STEC) in Japan was examined by using stool samples from 87 calves, 88 heifers, and 183 cows on 78 farms. As determined by screening with *stx*-PCR, the prevalence was 46% in calves, 66% in heifers, and 69% in cows; as determined by nested *stx*-PCR, the prevalence was 100% in all animal groups. Of the 962 isolates picked by colony *stx* hybridization, 92 isolates from 54 farms were characterized to determine their O serogroups, virulence factor genes, and antimicrobial resistance. Of these 92 isolates, 74 (80%) could be classified into O serogroups; 50% of these 74 isolates belonged to O serogroups O8, O26, O84, O113, and O116 and 1 isolate belonged to O serogroup O157. Locus of enterocyte effacement genes were detected in 24% of the isolates, and enterohemorrhagic *E. coli* (EHEC) *hlyA* genes were detected in 72% of the isolates. Neither the bundle-forming pilus gene nor the enteropathogenic *E. coli* adherence factor plasmid was found. STEC strains with characteristics typical of isolates from human EHEC infections, which were regarded as potential EHEC strains, were present on 11.5% of the farms.

The importance of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) has increased since a food-borne infection caused by enterohemorrhagic *E. coli* (EHEC) was first described (25). Cattle have been implicated as a principle reservoir of STEC (3, 5, 31). The STEC strains are classified into a large number of O serogroups (4, 22). However, the majority of outbreaks and/or sporadic cases of hemorrhagic colitis and hemolytic uremic syndrome have been caused by members of only a few serogroups, such as O26, O111, and O157 (14, 26, 28). The Stx are known to be essential virulence factors of EHEC (6, 21), but the Stx alone may not be sufficient to cause disease (2). The virulence of STEC is known to be mediated through Stxs and attachment factors. The genes coding for these virulence-associated properties are located on phages, a pathogenicity island called the locus of enterocyte effacement (LEE), and plasmids (1, 9, 15). In particular, genes of the LEE are almost always detected in EHEC and are always detected in enteropathogenic *E. coli* (EPEC) and attaching and effacing *E. coli* (AEEC), and they express the attaching and effacing (AE) lesion for enteroepithelial cells (9). The LEE consists of genes for adhering intimin (*eae*), translocated intimin receptor (*tir*), a type III secretion system, and other secreted proteins (9). These recently discovered mediators of AE lesions are often examined to characterize STEC strains isolated from human clinical samples (29).

The aims of the present study were to describe the prevalence of STEC in healthy cattle in the central part of Japan and to genetically characterize various virulence genes of the STEC strains isolated.

E. coli O157:H7 strains ATCC 43889, ATCC 43890, ATCC 43894, and ATCC 35150, used as positive control strains for *stx* genes, were obtained from the American Type Culture Collection. *E. coli* 166, VR299-2, and EPEC108, used as positive control strains for intimin β, intimin ε, the bundle-forming pilus (*bfp*), and the "EPEC adherence factor" (EAF) plasmid (1, 19, 24), were derived from the stock culture collection of the National Veterinary and Food Research Institute. *E. coli* Fuku-07, Oki-04, Sai-11, and Shizu-19, previously isolated from cattle in Japan and identified as O157:H7 strains, were used as reference strains.

A total of 358 rectal stool grab samples were collected from healthy dairy cattle (87 calves less than 5 months old, 88 heifers 5 to 12 months old, and 183 cows) on 78 farms located in a region of central Japan called the Kanto-Koshinetsu region between May and October 1998. All rectal stools were sampled by veterinarians from governmental animal hygiene centers. The samples were placed in cool room (4 to 8°C) and taken to the laboratory for immediate processing (usually within 24 h). The stool samples were streaked on sorbitol-MacConkey agar (SMAC) (Eiken, Tokyo, Japan), and 1 g of each stool sample was enriched in 19 ml of modified *E. coli* broth (mEC) (Eiken). Both SMAC plates and mEC were incubated at 37°C for 16 to 18 h.

A 100-μl aliquot of an enriched mEC culture (described above) was used for *stx*-PCR (34). It was centrifuged at 13,500 × *g* for 1 min, and the pellet was washed with 1 ml of phosphate-buffered saline. The bacterial sediment was resuspended in 100 μl of distilled water and heated at 100°C for 5 min. Five microliters was used for *stx*-PCR performed with a pair of *stx* common PCR primers (Table 1) (34). (The positions of the *stx* conserved sequence primers for *stx* genes are as follows: sense primer VT com-u, positions 280 to 300; and antisense primer VT com-d, positions 778 to 797.) When a sample was negative in the *stx*-PCR, 1 μl of the PCR mixture

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TABLE 1. Oligonucleotide probe and primers and PCR programs to amplify specific fragments from the various pathogenic genes in *E. coli*

Oligonucleotide probe or PCR primer (orientation)	Target gene(s)	Sequence (5′-3′)	Reaction temp (°C)			Size of product (bp)	Reference
			Denaturation	Annealing	Extension		
VT com (oligonucleotide probe)[a]	*stx*1 and *stx*2	GAGCGAAATAATTTATATGTG					34
VT com-u (forward) VT com-d (reverse)	*stx*1 and *stx*2	GAGCGAAATAATTTATATGTG TGATGATGGCAATTCAGTAT	94 (45)[b]	55 (45)	72 (60)	518	34
VT com-nesF (forward) VT com-nesR (reverse)	*stx*1 and *stx*2	CGGACAGTAGTTATACCAC CTGCTGTCACAGTGACAA	94 (30)	55 (30)	72 (45)	171	34
VT1a (forward) VT1b (reverse)	*stx*1	CAGTTAATGTGGTGGCGAAG CTGCTAATAGTTCTGCGCATG	94 (60)	55 (60)	72 (60)	894	18
VT2a (forward) VT2b (reverse)	*stx*2	CTTCGGTATCCTATTCCCGG GGATGCATCTCTGGTCATTG	94 (60)	55 (60)	72 (60)	478	18
BFP1 (forward) BFP2 (reverse)	*bfp*	GATTGAATCTGCAATGGTGC GGATTACTGTCCTCACATAT	94 (60)	57 (60)	72 (60)	597	30
EAF1 (forward) EAF25 (reverse)	EAF plasmid	CAGGGTAAAAGAAAGATGATAA TATGGGGACCATGTATTATCA	94 (60)	57 (45)	72 (60)	399	8
Ehly AF (forward) Ehly AR (reverse)	EHEC-*hlyA*	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	94 (60)	60 (120)	72 (60)	534	20
eae-1 (forward) eae-2 (reverse)	*eae*	ACGTTGCAGCATGGGTAACTC GATCGGCAACAGTTTCACCTG	94 (60)	55 (60)	72 (60)	815	10
Int-α (forward) Int-Ru (reverse) Int-αR (reverse)	*eae*	CCTTAGGTAAGTTAAGT TTTATTTGCAGCCCCCCAT TGACTAGACTTATTATATTC[c]	95 (45)	45 (60)	72 (60)	557 588	1 This study
Int-β (forward) Int-Ru (reverse)	*eae*	TAAGGATTTTGGGACCC TTTATTTGCAGCCCCCCAT	95 (60)	45 (60)	72 (60)	563	1
Int-γ (forward) Int-Ru (reverse) Int-γR (reverse)	*eae*	ACAAACTTTGGGATGTTC TTTATTTGCAGCCCCCCAT AGTTCATAGAACTATAATGGC[c]	95 (45)	55 (60)	72 (60)	542 571	1 This study
Int-δ (forward) Int-Ru (reverse)	*eae*	TACGGATTTTGGGGCAT TTTATTTGCAGCCCCCCAT	95 (45)	45 (60)	72 (60)	563	1
SK-1 (forward) LP-5 (reverse)	*eae*[d]	CCCGAATTCGGGACAAGCATAAGC AGCTCACTCGTAGATGACGGCAAGCG	94 (45)	65 (45)	72 (120)	2,608	19
ESPA-F (forward) ESPA-R (reverse)	*espA*	ACTCGGTGTTTTTCAGGCTGC TGAAATAGTTCTATATTG	94 (40)	53 (45)	72 (60)	445[e]	This study
ESPB-F (forward) ESPB-R (reverse)	*espB*	GCCGTTTTTGAGAGCCAGAAT ATCATCCTGCGCTCTGCGAAC	94 (40)	63 (45)	72 (60)	633[e]	This study
ESPD-F (forward) ESPD-R (reverse)	*espD*	CGCTGGATTTACAACTGGTTA CCAGCTCAACCTTCGCACTCT	94 (40)	60 (45)	72 (60)	939[f]	This study
TIR-F (forward) TIR-R (reverse)	*tir*	CATTACCTTCACAAACCGAC CCCCGTTAATCCTCCCAT	94 (40)	57 (60)	72 (75)	1,550[g]	This study

[a] The oligonucleotide probe is specific for each *stx* variant, and an alkaline phosphatase was labeled at the 5′ end of an oligonucleotide molecule. Hybridization was performed at 50°C.
[b] The value in parentheses is the reaction time (in seconds).
[c] Improved PCR primer.
[d] Primers SK-1 and LP-5 are specific for intimin variant ε.
[e] Product size when *E. coli* E2348/69 DNA (National Center for Biotechnology Information accession no. AF022236) was used as the template.
[f] Product size when *E. coli* DA-EPEC-B6 DNA (National Center for Biotechnology Information accession no. Y17875) was used as the template.
[g] Product size when *E. coli* 955F2 DNA (National Center for Biotechnology Information accession no. AF070067.1) was used as the template.

was subjected to a nested *stx*-PCR (Table 1). (The positions of the *stx* conserved sequence primers for *stx* genes are as follows: sense primer VT com-nesF, positions 395 to 413; and antisense primer VT com-nesR, positions 547 to 565.)

An oligonucleotide probe specific for both *stx*1 and *stx*2 (Table 1) (34) was synthesized and labeled with alkaline phosphatase at the 5′ end by using an AP-Oligonucleotide labeling kit (Boehringer, Mannheim, Germany). The DNA probe was used to detect STEC by colony hybridization. For each stool sample 20 sorbitol-positive colonies typical of *E. coli* were picked from the SMAC plate. When sorbitol-negative coliform colonies appeared on the agar, we also picked up to 20 such colonies. The colonies were examined for the presence of any *stx* genes by colony hybridization techniques. All *stx*-positive isolates were confirmed to be *E. coli* strains by conventional biochemical tests, and if needed, API 20E and/or Micro ID techniques were employed. Then, up to three *stx*-positive colonies per sample were randomly chosen and subjected to classification as *stx* type *stx*1 or *stx*2 by PCR (Table 1) (18). One isolate per animal was selected for further study if all *stx*-positive isolates showed the same *stx* profile. The STEC isolates selected were characterized by PCR to determine the presence of *bfp*, the EAF plasmid, EHEC *hlyA*, and the LEE genes *eae*, *espA*, *espB*, *espD*, and *tir* (Table 1). Intimin subtyping (α, β, γ, δ, or ε) was carried out for *eae*-positive isolates. All PCRs included 25 or 30 cycles of amplification with the conditions described in Table 1.

Determination of the O-antigen group was carried out by the method described in the Denka Seiken protocol (Denka Seiken, Tokyo, Japan). A strain identified as an STEC strain was incubated on Trypticase soy agar (Difco) at 37°C for 18 h. Approximately 0.3 g of organisms was harvested and suspended in 3 ml of saline solution. After heating at 100°C for 30 min, the suspension was centrifuged at 900 × *g* for 20 min. The sediment was resuspended in 0.5 ml of saline and used as the O-antigen solution. Sera against each O-antigen group tested were obtained from Denka Seiken and the *E. coli* Reference Laboratory, Universidad de Santiago de Compostela, Lugo, Spain, or were prepared at the National Institute of Animal Health, Tsukuba, Japan.

Antimicrobial susceptibility was tested by using the following 14 antimicrobial agents, most of which are commonly used for therapeutic treatment in production animals in Japan (the exceptions are ciprofloxacin and nalidixic acid): aminobenzylpenicillin, ceftiofur, dihydrostreptomycin, kanamycin, thiamphenicol, chloramphenicol, oxytetracycline, chlortetracycline, erythromycin, josamycin, colistin, nalidixic acid, ciprofloxacin, and enrofloxacin. Aminobenzyl penicillin, dihydrostreptomycin, oxytetracycline, and chlortetracycline were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and the other drugs were supplied by the National Veterinary Assay Laboratory, Tokyo, Japan. In vitro susceptibility tests were carried out by the agar dilution method by using the recommendations of the National Committee for Clinical Laboratory Standards (17). Briefly, all drugs were serially twofold diluted, and 1 ml of each dilution was mixed with 9 ml of Mueller-Hinton agar (Difco). After inoculation of 10^3 to 10^4 CFU of organisms, the plates were incubated at 37°C for 24 h. The MIC was the lowest concentration of antimicrobial agent that suppressed visible bacterial growth. Two reference strains, *E. coli* NIH J2 and *Staphylococcus aureus* 209P, were included as internal controls in the susceptibility study. Since breakpoints for drugs used in animals have not been established, a strain was defined as resistant when its growth could not be suppressed with four times the MIC at which 90% of the isolates were inhibited or with 16 times the MIC at which 50% of the isolates were inhibited.

In this study, all *stx*-positive organisms isolated from cattle were identified as *E. coli* on the basis of biochemical properties. Therefore, an *stx*-PCR-positive sample was defined as STEC positive in this report. The STEC prevalence values for cows and heifers were 127 of 183 animals (69%) and 58 of 88 animals (66%), respectively, and thus were higher than the value for calves (40 of 87 animals [46%]). However, nested *stx*-PCR could detect STEC in all the samples examined. As determined by *stx* colony hybridization for 20 colonies per sample, we recovered 962 (12.4%) STEC colonies from a total of 7,745 coliforms. Sorbitol-negative STEC were detected only rarely among the sorbitol-negative colonies (20 of 585 colonies [3.4%]). STEC were recovered from 17 calves (20%), 27 heifers (31%), and 35 cows (19%). Of 962 STEC isolates, 92 from 74 animals on 54 farms were selected and examined by serotyping, virulence factor analysis, and antimicrobial susceptibility testing. STEC were isolated by colony hybridization from 76 of 79 (96%) *stx*-PCR-positive samples but only from 3 of 123 *stx*-PCR-negative samples. The latter three samples might have contained PCR inhibitors, because there were enough STEC organisms in the samples to be detected by colony hybridization.

The 92 STEC strains selected belonged to 25 different O serogroups, and 18 strains were nontypeable. Only one isolate was a member of serogroup O157, and 37 of the 74 typeable isolates could be classified into five serogroups (O8, O26, O84, O113, and O116) (Table 2). A connection was found between the serogroups and the types of *stx* genes; all of the serogroup O26 strains contained *stx*1, whereas the serogroup O84, O113, and O116 strains contained *stx*2. Another connection was found between serogroup and the presence of *eae*. The majority of the strains belonging to serogroups O26 (eight of nine strains), O84 (all six strains), O103 (all three strains), and O111 (both strains) contained *eae* and the LEE-related genes *espA*, *espB*, *espD*, and *tir* (Table 2). The serogroup O26, O111, and O157 strains, which are most frequently detected as causal agents of hemorrhagic colitis and hemolytic uremic syndrome, together accounted for more than 10% of the STEC isolates examined (12 of 92 strains). These STEC strains originated from nine farms. Thus, of 78 (11.5%) of the cattle farms analyzed were reservoirs of potentially EHEC. All 92 STEC strains were negative for either *bfp* or the EAF plasmid. The EHEC *hlyA* gene was prevalent in STEC strains; 66 of 92 (72%) of the strains were EHEC *hlyA* positive as determined by PCR. Of the 22 *eae*-positive STEC strains, 16 could be intimin typed and placed in established subtypes. The eight *eae*-positive isolates belonging to O serogroup O26 were intimin type β, and all three O serogroup O103 strains were intimin type ε; the other *eae*-positive isolates were intimin type β or nontypeable (Table 2). The only O157 isolate and the reference O157 strains were classified as intimin type γ; no intimin type γ STEC strains were detected among the non-O157 serogroup strains (Table 2).

TABLE 2. Characterization of 92 STEC strains isolated from cattle in Japan

O serogroup	No. of strains or strain	No. of strains from[a]:			No. of strains with the following genes:										Intimin type	Antimicrobial resistance[b]
		Calves	Heifers	Cows	*stx*1	*stx*2	*stx*1 and *stx*2	*ehlyA*	*bfp*	EAF plasmid	*eae*	*espA*	*espB*	*espD*	*tir*	
O1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	
O2	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	
O8	8	3	4	1	3	2	3	4	0	0	0	0	0	0	0	ABPC + DSM (1)[c] OTC + CTC (2)
O15	4	0	0	4	0	4	0	0	0	0	0	0	0	0	0	
O22	4	0	2	2	0	0	4	3	0	0	0	0	0	0	0	
O26	9	4	1	4	9	0	0	6	0	0	8	8	8	8	8	β (8)[c] CL (1)
28ac	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	
O38	3	0	1	2	0	0	3	2	0	0	0	0	0	0	0	
O55	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	
O73	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	
O84	6	1	1	4	6	0	0	6	0	0	6	0	6	6	5	NT[d]
O88	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	
O103	3	0	1	2	3	0	0	3	0	0	3	1	3	3	3	ε (3) OTC + CTC (1)
O104	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	
O111	2	0	0	2	0	2	0	2	0	0	2	2	2	2	2	β (2) DSM (1) DSM + OTC + CTC (1)
O113	8	3	2	3	0	8	0	5	0	0	0	0	0	0	0	
O116	6	1	3	2	0	6	0	6	0	0	0	0	0	0	0	
O119	2	0	2	0	1	1	0	1	0	0	0	0	0	0	0	
O123	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	
O125	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	
O136	4	1	1	2	4	0	0	4	0	0	0	0	0	0	0	
O153	2	0	0	2	0	2	0	2	0	0	0	0	0	0	0	
O157	1	0	0	1	0	0	1	1	0	0	1	1	1	1	1	γ (1)
O158	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	
O163	2	1	0	1	0	1	1	2	0	0	0	0	0	0	0	
NT	18	4	7	7	1	11	6	14	0	0	2	2	2	2	2	β (2) CL (1)
Total	92	19	28	45	28	42	22	66	0	0	22	14	22	22	21	
O157	Fuku-07			1			1	+[e]	−	−	+	+	+	+	+	γ
O157	Oki-04			1			1	+	−	−	+	+	+	+	−	γ
O157	Sai-11			1			1	+	−	−	+	+	+	+	+	γ
O157	Shizu-19			1			1	+	−	−	+	+	+	+	+	γ
O157	ATCC 43889 (human)					1		+	−	−	+	+	+	+	+	γ
O157	ATCC 43890 (human)				1			+	−	−	+	+	+	+	+	γ
O157	ATCC 43894 (human)						1	+	−	−	+	+	+	+	+	γ
O157	ATCC 35150 (human)						1	+	−	−	+	+	+	+	+	γ

[a] The calves were less than 5 months old, and the heifers were between 5 and 12 months old.
[b] ABPC, aminobenzylpenicillin; DSM, dihydrostreptomycin; OTC, oxytetracycline; CTC, chlortetracycline; KM, kanamycin; CL, colistin.
[c] The numbers in parentheses are numbers of strains.
[d] NT, the strains could not be typed.
[e] +, gene present; −, gene not present.

Only eight isolates were resistant to some of the antimicrobial drugs tested (Table 2). Six of these isolates were resistant to aminoglycosides (kanamycin and dihydrostreptomycin) and/or tetracyclines (chlortetracycline and oxytetracycline), and the other two showed resistance to colistin (Table 2). Resistance to these drugs, which are conventionally used in veterinary medicine, is common in *E. coli* strains from cattle. The LEE genes were found in all five drug-resistant strains which were members of O serogroups other than serogroup O8 but not in serogroup O8 strains.

In early reports workers described STEC infection rates in cattle of 8% for cows and 19% for calves in the United States (28), 17% for cows and 9% for bulls in Germany (16), and 9% for cows and 25% for calves in Canada (32). According to these reports, remarkable differences were found in the rates of recovery of STEC isolates for adult and young animals; however, these reports were published in the first half of the 1990s. In this study we recovered STEC by colony hybridization at slightly higher frequencies (20% for calves, 31% for heifers, and 19% for cows). Blanco et al. have reported that the STEC prevalence in Spain is 35% for cows and 37% for calves (5) and that there is not a significant difference between calves and cows. The use of different detection methods and the rapid development of the methods make it difficult to compare STEC prevalence rates reported for various geographic areas. For the same reason it is impossible to judge whether the prevalence rate in cattle has really increased as reflected in the reports from the last 10 years. However, the results of STEC

screening by PCR indicate that STEC are widespread in healthy cattle in central Japan. Using nested *stx*-PCR, we detected *stx* genes from each cow. We suggest that cattle always discharge various amounts of STEC, but when the number is low, the STEC cannot be detected by *stx*-PCR. We found that many calves were STEC positive and STEC negative as determined by *stx*-PCR when several samples collected at 2- to 8-week intervals were examined (data not shown).

The *stx* genes have been detected in members of approximately 90 to 100 *E. coli* O serogroups (4, 33). Most STEC strains of bovine origin seem to belong to 20 O serogroups: O4, O8, O22, O25, O32, O45, O82, O84, O103, O111, O113, O116, O121, O136, O146, O153, O157, O171, O172, and OX3 (O174) (4, 22). The 92 STEC strains used in our study were classified into 25 O serogroups, but only 10 serogroups (O8, O22, O84, O103, O111, O113, O116, O136, O153, and O157) were the same as the major bovine STEC O serogroups. Less than one-half of the Japanese isolates (44 of 92 isolates) belonged to these major O serogroups. Moreover, a comparison of the serogroup data for Spanish (5) and Japanese cattle showed that members of 13 of the 25 Japanese O serogroups (O1, O15, O28ac, O38, O55, O73, O88, O104, O111, O119, O123, O125, and O158) were found only in Japanese cattle. Similarly, of the 27 Spanish O serogroups, 18 (O4, O9, O20, O41, O74, O77, O78, O82, O90, O91, O92, O105, O132, O146, O150, O162, O165, and O171) were represented only by Spanish STEC strains. The distributions of O serogroups in cattle were thus markedly different in Spain and Japan. A comparison of cattle STEC strains from France (23) and Japan produced similar results. Based on STEC O serogroups, it seems that the cattle in Japan are isolated from the cattle in European countries in spite of the internationalization of the livestock market.

To assess the hazard of bovine STEC for human health, it is necessary to analyze a broad range of virulence-associated genes from isolates obtained from diseased humans and from cattle. Human EHEC typically possess the LEE genes together with *stx* (12) and sometimes the *per* (plasmid-encoded regulation) gene group on the EAF plasmid, which activate the LEE cassette (11, 27). The LEE is a gene group that is essential for microorganisms to cause AE lesions in intestinal cells (12). As most STEC strains isolated from diseased humans carry the LEE cassette (29), a detailed analysis of these genes is justified. The LEE was present in 22 of 92 STEC strains from cattle, whereas *bfp* or the EAF plasmid could not be found in any of these strains. Organisms with the EAF plasmid seem to be isolated very rarely from cattle, as no reports have described *E. coli* isolates with the EAF plasmid from cattle samples. Moreover, no reports have described AE lesion expression of LEE-possessing *E. coli* in weaned calves or adult cattle.

As the size of the LEE cassette is 35 kbp and the LEE cassette contains 41 predicted open reading frames (7), it is impossible to determine by molecular techniques alone whether a strain is an AEEC (including EHEC and EPEC). Therefore, observation of AE lesion expression in cell cultures is a common biological assay used to confirm the presence of AEEC (13). Although the biological assay is very important for confirming bacterial pathogenicity of a clinical sample, using this assay would be a very difficult task for epidemiological investigations because of the number of isolates to be examined. As all AE lesion-positive organisms are included among strains that possess the LEE, detecting LEE genes by a simple method such as PCR should be a very useful means of screening for epidemiological studies. In order to avoid false-negative results for LEE, we developed several PCRs to detect part of *espA*, *espB*, *espD*, and *tir*, genes which are essential parts of the type III secretion system of the LEE. In this study, all 22 STEC strains that possessed LEE could be detected by the *eae*-PCR alone. This result was confirmed by other PCRs for the LEE genes. One strain, however, was negative for *espA*, and another was negative for *tir*. Thus, the strains could be subtyped on the basis of their LEE genes by using the new PCR assays. We expect that these new PCRs will be useful tools for analyzing the LEE cassette.

Finally, as a method for direct detection of EHEC O157 in human samples by using multiplex PCR has been developed (22), a combination PCR for *stx* and intimin type γ may be an effective prescreening method for detection of EHEC O157 in cattle fecal samples because pathogenic O157 strains always contain a type γ *eae* gene in the LEE.

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