APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2003, p. 5690–5692 Vol. 69, No. 9
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.9.5690–5692.2003

Prevalence and Characteristics of *eae*-Positive *Escherichia coli* from Healthy Cattle in Japan

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Received 26 February 2003/Accepted 27 June 2003

The prevalence of *eae*-positive *Escherichia coli* (*eae*EC) in Japan was examined using rectal stool samples taken from 35 calves less than 1 month old, 107 calves more than 1 to 3 months old, 88 heifers more than 3 to 6 months old, 214 heifers over 6 months old, and cows from 95 farms. Screening with *eae* PCR revealed the prevalence to be, with increasing age, 31.4, 8.4, 26.1, and 14.5%, respectively. Of 51 selected *eae*EC strains, more than 40% were serotyped as O26, O103, O111, O145, or O157, which are frequently detected as enterohemorrhagic *E. coli* types. Four strains were identified as recently reported intimin types η, ι, and κ.

Enterohemorrhagic *Escherichia coli* (EHEC), enteropathogenic *E. coli* (EPEC), and attaching and effacing *E. coli* (AEEC) are food-borne pathogens that can cause diarrhea in humans (7, 11, 15). These pathogenic *E. coli* types often possess genes coding for Shiga toxins (*stx* genes) and for intimin (*eae*), an outer membrane protein. *E. coli* strains with *stx* genes are called Shiga toxin-producing *E. coli* (STEC). Cattle are considered to be the main reservoir of STEC strains, including EHEC strains (2, 3). STEC strains are classified into more than 200 O serotypes (4, 16); however, the majority of outbreaks and/or sporadic cases of hemorrhagic colitis and hemolytic-uremic syndrome in humans have been caused by the members of only a few serogroups, such as O26, O111, and O157 (10, 17, 19). Since the strains of these limited O serogroups almost all possess *eae* (3, 9), this gene may be a more useful target than the *stx* genes for screening EHEC strains in cattle fecal samples.

We used PCR to investigate the prevalence of *eae*-positive *E. coli* in cattle feces and genetically characterized the intimin types found, as well as various virulence genes seen in the isolated strains.

E. coli O157:H7 strain ATCC 35150 (American Type Culture Collection, Manassas, Va.) was used as the positive control for stx_1, stx_2, *eae*, and intimin type γ (intimin γ). The *E. coli* strains JS144, 166, VR299-2, and EPEC108, which were used as positive-control strains for intimins α, β, and ε and bundle-forming pilus (*bfp*), respectively, were derived from the stock culture collection of the National Institute of Animal Health, Tsukuba, Japan. A total of 444 rectal stool grab samples were collected from healthy dairy cattle (35 calves under 1 month old, 107 calves more than 1 to 3 months old, 88 heifers more than 3 to 6 months old, 214 heifers more than 6 months old, and cows) on 95 farms located in the western and central parts of Japan between May and November 2001. All rectal stool samples were sampled by veterinarians at regional governmental animal hygiene centers. The samples were placed in cool boxes (4 to 8°C) and taken to the laboratory for immediate processing (usually within 24 h). Each sample of 1 g of rectal stool was enriched in 19 ml of Trypticase soy broth (Eiken, Tokyo, Japan) at 37°C for 18 h. Ten microliters of the Trypticase soy broth culture was inoculated onto MacConkey agar (MAC; Eiken). The MAC plates were incubated at 37°C for 18 h, and a loopful of colonies from an area of confluent growth was tested using *eae*, stx_1, and stx_2 PCR (5, 12). From *eae*-PCR-positive samples we isolated *eae*-positive colonies by colony hybridization with an *eae* DNA probe or by *eae* PCR of randomly isolated individual colonies. The *eae* probes were prepared by labeling *eae*-PCR amplicons from *E. coli* strain ATCC 35150 with a DIG High Prime kit (Roche Diagnostics GmbH, Mannheim, Germany). The pair of primers for *eae* PCR were located at the 5′ end of the *eae* gene, a common region for intimin subtypes (5). For colony hybridization or individual colony PCR, up to 24 typical *E. coli* isolates were taken from the MAC plate. All *eae*-positive isolates were confirmed to be *E. coli* by conventional biochemical tests and, if needed, by an API 20E system. Up to three *eae*-positive colonies per sample were randomly chosen and subjected to O-serogroup typing and classification of intimin types α, β, γ (1), and ε (13) by PCR. If the intimin types of isolates could not be classified by the PCR technique described above, the isolates were analyzed by DNA sequencing of 800 bp at the 3′ end of the *eae* gene (1). One strain per farm was selected for further study from strains showing the same O serogroup and intimin type profile at each farm. The *eae*-positive strains selected were characterized by PCR to determine the presence of stx_1, stx_2, *bfp*, EHEC *hlyA* (14), and *irp2* located in the high-pathogenicity island (HPI) (6).

eae and *stx* were detected in 74 (16.7%) and 138 (31.1%) of 444 animals, respectively (Table 1). There were no regional variations seen in *eae* detection rates. The *eae* gene was detected more often in neonatal animals (~1 month old) than in older animals. The *stx* genes were detected more often in older animals but least often in animals over 6 months old. Interest-

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TABLE 1. Prevalence of *eae* and *stx* genes in different age groups of healthy cattle in Japan

Age of cattle (mo)	No. of cattle[a]	No. (%) of cattle with gene(s):			
		eae	stx_1	stx_2	stx_1 and stx_2
~1	35	11 (31.4)	3 (8.6)	6 (17.1)	5 (14.3)
>1–3	107	9 (8.4)	14 (13.1)	14 (13.1)	4 (3.7)
>3–6	88	23 (26.1)	23 (26.1)	13 (14.8)	16 (18.2)
>6	214	31 (14.5)	25 (11.7)	6 (2.8)	9 (4.2)
Total	444	74 (16.7)	65 (14.6)	39 (8.8)	34 (7.7)

[a] A total of 444 cattle rectal stool samples were collected from 95 farms.

ingly, animals aged >1 to 3 months showed the least frequent presence of both *eae* and *stx*; in contrast, those >3 to 6 months showed a generally higher prevalence. Intimin is known to be a strong immunogen (18), and it is classified into 10 types (21). Although several intimin type groups might generate immunological cross-reactions, *E. coli* strains possessing other intimin types can still infect animals. Therefore, we believe that neonates, which do not have an immunological history of contact with any intimin-expressing organisms, are likely to be the most easily infected by intimin-producing *E. coli*. According to this hypothesis, once an animal has acquired immunity against one intimin type, *E. coli* strains expressing the same type of intimin are eliminated around 2 months after the onset of infection. Consequently, other types of intimin-producing *E. coli* from neighboring or imported animals are likely to infect the animal at heifer age. If this hypothesis is true, intimin types β and γ, especially the intimin γ seen in O157:H7 strains, should act as an effective vaccine for eliminating the human EHEC strain from cattle.

Forty-five of the 51 *eae*-positive strains selected belonged to 20 different O serogroups, with 6 strains being nontypeable. Two isolates were members of serogroup O157, and 26 of the typeable strains could be classified into five serogroups (O26, O70, O103, O145, and O156) (Table 2). The O26, O103, O111, O145, and O157 serogroups, which are frequently detected as causal agents of hemorrhagic colitis and hemolytic-uremic syndrome, together accounted for more than 40% of the *eae*-positive *E. coli* strains examined (19 of 45 strains). These major human potential EHEC strains were isolated from various locations on farms. Almost all of these strains, except strains determined as O145, possessed one or more *stx* genes. On the other hand, when *stx* was used for screening STEC in cattle in 1998, only 20% of STEC strains could be classified into these potential EHEC serogroups (9). The O113 and O116 STEC strains, which have only rarely been isolated from humans, were the most predominant STEC strains in cattle in Japan (9). Moreover, except for two O157 strains, no isolates tested possessed stx_2. This suggests that most *E. coli* strains with stx_2 in cattle in Japan do not possess *eae*. This fact was confirmed in a previous study (9), in which only 3 of 22 STEC strains with stx_2 from cattle possessed *eae*. The three strains were classified as either O111 or O157 (9). Therefore, when potential EHEC strains are screened in cattle fecal samples by PCR, *eae* is likely to be a more useful target than the *stx* gene. However, O26 strains from humans are the most common O serogroup of *E. coli* strains possessing the HPI locus (6); *irp2* in the HPI was also detected in three strains belonging to serogroups O35, O49, and O70. In a recent study, the gene was found in O39 strains isolated from broiler chickens in Finland (8). The HPI locus might be prevalent in many types of *E. coli* in various kinds of animals. In contrast, *bfp* was not found in any of the strains. Organisms with *bfp* seem to be isolated very rarely from cattle, since there are no reports of *E. coli* isolates with *bfp* from cattle samples. In contrast, EHEC-*hlyA* was detected at a high rate of more than 75% (39 of 51 animals). This virulent gene was also detected at a higher rate in STEC in 71.7% (66 of 92) of cattle tested in Japan in 1998 (9). In this study, which confirmed the prevalence in cattle of the EHEC-*hlyA* gene in *E. coli* strains, regarded as gut flora, 23 strains from 23 cattle fecal samples at different farms, which were identified as neither STEC nor AEEC, were examined by PCR. Fourteen of these 23 strains (60.9%) possessed the gene. Therefore, the EHEC-*hlyA* gene is believed to have spread into gut flora *E. coli* in cattle in Japan. It may therefore no longer be an effective marker for identifying pathogenic *E. coli* strains in cattle.

Concerning the typing of intimin, 39 of 51 strains could be identified by established PCR (1, 13). In particular, any one of the strains typed as β, γ, or ε intimin could be amplified by PCR. However, of nine strains identified as α intimin, two strains could not be amplified but were instead identified by DNA sequence analysis. It appears that the DNA sequences of

TABLE 2. Characterization of 51 *eae*-positive *E. coli* strains isolated from healthy cattle in Japan

O serogroup	No. of strains	Subtype(s) of intimin	Presence of gene(s):			
			stx-genes	*bfp*	*irp2* (HPI)	*hlyA*
O15	1	β	−	−	−	−
O26	5	β	1	−	+	+
O26	1	β	−	−	+	+
O26	1	β	1	−	+	−
O35	1	NT[a]	−	−	+	+
O49	1	κ	−	−	+	+
O51	1	β	−	−	−	−
O63	1	ε	−	−	−	+
O70	1	β	−	−	+	+
O70	1	NT	1	−	−	+
O70	1	NT	−	−	−	−
O74	1	NT	−	−	−	+
O76	1	α	−	−	−	+
O84	1	α	1	−	−	+
O103	4	ε	1	−	−	+
O103	1	γ	1	−	−	+
O109	2	α, NT	−	−	−	+
O111	1	γ	1	−	−	+
O115	2	γ, NT	−	−	−	+
O117	1	β	−	−	−	−
O138	1	ε	−	−	−	+
O145	1	η	−	−	−	−
O145	3	γ	−	−	−	+
O153	2	ι	−	−	−	+
O156	4	γ	−	−	−	−
O156	1	α	1	−	−	+
O156	2	ε, NT	1	−	−	+
O157	2	γ	1, 2	−	−	+
ONT[b]	4	α	1	−	−	+/−[c]
ONT	2	α, β	−	−	−	+

[a] NT, could not be typed.
[b] ONT, the strain could not be O serotyped, including strains that were O:rough.
[c] Three strains possessed the relevant gene.

α-intimin-specific regions show greater variation than those of other intimins. This fact has also been recognized in *E. coli* strains with avian intimin (8). According to one report, the DNA homology among α-intimin-specific regions in strains was calculated to be 86 to 100%. On the other hand, the remaining 10 strains were determined as either not typeable (strains which could not be subcloned) or having recently reported intimin types (21). Intimins η, ι, and κ are newly categorized intimin types that have been reported in *E. coli* strains from patients. In this study, we also detected these new types not only from bovine *E. coli* strains but also from different O serogroups (O49, O145, and O153) of human origin (21). For example, Zhang et al. reported that intimin η was detected in O2, O12, and O125 and intimin κ was present in O118 and O157:H42 (21). On the other hand, in a previous report, 6 of 15 *eae*-positive *E. coli* strains isolated from wild birds could not be intimin typed by PCR (8). Since the nontypeable strains are of types that are either newly established or hitherto unknown, if some have newly established types, then the newly categorized intimin types are likely to have been more prevalent in various O serogroups than previously realized, since these strains were serotyped as O54, O72, O103, and O144, which are very rare types in mammals, except O103 (8). We conclude that *E. coli* strains with newly categorized intimin types have different serotypes according to the animal species from which they are derived.

We thank the staff of the Livestock Hygiene Centers for collecting the samples used in this study. We are very grateful to Mika Kanazaki for technical assistance.

This work was supported by grants from the National Agricultural Research Organization, Japan.

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