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Year-round Monitoring of Verotoxin-producing *Escherichia coli* from Feces of Dairy Cattle

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ABSTRACT : A PCR-aided monitoring of verotoxin-producing *Escherichia coli* (VTEC) was performed over the period of 12 months by using fresh feces collected monthly from 5 dairy cows that had been identified as VTEC carriers. The PCR products were confirmed to be verotoxin genes by Southern hybridization using a gene fragment of verotoxin 2 as a probe. Although seasonal variation of VTEC shedding seemed to depend on each cow, several factors may have influenced the frequency of detection. Shedding of VTEC tended to be reduced during grazing from the middle of May up to the beginning of October. Only one cow was positive for VTEC in August. Dry-off was also suggested to have a depressive effect on VTEC shedding, *i.e.* 3 of 4 dry cows showed no shedding of VTEC. Contrary to these factors, winter or indoor rearing tended to increase VTEC with only 5/24 samples being negative during the period from November to April. Total VFA concentration was higher ($p<0.05$) in VTEC-positive feces than in VTEC-negative feces, while fecal pH and VFA proportions were not different. Partial sequences of verotoxin genes from feces of 4 VTEC-positive cows were nearly identical (99-100%), suggesting that gut bacteria sharing the same gene were distributed among the cows. The present results indicate that grazing and dry-off could be factors which reduce VTEC shedding, while winter/indoor rearing may be a factor which increases the shedding, possibly through on-farm interactions. (**Key Words :** Dairy Cattle, Feces, Verotoxin Genes, *Escherichia coli*, PCR Detection, Diet Change)

INTRODUCTION

Verotoxin-producing *Escherichia coli* (VTEC) has attracted much attention since outbreaks of food-borne pathogenic VTEC have occurred all over the world (Gilgen et al., 1998; Sanz et al., 1998). Although causes of these outbreaks are still being argued, alimentary tracts of cattle are one of the most likely sources for VTEC contamination (Miyao et al., 1996; Grauke et al., 2002). If so, dietary condition could influence growth and acid-tolerance of *E.coli* or VTEC in cattle. Diaz-Gonzalez et al. (1998) pointed out that the shift of a feeding regimen from a high grain diet to a high hay diet reduced fecal shedding of VTEC. Subsequently, several studies have been conducted to determine how dietary condition affects VTEC shedding in cattle. However, results based on short term tracking studies were not consistent and conclusive. Fasting promoted growth and maintenance of acid-tolerant *E. coli* (Rasmussen et al., 1993), while it did not cause a significant change in ruminal abundance of VTEC (Harmon et al., 1999). Contrary to the initial statement of Diaz-Gonzalez et al. (1998), high roughage feeding was revealed to stimulate the shedding of VTEC (Hovde et al., 1999).

Based on the above contradictions, more detailed information of intestinal ecology of VTEC is clearly necessary, especially through longer term monitoring of VTEC-positive cattle, since shedding can fluctuate over time (Rasmussen et al., 1993). The mechanisms involved in such ecological variation need to be characterized and the influential factors defined. Research on these may provide clues for minimizing the risks of contamination of meat and milk products at the farm level.

We have attempted a long term experiment to collect feces on a monthly basis over the period of a year and assess VTEC positives as previously defined (Kobayashi et al., 2004).

MATERIALS AND METHODS

Animals, feeding and sampling

Five Holstein dairy cows (cows A-E, 612±37 kg in body weight) previously identified as VTEC-carriers (Kobayashi

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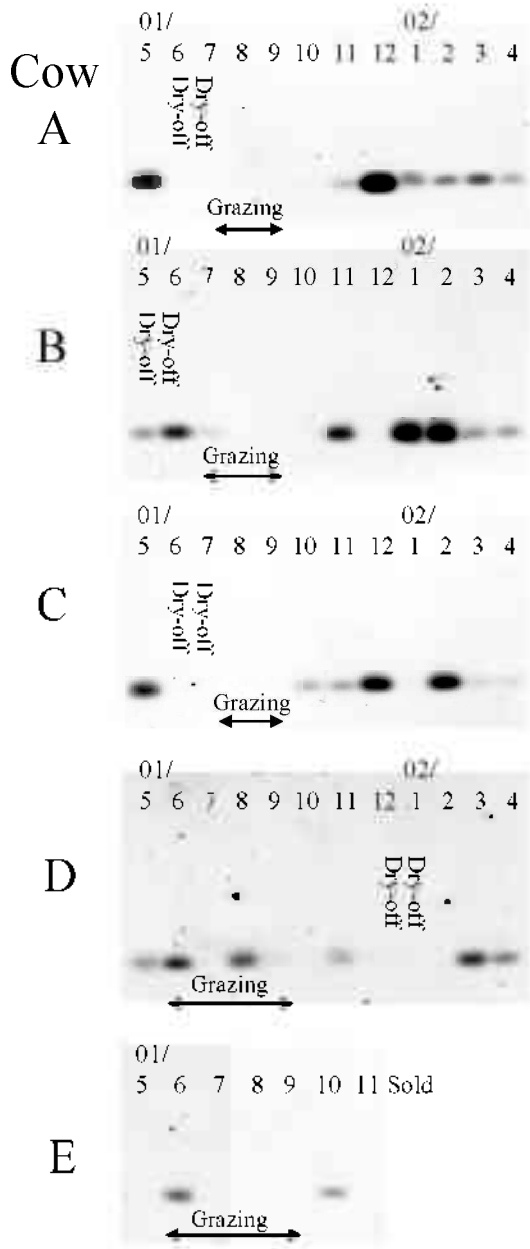

Figure 1. Seasonal detection of VTEC from fresh feces of 5 dairy cows that were previously identified as VTEC- carriers. Detection was carried out by PCR followed by Southern hybridization using verotoxin 2 gene fragment as a probe.

et al., 2004) were used. The cows had been confirmed to be VTEC-positive in 2-3 samples of the last 3 successive monthly fecal samplings before the start of the present experiment. The cows were individually stalled, based on the conventional feeding schedule for milking cows. Briefly, after calving, concentrate was provided at the level of 20% of the milk that each cow produced with *ad libitum* feeding of timothy hay or timothy grass silage. Part of the grass silage was replaced with corn silage and alfalfa silage when available. Cows were grazed on a grass pasture, mainly comprised of timothy and orchard grass (90%) with white clover (10%), for 2-4 h a day from the beginning of May to the beginning of October. Milking was done twice daily (0800 and 1700 h). Cows were dried off when their milk production became less than 10 kg/d (about 10-12 months after calving), and then the dry cows were maintained for about 2 months mainly on hay and grass silage with a minimal amount of concentrate (1 kg/d) but without being allowed to graze or to be kept outdoors. All the cows were held indoors continuously in winter.

Fresh feces (approximately 100 g/cow) were taken directly using the rectal grab technique. The sampling was carried out once a month (at 1200 h on the first Tuesday of each month) over a year from May, 2001 to April, 2002. Feces were thoroughly mixed and a portion (10 g) was frozen at -80°C until analysis.

Analyses

Feces were subjected for DNA extraction according to the method of Purdy et al. (1996). In brief, feces were mixed in a plastic tube with glass beads, Tris-EDTA buffer, phenol and sodium dodecyl sulfate by several shakings at 0°C. The tubes were centrifuged to separate DNA which was then purified by hydroxyapatite chromatography followed by gel-filtration. The purified DNA was employed as a template for PCR detection of VTEC using a commercially available kit (O157 detection kit, Takara) that allowed amplification of either of 4 different verotoxin genes (*vt1*, *vt2*, *vt2vha* and *vt2vpl*). Southern hybridization with Alkphos DNA hybridization kit (Pharmacia Biotech) was made to specifically detect VTEC at high sensitivity. DNA sequencing with a ThermoSequenase Cycle Sequencing kit (Amersham) and a DSQ2000L automated DNA sequencer (Shimadzu) was performed to predict phylogenetic relationships between the genes detected. The *vt2* from a mountain goat found in a previous study (Kobayashi et al., 2004) was used as a source for constructing a verotoxin gene probe for the hybridization. All the procedures for DNA manipulation were according to the manual of Sambrook and Russell (2001).

Sequences of verotoxin genes amplified by PCR were aligned by Clustal W ver.1.81 and a phylogenic tree showing the relatedness of the sequences of verotoxin genes was drawn by the neighbor-joining method with Tree View software. A boot strap value showing reliability of each branch of the phylogenic tree was obtained by repeating the calculation 1,000 times using Phylip package software available at a Web site (http://evolution.genetics.washington.edu/phylip.html). The sequence of *vt2* gene used as a probe and the sequences of verotoxin genes detected in feces from cows A-D during the winter indoor rearing period (January-April, 2002) in the present study were submitted to the DDBJ data base under the following accession numbers: Probe, AB259615; A1, A2, B1, B2, B3, B4, C1, D1, D2,

Table 1. Comparison between VTEC-negative and -positives feces in pH, total VFA and VFA proportions

VTEC carriage	pH	Total VFA (mmol/g)	Acetate	Propionate	Butyrate
			molar %		
Nagatives (n = 26)	7.19±0.18	29.2±11.7	72.1±4.1	18.1±2.5	5.2±1.8
Positives (n = 29)	7.12±0.28	39.4±14.3***	72.4±2.7	18.4±1.3	4.7±1.3

Five experimental cows previously identified VTEC-carriers were employed as year-round donors of feces that were used for analyses of VTEC detection, fecal pH and VFA.

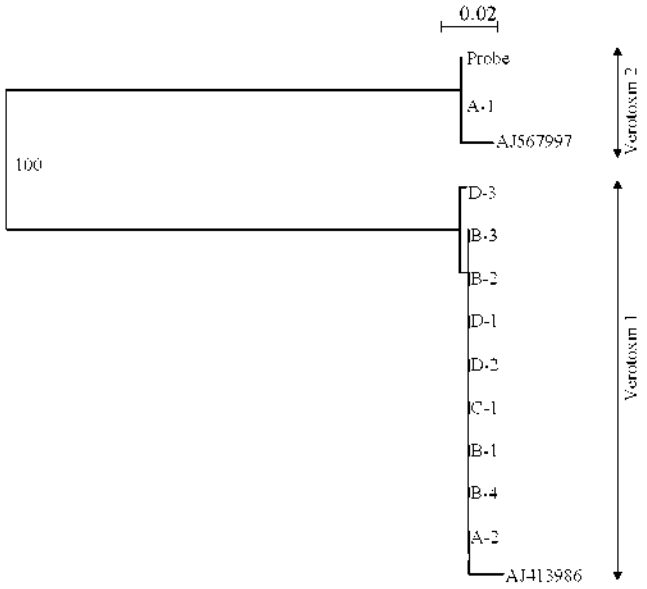

Figure 2. Phylogenic tree showing relatedness of verotoxin genes obtained from the experimental cows and other sources. Probe, verotoxin 2 gene from mountain goat used as a probe for hybridization; AJ413986, shiga toxin 1 gene; AJ567997, shiga toxin 2 gene; A1-2, genes cloned from cow A; B1-4, genes cloned from cow B; C1: a gene cloned from cow C; D1-3: genes cloned from cow D.

and D3, AB259605 to AB259614, respectively.

Feces (2 g) were suspended in distilled water in a volumetric flask (25 ml) and filtered to measure pH with a glass electrode (Horiba, Japan) and VFA on a gas chromatograph equipped with a capillary column and FID (Shimazu GC14B, Japan). Differences in fecal pH, total VFA and VFA molar proportions between VTEC-negative and -positive feces were analyzed by Student's t test.

RESULTS

Figure 1 shows monthly PCR monitoring of VTEC in feces of individual cows with more sensitive and specific detection by Southern hybridization using the *vt2* fragment as a DNA probe. Since seasonal variation of VTEC shedding seemed to be dependent on each individual, the detection results are described for each cow as follows.

In cow A, VTEC became undetectable during the 2 month dry-off period (June-July, 2001) followed by a 3 month period (Aug-Oct) which included a grazing period. However, VTEC was restored in the period from November, 2001 through April, 2002 which was the winter indoor-rearing season during which there was no grazing or outdoor housing. Cow B was VTEC-positive from May through June, 2001 which was a dry-off period. During the period from July up to October, VTEC was not detectable for this cow, similarly to cow A. During the indoor season from November up to April (except December), VTEC was restored in cow B, again similarly to cow A. Cow C showed similar results almost to those of cow A, *i.e.* VTEC became undetectable in the 2 months dry-off period after June, 2001, but was restored in the indoor rearing season from October, 2001 through April, 2002 excluding January. Although inconsistent detection results were obtained for cow D, the dry-off period (January to February, 2002) was VTEC-negative even in the indoor winter rearing season. This cow was VTEC-positive even during the grazing period (August) and VTEC-negative during the indoor period (December). VTEC was not detectable in cow E during the grazing period from July to September; thereafter, cow E was excluded from the experiment by being sold.

Overall, grazing, summer or outdoor rearing, from the middle of May up to the beginning of October, tended to reduce VTEC shedding. Especially, during the period from July to September, all 5 cows were VTEC-negative except for cow D in August. Dry-off also tended to lower VTEC shedding, *i.e.* 3 of 4 dry cows (cows A, C and D) showed no shedding of VTEC. Contrary to these factors, winter or indoor rearing seemed to be a stimulating factor for VTEC shedding, *i.e.* 4 cows (cows A-D) shed VTEC during the period from November to April, except for 5 cases out of a total of 24 samples.

Fecal pH was in a range from 6.36 to 7.71. Total VFA showed high variation, ranging from 9.3 to 74.5 μmol/g feces. Molar proportions of VFA varied within the range of 68 to 74%, 15 to 20% and 3 to 6% for acetate, propionate and butyrate, respectively. Since these parameters showed no notable change with seasons and animals, the data were pooled for VTEC-negative and -positive feces to obtain averages and standard deviations for comparison (Table 1). Although differences were not observed for fecal pH and VFA molar proportions between VTEC-positive and -negative samples, total VFA concentration was significantly higher in the positives than in the negatives ($p<0.001$).

Relatedness of the cloned verotoxin genes from 4 cows

Figure 3. Comparison of deduced amino acid sequences of verotoxins obtained from the experimental cows and other sources. Although amino acid residues different between verotoxins 1 and 2 are shaded, those within each verotoxin are identical. Probe, verotoxin 2 gene from mountain goat used as a probe for hybridization; AJ413986, shiga toxin 1 gene; AJ567997, shiga toxin 2 gene; A1-2, genes cloned from cow A; B1-4, genes cloned from cow B; C1: a gene cloned from cow C; D1-3: genes cloned from cow D.

(total 10 genes obtained in winter indoor season) to known genes is presented as a phylogenetic tree in Figure 2. The cloned sequences were separable into 2 clusters with 100% bootstrap value. One clone from cow A (A1) was a verotoxin 2 gene that showed 100% sequence identity with the formerly found *vt2* from a mountain goat (Probe) and 99% identity with shiga toxin 2d subunit A (AJ567997). The other 9 cloned sequences from 4 different cows (A2, B1-4, C1, D-3) were verotoxin 1 genes, sharing 99-100% identity within them and also 98% identity with known shiga toxin 1A variant OX3 (AJ413986).

Deduced amino acid sequences of the cloned verotoxin genes are aligned in Figure 3. All the sequences from the clones obtained in the present study were perfectly (100%) identical to known sequences, *i.e.* one sequence was verotoxin 2, while 9 sequences were verotoxin 1, as assumed from DNA sequence. The high sequence identity of verotoxin 1 within the clones obtained from 4 cows, in particular the high identity at DNA level, suggests that all the genes originated from a common source(s) and were transferred among cows reared on the same farm.

DISCUSSION

In the previous study, we developed a protocol for PCR detection of VTEC, by which six dairy cows were identified as VTEC carriers on a farm (Kobayashi et al., 2004). Five of these 6 cows remaining on the farm were seasonally monitored in their shedding of VTEC under practical feeding conditions over a year including the periods for calving, milking, grazing and dry-off. VTEC is basically a minor member of the intestinal microbiota of cattle and is considered to vary with changes of intestinal environment. Such changes are caused mainly by variation in diet and consequently could be influenced by stage of lactation. After the analyses of 54 samples, two factors were proposed to decrease the occurrence of VTEC in the experimental cows. Shedding of VTEC was lower during grazing (outdoor rearing) and dry-off.

VTEC became undetectable in feces by PCR and hybridization analyses during the grazing period from the middle of May up to the beginning of October. Over this period, 12 of the total 15 samples were VTEC-negative (Figure 1). Especially, during the period from July to September, all the five cows were negative except for one cow in August. Therefore, grazing or outdoor rearing, may be a factor to reduce VTEC shedding. Jonsson et al. (2001) similarly reported that summer grazing decreased occurrence of VTEC-positives, pointing out there was less opportunity for access of negative cattle to positive cattle and more opportunity for clearance of VTEC by ingesting fresh forage. Another possible cause of the depressive effect of grazing is the presence of saponin in fresh clover that is known to prevent the growth of VTEC (Rasmussen et al., 1999). Softer feces, often observed during grazing, also may facilitate clearance of VTEC from the large intestine, possibly by faster passage of digesta (Jonsson et al., 2001). Contrary to grazing in summer, indoor rearing in winter enhances the opportunity for uninfected cattle to get access to VTEC-infected cattle (Thran et al., 2001). In fact, a high

occurrence of VTEC during the winter period from November to April was demonstrated, with 19 of a total 24 samples which were positive (Figure 1).

Of 4 cows in the dry-off period, 3 cows were negative throughout the period (Figure 1). In these cases the verotoxin genes were not detected at all even after PCR/Southern hybridization that allows at least 100 times more sensitive detection in comparison with a normal PCR (Kobayashi et al., 2004). This indicates that VTEC disappeared or decreased to undetectable level in feces, compared to those detected during the milking period. In the dry-off period, the dietary condition drastically shifts toward reduction of concentrate supply, cessation of corn silage feeding and an increase in the intake of hay. Such shifts could cause lowering of the number of *E. coli* including VTEC with limited supply of starch to animal large intestine as a growth substrate for VTEC (Diez-Gonzalez et al., 1998). Significantly higher total VFA concentration in VTEC-positive feces (Table 1) may support this assumption, while fecal pH did not respond significantly to VTEC incidence in feces. The reason for no difference in pH is not clear. Ammonia may be higher in VTEC-positive feces, even though it was not determined.

Changes in gastro-intestinal environments and microbiota within the experimental cows are brought by complex factors including diet, management and physiological status of animals. It is, therefore, difficult to identify what factor should be mainly manipulated to minimize VTEC occurrence. In addition, as pointed out in the previous report, the PCR targets are not VTEC themselves but verotoxin genes that are also distributed in other organisms as well as *E. coli* (Schmidt et al., 1993). Careful consideration should be taken if the data are used for controlling these microorganisms.

One of the important results in the present study is that the partial sequences of verotoxin genes detected are nearly identical (99-100%) to each other (Figure 2). This suggests that the same gene, probably originated from the same source, is widespread on the same farm and the infection is probably transferred as a result of contact between animals. This could occur through environmental sources such as bedding, feed or water (Davis et al., 2005). It is, therefore, reasonable that indoor and outdoor management cause opposite results on VTEC occurrence as observed in the present study (Figure 1). Also, a more comprehensive survey is necessary when the hygiene of farm and rural environments is considered (Johnson et al., 1999).

In conclusion, VTEC shedding in dairy cows showed variations within seasons and animals, though grazing and dry-off are thought to be factors to reduce the shedding, while winter/indoor rearing may be a factor to increase the shedding. On-farm direct and/or indirect interaction among cows could increase shedding opportunity, since the partial sequence of verotoxin genes detected are nearly identical.

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