Molecular Detection of Verotoxigenic *Escherichia coli* (VTEC) from Animal Feces for Screening VTEC-shedders

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ABSTRACT : Seventy-six animals including cattle, sheep, horses, 6 species of zoo animals were employed for collection of fresh feces in order to detect verotoxigenic *Escherichia coli* (VTEC) by safe, quick and sensitive PCR-based molecular methods. Bacterial cell disruption with bead-beating followed by bacterial DNA purification with hydroxyapatide chromatography and gel filtration allowed DNA preparation from animal feces with high recovery and purity. A mountain goat was firstly shown by PCR and sequencing to shed verotoxin 2 gene (*vt2*) that was used to generate *vt2* probe and second primer set for nested PCR to attempt more sensitive detection. Most sensitive nested PCR revealed that 45% of tested cattle and 47% of tested zoo animals were VTEC-positive, while least sensitive normal PCR detected VTEC from none of these animals except a mountain goat. Moderately sensitive detection by PCR in combination with hybridization suggested that the VTEC density varied between the VTEC-positive cattle. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 3 : 423-427)***

Key Words : Verotoxin, *E. coli*, Cattle, Mountain Goat, PCR, Hybridization

INTRODUCTION

Verotoxigenic *Escherichia coli* (VTEC) is an acid-tolerant pathogenic bacterium, causing acute illness by infecting into human intestinal tissues via food (Johnson et al., 1999). One of such VTEC strains is represented by *E. coli* O157:H7 that has been known in several outbreaks all over the world. Beef is thought to be major carrier of VTEC that could be originated from alimentary tract of cattle (Gilgen et al., 1998). Recent reports say that animal gut such as rumen and large intestine is a reservoir of VTEC, occurrence of which could be elevated by some dietary conditions such as high grain feeding (Diez-Gonzalez et al., 1998) and fasting (Rasmussen et al., 1993). Meanwhile, there is an opponent report saying that a high roughage diet could promote shedding of VTEC in experimentally inoculated cattle (Hovde et al., 1999). Apparently, this controversy on dietary factors remains to be concluded.

We have been motivated to explore possible dietary control of VTEC occurrence in alimentary tract of cattle in order to minimize the risk of VTEC infection through beef that could be contaminated with VTEC when slaughtered. In the present study, as the first step, a safe, rapid and sensitive protocol for detecting VTEC was developed by using molecular methods targeting verotoxin genes, which did not require hazardous process and special experimental facility for culturing enumeration of VTEC as most of the previous studies involved. Next, the protocol was applied to screening of VTEC-positive animals that could be used for further investigation to determine dietary factors influencing the occurrence of this pathogen.

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MATERIALS AND METHODS

Animals, sampling and DNA preparation

Seventy-six animals in total were employed for collection of fresh feces. These consisted of 20 fattening beef steers (Holstein×Japanese black) and 12 sheep (Corridale×Suffolk) in Farm A, 22 dairy cows (Holstein) in Farm B, 5 horses (Thoroughbred) in Farm C and 17 animals belonging to the genus *Bovidae* in Zoo D that were 3 Japanese serows (*Capricornis crispus*), 3 Formosan serows (*Capricornis swinhoei*), 3 mountain goats (*Oreamnos americanus*), 3 chamois (*Rupicapra rupicapra*), 3 gorals (*Nemorhaedus goral*) and 2 musk oxen (*Ovibos moschatus*). The feces were frozen at -80°C until analysis. Beef cattle were on concentrate and rice straw diet (approximately, 8:2), while dairy cows were on concentrate and an orchardgrass hay/silage diet (approximately, 1:9 to 3:7). Sheep, horses and all zoo animals were fed a timothy hay with small amount of wheat bran.

The following 5 different protocols for DNA extraction and purification from feces were tested to know which bore the best PCR results. A boiling method (Stewart et al., 1998) (protocol 1), a bead-beating method (Stahl et al., 1988) (protocol 2), a hydroxyapatite (Bio-Gel HTP, Biorad) purification after bead-beating (Purdy et al., 1996) (protocol 3), a gel-filtration (Microspin S-200HR columns, Pharmacia biotech) after this (protocol 4), and a repeated phenol-chloroform purification after bead-beating (protocol 5) were compared for PCR amplification of partial 16S rRNA gene of *E. coli* (*see* bellow).

(A)

Protocol	1	2	3	4	5
Recovered DNA from feces (ng/g feces)	83	246	249	266	74

(B)

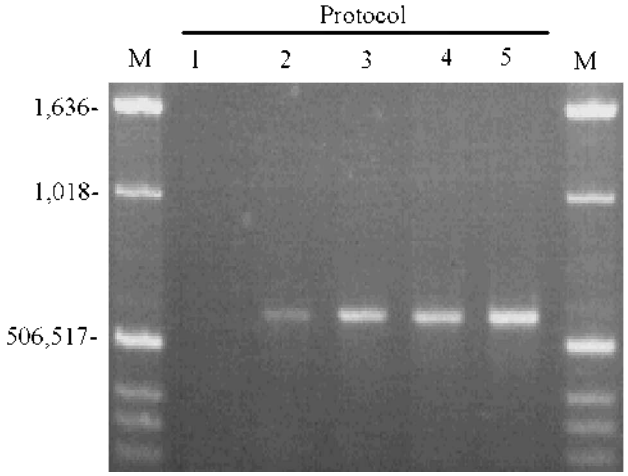

Figure 1. Comparison of fecal DNA recovery (A) and purity as judged by PCR detection of *E. coli* 16S rRNA gene (B) between 5 different DNA extraction/purification protocols. M: molecular size marker, Protocol 1: boiling, 2: bead beating, 3: bead beating+hydroxyapatite chromatography, 4: bead beating+ hydroxyapatite chromatography+gel filtration, 5: bead beating+ repeated phenol/chloroform extractions.

Molecular detection

E. coli in feces was attempted to detect by PCR amplification of a part of 16S rRNA gene (a range from 452nd to 1.035th nucleotide position) by using primers, the sequences of which are specific to *E. coli* (Tsen et al., 1998). Detection of VTEC was performed by using a commercial kit (Takara 0-157 PCR Screening Set, Tokyo) targeted for a partial region (170 bp from 395 th to 565 th nucleotide position) of 4 different verotoxin genes (*vt1*, *vt2* and the two *vt2* variants, *vt2vha* and *vt2vpl*) according to the condition of manufacturer's recommendation. PCR products were validated their sizes with electrophoresis of 2% Metaphor agarose (Nakalai, Kyoto) after visualized with an ethidium bromide staining.

The PCR products for detecting VTEC were employed for Southern hybridization using *vt2* found in the present study (see Results) as a DNA probe. Then the probe was labeled with alkaline phosphatase (AlkPhos direct labeling and detection kit, Amersham Pharmacia Biotech). DNA blotting from a gel to a nylon membrane (Boehringer, Germany) was performed via a capillary suction (Sambrook and Russell, 2001). The PCR products were also used as templates for a nested PCR for more sensitive detection of verotoxin genes by using a primer set newly designed at internal region of the sequence of the first PCR products. Sequences of the designed primers for nested PCR were 5'-TGC AAC GTG TCG CAG CGC-3' (a forward primer targeting the 416th to 433rd position of *vt2*) and 5'-GAC AAA ACG CAG AAC TGC TC-3' (a reverse primer targeting the 533rd to 552nd position of *vt2*). Sensitivity of VTEC detection by PCR plus hybridization and nested PCR was roughly validated by performing the detection using serially diluted sample DNA as a template. The PCR products targeted for *vt* genes were confirmed as a part of a *vt* gene by sequencing (ABI PRISM 310), in which a cycle sequencing kit (DYEnamic ET terminal cycle sequencing kit, Amersham Pharmacia Biotech) was used. DNA and deduced amino acid sequences were processed and aligned via a software of Genetyx (Tokyo Software Co.) and a web site of BLAST and Clustal W (http://www.ddbj.nig.ac.jp/E-mail/clustalw-j.html).

RESULTS

DNA amount recovered from feces of a beef cattle was higher in the protocols 2, 3 and 4 than the protocols 1 and 5 (Figure 1A), while purity of the DNA as judged by visualized PCR products on the agarose gel was higher in

Table 1. PCR detection of total *E. coli* and verotoxigenic *E. coli* (VTEC) from various animals

Animal (number)	Source	Total *E. coli* by PCR	VTEC*	
			by PCR	by nested PCR
Beef cattle (20)	Farm A	12	0	7
Dairy cattle (22)	Farm B	12	0	12
All cattle (42)		24	0	19
Sheep (12)	Farm A	0	nd	nd
Horses (5)	Farm C	0	nd	nd
Japanese serow (3)	Zoo D	1	0	1
Formosan serow (3)	Zoo D	1	0	1
Mountain goat (3)	Zoo D	2	1	1
Chamois (3)	Zoo D	3	0	3
Goral (3)	Zoo D	3	0	2
Musk ox (2)	Zoo D	0	nd	nd
All zoo animals (17)		10	1	8
Total (76)		34	1	27

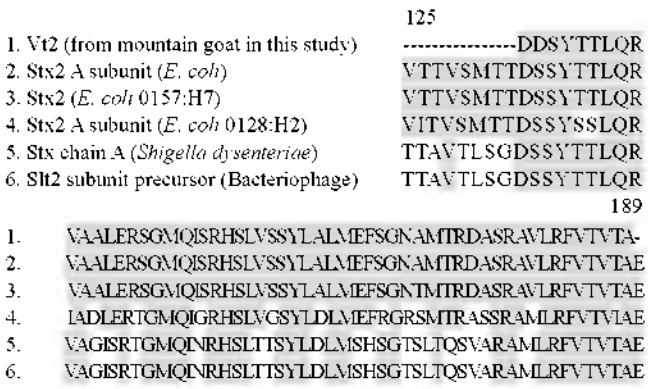

Figure 2. Multiple alignment of amino acid sequences of Verotoxin 2 from feces of a mountain goat and other sources. Amino acids sharing 100% identity are shaded.

the protocols 3, 4 and 5 (Figure 1B). Taking into account of these results, the protocol 4 was chosen as the best of the five protocols for DNA extraction and purification, and then used for all the following analyses.

Detection of total *E. coli* and VTEC in feces of all tested animals by normal PCR and nested PCR is shown in Table 1. For total *E. coli*, fecal DNA from 12 of 20 beef cattle, 12 of 22 dairy cows and 10 of 17 zoo animals showed amplification of a partial 16S rRNA gene of *E. coli* that was visible on an ethidium bromide-stained agarose gel. However, all feces of 12 sheep and 5 horses contained no or quite less *E. coli*, DNA of which was not visible enough even after PCR. By using all these *E. coli*-positive samples, PCR detection of VTEC was attempted. Then, 6 feces only from zoo animals showed amplified DNA on a gel, sizes of which were 140, 170 and 190 bp. These amplificants were sequenced and then only one of the six sequences was found as a partial verotoxin gene, which was originated from a mountain goat.

This gene fragment showed high similarity in DNA sequence with known verotoxin genes, sharing 95-98% DNA indentities. The deduced amino acid sequence showed 100% identity with Verotoxin 2 (VT2) of *E. coli* and 95-97% with other known sequences of Shiga toxin 2A (STX2A) or Shiga-like toxin 2 (SLT2) (Figure 2). Therefore, the obtained gene sequence was the first confirmation of *vt2* from a mountain goat.

Table 2 gives comparison in sensitivity for VTEC detection between 3 different methods (normal PCR, PCR plus Southern hybridization and nested PCR) that were applied to 12 dairy cow samples showing *E. coli*-positive. None was VTEC-positive by normal PCR, while 6 and 12 cows were positive by the hybridization and nested PCR, respectively. Then, molecular sizes of the hybridized signals and the amplificants were exactly as predicted (171 and 137 bp, respectively). PCR plus hybridization and nested PCR could detect verotoxin genes even in 10^2 to 10^3 fold- and 10^4 to 10^5 fold-diluted DNA from these samples, respectively.

DISCUSSION

Extraction and purification of fecal DNA

Several protocols for DNA extraction and purification from animal digesta have been proposed (Stahl et al., 1988; Stewart et al., 1998) and even a commercial kit (Qiagen) is available. Our assessment revealed that one of them developed by Purdy et al. (1996) bore satisfactory results of *E. coli* 16SrDNA-targeted PCR in the respects of DNA recovery and purity, i.e. cell breaking by a bead-beating followed by DNA purification with a hydroxyapatite chromatography and a gel filtration was enough for obtaining DNA high in quantity and quality as a template for PCR (Figure 1). PCR can be inhibited by several contaminants in environmental samples including feces that are proteins, organic acids and polysaccharides (Purdy et al., 1996). These are considered to be fairly well eliminated through two resins in the present study. By using this protocol, it might be expected that bacteria present at a level of 10^5/g may be detected, since *E. coli* inoculated to supernatant of squeezed and centrifuged feces was detected up to this level by PCR (data not shown). In this regard, all the sheep and horses do not seem to shed *E.coli* above this minimum detection level ($\times 10^5$/g). Although a boiling method is simpler and less time-consuming, it was not suitable for the present PCR, especially PCR targeting quite minor bacteria in animal feces.

VTEC distribution among various animals

All 34 samples showing positive in PCR targeting total *E.*

Table 2. Comparison between 3 difference methods in sensitivity of VTEC detection

Method	Detection signal	DNA size	Detection frequency[a] (VTEC/*E. coli*)	Relative sensitivity[b]
Normal PCR		←171 bp	0/12	1
PCR plus southern hybridization		←171 bp	6/12	10^2-10^3
Nested PCR		←137 bp	12/12	10^4-10^5

[a] Detection frequency is shown by numbers of VTEC positive animal/numbers of *E. coli* positive animal.
[b] Minimum dilution of sample DNA by which VTEC turns undetectable with corresponding detection method.

coli were employed for detection of VTEC by normal PCR. Then, one of 17 zoo animals, a mountain goat, was finally confirmed VTEC-positive by sequencing (Figure 2), which is the first demonstration of presence of *vt2* from this animal species. Other zoo animals also exhibited possession of *vt2* by nested PCR (Table 1), suggesting wide distribution of VTEC among various herbivorous animals. Besides herbivores, dogs, cats and birds are so far known as potential hosts of VTEC (Johnson et al., 1999). Although there is no idea about infection route of VTEC to the present zoo animals, feeds such as hay and wheat bran would be carriers of VTEC. In addition, these animals might had contacted with other VTEC-infected animals before transported and/or captured. Actually, some wildlife including Japanese sika deer (Asakura et al., 1998) and North American moose (Todd et al., 1999) are reported to shed VTEC, which might be partly caused by horizontal transfer from domestic animals living closer to their natural habitat.

VTEC occurrence and presence level in cattle

Frequency of VTEC occurrence in cattle has been reported in many countries. The highest occurrence, 44% (40 of 91 healthy cows), is recorded in feces of Argentine cows just prior to slaughter (Sanz et al., 1998), which is followed by 42% (38 of 90 healthy cattle) in rectal samples of Japanese cattle (Miyao et al., 1996). Meanwhile, VTEC was only detected during winter with an occurrence rate of 9.5% in the case of grazing heifer of USA (Thran et al., 2001). The occurrence rate obtained by nested PCR in the present study (36% for 27 of 74 animals) (Table 1) is within a range of the reported percentages.

All VTEC-shed cattle detected by most sensitive nested PCR was initially negative by least sensitive normal PCR, whether they are beef cattle on high concentrate diet or dairy cow on high roughage diet (Table 1). This suggests that VTEC might not flourish under these practical feeding conditions. In the mean time, VTEC density seemed different between the VTEC-positive cows, judging from the results with moderately sensitive detection by PCR plus hybridization, i.e. 6 dairy cows were considered to shed more VTEC than another 6 cows (Table 2). It is of interest to determine what factor causes such variation of VTEC density between the positive animals.

Opportunities and problems

We firstly found *vt2* from a mountain goat that was useful as a material for generating a DNA probe and inner primers in order to screen VTEC-shedding animals with higher sensitivity. Many positive animals including cattle and several species of zoo animals were finally screened, suggesting that all these animal species could be reservoir of VTEC. The safe, rapid, sensitive and culture-independent detection established in the present study may allow more extensive research dealing with large number of samples from wider range of animals, plants and even surrounding environments. Meanwhile, the present detection is less quantitative, and is targeted for verotoxin genes that could be present in other bacteria besides VTEC (Schmit et al., 1993). Even in VTEC, only subset of VTEC strains is pathogenic for humans (Panton et al., 1997). Therefore, further interpretation of the detection results should be careful, especially about details of the presence level and the possible human risk of VTEC.

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