Long-Term Excretion of Shiga Toxin-Producing *Escherichia coli* (STEC) and Experimental Infection of a Sheep with O157

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(Received 4 March 2002/Accepted 12 July 2002)

**ABSTRACT.** To investigate a long-term shedding of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) from sheep, a fifteen-month study for STEC isolation from a sheep, which had yielded STEC before, was attempted. The sheep continued to shed STEC and 39 STEC were isolated. The number of STEC in the feces was estimated at 1.7 × 10^6 per gram. In addition, although Stx1-negative O157 and stx2-encoding bacteriophage were experimentally infected to the sheep, Stx-negative O157 or Stx2-producing bacterial cells were not detected. The genetical and biochemical characterization of those 39 STEC strains showed that all STEC strains produced Shiga toxin 1 (Stx1) and were divided into three classes (I to III). From phylogenetic analysis of their amino acid sequences, class-I STEC was classified as group 1 comprising mainly human STEC, and classes II/III were as group 2 comprising sheep STEC. Our results suggest that STEC easily colonized in sheep and that the sheep continued to shed STEC, showing that sheep might be an important reservoir for human STEC infection.

**KEY WORDS:** bacteriophage, feces, O157, ovine, STEC.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is associated with infectious diseases in humans and animals. Although the predominant STEC serotype associated with human infection is O157:H7 [6], non-O157 STEC can also cause human diseases [1, 11]. Most STEC are non-pathogenic for animals, which result in such healthy animals as cattle, sheep, goats, pigs, poultry, dogs and cats [5, 15, 21, 25, 27], harboring STEC asymptomatically, while several STEC serotypes cause diseases in pigs and calves [4]. STEC survive in bovine and ovine slurry for a long time and can retain the potential to produce Stx [10, 12]. Therefore, animals with STEC may be a major reservoir of human STEC infection. Generally, STEC is detected in 0.3–9.9% of cattle and in 0.9–49% of sheep [3, 6, 15, 30]. When STEC was experimentally inoculated to sheep, it was excreted for up to 50 days [13, 14, 16]. These results strongly suggest that, as STEC might colonize easily in sheep, sheep might be an important reservoir of STEC that infect humans. In this study, we focused on the sheep from which STEC (serotypes O153:H25 and O2:Hnt) was isolated in July and November 1997, respectively [3], to examine its continued shedding of STEC, and also, the role of sheep in human STEC infection was discussed from the DNA analysis.

**MATERIALS AND METHODS**

*Sheep*: A three-year-old male sheep, which was identified as a STEC carrying natural host in our previous paper [3].

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teriophage to the sheep: Approximately 10⁹ cells of Stx
non-producing O157:H7 (Stx-negative O157) originating
from house fly [9], which was grown in BHI at 37°C for 16
hr with shaking, were orally inoculated to the sheep after
the above experiments to isolate STEC. Then, five pieces of
fresh feces were weekly collected for 20 weeks and directly
plated on DHL and Sorbitol MacConkey (SMC; Eiken Co.,
Ltd.) agar plates, and O157 colonies were counted as
described above. At 20 weeks post infection with O157,
10¹¹ plaque-forming units of stx²-encoding bacteriophage,
Stx²φ-K7 [19], were orally infected to the sheep. Then, the
fecal shedding of Stx-positive O157 or E. coli was examined
for additional 10 weeks in the above-mentioned manner.

Other procedures: Disk diffusion susceptibility tests by
using Sensi-Disc (Becton Dikinson, U.S.A.), pulsed field
gel electrophoresis (PFGE) by using XbaI, isolation of total
DNA and DNA sequencing were carried out as described
previously [3, 19]. Nucleotide sequences have been submit-
ted to the DDBJ data bank as accession numbers AB071619
and AB071620 for class-I, AB071621 and AB071622 for
class-II, and AB071623 and AB71624 for class-III.

RESULTS

Isolation of STEC from a sheep: Fecal samples were col-
clected from a sheep for 13 months, followed by enrichment
and PCR amplification. A total of 19 non-O157 STEC were
isolated (Table 1), but O157 cells were not (data not shown),
showing that STEC was shed continuously every month
from the sheep, except September (Table 1). In September,
isolation of STEC was attempted three times, but no STEC
was detected.

Numbers of STEC in sheep feces: A total of 500 red E.
coli colonies on the DHL agar plates were randomly
selected for detection of STEC by colony hybridization
using a 518 bp stx-specific probe to count the number of
STEC in sheep feces. Twenty colonies were identified as
non-O157 STEC (Table 2), estimating that approximately
1.7 × 10³ of STEC cells might be present in one gram of
sheep feces, which contained 4.3 × 10⁵ red colonies (data
not shown).

Experimental infection with Stx-negative O157 and bac-
teriophage: When 10⁹ cells of Stx-negative O157:H7 were
orally inoculated into the sheep, the level of fecal shedding
of O157 cells from the 3rd to 14th weeks ranged from 10² to
10³ cells/g (data not shown). However, after the 15th week,
over 10⁴ cells of O157 were constantly detected in one gram
of feces (data not shown). In addition, all O157 colonies
were Stx-negative and some of them were identical to the
parental strain by PFGE (data not shown). In addition,
although stx²-encoding bacteriophage was experimentally
inoculated to the sheep, Stx2-positive E. coli or O157 were
not isolated during the experiments (data not shown).

Genetical characterization of STEC isolates: All 39
STEC isolates produced only Stx1, but no STEC isolates
were eaeA-positive and 22 isolates were hlyA-positive
(Tables 1 and 2). Those hlyA+ 22 isolates were resistant to
both streptomycin (Sm) and tetracycline (Tc), but other iso-
lates were sensitive to all antibiotics (Tables 1 and 2). Their
serotypes were grouped into three, ONT:H8, ONT:NM and

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sampling date</th>
<th>Serotypea)</th>
<th>Stxb)</th>
<th>PCRc) stx¹ stx² eaeA hlyA</th>
<th>Antibiotic resistance</th>
<th>Plasmid profile</th>
<th>PFGE</th>
<th>Class</th>
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<td>ONT:NM</td>
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<td>+</td>
<td>Te,Sm</td>
<td>A</td>
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<td>+</td>
<td>Te,Sm</td>
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<td>ONT:NM</td>
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<td>+</td>
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<td>+</td>
<td>Te,Sm</td>
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<tr>
<td>110502</td>
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<td>–</td>
<td>Te,Sm</td>
<td>A</td>
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<td></td>
<td>O157:H7</td>
<td>1,2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>NT</td>
</tr>
<tr>
<td>C600</td>
<td></td>
<td>DN</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
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</table>

a) ONT, O-nontypable; NM, nonmorbidity.
b) Stx typing was by VTEC-RPLA “SEIKEN”.
c) +, positive; -, negative.
ND: Not done.
NT: Not typable.

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ONT:H34 (Tables 1 and 2). Plasmid profiles were also classified into three groups (Fig. 1, Tables 1 and 2), but PFGE patterns with $X_{ba}b$ were divided into two (Fig. 1, Tables 1 and 2). Finally, we classified the isolates into three classes: 22 in class I ($eaeA^-$, $hlyA^+$, plasmid type A, Tc- and Sm-resistant, PFGE type A), 15 in class II ($eaeA^-$, $hlyA^-$, plasmid type B, antibiotics-sensitive, PFGE type B), and 2 in class III ($eaeA^-$, $hlyA^-$, plasmid type C, antibiotic-sensitive, PFGE type B) (Tables 1 and 2).

Comparison of Stx1 amino acids sequences: Based on the DNA sequences of $stx1$ genes for six representative STEC isolates; two of class I (strains 110201 and 1205002), two of class II (strains 110401 and 1105003), and two of class III (strain 1205006 and 1205010), amino acid sequences were compared with each other, resulting in that the sequences of the same class were completely identical to each other and over 96% identical to the other classes (Fig. 2). Their amino acid sequences were classified into two groups based on the phylogenetic analysis (Fig. 2) [4]; class II/III STEC were placed in group 2 of sheep STEC isolates and class I STEC were classified into group 1 of mainly human STEC [4] (Fig. 2).

DISCUSSION

Generally, healthy domestic animals are important natural reservoirs of human STEC infection [6], and direct or indirect contact with animals and their products, such as feces and slurry, have been implicated in several human cases of STEC infection [7, 12, 20, 31]. We genetically examined different types of STEC, isolated from four sheep

Table 2. Characterization of STEC strains directly isolated from a sheep in May, 2000

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>PCR $^a$</th>
<th>Antibiotic resistance</th>
<th>Plasmid profile</th>
<th>PFGE</th>
<th>Class</th>
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<tr>
<td>1205001 ONT:H8</td>
<td>1</td>
<td>+</td>
<td>– – – – –</td>
<td>–</td>
<td>B</td>
<td>B</td>
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<tr>
<td>1205002 ONT:NM</td>
<td>1</td>
<td>+</td>
<td>– – – +</td>
<td>Tc,Sm</td>
<td>A</td>
<td>A</td>
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<tr>
<td>1205003 ONT:H8</td>
<td>1</td>
<td>+</td>
<td>– – – –</td>
<td>–</td>
<td>B</td>
<td>B</td>
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<tr>
<td>1205004 ONT:NM</td>
<td>1</td>
<td>+</td>
<td>– – – +</td>
<td>Tc,Sm</td>
<td>A</td>
<td>A</td>
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<tr>
<td>1205005 ONT:NM</td>
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<td>+</td>
<td>– – – +</td>
<td>Tc,Sm</td>
<td>A</td>
<td>A</td>
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<tr>
<td>1205006 ONT:H34</td>
<td>1</td>
<td>+</td>
<td>– – – –</td>
<td>–</td>
<td>C</td>
<td>B</td>
</tr>
<tr>
<td>1205007 ONT:NM</td>
<td>1</td>
<td>+</td>
<td>– – – +</td>
<td>Tc,Sm</td>
<td>A</td>
<td>A</td>
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<tr>
<td>1205008 ONT:NM</td>
<td>1</td>
<td>+</td>
<td>– – – +</td>
<td>Tc,Sm</td>
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<td>A</td>
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<tr>
<td>1205009 ONT:NM</td>
<td>1</td>
<td>+</td>
<td>– – – –</td>
<td>–</td>
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<td>B</td>
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<tr>
<td>1205010 ONT:H34</td>
<td>1</td>
<td>+</td>
<td>– – – –</td>
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<td>B</td>
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<tr>
<td>1205011 ONT:H8</td>
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<td>+</td>
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<td>B</td>
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<tr>
<td>1205012 ONT:NM</td>
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<td>+</td>
<td>– – – +</td>
<td>Tc,Sm</td>
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<td>A</td>
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<td>B</td>
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<tr>
<td>1205014 ONT:H8</td>
<td>1</td>
<td>+</td>
<td>– – – –</td>
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<td>B</td>
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<tr>
<td>1205015 ONT:H8</td>
<td>1</td>
<td>+</td>
<td>– – – –</td>
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<td>Tc,Sm</td>
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<td>+</td>
<td>– – – +</td>
<td>Tc,Sm</td>
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<td>+</td>
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<td>– – – –</td>
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<tr>
<td>1205020 ONT:NM</td>
<td>1</td>
<td>+</td>
<td>– – – +</td>
<td>Tc,Sm</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

a) ONT, O-nontypable; NM, nomborlty.
b) Stx typing was by VTEC-RPLA “SEIKEN”.
c) +, positive; –, negative.

Fig. 1. The representative plasmid (A) and PFGE (B) patterns. (Panel A) : lanes 1 to 3, plasmid type A to C, respectively. (Panel B) : lanes 1 and 2, PFGE patterns A and B, respectively. An arrow shows a 90-kb virulence plasmid of STEC O157:H7, pO157 [26].

Fig. 2. (Panel A) : lanes 1 to 3, plasmid type A to C, respectively. (Panel B) : lanes 1 and 2, PFGE patterns A and B, respectively. An arrow shows a 90-kb virulence plasmid of STEC O157:H7, pO157 [26].
Therefore, in this study, we focused only on one sheep, and investigated continued shedding of STEC. As a result, three classes of STEC were continuously excreted over 13 months, suggesting that sheep might be a natural shedder of STEC. Those STEC were non-O157, but O157 STEC easily colonized in the intestine and was continuously excreted in the feces after experimentally infected with O157 to the sheep. In this case, about 10^4 O157 cells were contained by 1.0 g of sheep feces as the normal flora. Since the infectious dose for humans is statistically estimated as low as 10 STEC cells [4, 31], the presence of such numerous STEC in feces suggests that sheep might be critically important reservoir for human STEC infection. In addition, we isolated two kinds of STEC from the sheep used in this study in July and November 1997 [3], but we isolated here different types of STEC from the same sheep, whose serotypes and DNA sequences were completely different from those of previous STEC [3] (Tables 1 and 2, Fig. 2), suggesting that sheep would retain many kinds of STEC depending on circumstances. If once sheep could get O157:H7 from the outside into the body, it would continuously shed like calves [23].

As bacteriophages convert other enterobacteria to Stx-positive bacterial cells [2, 22, 28], we speculated that such a phage conversion might occur in sheep. Therefore, stx-encoded bacteriophage [19] was orally inoculated into the sheep after the experimental infection with Stx-negative O157, but no Stx2-positive enterobacteria were detected. As this phage conversion was at a frequency of 2.4 × 10^-1 in E. coli C600 in vitro [19], the phage conversion might rarely occur in sheep at a low frequency. Although STEC was widely distributed in many kinds of animals, such a diffusion would be caused by the transmission of STEC but not in vivo phage conversion.

Several studies have been made on the relationship between ruminant diet and STEC [8, 13, 16, 17]; grain-fed animals shed STEC for markedly shorter periods than hay-fed animals. In this study, although we isolated no STEC in September, we changed the diet from grass to hay in this month. Although the relationship of volatile fatty acid (VFA) concentration and STEC excretion was not examined in this case, artificial diet change might add stress to the sheep, perhaps resulting in normal flora changing and no STEC being isolated.

All STEC producing Stx1 in this study were biochemically and genetically classified into three classes (I to III) (Tables 1 and 2). Based on the amino acid sequences of their stx1 genes, class I was closely related to human STEC of group 1, but the other classes were unique to sheep STEC.
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of group 2 (Fig. 2) [4]. STEC isolates of class 1 were resis-
tant to Tc and Sm (Tables 1 and 2). Since animal-specific
STEC was mostly sensitive to antibiotics and human STEC
were often resistant to the antibiotics [4], Tc- and Sm-resis-
tant STEC of class I might be cycling between humans and
sheep. In our previous study [18], we showed large R plas-
mids encoding Tc and Sm in O157, but the largest plasmid
in class I STEC (Fig. 1) might be R plasmid. Thus, sheep
STEC of class-I might be a human pathogen, and sheep also
would be an important reservoir of human STEC. There-
fore, sheep might be an important animal model of human
STEC infection.

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