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Prevalence and characterization of Shiga toxin-producing *Escherichia coli* O157 and O26 in beef farms

Y. Sasaki *, Y. Tsujiyama, M. Kusukawa, M. Murakami S. Katayama, Y. Yamada

Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries, 1-2-1 Kasumigaseki, Chiyoda-ku, Tokyo 100-8950, Japan

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ABSTRACT

Rectal content grab samples were collected from 2436 beef cattle reared on 406 beef farms in Japan between November 2007 and March 2008. STEC strains O157 and O26 were isolated from 110 (27.1%) and 7 (1.7%) farms, respectively. Farms that tested positive for STEC O157 were located in 35 out of all 47 Japanese prefectures. This indicates that STEC O157 strains are widespread on beef farms nationwide. Of the 2436 tested beef cattle, 218 (8.9%) and 10 (0.4%) had STEC strains O157 and O26 in the rectal content, respectively. The most common Shiga toxin genes detected in the isolated STEC O157 strains were: stx_{2c} alone (32.1%), stx_2/stx_{2c} (27.2%), and stx_1/stx_2 (21.8%). Almost all of the STEC O157 and STEC O26 strains expressed Shiga toxins (Stx). Most of the STEC O157 and STEC O26 strains possessed *eaeA* and EHEC-*hlyA*. These results strongly suggest that STEC strains O157 and O26 from beef cattle would be pathogenic to humans. Therefore, it is important to reduce STEC strains O157 and O26 in beef cattle in order to prevent foodborne disease caused by STEC. The presence of dogs and/or cats on a farm was significantly ($P = 0.02$) associated with the prevalence of STEC O157. More research is needed to clarify the role of dogs and cats.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is an important human foodborne pathogen worldwide (Mainil and Daube, 2005). Although many STEC O serogroups cause human STEC infections, STEC O157 strain has been the cause of most outbreaks of the infection in Japan and STEC O26 to a lesser extent (Infectious Disease Surveillance Center, 2009). As cattle are considered the main reservoir of STEC, the reduction of STEC infections in cattle may be the key to prevent human STEC infection. The prevalence of STEC strains O157 and O26 in beef and dairy cattle has been studied in Japan (Kushima et al., 2001; Miyao et al., 1998; Shinagawa et al., 2000). In these studies, samples were obtained at slaughterhouses, and the prevalence rates of STEC strains O157 and O26 in the originating farms could not be estimated because the samples did not have traceability information. Epidemiological information about beef farms is needed in order to identify biosecurity measures that may be associated with the STEC O157 and O26 prevalence in beef farms.

The major virulence factors implicated in human STEC infection are potent Shiga toxins (Stx), which are categorized into 2 groups: Stx1 and Stx2 (Gyles, 2007). Additional factors described to contribute to virulence include intimin (encoded by the *eae* gene) and enterohaemorrhagic *E. coli* (EHEC) haemolysin (encoded by EHEC-*hlyA*) (Paton and Paton, 1997). STEC can be characterized by the possession of these virulence factors.

The objective of the present study was to estimate the prevalence of STEC O157 and O26 in beef farms in Japan, to identify risk factors associated with STEC O157 and O26 prevalence in beef farms, and to characterize STEC O157 and O26 strains from beef cattle.

* Corresponding author. Tel.: +81 3 3502 5722; fax: +81 3 3597 0329.
E-mail address: yoshimasa_sasaki@nm.maff.go.jp (Y. Sasaki).

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2. Materials and methods

2.1. Animals, samples and farm management survey

This study intended to obtain the prevalence among beef farms rearing 50 or more beef cattle, with a precision of 5% and 95% confidence limits. Assuming the prevalence of 25%, the minimum necessary number of beef farms in the study was calculated as 288. Since the participation in this study was voluntary, twice the number of farms were provisionally selected as subjects of this study from all of the 47 prefectures in Japan, in proportion to the number of beef farms of the corresponding scale based on the national statistics on livestock production. Contacts were made through respective prefectural governments. Four hundred and six of the above farms expressed willingness to participate in the study, covering all but 2 Japanese prefectures. For each farm, 6 healthy beef cattle, whose scheduled dates of slaughtering were closest among all animals in the farm to the date of sampling were selected. From each selected animal a rectal content grab sample was collected over the period of November 2007 to March 2008. The cattle tested included 1460 (59.9%) Japanese Black, 349 (14.3%) Holstein-Friesian, 448 (18.4%) first-generation hybrid (F1) of Japanese Black and Holstein-Friesian, and 179 (7.3%) other varieties, including Japanese Shorthorn and Japanese Brown. The average age of the tested cattle was 25.8 months, and the age of 95% of the cattle was between 17 and 36 months. All animals were sampled by veterinarians of prefectual Livestock Hygiene Centres. The samples were sent to a laboratory, the Institute for Food and Environmental Science (IFES), by express delivery under refrigeration. At the laboratory, the samples were kept refrigerated at 4 °C for up to 7 days before analysis.

The farmers were asked to fill in a questionnaire on farm management with the assistance of veterinarians from the prefectual Livestock Hygiene Centre. Duly filled questionnaire forms were submitted to the IFES along with the samples. To examine the relationship between the prevalence of STEC O157 and STEC O26 and farm management factors, univariable analyses were performed. If the expected value of a variable was greater than 5, then the chi-square test was used; otherwise, Fisher's exact test was used.

2.2. Bacteriological examination

Each rectal content sample (25 g) was incubated in 225 mL of mEC broth supplemented with 25 μg/mL of novobiocin (Kyokuto, Japan) at 42 °C for 24 h with shaking. In 1 mL of each enrichment broth, *E. coli* O157 and O26 were concentrated with anti-O157 and anti-O26 immunomagnetic beads (Denka Seiken Co., Japan), respectively. Subsequently, the O157-specific bead-bacteria complex was plated onto sorbitol-MacConkey agar with cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L) and onto CHROMagar O157 (Kyokuto, Japan) agar supplemented with potassium tellurite, and incubated at 37 °C for 18 h. Five suspected colonies were subcultured to a cellobiose–lactose–indole–β-D-glucuronidase agar. The O26-specific bead-bacteria complex was plated onto a cefixime potassium tellurite–rhamnose MacConkey agar (CT-RMAC, Denka Seiken Co.) and onto ViRX-O26 agar medium (Eiken Chemical, Japan) supplemented with cefixime (50 mg/L) and potassium tellurite (2.5 mg/L), and incubated at 37 °C for 18 h. Five suspected colonies were subcultured onto a nutrient agar. The colonies were screened for O157 or O26 antigen by agglutination tests using anti-O157 or anti-O26 serum (Denka Seiken Co.) according to the accompanying instruction manual. The colonies suspected to be *E. coli* O157 or *E. coli* O26 were examined on triple-sugar iron agar, lysine indole motility semisolid agar, Voges–Proskauer semisolid medium, and Simmon's citrate agar for the identification of *E. coli*.

2.3. Characterization of E. coli strains O157 and O26 from beef cattle

E. coli strains O157 and O26 were first characterized in motility and by an agglutination test using anti-H sera (Denka Seiken Co.) according to the accompanying instruction manual. DNA from the *E. coli* O157 and *E. coli* O26 strains was extracted using a commercially available DNA extraction kit (Extragen II, Tosoh, Japan) according to the accompanying instruction manual. A LAMP assay was performed using a loop-mediated isothermal amplification kit (Loopamp® Verotoxin Typing Kit, Eiken Chemical) to detect the presence of *stx* genes in the *E. coli* O157 and *E. coli* O26 strains according to the accompanying instruction manual. The LAMP assay kit contained a VT1 set for the detection of stx_1 and a VT2 set for the detection of stx_2 and could detect stx_1 and stx_2 separately.

For STEC O157 and STEC O26 strains that were positive for stx_1 and stx_2 in the LAMP assays, the types of the *stx* genes (stx_1, stx_2, stx_{2c}, stx_{2d}, stx_{2e}, and stx_{2f}), EHEC-*hlyA*, *eaeA*, $rfbE_{O157}$, and $fliC_{H7}$ were investigated by PCR analysis. The primer sets used in this study are shown in Table 1. The production of Stx1 and Stx2 was confirmed by reverse passive latex agglutination (RPLA) with a Shiga toxin detection kit (VTEC-RPLA SEIKEN, Denka Seiken Co.).

PCR analysis and RPLA for Stx production were also conducted on 11 *E. coli* O157 and 15 *E. coli* O26 strains, which were negative for stx_1 and stx_2 genes in the LAMP assays, to confirm the absence of their variant genes, which may not be detectable by this assay kit, as well as the products thereof.

3. Results

3.1. Prevalence of STEC strains O157 and O26 in beef cattle farms

From the 406 beef farms surveyed, 254 *E. coli* O157 strains were isolated from 226 (9.3%) cattle on 113 (27.8%) farms (Table 2). *E. coli* O157 strains were obtained from 35 (77.8%) of the 45 prefectures participating in this study. For *E. coli* O26, 26 strains were isolated from 24 (1.0%) cattle on 19 (4.7%) beef farms. *E. coli* O26 strains were obtained from 15 (33.3%) of the 45 prefectures.

To investigate the presence of *stx* genes of these strains, their extracted DNAs were tested by LAMP assay. Out of the 2436 beef cattle, 218 (8.9%) excreted STEC O157 strains,