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Detection of Verotoxigenic *Escherichia coli* O157 and O26 in food by plating methods and LAMP method: A collaborative study

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Abstract

In order to establish a rapid and sensitive method for the detection of Verotoxigenic *Escherichia coli* O157 and O26, a collaborative study was conducted focusing on a comparison of the efficiency of loop-mediated amplification (LAMP) assay targeting the Verocytotoxin (also called Shiga toxin) gene, utilizing a direct plating method and a plating method with immunomagnetic separation (IMS-plating method) using various agar media. In combination with enrichment with the modified EC supplemented with novobiocin, *E. coli* O157 was detected in most samples of ground beef and alfalfa sprouts by LAMP assay, the direct plating method and the IMS-plating method. *E. coli* O26 was detected in approximately 100% of the food samples by LAMP assay. However, the IMS-plating and direct plating methods recovered 80 and 50% in ground beef samples, respectively. As a result, it was demonstrated the LAMP assay is superior to the IMS-plating method. Based on these results, it appears LAMP assay is effective as a screening assay to detect *E. coli* O157 and O26 from positive samples.

Keywords: Verotoxigenic *Escherichia coli*; O157; O26; Detection; Collaborative study

1. Introduction

Numerous infections with Verotoxigenic *Escherichia coli* (VTEC) serotype O26 have occurred worldwide, although serotype O157 is the most major serotype of the pathogen (Tozzi et al., 2003; Infectious Diseases Surveillance Center, National Institute Infectious Diseases, 2006). Many researchers and companies have superior developed detection methods for serotype O157 using selective culture media, immunomagnetic separation method or immune kits (Jung et al., 2005; O'Brien et al., 2005; Onoue et al., 1999). However, effective detection methods to isolate serotype O26 are a few. Isolation by culture methods is carried out in a combination with the immunomagnetic separation method. As one approach, the culture methods are employed in combination with anti-O157 and anti-O26 immunomagnetic separation method and plating are useful for detection of the serotypes O157 and O26. However, these methods consume a lot of time. Recently, molecular methods have been applied in detection of virus in food (De Medici et al., 2004). In the clinical field, molecular methods are typically used for diagnosis of infection with bacteria and viruses (Espy et al., 2006; Iijima et al., 2004). Conventional PCR is already popular, although the detection of an amplicon by PCR requires electrophoresis in an agarose gel. Real-time PCR is more sensitive due to the detection of fluorescence from amplification (Mullah et al., 1998). In addition, the reaction is monitored in real-time. Furthermore, loop-mediated isothermal amplification (LAMP) assay has been applied to detect pathogens targeting DNA specifically,

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sensitively and rapidly (Mekata et al., 2006; Ohtsuka et al., 2005). LAMP is a novel nucleic acid amplification method that relies on an auto-cycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (Nagamine et al., 2001; Nagamine et al., 2002; Notomi et al., 2000). LAMP is different from PCR in that: four or six primers perform the amplification of the target gene though the design of primers for LAMP assay are complicated, the amplification uses a single temperature step at 60–65 °C for about 60 min, and the amplification products have many types of structures in large amounts. Thus, LAMP is faster and easier to perform than PCR, as well as being more specific. Furthermore, gel electrophoresis is not needed, because the LAMP products can be detected indirectly by the turbidity that arises due to the formation of insoluble magnesium pyrophosphate (Mori et al., 2001). In addition, the preparation steps pf LAMP assay are fewer than PCR and real-time PCR assays, and LAMP assay needed lesser than those assays. A large amount of by-product, pyrophosphate ion, is produced, yielding white precipitate of magnesium pyrophosphate in the reaction mixture. Since the increase in the turbidity of the reaction mixture according to the production of precipitate correlates with the amount of DNA synthesized, real-time monitoring of the LAMP reaction can be achieved by real-time measurement of turbidity.

In a preliminary study, we determined the effectiveness of molecular detection methods such as a real-time PCR or LAMP assay targeting the Verocytotoxin (VT; also called Shiga toxin) gene to detect VTEC in food. As a result, it was demonstrated that certain molecular methods are sufficiently sensitive and specific to detect VTEC in food. Because beef products and vegetables such as sprouts are often related to VTEC outbreaks (Meng et al., 2001), ground beef and alfalfa sprouts were chosen as samples in the present study.

The purpose of this study was to evaluate the utility of a molecular detection method using LAMP assay and culture methods in combination with immunomagnetic separation and plating onto agar media for the detection of *E. coli* O157 and O26 in ground beef and alfalfa sprouts. Different laboratories participated in the study by conducting the trials to detect *E. coli* O157 and O26 from artificially inoculated samples using the same detection procedure.

2. Materials and methods

2.1. Design

Participants were 22 laboratories (16 local governments, three private, two quarantine station and a national government laboratory) belonging to food research institutions. After media and other materials, each of the same lot, had been sent to the participants, each test for *E. coli* O157 and O26 was carried out separately. Within a few hours after receiving the *E. coli* O157 or O26-inoculated or uninoculated samples, the participants initiated. After performing the analysis, they sent the collected data sheets to the reference laboratory, the Division of Microbiology, National Institute of Health Sciences.

2.2. The preparation of test sample

E. coli O157:H7 strains (no. 212, VT1 and VT 2) (Michino et al., 1999) and *E. coli* 026:H11 (no. 26016, VT1 and VT 2) isolated from patients of the outbreaks in Japan were separately grown overnight in tryptic soy broth (TSB, Difco™, Becton, Dickinson and Company, Sparks, MD, USA) at 37 °C. Each strain was diluted with phosphate buffered saline (PBS, pH7.4) to give the appropriate cell numbers. Ground beef and alfalfa sprouts were purchased from a retail shop in Tokyo. Prior to inoculation, these food samples were examined for no contamination with VTEC O157 and O26 by LAMP assay and the IMS-plating method in combination with modified EC broth with novobiocin (mEC+n) enrichment, as described below. Aerobic bacteria and coliform bacteria counts were approximately 10^8 and 10^5 cfu/g and 10^7 and 10^2 cfu/g in ground beef and alfalfa sprouts, respectively.

The 0.2 ml suspension of *E. coli* O157 or O26 was spiked into 25 g ground beef or alfalfa sprouts in a stomacher bag. Inoculated cell numbers were counted on tryptic soy agar (TSA, Difco™) after 24 h culture at 35 °C. In *E. coli* O157, the numbers were 11.7 CFU/25g (5–17 CFU/25g, $n=10$) for ground beef and alfalfa sprouts samples at a low level of inoculation, and 46.8 CFU/25g for alfalfa sprouts samples at a high level. In *E. coli* O26, the numbers were 10.7 CFU/25g (3–17 CFU/25g, $n=10$) for ground beef and alfalfa sprouts samples at a low level of inoculation, and 42.8 CFU/25g for alfalfa sprouts samples at a high level. Immediately after being spiked, the inoculated and uninoculated samples were immediately packed with ice packs. The samples were monitored by a thermorecorder (Tomprobe, AES, Combourg, France) so as to maintain them at 0–5 °C during transportation to participants. Within 24 h of packing each participant had received the packed samples inoculated with *E. coli* O157 or O26. A total of 16 samples (ground beef samples inoculated at a low level and uninoculated, alfalfa sprout samples inoculated at low and high levels and uninoculated in triplicate, and one ground beef sample to be inoculated by each participant as a positive control) were assigned a random number by staff members who did not participate in the test.

2.3. Samples as a positive control

Each strain of *E. coli* O157 and O26 was separately cultured in a slant of TSA at 36±1 °C for 18–20 h in each laboratory of the participants. After maintenance under room temperature for 4 days, cells were suspended in PBS (approximately 10^4 cfu/ml) on the day enrichment was started. A portion (0.1 ml) of the suspension was inoculated into a ground beef sample as a positive control, and then the sample was enriched the same way as the 15 test samples.

2.4. Enrichment

Two hundred twenty five ml of modified EC broth with novobiocin (mEC+n, Merck, Darmstadt, Germany) were added