Sensitive and rapid detection of Vero toxin-producing *Escherichia coli* using loop-mediated isothermal amplification

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A loop-mediated isothermal amplification (LAMP) assay was developed to detect Vero toxin (VT)-producing *Escherichia coli* rapidly (within 60 min). The 24 strains of VT-producing *E. coli* were successfully amplified, but 6 strains of non-VT-producing *E. coli* and 46 bacterial species other than *E. coli* were not. The sensitivity of the LAMP assay was found to be >0.7 c.f.u. per test using serogroups O157, O26 and O111 of VT-producing *E. coli*; this sensitivity is greater than that obtained by PCR assay. Furthermore, the LAMP assay was examined for its ability to detect VT-producing *E. coli* in food because of the difficulty of detection in food samples. The recovery of VT-producing *E. coli* by LAMP assay from beef and radish sprouts inoculated with the pathogen was high, similar to that obtained using culture methods with direct plating and/or plating after immunomagnetic separation. Although PCR assay was unable to recover VT-producing *E. coli* from half of the radish samples, LAMP assay was successful in most samples. In addition, VT-producing *E. coli* was successfully detected in cultures of the beef samples by LAMP assay, but not by the culture method. The LAMP products in naturally contaminated beef samples were analysed to confirm the specific amplification of the VT-encoding gene, and were found to show a specific ladder band pattern on agarose gel after electrophoresis. Additionally the sequences of the LAMP products coincided well with the expected sequences of the VT-encoding gene. These results indicate that the proposed LAMP assay is a rapid, specific and sensitive method of detecting the VT-producing *E. coli*.

INTRODUCTION

Sensitive and rapid detection methods for Vero toxin (VT)-producing *Escherichia coli* are required. VT-producing *E. coli* has many serotypes, although O157 predominates. Thus, the early detection of VT is important to save both time and cost. The present culture methods require more than 3 days, and rapid detection methods like enzyme immunoassay require a high population of the target pathogen (Bennett et al., 1996; Chapman et al., 2001). A PCR assay was developed to rapidly detect target pathogens in enrichment cultures; however, the assay generally requires electrophoresis to detect the amplicon, which takes several hours. Recently, real-time PCR assays have been applied to detect food-borne pathogens, but such assays requires an expensive thermal cycler with a fluorescence detector (Mullah et al., 1998).

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that relies on an autocycling strand displacement DNA synthesis performed by the Bst DNA polymerase large fragment (Nagamine et al., 2001, 2002; Notomi et al., 2000). LAMP is different from PCR in that four or six primers perform the amplification of the target gene, 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 a large amount of by-product, pyrophosphate ion, being produced, yielding an insoluble white precipitate of magnesium pyrophosphate in the reaction mixture (Mori et al., 2001). 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.
VT is classified into two types: VT1 and VT2 (Jackson et al., 1987), and VT1- and VT2-producing E. coli are regarded as pathogens. VT1 is identical with Shiga toxin. VT2 differs from VT1 in its physico-chemical, immunological and molecular genetic properties. Several variants of VT, especially VT2, have been reported. Sensitive detection methods for VT must target VT1 and VT2, including VT2vha, VT2vhp (Ito et al., 1990, 1991) and VT2vp1 (Lin et al., 1993; Weinstein et al., 1988). In the present study, we developed a LAMP assay to detect VT1 and/or VT2-producing E. coli rapidly and sensitively.

METHODS

Bacterial strains. In this present study, 24 strains of VT-producing E. coli, 6 strains of non-VT-producing E. coli and 46 bacterial species other than E. coli (Table 1) were tested using the LAMP assay to show specificity. Vibrio spp. were cultured on tryptcase soy agar (TSA) medium (Difco) containing 3% NaCl for 18 h at 37 °C. The other bacteria were cultured on brain heart infusion agar (Difco) at 37 °C for 18 h. Colonies were suspended to 2 McFarland standard turbidity in 5 ml PBS. The bacterial suspension was diluted in PBS to 10^(-10) c.f.u. ml^-1. The dilutions were used for the LAMP assay.

Cultures of five strains of VT-producing E. coli (nos. 157-891, ATCC43890, 157-120, BFR 26015 and 97353) were tested to investigate tenfold in PBS to 10^(-1) (Table 2) were used for the LAMP reactions (Notomi et al., 2000). The LAMP reaction was performed using a Loopamp DNA amplification kit (Eiken Chemical). Each 50 μl bacterial dilution or culture sample was added to 42 μl extraction solution (50 mM NaOH, pH 12.5) (Beige et al., 1995) in order to extract DNA before heating at 100 °C for 5 min. After flash heating, the samples were added to 8 μl 1 M Tris/HCl (pH 7.0) and centrifuged (Tomy) at 2000 g, and the supernatant was transferred to a new micro-tube and used as the template DNA solution for the LAMP assay. The LAMP reaction mixture contained the primers for VT detection (20 μl), Bst polymerase (1 μl) and template DNA solution (5 μl). The reaction components were mixed in a tube, incubated at 65 °C for 60 min using a Loopamp LA-200 (Teramecs) and then heated to 80 °C for 2 min to terminate the reaction. LAMP amplification was detected as a value of turbidity at 650 nm using a Loopamp LA-200 in real-time. In addition, turbidity produced by the by-product magnesium pyrophosphate was noted visually. VT-producing E. coli DNA extracted from a suspension of the pathogen by heating at 95 °C for 10 min was used as a positive control each time.

Aliquots of 1 μl LAMP products were electrophoresed in 2% agarose gel. After staining with ethidium bromide, the products were detected under UV light.

PCR assay. The bacterial dilutions in PBS of five strains of VT-producing E. coli, described above, were heated at 95 °C for 10 min, and centrifuged at 10 000 g for 5 min. The supernatant was transferred to a new micro-tube as the template DNA solution for PCR. PCR targeting VT-encoding genes was performed as follows. Primer sets EVC-1 and EVC-2 (0.5 μl each) (Takara Bio), dNTP mixture (4 μl), 10 × Taq buffer (5 μl), Taqara Taq (0.25 μl), template DNA solution (2.5 μl) and distilled water (37.25 μl) were mixed in a reaction tube. The reaction was performed at 94 °C for 1 min for denaturing, at 55 °C for 1 min for annealing and at 72 °C for 1 min for extension using a thermal cycler (ABI7000, Applied BioSystems). After 35 cycles of the reaction, followed by heating to 72 °C for 10 min, the PCR products were subjected to electrophoresis in 3% agarose gel. After staining with ethidium bromide, the PCR product (171 bp) was detected under UV light.

Food samples. Ground beef and radish sprouts were purchased from retail shops in Tokyo for inoculation with VT-producing E. coli. The aerobic bacterial counts in the ground beef and radish sprouts were 5.3 and 8.2 log c.f.u. g^-1, respectively.

LAMP assay. In the present study, five primers (two inner primers, two outer primers and one loop primer) targeting the VT1 gene and six primers (two inner primers, two outer primers and two loop primers) targeting the genes of VT2, VT2vha, VT2vhp and VT2vp1 (Table 2) were used for the LAMP reactions (Notomi et al., 2000); the domains targeted by these primers are shown in Fig. 1. The LAMP reaction was performed using a Loopamp DNA amplification kit (Eiken Chemical). Each 50 μl bacterial dilution or culture sample was added to 42 μl extraction solution (50 mM NaOH, pH 12.5) (Beige et al., 1995) in order to extract DNA before heating at 100 °C for 5 min. After flash heating, the samples were added to 8 μl 1 M Tris/HCl (pH 7.0) and centrifuged (Tomy) at 2000 g, and the supernatant was transferred to a new micro-tube and used as the template DNA solution for the LAMP assay. The LAMP reaction mixture contained the primers for VT detection (20 μl), Bst polymerase (1 μl) and template DNA solution (5 μl). The reaction components were mixed in a tube, incubated at 65 °C for 60 min using a Loopamp LA-200 (Teramecs) and then heated to 80 °C for 2 min to terminate the reaction. LAMP amplification was detected as a value of turbidity at 650 nm using a Loopamp LA-200 in real-time. In addition, turbidity produced by the by-product magnesium pyrophosphate was noted visually. VT-producing E. coli DNA extracted from a suspension of the pathogen by heating at 95 °C for 10 min was used as a positive control each time.

A recovery of VT-producing E. coli from food inoculated with the pathogens. For inoculation, serogroups O157 (no. 157-212) and O26 (no. BFR 26015) of VT-producing E. coli were cultured in TSA at 37 °C for 18 h. Cultures were diluted in PBS to a 10^-6 dilution, and 0.1 ml was inoculated into each 25 g of ground beef and radish sprouts. The food samples were homogenized in 225 ml modified EC including novobiocin (mEC) broth as an effective enrichment broth for serogroups O157 and O26 (Hara-Kudo et al., 1999, 2000; Okrend et al., 1990) with a Stomacher (model 400; AJ Seward) prior to incubation, and then incubated statically at 42 °C for 18 h.

The cultures of food inoculated with serogroup O157 were plated onto each of two plates of sorbitol MacConkey agar (Oxoid) supplemented with cefixime (0.05 mg l^-1) and potassium tellurite (2.5 mg l^-1) (CTSMAC) and CHROMagar O157 (CHROMagar) directly, or after immunomagnetic separation (IMS) performed according to the manufacturer’s instructions with Dynabeads anti-E. coli O157 (Dyna). At least three colonies suspected to be O157 were tested for agglutination using an E. coli O157: H7 UNI latex kit (Unipath Oxoid). Production of lactose, acid non-production from cellobiose and non-fluorescence under UV light were observed in cellobiose-lactose-indole-β-D-glucuronidase (CLIG) agar (Kyokuto).

The cultures of food inoculated with serogroup O26 were plated onto each of two plates of rhamnose MacConkey agar (Oxoid) supplemented with cefixime (0.05 mg l^-1) and potassium tellurite (2.5 mg l^-1) (mEC) and CHROMagar O157 (CHROMagar) directly, or after IMS performed according to the manufacturer’s instructions using immunomagnetic beads coated with anti-O26 antibody (Denka Seiken). At least three colonies suspected to be O26 were tested for agglutination using the E. coli O26 antisem (Denka Seiken).

For PCR assay, 0.1 ml mEC + n culture was transferred to a tube and centrifuged at 10 000 g for 10 min. After removing the supernatant, 0.1 ml sterilized distilled water was added to the tube and the mixture was heated at 95 °C for 5 min. After centrifuging at 10 000 g for 10 min, the supernatant was used as template for the PCR described above.

For the LAMP assay, 50 μl culture was added to 50 μl extraction solution to extract DNA, and the LAMP reaction was then carried out as described above.

Analysis of LAMP products. Using meat obtained in an inspection carried out by the local government of Saitama prefecture,
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Japan, 25 g surface portions of beef loaf samples were incubated in mEC+n at 42 °C for 18 h. The food cultures were plated onto each of two plates of CT-SMAC and CHROMagar O157 directly, or after IMS with Dynabeads anti-

*E. coli* O157. The culture (1 ml) was tested by the PCR and LAMP assays described above. In four samples, the VT-encoding gene was detected in the enrichment culture by LAMP assay, although VT-producing *E. coli* O157 was not isolated. The enrichment cultures of four purchased beef samples (samples 03-150, 03-156, 03-266 and 03-304) were analysed by LAMP assay. The LAMP products from the samples were purified using a QIAquick PCR purification kit (Qiagen) and sequenced directly with the Vt1F2Cy5 (5'-CAT TCG TTG ACT TCT TAT CTG G-3') and Vt2F2Cy5 (5'-CAC TCA CTG GTT TCA TCA TAT CTG G-3') primers for VT1 and VT2 using an ABI 310 genetic analyser (Applied Biosystems) with a BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems). The PCR amplification products were

Table 1. cont.

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<th>Species</th>
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* Determined by a reversed passive latex agglutination assay.

Table 2. LAMP primers

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<tbody>
<tr>
<td>VT1</td>
<td></td>
</tr>
<tr>
<td>FIP</td>
<td>GCTTTGCCACAGACTGCAATCATTGGTGAATCTTCTTATCTGG</td>
</tr>
<tr>
<td>BIP</td>
<td>CTGTGACAGCTGAAGCTTTACGCGAAATCCCCTCTGAATTTGCC</td>
</tr>
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<td>F3</td>
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<td>Loop F</td>
<td>AGGTCCGCTATGCCGACATTAAAT</td>
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<tr>
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<td></td>
</tr>
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<tr>
<td>BIP</td>
<td>CTGTCAAGGAGCCTTACGGGACAAATTCTCCCTGTATCTGCC</td>
</tr>
<tr>
<td>F3</td>
<td>CAGTTATACACTCTGAAGCTG</td>
</tr>
<tr>
<td>B3</td>
<td>CTGTAGGAGCCGAGTTC</td>
</tr>
<tr>
<td>Loop F1</td>
<td>TGTATTACGACTGAACTCCATTAACG</td>
</tr>
<tr>
<td>Loop F2</td>
<td>GCCATTCCACTAAACTCCATTAACG</td>
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</tbody>
</table>
purified using a Montage PCR centrifugal filter device (Millipore) according to the manufacturer’s instructions. The purified DNA was sequenced using an ABI310 Genetic Analyser (Applied Biosystems) with a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

Aliquots 1 μl LAMP products were electrophoresed in 2 % agarose gel. After staining with ethidium bromide, the products were detected under UV light.

**RESULTS**

Specificity of the LAMP assay

Twenty-four strains of VT-producing *E. coli*, including either VT1- or VT2-producing *E. coli* or both were positive in the LAMP assay (Table 1). The serogroups were O157, O26, O111 and O145, and the serogroup did not affect the assay. Six strains of non-VT-producing *E. coli*, including serogroup O157, were negative in the assay. Additionally, 46 species of non-VT-producing bacteria other than *E. coli* were not detected.

Sensitivity of the LAMP assay

The sensitivities of the LAMP and PCR assays were tested on five strains of VT-producing *E. coli* at various cell densities. In the LAMP assay, all strains were detected in the $10^{-6}$ dilution (0.7–2.2 cells per test tube) (Table 3). In the PCR assay, $10^{-6}$ dilutions of all strains, $10^{-5}$ dilutions of two strains and $10^{-4}$ dilutions of one strain were negative, even though they had been positive in the LAMP assay. The sensitivity of the LAMP assay was higher than that of the PCR assay for all strains tested in the present study.

Recovery of VT-producing *E. coli* from food samples inoculated with the pathogen

The inoculation levels of serogroups O157 and O26 were 8.9 and 5.9 c.f.u. per 25 g of ground beef, and 36.8 and 20.8 c.f.u. per 25 g of radish sprouts, respectively (Table 4). In the ground beef samples, serogroup O157 was detected perfectly by all methods (Table 4). Serogroup O26 was also perfectly detected by the PCR and LAMP assays, and the IMS culture method. However, the culture method with direct plating failed to detect serogroup O26 in one of ten samples. In the radish sprout samples, the PCR and LAMP assays, and the culture method using plating onto CT-SMAC, were successful in detecting serogroup O157 in all samples (Table 4). However, the detection ratio of plating onto CHROMagar O157 was less than that of plating onto CT-SMAC. Serogroup O26 was detected in nine of ten samples using the LAMP assay and culture with IMS, and in five of ten samples with PCR assay.

Analysis of LAMP products of naturally contaminated beef samples

Fig. 2 shows the results of the LAMP assay by three detection methods of LAMP products in four samples of beef culture and a strain culture.
of the negative control, showed an increase in turbidity, which occurred over time (Fig. 2a). Visible turbidity was detected in the test tube (Fig. 2b). The amplicons in the tubes were analysed by agarose gel electrophoresis, and the ladder patterns of four samples showed specific amplification of the target sequence (Fig. 2c). Additionally, the LAMP products were analysed by sequencing. In the LAMP products for the VT1 gene, sequences from a part of the F1 region to a part of the B1 region were mostly homologous to the expected sequences (Fig. 3a). In the LAMP products for the VT2 gene, sequences from a part of the F1 region to a part of B2 were also mostly homologous to the expected sequences (Fig. 3b).

**DISCUSSION**

The specificity of LAMP was confirmed for VT1- and VT2-producing *E. coli* of various serogroups (Table 2). In the present study, a loop F primer designed for VT2 contained two types. Six strains of VT2- but not VT1-producing *E. coli* showed a positive reaction. To confirm the sensitivity of LAMP, serogroups O157, O26 and O111, as three predominant serogroups in many countries including Japan (National Institute of Infectious Diseases & Infectious Diseases Control Division, Ministry of Health and Welfare of Japan, 2005), were tested at various concentrations. The minimum dilution for the detection of five strains of three serogroups was $10^{-6}$. However, the concentrations of the *E. coli* cells were different among strains. These results demonstrate that the detection limit of the LAMP assay is between 0.7 and 2.2 cells per test tube (Table 3). The *E. coli* concentration of the sample was 140–440 cells ml$^{-1}$. Because the contamination level of VT-producing *E. coli* in food is usually low, enrichment is necessary to detect it. After enrichment, the population of VT-producing *E. coli* should reach the minimum level detectable by the LAMP assay. In the detection of VT-producing *E. coli* by PCR, the PCR sensitivity found in the present study was similar (Table 3) to that obtained in previous studies (10–17 cells per test tube) (Kawasaki et al., 2005; Read et al., 1992), though the conditions of the PCR assay differed to some extent. Because the DNA extraction conditions were different between the PCR and LAMP assays, these conditions might affect the sensitivity. In an elementary experiment, the sensitivity of PCR assay using DNA extracted by heat treatment at 100°C for 5 min with alkaline extraction solution and at 95°C for 5 min without extraction solution was compared. There was no difference in a test for cells without food culture or with sprouts culture.

Thus, in comparison with other assays, it was suggested that the LAMP assay is more sensitive because the amplification of the target gene using LAMP is faster and greater in yield.

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**Fig. 2.** The detection of VT-producing *E. coli* in naturally contaminated beef samples by turbidity and agarose electrophoresis in the LAMP assay. (a) Enterohaemorrhagic *E. coli* (EHEC) detection by real-time turbidity at 650 nm. Cell suspension of strain no. 157-212 (●) was used as a positive control, the samples were cultures in mEC+n of naturally contaminated beef samples 03-150 (○), 03-156 (□), 03-266 (■) and 03-304 (△), a negative control was used (△). (b) Visual EHEC detection by observation of turbidity: tube 1, strain no. 157-212; tube 2, beef sample 03-150; tube 3, beef sample 03-156; tube 4, beef sample 03-266; tube 5, beef sample 03-304; tube 6, negative control. (c) EHEC detection by agarose gel electrophoresis: lane 1, strain no. 157-212; lane 2, beef sample 03-150; lane 3, beef sample 03-156; lane 4, beef sample 03-266; lane 5, beef sample 03-304; lane 6, negative control; lane M, 100 bp ladder size markers (Takara).
To confirm the LAMP amplification of the target, an examination of the ladder patterns by agarose gel electrophoresis is useful. Furthermore, the sequences of the product are useful to confirm the PCR amplification of the target. Notomi et al. (2000) confirmed the structures of the LAMP amplified products for HBs regions of hepatitis virus B by cloning and sequencing. The results of the present analysis show that the sequences of LAMP products from naturally contaminated beef samples coincide well with the expected sequences.

**Fig. 3.** Analysis of the sequences of products of LAMP for VT-producing *E. coli* in naturally contaminated beef samples: (a) VT1 gene, (b) VT2 gene. The cultures of four samples and a control strain (no. 157-212) were tested. Identical (-) and missing (*) nucleotides compared with the expected sequences are indicated.

**Table 3.** Sensitivity of the LAMP assay in enterohaemorrhagic *E. coli*

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Serogroup</th>
<th>VT</th>
<th>Dilution of cultures for assay</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>10⁻³</td>
</tr>
<tr>
<td>157-891</td>
<td>O157</td>
<td>VT1 and VT2</td>
<td>1113</td>
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<td></td>
<td>LAMP assay</td>
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<td>PCR assay</td>
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<tr>
<td>ATCC 43890</td>
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<td>VT1</td>
<td>800</td>
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<td>157-120</td>
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<td>VT2</td>
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<td></td>
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<td>PCR assay</td>
</tr>
<tr>
<td>BFR 26015</td>
<td>O26</td>
<td>VT1 and VT2</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>PCR assay</td>
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</tbody>
</table>
VT-producing *E. coli* is known to include many serogroups (Bettelheim, 2000) and new serogroups might be added in the future. In fact, 1% of isolates of VT-producing *E. coli* in Japan are currently untypable using commercially available antisera (National Institute of Infectious Diseases & Infectious Diseases Control Division, Ministry of Health and Welfare of Japan, 2004). Methods of detecting VT-producing *E. coli* were developed using culture and molecular techniques. The isolation of VT-producing *E. coli* is essential for the typing of strains and identification of the cause of food-borne infections. Effective media to isolate serogroup O157 are available worldwide, but there are few effective media for other VT-producing *E. coli* (Bettelheim, 1995; Hara-Kudo et al., 2002).

The present study demonstrates that the LAMP assay is effective in detecting VT-producing *E. coli* rapidly and with high sensitivity; we thus recommend it as an effective and time-saving method. Firstly the presence of the VT-encoding gene is tested in enrichment culture with LAMP assays. Immediately after the result is obtained, VT-encoding gene positive culture is plated onto agar media for serogroup O157 and other serogroups of *E. coli* (Bettelheim, 1995; Hara-Kudo et al., 2002).

Table 4. Recovery of enterohaemorrhagic *E. coli* O157 and O26 by the culture method, and detection by PCR and LAMP assays

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Detection method</th>
<th>IMS</th>
<th>Agar medium</th>
<th>Food</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ground beef</td>
</tr>
<tr>
<td>O157</td>
<td>Culture method</td>
<td>–</td>
<td>CT-SMAC</td>
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<tr>
<td></td>
<td>–</td>
<td></td>
<td>CHROM agar O157</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>CT-SMAC</td>
<td>10</td>
</tr>
<tr>
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<td></td>
<td>CHROM agar O157</td>
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<td></td>
<td></td>
<td></td>
<td>10</td>
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<tr>
<td>LAMP assay</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>O26</td>
<td>Culture method</td>
<td>–</td>
<td>CT-RMAC</td>
<td>9</td>
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<tr>
<td></td>
<td>–</td>
<td></td>
<td>RX O26</td>
<td>9</td>
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<tr>
<td></td>
<td>+</td>
<td></td>
<td>CT-RMAC</td>
<td>10</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>RX O26</td>
<td>10</td>
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<tr>
<td>PCR assay</td>
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<td>LAMP assay</td>
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</tbody>
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**ACKNOWLEDGEMENTS**

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**REFERENCES**


