A Simple Filtration Technique To Detect Enterohemorrhagic 
Escherichia coli O157:H7 and Its Toxins in Beef 
by Multiplex PCR

KASTHURI VENKATESWARAN,1* YURI KAMIOH,1 EIJI OHASHI,1 
AND HISAO NAKANISHI2

Central Research Laboratory, Nippon Suisan Kaisha, Ltd., Hachioji City, Tokyo 192,1 
and 
Public Health Research Institute of Kobe, Minatojima-Nakamachi, Kobe 650,2 Japan

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Primers, specific for a unique base substitution in uidA of Escherichia coli O157:H7, were coupled with oligonucleotides for the shiga-like toxin I (SLT-I) and SLT-II genes in a multiplex PCR assay. A minimum of 10^2 CFU per PCR (10 μl) was necessary to amplify E. coli O157:H7-specific bands by multiplex PCR. Food particles as well as various unknown metabolic by-products of bacteria inhibited the PCR, but a simple two-step filtration procedure eliminated this inhibition. To reliably generate PCR products, an E. coli inoculum of 10^4 CFU g of food slurry−1 in a nonspecific medium was required with 6 h of enrichment at 37°C. However, when the food homogenate was incubated overnight, E. coli O157:H7 at an initial inoculum of even 1 CFU g−1 was detected.

Most Escherichia coli strains are harmless commensals in the human gut. However, some strains, such as E. coli O157:H7, can cause severe food-borne disease and are referred to as enterohemorrhagic E. coli (EHEC) (12, 18, 19). The EHEC strains of serotype O157:H7 cause hemorrhagic colitis, which may develop into life-threatening hemolytic-uremic syndrome.

EHEC strains produce toxins known as verotoxins or Shiga-like toxins (Shiga-like toxin I [SLT-I] and SLT-II) because of their similarity to the toxins produced by Shigella dysenteriae (13, 16). A large food-poisoning outbreak in 1993, traced to the consumption of undercooked hamburgers contaminated with O157:H7, infected over 700 persons in four states of the United States, resulting in 51 cases of hemolytic-uremic syndrome and four fatalities (5). A waterborne O157:H7 outbreak involving 200 children in Saitama Prefecture, Japan, resulting in 2 fatalities was reported. Recently, more than 8,000 children (including seven fatalities), in 43 of the 47 total Japanese prefectures, were shown to excrete O157:H7 after consuming the midday school meal.

One of the major challenges in diagnosing such problems is identifying the strain of E. coli responsible. Bioassays and conventional methods are commonly used to differentiate toxigenic from nontoxic E. coli strains (20, 22), but there are limitations with these methods. Recently, the multiplex PCR has been used for the detection of various toxin-producing E. coli (4, 12, 17) strains. In the multiplex PCR method, two or more primer sets are used to simultaneously amplify multiple target sequences. Cebula et al. (4) developed an assay that simultaneously identifies isolates of O157:H7 and the types of toxin they encode. The first set of primers is specific for the uidA gene, which encodes β-glucuronidase in E. coli (2). Although O157:H7 isolates fail to exhibit β-glucuronidase activity, they do carry the uidA gene (9, 10). Exploiting the uniqueness of a 92-base change in the uidA gene, Cebula et al. (4) designed a second set of primers in a mismatch amplification mutation assay format (6) to preferentially amplify the uidA allele in O157:H7 strains. The third and fourth sets of primers were directed to the conserved regions within the genes encoding SLT-I and SLT-II, respectively (14).

Multiplex PCR studies have largely concentrated on the identification of bacterial strains or toxins with DNA extracted from pure cultures (2, 4, 14). PCR amplification of enrichment broth was successful in foods only when an immunomagnetic separation step for E. coli O157:H7 was performed (15), but PCR methods for detection of bacteria directly from food samples need at least partially purified DNA (12, 15, 27). The potential of the multiplex PCR assay as a rapid diagnostic tool for screening O157:H7 isolates directly from beef is described in this work. The assay requires an enrichment step followed by a two-step filtration procedure and multiplex PCR-based amplification of four sets of target DNAs. Since no DNA extraction step is required, this method has greater convenience and speed than many established procedures (12, 15). The time needed for detection, including sample preparation, PCR, and agarose gel electrophoresis, is less than 24 h. This method is therefore more rapid and sensitive than verotoxin assay (20), enzyme-linked immunosorbent assay (ELISA) (23), DNA hybridization (8), and direct-sample PCR (15) techniques.

Bacterial isolates. Table 1 includes a list of all strains tested in this study. These strains were purchased from the American Type Culture Collection (Rockville, Maryland) or received as gifts from the National Children’s Medical Research Center (Tokyo, Japan) and the National Institute of Health—Japan (Tokyo).

Biochemical, serological, and immunological characterization of toxigenic strains of E. coli. The U.S. Food and Drug Administration procedure was used to verify the identity of all E. coli isolates (24). Isolates were streaked onto Levine’s eosine methylene blue (EMB) agar (Nissui, Tokyo, Japan), Fluorocult violet-red bile agar (VRB-MUG; Merck, Darmstadt, Germany), and Fluorocult E. coli O157:H7 agar (Merck), Colonies that were sorbitol negative (green coloration) in Fluorocult E. coli O157:H7 agar were presumptively identified as E. coli O157:H7. All strains were examined to determine their characteristics on EMB agar, for gas production at 44.5°C in EC medium (Nissui), and for β-glucuronidase activity (24).
The β-glucuronidase activity was monitored by using Bactident E. coli (Merck) as recommended by the manufacturer. Biochemical characteristics of the bacterial isolates were determined by using the EB-20 system (Nissui) and the Biolog identification system as described earlier (24, 25). Serotyping of the O somatic antigen (O157) and H flagellar antigen (H7) was carried out with commercially available kits (Denka Seiken antisera; Tokyo, Japan). The ELISA for LT antigen (H7) was carried out with commercially available kits (Denka Seiken) as described earlier (24). Serotyping of the O somatic antigen (O157) and H flagellar antigen (H7) was carried out with commercially available kits (Denka Seiken antisera; Tokyo, Japan). The ELISA for LT antigen (H7) was carried out with commercially available kits (Denka Seiken antisera; Tokyo, Japan).

Morphological and biochemical characteristics of various toxigenic strains of E. coli and Shigella species used in this study are tabulated in Table 1. All bacterial strains tested except four isolates of E. coli and S. dysenteriae ATCC 13313 showed the typical metallic-sheen colonies on EMB agar plates. Gas was produced from lactose at 44.5°C by all O157:H7 strains but not by nine of the other E. coli strains. Sorbitol was not utilized by E. coli O157:H7, whereas other toxigenic strains utilized this carbohydrate. Neither S. dysenteriae nor Shigella sonnei utilized sorbitol, but both exhibited green coloration on Fluorocult E. coli O157:H7 agar. Cross-reaction of the somatic antigen O157 with the flagellar antigen H7 was not seen between O157 and H11, nor was it seen between H7, H11, and H. An immunological test for the production of verotoxins by the various strains showed that SLT-I was not produced at a titer higher than that of SLT-II (data not shown). Groupwise variation in the production of verotoxins revealed that O157:H7 strains produced SLT-II predominantly over SLT-I. However, this group could not be differentiated based solely on its serological and immunological characteristics.
The absence of sorbitol fermentation by O157:H7 is a phenotypic characteristic used to isolate this organism from clinical and food specimens. Though it is a useful test, confirmation with O157 and H7 antisera is still required since other bacteria share this serotype and a number of O157:H7 strains ferment sorbitol (11). Antibodies to the O157 antigen are also used in many assays to detect O157:H7 in clinical and food samples. These tests are not specific and provide no information about the toxin types produced by the isolates, since the O157 antigen is present on other E. coli species (1, 21). Additionally, anti-O157 serum often cross-reacts with Citrobacter freundii, Escherichia hermanii, and other bacteria (3). Analyses of food products with anti-O157 serum have recognized O157 isolates that neither produced SLT nor were of the H7 serotype (26). We have confirmed in the present study a previous report (7) that the O157:H7 serogroup does not exhibit β-glucuronidase activity. In addition to O157:H7 strains, one heat-labile toxin (LT)-producing E. coli (ATCC 43886) had no fluorescence on VROB-MUG agar containing lactose. However, this strain showed fluorescence on Fluorocult E. coli O157:H7 agar, in which sorbitol is used as carbon source. Such lactose suppression of β-glucuronidase activity in E. coli has been previously reported (7, 24). Therefore, differentiation of E. coli O157:H7 from other toxigenic and nontoxigenic strains on the basis of biochemical characteristics is dubious.

**Specificity of multiplex PCR primers.** Bacterial strains were grown on Trypticase soy agar (Nissui) plates overnight at 37°C. The bacterial cells in a well-isolated colony were resuspended and grown on Trypticase soy agar. The typical contamination. Total viable counts were enumerated in Trypticase soy broth (TSB) (Nissui) to produce a beef homogenate containing lactose. However, this strain showed fluorescence on Fluorocult E. coli O157:H7 agar, in which sorbitol is used as carbon source. Such lactose suppression of β-glucuronidase activity in E. coli has been previously reported (7, 24). Therefore, differentiation of E. coli O157:H7 from other toxigenic and nontoxigenic strains on the basis of biochemical characteristics is dubious.

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All bacterial DNA amplification was carried out in a final volume of 100 μl. Ten microliters from a 10^5 CFU ml^{-1} bacterial suspension prepared in PBS, or food homogenate as described below, was added to the PCR mixture, and amplification conditions were employed as detailed elsewhere (24). Following DNA amplification, 15-μl aliquots from each PCR mix were analyzed by submarine gel electrophoresis on 2% agarose (Nusieve GTG; FMC Bioproducts, Rockland, Maine) gels.

Amplicons corresponding to the uidA gene (147 bp), the uidA gene specific for the E. coli O157:H7 serovar (252 bp), the SLT-I gene (348 bp), and the SLT-II gene (584 bp) were generated with suitable PCR primers from toxigenic and nontoxigenic E. coli strains isolated from various outbreaks (Fig. 1). The uidA gene was observed in all 37 E. coli strains and both Shigella strains tested. E. coli strains with the O157:H7 serotype were distinguished simultaneously with the SLT type known to be produced by these strains. The amplicons generated approximated the sizes predicted on the basis of the selected primer sets. As anticipated, only the uidA gene (147 bp) was amplified from wild-type E. coli, whereas the expected toxin gene-specific products, but not O157:H7-specific products (252 bp), were amplified from the SLT-producing non-O157:H7 serotypes examined. PCR results indicated that ATCC strains 43890, 43894, and 43895 were positive for an SLT-I-homologous sequence (348 bp) and that ATCC strains 43894 and 43895 were positive for SLT-II (584 bp). Several of the SLT-producing, non-O157:H7 serotypes of E. coli O157:H7 strains were counted in Fluorocult E. coli O157:H7 agar, when necessary.

The sensitivity of the PCR assay for detecting artificially inoculated E. coli O157:H7 43895 in beef is presented in Table 2. When whole bacterial cells were resuspended in sterile PBS, 10^2 CFU of E. coli in a 10-μl PCR mixture was needed to amplify all four amplicons. Amplicons specific for E. coli or toxigenic genes were not amplified for the un inoculated food sample (Fig. 2, lanes F). At time zero, 10^2 CFU g of beef homogenate failed to yield the desired PCR products. Although a faint band at 147 bp (E. coli specific) was noticed after 18 h of enrichment in TSB, the amplicons generated by toxigenic strain- and O157:H7-specific probes were not distinct. This indicates that food particles and/or other unknown metabolic by-products are inhibitory to the PCR.

To remove any PCR-inhibitory substances from food, subsamples drawn from the microcosms were subjected to two-step filtration. A 400-μl sample of a sterile-PBS-washed sample was pass through a 5-μm-diameter Ultrafree filter tube (cat-
Influence of competitive food microflora on the sensitivity of multiplex PCR. The results presented here indicate that the E. coli O157:H7 detection was enhanced when the beef homogenate was subjected to filtration. Although 8 to 10% of the bacterial population was removed along with particulate matter, the majority of the bacterial cells were collected after 5-μm-pore-size filtration. This was evident at any preenrichment incubation level. When 6-h, TSB-enriched beef homogenate was passed through 5-μm-pore-size filters, a minimum of 10² CFU of E. coli O157:H7 cells per g was required to amplify all four bands (faint bands), or the ratio of E. coli O157:H7 to other, competitive microflora in the sample had to be in the range of 10³/1 (Table 2). However, a combination of 18 h of enrichment in TSB and a simple two-step filtration treatment generated all four bands clearly, even when E. coli O157:H7 and other food microflora were present at a ratio of 1/10².

TABLE 2. Sensitivity of filtration method in the amplification of various bands specific to E. coli O157:H7 serovar and its toxins

<table>
<thead>
<tr>
<th>Sample or parameter</th>
<th>Preenrichment incubation in TSB (h)</th>
<th>PCR band(s) amplified when the initial inoculum level was in the range of (CFU g of food slurry⁻¹)²:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10⁰</td>
</tr>
<tr>
<td>Untreated food slurry</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>E, O</td>
</tr>
<tr>
<td>Beef slurry (5-μm pore size filtered)</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>O, I, II</td>
</tr>
<tr>
<td>Beef slurry (5-μm pore size filtered; retained on 0.2-μm-pore-size filters)</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>Population²</td>
<td>0</td>
<td>10⁵/10</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10⁷/10</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>10⁹/10</td>
</tr>
</tbody>
</table>

- Abbreviations: E, E. coli band (147 bp); O, E. coli O157:H7 band (252 bp); I, SLT-I band (348 bp); II, SLT-II band (584 bp).
- The numerator and denominator denote E. coli O157:H7 serovar and food microflora populations (CFU per gram), respectively.
(Table 2). However, successful PCR amplification after an overnight incubation was due to not only the proliferation of the target organism but the removal of PCR-inhibitory substances as well.

The development of molecular methodologies to detect pathogenic microorganisms in food, clinical, and environmental samples has led to improved patient diagnosis and the more precise determination of public health risks associated with food consumption and environmental exposure. The standard bioassays used for identification of pathogenic E. coli, such as assays for cytoeffectic effects on Vero cells and rabbit ileal loop assays, are difficult to adapt for the screening of large numbers of E. coli isolates. Bioassays are labor-intensive and costly, and the results can take several weeks. The application of molecular approaches in food microbiology offers a more efficient alternative to these labor-intensive methods.

The multiplex PCR method described here is a highly effective means for specifically detecting and characterizing EHEC organisms directly from food. The major advantage of this protocol over existing assays is that it can identify the types of SLT encoded by the strain and simultaneously discriminate other SLT-producing E. coli strains from O157:H7, the predominant serotype implicated in disease.

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REFERENCES