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# Use of the Duplex TaqMan PCR System for Detection of Shiga-Like Toxin-Producing *Escherichia coli* O157

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**Real-time PCR assays have been applied for the detection and quantification of pathogens in recent years. In this study two combinations of primers and fluorescent probes were designed according to the sequences of the $rfb_{Escherichia\ coli\ O157}$ and $stx_2$ genes. Analysis of 217 bacterial strains demonstrated that the duplex real-time PCR assay successfully distinguished the *Escherichia coli* O157 serotype from non-*E. coli* O157 serotypes and that it provided an accurate means of profiling the genes encoding O antigen and Shiga-like toxin 2. On the other hand, bacterial strains that lacked these genes were not detected by this assay. The quantitative ranges of the real-time PCR assay for these two genes were linear for DNA concentrations ranging from $10^3$ to $10^9$ CFU/ml of *E. coli* O157:H7 in pure culture and milk samples. The real-time PCR allowed the construction of standard curves that facilitated the quantification of *E. coli* O157:H7 in feces and apple juice samples. The detection sensitivity of the real-time PCR assay ranged from $10^4$ to $10^9$ CFU/g (or $10^4$ to $10^9$ CFU/ml) for feces and apple juice and $10^5$ to $10^9$ CFU/g for the beef sample without enrichment. After enrichment of the food samples in a modified tryptic soy broth, the detection range was from $10^0$ to $10^3$ CFU/ml. The real-time PCR assays for $rfb_{E.\ coli\ O157}$ and $stx_2$ proved to be rapid tests for the detection of *E. coli* O157 in food matrices and could also be used for the quantification of *E. coli* O157 in foods or fecal samples.**

*Escherichia coli* is a gram-negative bacterium that generally inhabits the intestinal tract of humans and animals. However, some of isolates of this organism are pathogenic, and these enterovirulent *E. coli* isolates are important food-borne pathogens associated with severe gastrointestinal and circulatory system diseases, such as hemorrhagic colitis (HC), hemorrhagic-uremic syndrome (HUS), and thrombotic thrombocytopenic purpura, in humans (17, 19). *E. coli* O157:H7 is a major strain which causes these kinds of food-borne outbreaks all over the world. In 1975, *E. coli* O157:H7 was first isolated from clinical samples, but it was not reported in association with outbreaks until 1982 (18). In 1996 there were some large outbreaks in Japan which originated in Sakai City, Osaka (22). These outbreaks affected more than 17,000 people. A total of 106 children developed HUS, and 13 of these children died (18). Similar outbreaks have been reported in Australia, Canada, the United States, various European countries, and Africa (6, 8, 10, 22, 26, 28).

The pathogenicity of *E. coli* O157:H7 is associated with a number of virulence factors, including Shiga-like toxins 1 and 2 (encoded by the $stx_1$ and $stx_2$ genes, respectively) and intimin (encoded by the *eaeA* gene). Shiga-like toxins are believed to play a major role in the pathogenesis of HC and HUS through a cytopathic effect on the vascular endothelial cells of the kidneys and intestines (29). Strains isolated from patients with HC usually produce both Shiga-like toxins 1 and 2, and strains that produce only $stx_1$ are uncommon (11).

In Taiwan, infection with *E. coli* O157:H7 is a reportable infectious disease. No cases were reported in Taiwan until 2001. In the summer of 2001, a patient presented with symptoms that included bloody diarrhea, HC, and HUS. This patient's diarrhea stools, other suspected stools, and environmental samples were collected. We analyzed and confirmed that the infectious strain was *E. coli* O157:H7. This was the first infectious case caused by *E. coli* O157:H7 in Taiwan (35).

Cattle are generally considered the major reservoir for this organism, although it has also been isolated from sheep (20), goats (3), dogs, deer, horses, and seagulls (18). An important aspect of this organism is the fact that the ingestion of 10 to 100 of these organisms may be sufficient to cause an infection (33). Among the most important sources of human infection are direct contact with cattle and other ruminants, contaminated bathing water, beef products, unpasteurized milk, vegetables, fruits, and drinking water (7). The detection and correct identification of this strain are important parts of food hygiene. Traditional methods for the identification of *E. coli* O157:H7, such as biochemical and serotype tests, used to take 5 to 7 days. In recent years, some molecular methods were developed to detect and identify this food-borne pathogen, such as PCR and enzyme-linked immunosorbent assay. PCR is a rapid and easy-to-use method and can provide a preliminary characterization (5, 9). The use of the PCR method to detect pathogens, however, has some shortcomings, such as some false-positive or false-negative results for more complex samples and a low sensitivity with more primer sets. At the same time, the ethidium bromide used to stain the electrophoresis gel after PCR is a harmful chemical and its application is time-consuming. The TaqMan detection system (Applied Biosystems, Foster City, Calif.) is a new qualitative and quantitative system that uses a fluorogenic hybridization probe to detect the target genes; and it has previously been demonstrated to be a rapid, high-throughput, semiautomatic PCR scheme for the identification of *E. coli* (23, 29), *Salmonella* (28), and *Listeria* spp. (1).

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TABLE 1. Sequences of primers and probes used in this study

| Primer name | Sequence (5′→3′) | Source or reference |
|---|---|---|
| Stx2F[a] | ACC ACA TCG GTG TCT GTT ATT AAC C | This study |
| Stx2R[a] | CGG TAG AAA GTA TTT GTT GCC GTA TT | This study |
| Stx2P[a] | TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC TAT | This study |
| rfbF[b] | ATG CTG CCC ACA AAA ATA ATG TAAA | This study |
| rfbR[b] | TTC CAT AAT CGG TTG GTG TGC TAA | This study |
| rfbP[b] | AAC TGC TTT TCC TCG GTT CGT CGT GTA T | This study |
| NS5[c] | CTT CGG TAT CCT ATT CCC GG | 34 |
| NS7[c] | CGC TGC AGC TGT ATT ACT TTC | 34 |
| $VT_2S_4F$[d] | ATC CAG TAC AAC GCG CCA | 4 |
| $VT_2S_4R$[d] | CAC AGA CTG CGT CAG TGA GG | 4 |

[a] Primers Stx2F, Stx2R, and Stx2P were developed to amplify the Shiga-like toxin 2 gene, and 6-carboxyfluorescein was added as a label to the 5′ end of Stx2P.
[b] Primers rfbF, rfbR, and rfbP were developed to amplify the *rfb* gene, which was part of the O157 antigen, and 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein was added as a label to the 5′ end of rfbP.
[c] Primers NS5 and NS7 were designed to amplify Shiga-like toxin 2.
[d] Primers $VT_2S_4R$ and $VT_2S_4F$ were designed to amplify Shiga-like toxin 2.

The objective of this study was to assess the utility of the TaqMan PCR system for the detection and identification of the $stx_2$ gene (which is responsible for the biosynthesis of Shiga-like toxin 2) and the $rfb_{E.\ coli\ O157}$ gene (which is responsible for the biosynthesis of the O antigen) (31). We developed some modifications to improve the pretreatment and purification of food samples for PCR. These improvements were designed to increase the specificity and the accuracy of the PCR.

## MATERIALS AND METHODS

**Bacterial strains, culture media, and growth conditions.** *E. coli* O157:H7 strain Tw04 was used throughout this study. Strain Tw04 was isolated from a clinical sample in Taiwan in 2001 by our laboratory (35). In addition, a further 217 bacterial strains were used to evaluate the specificity of the real-time PCR assay for *E. coli* O157 detection in this study. These strains were *E. coli* O157:H7, clinical *E. coli* strains, and other non-*E. coli* strains. They were purchased from the Bioresources Collection and Research Center (Hsinchu, Taiwan) and the American Type Culture Collection, and some came from among the clinical isolates kept in our laboratory and the Centers for Disease Control, Taiwan. Before testing of the bacterial strains, they were retrieved from frozen storage and cultured in nutrient broth (Difco Laboratories, Detroit, Mich.) at 37°C overnight. The cultures were then transferred to tryptic soy broth (TSB; Difco) and incubated at 37°C until the optical density at 600 nm reached 1.2 to 1.4. The cells were subsequently harvested and used for DNA preparation and other tests. In addition, before they were tested they were checked for their genotype ($stx_2$, $rfb_{E.\ coli\ O157}$) by PCR (24).

**Genomic DNA preparation.** Two different methods were used for the preparation of genomic DNA. In one method the genomic DNA was prepared by direct purification of boiled cells lysed by the double-distilled water method. In the other method the genomic DNA was prepared with a Wizard Genomic DNA purification kit (Promega Corporation, Williamsburg, Iowa). The direct boiled cell method was performed as follows: 1 ml of log-phase cultured bacterial broth was centrifuged at 15,000 × *g* for 10 min. After centrifugation, the cell pellets were resuspended in 250 μl sterile distilled water and boiled for 10 min. The lysed cell debris was then removed by centrifugation (15,000 × *g* for 5 min), and the DNA in the supernatant was transferred to a fresh and sterile Eppendorf tube.

The preparation with the kit was performed according to the manufacturer's instructions. Bacterial cultures were centrifuged at 12,000 × *g* and mixed with nucleic lysis buffer and proteinase K at 37°C for 15 to 60 min to lyse the cell wall. The protein precipitation buffer was then used to bind the protein and precipitate it. After centrifugation, the supernatant was mixed with isopropanol to precipitate the DNA. The extracted DNA was then washed with ethanol and resuspended in double-distilled water. All DNA samples were used immediately or stored at −30°C.

**Sequencing of the Shiga-like toxin 2 gene of *E. coli* O157:H7 isolated from Taiwan.** Five primers were used to sequence the Shiga-like toxin 2 gene of *E. coli* O157:H7 strains which were isolated in Taiwan. The sequences of the primers are shown in Table 1. The PCRs were carried out in a total volume of 25 μl which included 1× reaction buffer, 200 nM of deoxynucleoside triphosphates, 200 nM of each of the primers, and 1.5 U of *Taq* polymerase (Takara, Tokyo, Japan). The PCR products were analyzed with a genetic analyzer (Applied Biosystems).

**Cloning.** To determine the detection limit of the TaqMan PCR assay, the 862-bp PCR product from the $stx_2$ gene of *E. coli* strain Tw04, obtained with primers NS5 and NS7, was cloned into *E. coli* strain DH5α by using the pGEM-T and pGEM-T Easy Vector systems kit (Promega Corporation) (25).

**Plasmid preparation.** Plasmid DNA was purified by using the Plasmid Miniprep Purification Kit II (Genemark Technology Corporation, Tainan, Taiwan). The extracted plasmid DNA was then assayed on 1.5% (wt/vol) agarose gels to make sure that the product was pure. Following the gel assay, the product was repurified with a Gene-Spin 1-4-3 DNA extraction kit (Protec Technology Enterprise Corporation, Taipei, Taiwan). The concentration of plasmid was determined by a fluorescent dye, bisBenzimide Hoechst 33258 (Sigma, St. Louis, Mo.), and with a fluorescence meter (Bio-Tek, Winooski, Vt.).

**Design of primers and fluorogenic probes.** The nucleotide sequences of the primers and fluorogenic probes are listed in Table 1. The primers and fluorogenic probes were designed according to the sequences listed in the instructions of the BLAST kit by the Primer Express software (v1.5; Applied Biosystems).

The sequence accession numbers in the GenBank database are X07865 for $stx_2$ and AF049343 for $rfb_{E.\ coli\ O157}$. 6-Carboxyfluorescein and 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein were used as fluorescent reporter dyes and were conjugated to the 5′ ends of the probes to detect amplification products specific for $stx_2$ and $rfb_{E.\ coli\ O157}$, respectively. The quencher dye 6-carboxytetramethylrhodamine was attached at the 3′ ends of these probes. The primers were synthesized by the Purigo Biotech Corporation (Taipei, Taiwan), and the probes were synthesized by the MWG Biotech Corporation (Ebersberg, Germany).

**TaqMan PCR assay for detection of *E. coli* O157.** PCR was performed in a reaction mixture with a total volume of 25 μl containing 1 μl of extracted DNA, 0.5 mM of $stx_2$ and $rfb_{E.\ coli\ O157}$ primers, 0.2 mM of each fluorogenic probe, and TaqMan Universal Master Mix (Applied Biosystems). The Master Mix contained AmpErase uracil-*N*-glycosylase (UNG), deoxynucleoside triphosphate with dUTPs, 6-carboxyrhodamine as an internal passive fluorogenic reference, and an optimized buffer component. Amplification and detection were carried out in optical-grade 96-well plates in an ABI Prism 7700 sequence detection system (Applied Biosystems) with an initial step of 50°C for 2 min, which is the required optimal AmpErase UNG enzyme activity, and then at 95°C for 10 min, to activate the AmpliTaq Gold DNA polymerase and to deactivate the AmpErase UNG enzyme, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The reaction conditions for amplification and the parameters for fluorescence data collection were programmed into a Power Macintosh 4400/20 computer (Apple Computer, Santa Clara, Calif.) linked directly to the ABI Prism 7700 sequence detection system by using the SDS 1.6 application software, according to the manufacturer's instructions. After real-time data acquisition, the threshold, which was defined as being 10-fold higher than the baseline, was determined; and the cycle threshold ($C_T$) value was manually set so that it intersected the amplification curves in the linear region of the semilog plot. The quantity of target gene copies in the PCR sample is predicted by the $C_T$ value (12).

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TABLE 2. Similarities of $stx_2$ gene sequences between each pair of strains isolated in Taiwan

| Strain | % Similarity | | |
|---|---|---|---|
| | Tw01 | Tw02 | Tw03 |
| Tw01 | | | |
| Tw02 | 97 | | |
| Tw03 | 99 | 97 | |
| Tw04 | 97 | 95 | 97 |

**Food sample preparation.** The pasteurized milk, beef, and apple juice were obtained from a local supermarket. All of the samples were initially tested by culture on sorbitol-MacConkey agar plates and by the PCR method. The microorganisms were prepared from 10-fold serial dilutions of a log-phase culture of *E. coli* O157:H7 strain Tw04, which contained $10^9$ to $10^0$ CFU/ml viable cells, for mixing with the food samples. The direct boiling cell lysis method and modified QIAamp DNA stool mini kit (QIAGEN Companies) method were used to extract DNA from samples which contained different concentrations of pathogens. The direct boiling cell lysis method was used for preparation of pasteurized milk samples and was performed as follows. One milliliter of milk sample was prepared, and cells were allowed to lyse for 10 min at 100°C. After boiling of the suspension, it was cooled to room temperature, spun at 16,000 × *g* for 30 s, and transferred to a new tube and spun again. The supernatant was then used as the PCR template in this study. The modified QIAamp DNA stool mini kit method used for the apple juice sample was performed as follows: 1 ml of intermixed apple juice sample was spun at 16,000 × *g* for 10 min, and then 850 μl of the supernatant was removed. After this step, all the follow-up steps were the same as those described in the original protocol for the kit until the wash step, when 2× washing buffer was added and the mixture was eluted with 50 μl of double-distilled water. The DNA was then extracted from imitated beef samples by the QIAamp DNA stool mini kit method, which was modified in this study. The constructed plasmid, the concentration of which was determined by use of the fluorescent dye bisBenzimide (Hoechst 33258), was placed in the extracted DNA as an internal standard in order to contrast the results of quantification.

**Human stool sample preparation.** A stool sample was collected from a 24-year-old healthy man. We screened the stool sample for *E. coli* O157:H7 by culture on sorbitol-MacConkey agar plates and PCR before the sample was seeded. The microorganisms were prepared from 10-fold serial dilutions of a log-phase culture of *E. coli* O157:H7 strain Tw04 containing $10^9$ to $10^0$ CFU/ml viable cells for mixing with the stool sample. The QIAamp DNA stool mini kit method was used to extract DNA from samples which contained different concentrations of the pathogen. The constructed plasmid, the concentration of which was determined by use of the fluorescent dye bisBenzimide (Hoechst 33258), was placed in the extracted DNA as the internal standard in order to contrast the results of quantitation.

**Enrichment of food samples in mTSB medium.** Different concentrations of freshly grown and enumerated cultures of *E. coli* O157:H7 strain Tw04 were used to inoculate 1 ml of apple juice or raw milk samples. The inoculated samples were then added to the enrichment medium, modified TSB (mTSB; Merck, Germany), to 10% (vol/vol) and incubated at 37°C.

## RESULTS

**Nucleotide sequences of Shiga-like toxin 2 in *E. coli* strains Tw02, Tw03, and Tw04.** In this study we used five primers, including primers NS5 and NS7 (34), primers $VT_2S_4F$ and $VT_2S_4R$ (4), and primer stx2P, to identify DNA sequences of about 1,274 bp of Shiga-like toxin 2 in *E. coli* strains Tw02, Tw03, and Tw04. These *E. coli* O157:H7 strains were isolated from environmental samples (strains Tw02 and Tw03) and a clinical sample (strain Tw04) in Taiwan. The sequence databases were aligned by use of the BLAST program (available at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). We compared the results obtained in our laboratory for *E. coli* O157:H7 strain Tw01 (GenBank accession number AF291819) in 2000, as shown in Table 2. The range of similarities of the $stx_2$ gene sequences between each pair of strains was 95% to 99%.

**Specificity of TaqMan PCR assay.** Genomic DNA from *E. coli* O28ac ($stx_2^+$ and O157$^-$), O157:H7 ($stx_2^-$ and O157$^+$), O78:H11 ($stx_2^-$ and O157$^-$), and O157:H7 ($stx_2^+$ and O157$^+$) was initially tested to evaluate the primers and probes used in the PCR assay for their abilities to amplify and detect PCR products specific for the $stx_2$ and $rfb_{E.\ coli\ O157:H7}$ genes. A fluorescent signal 10 times higher than the standard deviation of the mean baseline emission was indicative of a positive detection result. $Stx_2$- and $rfb_{E.\ coli\ O157}$-specific probes produced exponential increases in fluorescence only when the DNA from strains containing these genes was used as a template in the PCR assay. The amplified products generated in the samples were also analyzed on a 4% agarose gel by standard horizontal gel electrophoresis. Those samples that resulted in an exponential increase in fluorescence with a particular probe also contained an amplicon that was of the predicted size and that corresponded to the gene detected by the probe (data not shown).

Based on detection of the characteristics of toxin and O-antigen genes of *E. coli* O157, a duplex TaqMan PCR assay was developed. The specificity of the assay was tested against a panel of bacterial templates from 217 *E. coli* or non-*E. coli* strains, including 35 *E. coli* O157:H7 strains, 2 $stx_2$ and 158 non-$stx_2$ *E. coli* strains isolated from clinical cases in Taiwan, and 22 strains of other bacterial species. All Shiga-like toxin 2-producing *E. coli* O157:H7 strains were detected by the duplex TaqMan PCR assay, whereas the other strains of bacteria were negative, as shown in Table 3. The positive samples gave $C_T$ values lower than 16 and a high endpoint fluorescence usually of >1.0 (data not shown).

**Sensitivity of TaqMan PCR assay.** Genomic DNA prepared from 10-fold serial dilutions of a log-phase culture of *E. coli* O157:H7 strain Tw04 was used as the template to determine the detection sensitivity of this multiplex real-time PCR and to construct standard curves by plotting the numbers of CFU versus the $C_T$ values produced for both of the target genes. Standard curves showed a linear relationship between the $\log_{10}$ input CFU and the $C_T$ (the PCR cycle at which the fluorescence intensity rises above the threshold). The lowest detection limit of the PCR assay for the $rfb_{E.\ coli\ O157}$ and $stx_2$ genes was approximately $10^3$ CFU/ml ($10^3$ copies/ml), as shown in Table 4.

**Sensitivity of TaqMan PCR with food samples.** In this study, we also tried to detect *E. coli* O157:H7 DNA in three kinds of food samples, including beef, apple juice, and raw milk, which were associated with *E. coli* O157:H7 infections in the past. The DNA isolated from food samples was mixed with 10-fold serial dilutions of *E. coli* O157:H7 strain Tw04 and was used in this assay to construct a standard curve for the $stx_2$ gene. This quantification was linear over a range of initial target concentrations from $10^3$ to $10^9$ CFU/ml in a raw milk sample. In apple juice samples it was linear over a range of initial target concentrations from $10^4$ to $10^9$ CFU/ml, as shown in Table 4. In beef samples it was linear over a range of initial target concentrations from $10^5$ to $10^9$ CFU/g.

**Sensitivity of TaqMan PCR with stool samples.** Human feces containing different concentrations of *E. coli* O157:H7 strain Tw04 ($stx_2^+$ and O157$^+$) were tested to evaluate the

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TABLE 3. Strains used in this study and associated multiplex real-time PCR results

| Phenotype of strains | Genotype | Total no. of subjects ($n$ = 217[j]) | No. of positive results with probe specific for: | |
|---|---|---|---|---|
| | | | O157[h] | *stx2*[i] |
| *E. coli* O157:H7[a] | $stx_2$ | 30 | 30 | 30 |
| *E. coli* O157:H7 | $stx_1$ | 4 | 4 | 0 |
| *E. coli* O157:H7 | Non-*stx* | 1 | 1 | 0 |
| Non-O157 clinical *E. coli*[b] from Taiwan | Others[f] | 158 | 0 | 0 |
| Non-O157 clinical *E. coli*[c] with $stx_2$ gene from Taiwan | O28 and $stx_2$[g] | 2 | 0 | 2 |
| *Salmonella* Enteritidis strains[d] | | 10 | 0 | 0 |
| *Shigella* spp.[e] | | 10 | 0 | 0 |
| *Pseudomonas*[e] | | 1 | 0 | 0 |
| *Citrobacter freundii* ATCC 8090[e] | | 1 | 0 | 0 |

[a] All strains were isolated from the United States (10 strains), Canada (12 strains), Japan (9 strains), and Taiwan (4 strains).
[b] All strains were isolated from clinical samples of diarrhea cases in Taiwan.
[c] All strains were isolated from clinical samples in Taiwan by the Centers for Disease Control, Taiwan.
[d] All strains were isolated from food-borne cases by the Centers for Disease Control, Taiwan.
[e] All strains were from Bioresource Collection and Research Center, Hsinchu, Taiwan.
[f] All genotypes are non-$stx_2$; and their phenotypes include the following: O1, O3, O6:H?, O6:H12, O8:H?, O8; O14; O15:H21 O15:NM, O18, O20:H20, O23, O25:H51, O25:K98:NM, O26:H?, O26:H11, O27, O28:H?, O28ac:H?, O28ac, O29:H?, O29:NM, O30, O38, O44, O48, O55:H?, O55:H12, O75, O78:H11, O78:H2, O78:K80:H12, O86a, O90, O111ab:H21, O112ac:H?, O114, O115, O123, O124:H?, O125, O127a:H1, O127a:H16, O136:H19, O138, O142:H6, O144, O146:NM, O148, O153:NM, O158, O159:H21, O166, O168, O169:H28, and unknowns.
[g] All genotypes are $stx_2$, and the serotype was O28ac.
[h] Numbers of positive results by the multiplex real-time PCR assay for the $rfb_{E.\ coli\ O157:H7}$ gene.
[i] Numbers of positive results by the multiplex real-time PCR assay for the $stx_2$ gene.
[j] Total numbers of strains in this study.

utility of this real-time PCR assay for the detection of *E. coli* O157:H7 in humans. The DNA isolated from stool samples was mixed with 10-fold serial dilutions of *E. coli* O157:H7 strain Tw04 and was used in this assay to construct standard curves for the $stx_2$ and $rfb_{E.\ coli\ O157}$ genes. The numbers of CFU of *E. coli* O157:H7 present per gram of feces were interpolated from these standard curves and then compared to the bacterial counts determined by plating the same fecal samples on sorbitol-MacConkey agar-streptomycin plates. The numbers of CFU per gram of feces estimated by this real-time PCR assay by using $stx_2$- and $rfb_{E.\ coli\ O157}$-specific probes were similar to those determined by plating the same samples on sorbitol-MacConkey agar. This quantification was linear over a wide range of initial target concentrations ($10^4$ to $10^9$ CFU/g), as shown in Table 4. On the other hand, we detected *E. coli* O157:H7 strain Tw04 in seeded samples for evaluation of the assay methods that we had set up. In all experiments, the estimated values and the actual values were similar. The results are shown in Table 5.

TABLE 4. Sensitivity of the multiplex real-time PCR assay for detection of *E. coli* O157:H7 strain Tw04 in milk, apple juice, beef, and feces

| Sample | Result of this study[a] | $R^2$[b] | Previous result (reference)[c] |
|---|---|---|---|
| Pure culture | $10^3$ CFU/ml | 0.992 | $10^3$ CFU/ml (15) |
| Feces | $10^4$ CFU/g | 0.992 | $10^4$ CFU/g (27), $3.5 \times 10^4$ CFU/g (15) |
| Apple juice | $10^4$ CFU/ml | 0.997 | $10^8$ CFU/ml (9) |
| Milk | $10^3$ CFU/ml | 0.998 | $10^3$ CFU/ml (21) |
| Beef | $10^5$ CFU/g | 0.990 | |

[a] The limit of detection for *E. coli* O157:H7 in each sample by real-time PCR, for which the combinations of primers and probes were designed in this study.
[b] The results of linear regression by each concentration of each strain.
[c] The limit of detection for *E. coli* O157:H7 in each sample by real-time PCR in other studies.

**Enrichment of food samples in mTSB.** In this study, the effects of the length of the enrichment time on the sensitivity of the multiplex real-time PCR for detection of *E. coli* O157 in milk and apple juice samples were investigated. The results are shown in Table 6. After being enriched for 4 h, the sensitivity for the detection of *E. coli* O157 in milk samples was $10^0$

TABLE 5. Detection of *E. coli* O157:H7 strain Tw04 in seeded samples

| Sample | Real-time PCR result with probe specific for: | | Plate count |
|---|---|---|---|
| | O157 | $stx_2$ | |
| Plasmid | | | |
| P1[c] | ND[a] | 6.9[b] | 7.1[b] |
| P2[c] | ND | 5.1 | 4.3 |
| Milk | | | |
| M1[d] | 8.0 | 8.0 | 8.0 |
| M2[d] | 3.6 | 3.6 | 4.0 |
| Apple juice | | | |
| A1[e] | 7.8 | 7.8 | 8.0 |
| A2[e] | 5.1 | 5.1 | 5.1 |
| Human feces | | | |
| F1[f] | 6.7 | 6.7 | 7.0 |
| F2[f] | 4.9 | 4.9 | 5.1 |

[a] ND, not detectable; $stx_2$-specific probe has only the $stx_2$ gene.
[b] $\log_{10}$ CFU/ml or $\log_{10}$ CFU/g of sample.
[c] P1 and P2 were different concentrations of the plasmid in the imitated samples.
[d] M1 and M2 were different cells of *E. coli* O157:H7 strain Tw04 in the imitated milk samples.
[e] A1 and A2 were different cells of *E. coli* O157:H7 strain Tw04 in the imitated apple juice samples.
[f] F1 and F2 were different cells of *E. coli* O157:H7 strain Tw04 in the imitated fecal samples.

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TABLE 6. Sensitivity of the multiplex real-time PCR assay for detection of *E. coli* O157:H7 strain Tw04 in raw milk and apple juice after enrichment

| No. of CFU/ml | Sensitivity for detection in[a]: | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Milk | | | | | | Apple juice | | | | | |
| | 0 h | 2 h | 3 h | 4 h | 5 h | 10 h | 0 h | 2 h | 3 h | 4 h | 5 h | 10 h |
| $10^3$ | + | + | + | + | + | + | − | − | + | + | + | + |
| $10^2$ | − | − | + | + | + | + | − | − | + | + | + | + |
| $10^1$ | − | − | − | + | + | + | − | − | − | + | + | + |
| $10^0$ | − | − | − | + | + | + | − | − | − | − | − | + |
| 0 | − | − | − | − | − | − | − | − | − | − | − | − |

[a] The plus or minus sign indicates whether or not the relative fluorescent intensity after 40 cycles was greater than the threshold value for the negative control.

CFU/ml. In the apple juice samples, the same sensitivity of $10^0$ CFU/ml was obtained after a 10-h enrichment period.

## DISCUSSION

Traditional methods based on biochemical characteristics are labor-intensive, and the total time required for determination of the identities of the pathogens is typically about 72 h. Rapid detection techniques directed at similar immunological and genetic targets are therefore of great interest, especially since it is very difficult to determine the total number of bacteria present in foods or fecal samples. Immunological methods based on the detection of Shiga-like toxins have been developed. However, these methods cannot differentiate *E. coli* O157:H7 from other less virulent enterohemorrhagic and enteropathogenic *E. coli* strains. Other methods, based on the detection of O157 somatic and H7 flagellar antigens, are equally inadequate because of their lack of specificity.

Real-time PCR offers the ability to determine the absolute and relative amounts of pathogens in complex matrices, and assays that were recently developed for the identification of *E. coli* O157:H7 are based on the detection of genes encoding Shiga-like toxins (2, 21), intimin (23), and O antigen (9). However, those single real-time PCR methods sometimes lack specificity. For example, the targets for the H7 flagellar antigen genes may cross-react with *E. coli* O55:H7 and so will fail to identify *E. coli* O157:NM (where NM is nonmotile). Although some published reports have shown that a real-time PCR designed for use with two or three combinations of primers and probes could provide good specificity and sensitivity for the detection of enterovirulent *E. coli* or *E. coli* O157:H7 (15, 27), we decided that less effort for detection would be required by use of combinations of primers and probes which amplified the O antigen and Shiga-like toxin. The $rfb_{E.\ coli\ O157}$ gene encodes an enzyme that is necessary for O-antigen biosynthesis and that is highly conserved among isolates of the *E. coli* O157 serovar. According to previous reports, strains of *E. coli* O157 isolated from patients with HC usually produce both Shiga-like toxins 1 and 2. Isolates that produce only $stx_1$ are uncommon (11). Therefore, in this study we designed two combinations of primers and probes to detect and identify *E. coli* O157 isolates producing Shiga-like toxin 2 by targeting the particular genes $stx_2$ and $rfb_{E.\ coli\ O157}$.

The specificity of the real-time PCR was evaluated with 217 strains, including *E. coli* O157:H7 strains and other isolates. The results have shown that the combinations of primers and probes designed in this study correctly detected *E. coli* O157:H7 and that there were no false-positive or false-negative results by any of the tests. Comparison of the detection limits of these combinations of primers and probes showed that the results were better or the same as those published in the literature (9, 15, 21, 27). The detection range of the duplex real-time PCR assay for $stx_2$ and $rfb_{E.\ coli\ O157}$ was linear when DNA was prepared from pure culture samples containing from $10^3$ to $10^9$ CFU/ml of *E. coli* O157:H7. This result was the same in the raw milk sample. However, in the apple juice samples, the results were linear for pathogen concentrations ranging from $10^4$ to $10^9$ CFU/ml. The same results ($10^4$ to $10^9$ CFU/g) were evident for the human stool samples. According to the results of published papers, some PCR inhibitors are present, such as polyphenolic compounds in apple juice (30) and bile salts, heparin, and bilirubins in human stools (14, 32). Because of the presence of these inhibitors in apple juice and human stool samples, the DNA extracted from these samples needed to be purified before the real-time PCR was carried out. On the other hand, there were no inhibitors in the milk, and therefore, we could carry out the real-time PCR with lysed boiled cells. Although the range of linear concentrations in human stool samples was $10^4$ to $10^9$ CFU/g, the stools of food-poisoning patients usually harbor $10^6$ CFU/g bacteria and more (13). We used this combination of probes and primers to correctly detect *E. coli* O157 in stool samples.

Traditional culture-based approaches for the detection of *E. coli* O157:H7 in beef, such as growth on sorbitol-MacConkey agar and further screening for the production of Shiga-like toxin, may take several days (16). In this study, we extracted the DNA of the pathogen directly from the beef and the reaction was completed in 3 h. The detection range was linear from $10^5$ to $10^9$ CFU/g.

In summary, we have described a duplex TaqMan real-time PCR method for the detection of *E. coli* O157 that showed good specificity and sensitivity and that also saved substantial time because of the preparation of samples without preculture. The key points for correct detection in this study were the DNA extraction efficiency and the removal of PCR inhibitors from different materials. In the future, we will try to create other methods for the isolation of the DNA of pathogens and for the removal of PCR inhibitors from other, different samples which are related to *E. coli* O157:H7. The shortening of the processing time and the increase in the specificity for pathogen detection are critical for the safety and sanitation of our food supply.

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