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Use of Real-Time Polymerase Chain Reaction and Molecular Beacons for the Detection of *Escherichia coli* O157:H7

Nathalie Y. Fortin,[1] Ashok Mulchandani, and Wilfred Chen[2]
Department of Chemical and Environmental Engineering, University of California, Riverside, California 92521

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Molecular beacons (MBs) are oligonucleotide probes that fluoresce upon hybridization. In this paper, we described the development of a real-time PCR assay to detect the presence of *Escherichia coli* O157:H7 using these fluorogenic reporter molecules. MBs were designed to recognize a 26-bp region of the *rfbE* gene, coding for an enzyme necessary for O-antigen biosynthesis. The specificity of the MB-based PCR assay was evaluated using various enterohemorrhagic (EHEC) and Shiga-like toxin-producing (STEC) *E. coli* strains as well as bacteria species that cross-react with the O157 antisera. All *E. coli* serotype O157 tested was positively identified while all other species, including the closely related O55 were not detected by the assay. Positive detection of *E. coli* O157:H7 was demonstrated when $>10^2$ CFU/ml was present in the samples. The capability of the assay to detect *E. coli* O157:H7 in raw milk and apple juice was demonstrated. As few as 1 CFU/ml was detected after 6 h of enrichment. These assays could be carried out entirely in sealed PCR tubes, enabling rapid and semiautomated detection of *E. coli* O157:H7 in food and environmental samples.

Key Words: **rapid microbial detection; pathogens; food.**

Enterohemorrhagic *Escherichia coli* (EHEC)[3] O157:H7 is an important pathogen that is predominantly associated with hemorrhagic colitis and the more severe complications of hemolytic uremic syndrome (1). The pathogenicity of EHEC O157:H7 is associated with a number of virulence factors, such as Shiga toxins 1 and 2 (2). Although it has been estimated that 200 different EHEC serotypes produce the Shiga-like toxin (SLTs), EHEC O157:H7 is implicated in the vast majority of outbreaks and sporadic cases of bloody diarrhea (3, 4).

With an incubation period as short as 3 h and an infectious dose as low as 1 to 10 colony-forming units (CFU), EHEC O157:H7 is characterized by a high rate of attack and person-to-person transmission, which enables both food-borne and water-borne outbreaks (5, 6). EHEC O157:H7 is acid resistant and can survive at pH as low as 2.5; recent outbreaks originating from acidic food (i.e., commercial apple juice) have been reported (7). As sporadic manifestations can rapidly turn into major outbreaks, the development of rapid, automated, yet sensitive and specific diagnostic assays for the detection of EHEC O157:H7 has become a major concern worldwide. Traditional culture methods based on biochemical characteristics are labor intensive and typically require up to 72 h before completion. Rapid detection techniques directed at immunological and genetic targets are therefore of great interest (8).

Immunological methods based on the detection of Shiga toxins have been developed. However, these methods cannot differentiate O157:H7 from other less virulent EHEC and some Shiga toxins may not be detected at all (9). Other methods based on detection of the O157 somatic and H7 flagellar antigens are also inadequate because of the lack of specificity (10).

PCR-based assays present the advantage of being readily optimized and various strategies have been assessed for the detection of EHEC *E. coli* O157:H7. While amplifications of the major virulent genes *stx1* and *stx2*, the highly conserved 5′ end of the *eaeA* gene

[1] Current address: Department of Chemical Engineering, University of Bath, Bath BA2 7AY, UK.
[2] To whom correspondence should be addressed. Fax: (909) 787-2425. E-mail: Wilfred@engr.ucr.edu.
[3] Abbreviations used: EHEC, enterohemorrhagic *Escherichia coli;* SLT, Shiga-like toxins; CFU, colony-forming units; MB, molecular beacon; CTAB, hexadecyltrimethylammonium chloride; cpc, CTAB-phenol-chloroform; IGm, InstaGene matrix; SDS, sodium dodecyl sulfate; DABCYL, 4-(4′-dimethylaminophenylazo)benzoic acid; STEC, Shiga toxin-producing *E. coli*.

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or its more specific 3′ end (11, 12), or the plasmid-encoded hly_{933} gene (13) have been used to indicate the potential presence of pathogenic *E. coli*, these approaches did not lead to the identification of EHEC O157:H7 from non-O157 serotype. Recently, the *rfbE* gene coding for production of the lipopolysaccharide O side chain of *E. coli* O157:H7 has been elucidated (14, 15) and exploited to specifically detect the presence of the *E. coli* O157:H7 by PCR (16–18).

Recent development of fluorogenic nucleic acid probes, such as the molecular beacon (19), confers a new dimension to PCR for which results have become quantitative and available in a real-time manner. During the real-time PCR assay the target gene is amplified and simultaneously recognized and monitored by the fluorescent probe moiety. Highly reliable, real-time PCR gains all the advantages of a regular PCR (i.e., sensitivity, rapid, automated) in addition to a supplementary level of specificity; allele discrimination has been demonstrated (20). As the reaction is homogeneous, the risks of cross-contamination are minimized and downstream analyses are eliminated.

Molecular beacon (MB) possesses differential fluorescent properties based on the relative stability between its two duplex forms (hairpin and the probe-target hybrid). In the hairpin conformation, the fluorescent reporter is immediately adjacent to the quencher, so that the intensity of the background signal is minimized. When the probe encounters a single-strand target, it forms a hybrid with the target, undergoing a spontaneous conformation change that forces the arm sequences apart and causes fluorescence to occur. Perhaps the biggest advantage of molecular beacons is their extraordinary specificity. No increase in fluorescence is observed even in the presence of a target strand that contains only a single nucleotide mismatch (19).

Using the newly introduced MB concept, we developed a fluorogenic, real-time PCR assay for the detection of EHEC O157:H7 based on the *rfbE* locus. We demonstrated that this real-time PCR assay could be used to specifically detect the presence of EHEC O157:H7 in contaminated milk and apple juice samples. The specificity and the sensitivity of the assay were described and compared to previous fluorogenic PCR assays designed for O157:H7 detection (21–23).

MATERIALS AND METHODS

Bacterial Strains, Medium, and Culture Conditions

Bacterial strains evaluated in this paper are listed in Tables 1 and 2. *E. coli* strain O157:H7 ATCC 43895 obtained from Dr. W. Yanko (LA Sanitation District, Whittier, CA) was used as the reference strain in all optimization and sensitivity experiments. Bacterial strains were cultivated at 37°C at 300 rpm in modified EC medium (Difco Laboratories, Detroit, MI). Twenty mg/L of novobiocin (mEC+n) was added to enrichment culture of milk and apple juice samples. Prior to DNA extraction, 100 μl of 10-fold serial dilutions of freshly grown cells was spread on an mEC+n plate for colony enumeration.

DNA Preparation

Two methods were used for the preparation of chromosomal DNA. The CTAB (hexadecyltrimethylammonium chloride) phenol-chloroform (cpc) method (1) and the direct purification of boiled cell lysate by the InstaGene matrix (IGm) kit (Bio-Rad Laboratories, Hercules, CA). The cpc method was performed as follows: 1.5 ml of overnight grown bacteria was spun at 1400 rpm for 2 min and resuspended in 600 μl TE buffer (pH 8.0) containing 1% SDS and 1 μg/ml of proteinase K. Cells were allowed to lyse for 1 h at 37°C. One hundred microliters of 5 M NaCl was added to adjust the salt concentration to 0.5 M. The solution was subjected to three cycles of freezing (−70°C) and thawing (65°C), followed by the addition of 80 μl of 10% CTAB in 0.7 M NaCl solution (1% final) and incubation at 65°C for 10 min. After the first extraction with 800 μl of phenol: chloroform:isoamyl alcohol (25:24:1), the solution was briefly vortexed and spun at 12,000*g* for 15 min. The supernatant was transferred to a clean tube and subjected to a second chloroform:isoamyl alcohol (24:1) extraction. Chromosomal DNA was precipitated by adding 400 μl of isopropanol. The pellet was washed with 500 μl of iced 70% ethanol, dried, resuspended in 20 μl TE buffer, and stored at −20°C. Samples used for all experiments were extracted using the cpc method unless stated otherwise.

The principle of the InstaGene matrix kit is based on the removal of divalent cations that interfere with the *Taq* polymerase. Two hundred microliters of overnight grown bacteria was boiled for 10 min prior to the addition of 200 μl of IGm. DNA was recovered using the supplier's instructions. Samples were kept at −20°C to avoid rapid degradation. With the IGm method, DNA was ready for PCR in approximately 1 h.

Target Gene, PCR Primers, and Molecular Beacon

The first set of primers (O157AF 5′-AAGATTGCGCTGAAGCCTTTG-3′ and O157AR 5′-CATTGGCATCGTGTGGACAG-3′) was designed to amplify a 497-bp fragment of the *rfbE* gene as previously described (12). A second set of primers (O157BF 5′-AAATATAAAGGTAAATATGTGGGAACATTTGG-3′ and O157BR 5′-TGGCCTTTAAAATGTAAACAACGGTCAT-3′) was designed to amplify a smaller 149-bp fragment of the same *rfbE* region. The PCR primer set B and the probe moiety of the beacon target the gene

coding for the O157 antigen were designed based on the published sequence data for the *rfbE* gene (Accession Number S83460) of *E. coli* strain O157:H7 86-24 (3).

MB (0157rfbe) 5′-FAM-CGCTATGGTGAAGGTG-GAATGGTTGTCACGAATAGCG-3′-DABCYL (4-(4′-dimethylaminophenylazo)benzoic acid) was purchased from Midland Certified Reagent Company (Midland, TX). The 25-bp probe moiety was designed to perfectly match the *rfbE* gene of *E. coli* O157:H7 strain 86-24 and was flanked by two 6-bp arms. Fluorescein (6-FAM) was chosen as the chromophore (excitation at 488 nm and emission at 518 nm) and DABCYL was used as the quencher. Beacons were resuspended in TE buffer, stored at −20°C, and protected from light.

Thermal Denaturation Profiles

The thermal denaturing profile and signal-to-background ratio (*S/B*) of the molecular beacon were investigated to determine the optimal recording temperature of the fluorescent "probe-target" hybrid for real-time PCR. The changes in fluorescence of a 50-μl solution containing varying amount of $MgCl_2$ and 0.3 μM beacon with or without 0.9 μM of a perfectly complementary single-stranded oligo were measured. Solutions were heated in the ABI Prism 7700 sequence detector System (Perkin-Elmer, Foster City, CA) at 95°C for 10 min and gradually decreased to 10°C. The fluorescence intensity was recorded for each temperature increment. The (*S/B*) ratio was then determined using the following relation,

$$(F_{\text{probe-target}} - F_{\text{buffer}})/(F_{\text{hairpin}} - F_{\text{buffer}}),$$

where $F_{\text{probe-target}}$, F_{buffer}, F_{hairpin} are the fluorescence of the probe-target hybrid, the buffer, and the hairpin structure, respectively.

PCR Conditions

For each PCR, 2.5 μl of template DNA was added to 27.5 μl of PCR master mix (3.5 mM $MgCl_2$, 0.5 μM of each primers, 0.2 μM dNTPs mix, 1 μM MB, 5 U AmpliTaq Gold DNA polymerase, 2.5 μl TaqMan buffer A, and the rest water). Both the polymerase and the amplifying buffer A were purchased as part of the TaqMan PCR core reagent kit (PE Biosystems, Foster City, CA). All reactions were performed in the 200-μl MicroAmp optical tubes sealed with the MicroAmp optical caps (PE Biosystems). The Perkin-Elmer ABI Prism 7700 sequence detection system was used for real-time analyses.

For the PCR reactions, samples were heated at 95°C for 10 min, followed by 40 cycles of melting at 94°C for 45 s, fluorescent measurement at 41°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 45 s. At the end of each PCR run, data were automatically analyzed by the system and amplification plots were obtained. The threshold (C_t) cycle of each amplification reaction was calculated based on the first PCR cycle at which the fluorescence was 10-fold higher than the standard deviation of the mean baseline emission.

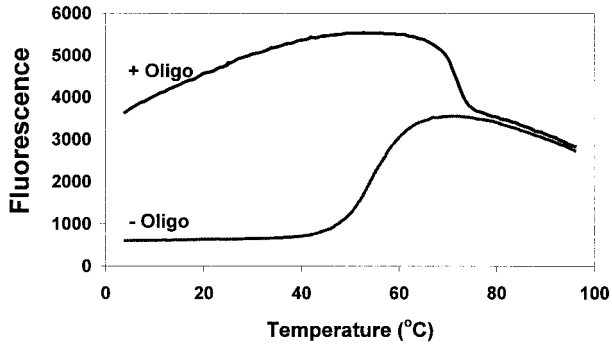

FIG. 1. Thermal denaturing profiles. The fluorescence intensity of the molecular beacon in the presence or absence of an excess amount of complementary oligo was monitored at different temperatures. The temperature range that provided the highest signal to background ratio was used as the annealing temperatures for the PCR.

Detection of E. coli O157:H7 in Milk and Apple Juice Samples

Unfiltered, pasteurized apple juice with no preservative, no sweeteners, and no artificial color (pH 3.73) and nonhomogenized organic whole milk, grade A pasteurized, processed in accordance with the California Organic Food Act (1990) (pH 6.71), were used. One hundred microliters of freshly grown and enumerated culture of *E. coli* O157:H7 ATCC 43895 were used to inoculate 1 ml of apple juice or raw milk samples. Inoculated samples were then added to the enrichment medium to 10% (v/v). The pH of mEC medium was adjusted to 7.15 and 20 mg/L of novobiocin was added. Five milliliters of this mixture was distributed into enrichment tubes and cultured at 37°C and 300 rpm for varying times. The enrichment was stopped by centrifuging the entire culture volume at 3500*g* for 10 min at 4°C and the pellet was resuspended in 250 μl of 9% NaCl solution. Concentrated bacterial suspension was transferred in a 1-ml Eppendorf tube and boiled for 10 min, and 100 μl of IGm matrix was added to the DNA preparation. DNA samples were stored at −20°C until use in PCR.

RESULTS

Thermal Denaturation Profiles

To determine the optimal recording temperature for the real-time PCR assay, the thermal denaturing pro-

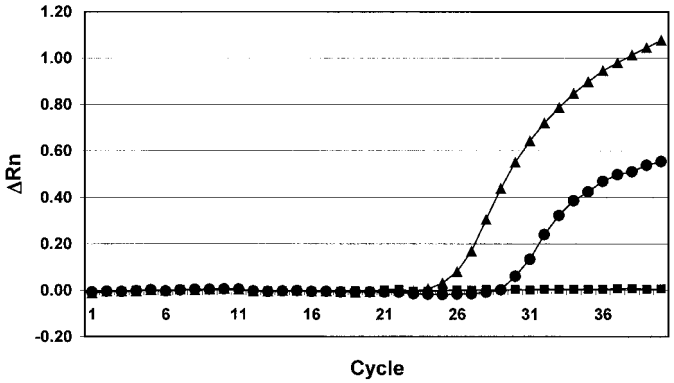

FIG. 2. Effect of amplicon size on the sensitivity of the real-time PCR assay. DNA extracted from *E. coli* O157:H7 ATCC 43985 was amplified with two different sets of primers, which produced amplicons of 149 and 496 bp, respectively. (■) No template control; (▲) 149-bp amplicon; (●) 496-bp amplicon.

files of the beacon in the presence or absence of a perfectly complementary oligo were investigated using a PE/ABI 7700 fluorescence reader. Figure 1 shows the fluorescence variation as a function of temperature. MB O157rfbe remained as a dark hairpin at temperatures below 40°C while the probe-target hybrid emitted high intensity of fluorescence. The maximum signal-to-background ratio was recorded at around 40°C. As the temperature increased from 40 to 73°C, the stem gradually melted and the signal-to-background ratio decreased dramatically. At 73°C, the probe-target hybrid denatured as indicated by a drop in the fluorescence. This result indicates that the optimal recording temperature window of MB O157rfbe should be at a temperature lower than 43°C.

Development of Real-Time PCR Assays

The ability of MB O157rfbe to detect *E. coli* O157:H7 in real-time PCR assays was investigated. DNA isolated from *E. coli* O157:H7 ATCC 43985 was used as the template. A 4-step PCR with a recording temperature at 41°C and an annealing step at 52°C was found to provide better sensitivity than a 3-step PCR with annealing and recording all at 45°C. Figure 2 shows the normalized fluorescent measurement vs the PCR cycle using the 4-step PCR. This plot clearly shows the progression of the amplification reaction. The real-time PCR assays were further optimized by monitoring the relative fluorescence intensity (ΔR_n) and the threshold cycle (C_t). We first adjusted the concentration of $MgCl_2$ to 3.5 mM, which provided the maximum signal-to-background ratio without drastically compromising the yield and specificity. Varying MB concentration from 125 pM to 1 μM resulted in a 3.5-fold increase in the ΔR_n value but had very little effect on the C_t value. More importantly, the length of the amplicon has a dramatic effect on the real-time PCR assay. Reducing the amplicon size from 479 to 146 bp reduced the C_t value by 3 and increased the ΔR_n value by 2-fold (Fig. 2). Optimal conditions were determined as stated under Materials and Methods.

Sensitivity of the Real-Time PCR Assay

The sensitivity of the real-time PCR assay was evaluated. Tenfold serial dilutions of template DNA isolated from *E. coli* O157:H7 strain ATCC 43985 were used for the assay (Fig. 3a). Any fluorescent signal that is 10-fold higher than the standard deviation of the mean baseline emission was indicative of a positive detection. Using this criterion, the detection limit of the real-time PCR assay was determined to be 5×10^3 CFU/ml, a value comparable to that reported using other fluorogenic PCR assays for *E. coli* O157:H7 (21, 22). Since the C_t value represents the PCR cycle at which the fluorescent intensity raises above the threshold, it can be used to quantify the input target concentration (Fig. 3b). This quantification proved to be linear over a wide range of initial target concentrations (from 10^3 to 10^8 CFU/ml).

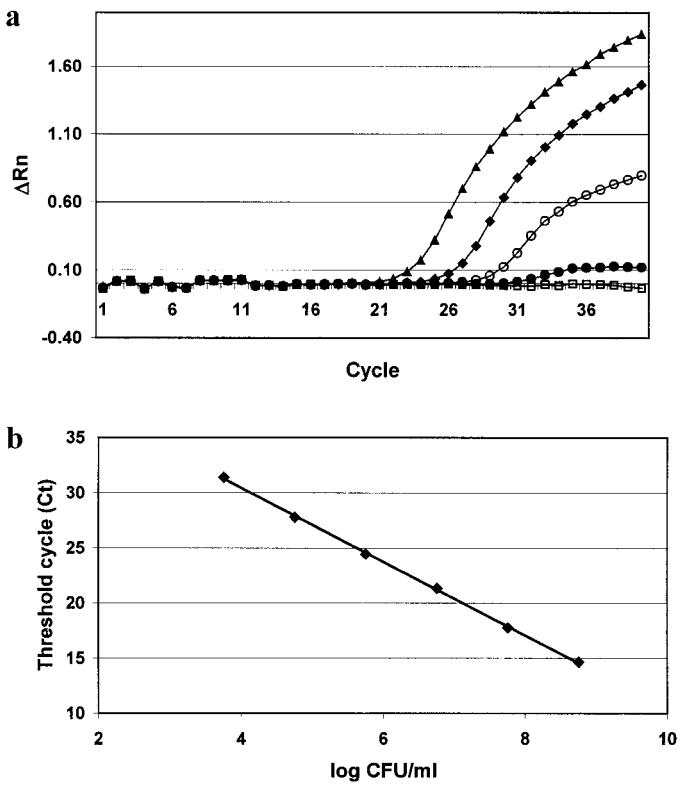

FIG. 3. Sensitivity (a) and standard curve (b) of the real-time PCR assay. Serial 10-fold dilutions of DNA template extracted from *E. coli* O157:H7 ATCC 43985 were used in each reaction. (□) No template control; (●) 5×10^3 CFU/ml; (○) 5×10^4 CFU/ml; (◆) 5×10^5 CFU/ml; (▲) 5×10^6 CFU/ml.

The method used to prepare the DNA template also influenced the sensitivity of the assay. When boiled-cell lysate was purified with IGm method, the C_t values obtained were reduced. Even 2×10^2 CFU/ml was detected but with less reliability. This detection limit is in line with the values previously published for the molecular detection of O157:H7 using a molecular beacon probe targeting the *stx2* gene (23).

Specificity of the Real-Time PCR

The specificity of the MB-based PCR assay relies on the selected sequence of the primer set and the probe moiety of the beacon, both of which were based on the *rfbE* gene of *E. coli* O157:H7 strain 86-24 (14). Genomic DNA recovered from 28 Shiga toxin-producing *E. coli* (STEC) strains and 7 other non-STEC strains was amplified to assess the ability of the real-time PCR assay to discriminate O157 serotype from others (Table 1). All of the 11 references strains of *E. coli* serotypes O157:H7 were positively recognized with the MB. Four non-H7 strains harboring the somatic O157 antigen were also positively detected. In contrast, the remaining non-O157 STEC strains did not emit fluorescence upon PCR amplification, regardless of their genetic profiles. This covered 11 different serovars including *E. coli* O55:H7, a serotype of EHEC that is genetically closely related to O157:H7. None of the non-STEC strains was detected by the real-time PCR assay.

Specificity of the MB was also tested against several bacteria that are known to cross-react with the O157 antisera, including *Escherichia hermanii, Citrobacter freundii, Vibrio cholerae, Yersinia enterocolitica,* and *Salmonella* group N. None of these bacteria produced a detectable fluorescent signal (Table 2). Although a similar *rfb* gene cluster has been cloned from *V. chloerae* and *Salmonella*, none of these bacteria was detected by the MB, indicating the specificity of the assay.

Detection of EHEC O157:H7 in Raw Milk and Apple Juice Samples

Application of the assay to detect *E. coli* O157 in food samples was assessed by using raw milk and apple juice seeded with known amount of *E. coli* O157:H7 cells and further enriched on selective mEc+n medium. In combination with the selective enrichment step, the real-time PCR assay could detect even 1 CFU/ml in raw milk only after a 6-h enrichment (Table 3). However, for apple juice samples, the sensitivity of detection was much reduced; a minimum of 11 h of enrichment was needed to achieve the same detection limit in raw milk (Table 3). This reduction in sensitivity is probably caused by the presence of polyphenolic compounds in apple juice that are inhibitory to the PCR (24). Addition of fining agents, such as BSA, slightly improved the PCR performance but did not fully resolved the inhibition.

TABLE 1

E. coli Serotypes Tested for the Specificity of the Molecular Beacon

Strain	Serotype	Genotype[a]	Detection[b]	Source[c]
E. coli ATCC 35150	O157:H7	*stx1, stx2*	+	ATCC
E. coli 4718	O157:H7	*stx1, stx2, eaeA*	+	1
E. coli 2725	O157:H7	*stx1, stx2, eaeA*	+	1
E. coli 2409	O157:H7	*stx1, eaeA*	+	1
E. coli 6363	O157:H7	*stx1*	+	2
E. coli ATCC 43895	O157:H7	Unknown	+	3
E. coli	O157:H7	Unknown	+	3
E. coli 1880	O157:H7	*eaeA*	+	4
E. coli 2977	O157:H7	*eaeA*	+	4
E. coli 5906	O157:H7	*eaeA, stx2*	+	4
E. coli B6-914	O157:H7	*eaeA*	+	4
E. coli 3048	O157:NM	*stx1, stx2, eaeA*	+	1
E. coli MF7123A	O157:NM	None	+	4
E. coli MF13180	O157:NM	*eaeA, stx1, stx2*	+	4
E. coli MF13180-25	O157:NM	*eaeA, stx1, stx2*	+	4
E. coli	O157:H$^-$	*stx1, stx2, eaeA*	+	4
E. coli DEC5A	O55:H7	Unknown	−	1
E. coli	O55:NM	*stx1*	−	4
E. coli 5710	O26:H11	*stx1, stx2, eaeA*	−	1
E. coli 13C60	O26:H11	*stx1*	−	2
E. coli 90.0105	O26:H11	*stx1*	−	4
E. coli 3128	O113	*stx1, stx2*	−	1
E. coli 3883	O111:NM	*stx1, eaeA*	−	1
E. coli 403	O111:NM	*stx1*	−	2
E. coli 13C62	O103:H2	*stx1*	−	2
E. coli 5702	O103:H2	*stx1*	−	1
E. coli 87.1468	O103:H2	*stx1*	−	4
E. coli 15597	Unknown	Unknown	−	3
E. coli Seff a	Unknown	Unknown	−	3
E. coli Seff b	Unknown	Unknown	−	3
E. coli FAMP	Unknown	Unknown	−	3
E. coli C	Unknown	Unknown	−	3
E. coli CN13	Unknown	Unknown	−	3
E. coli W3110	Unknown	Unknown	−	3

[a] The presence of the virulent genes were indicated by the donor.
[b] + or − indicates that the relative fluorescent intensity (ΔR_n value) after 40 cycles was greater than the threshold value. Each PCR was initiated with $\sim 10^7$ DNA molecules.
[c] 1, ARS, USDA, Ames, IA; 2, CFSAN, USFDA, Washington, DC; 3, LASCD, Whittier, CA; 4, ARS, USDA, Philadelphia, PA.

DISCUSSION

Because of the specificity and sensitivity, MB can be effectively incorporated into real-time PCR assays and provide a quick and accurate method for detection of specific nucleic acid sequences in homogeneous solutions (20). Reagents are mixed in one step and reactions are carried in closed tubes, thus preventing contamination. Data are recorded during each cycle and results are automatically analyzed immediately after the reaction is completed, usually within 2 h. Since the use of MB has previously been shown to detect very low

TABLE 2

Evaluation of Bacterial Strains That Cross-React with O157 Antisera

Bacteria	Cross-reaction	Detection[a]	Source[b]
E. hermannii strain 3390-90	+	−	1
E. hermannii strain 1000-80	+	−	1
E. hermannii strain 3018-85	−	−	1
E. hermannii strain 3607-89	−	−	1
C. freudii strain F90/2460-1	+	−	1
Salmonella strain 425-82	+	−	1
Salmonella strain 1552-82	+	−	1
Salmonella LT2	Unknown	−	2
V. cholerae Pacini O:1 ATCC 11623	+	−	ATCC
Yersinia enterocolitica ATCC 23715	Unknown	−	ATCC
Enterobacter cloacae ATCC 13040	Unknown	−	ATCC
Citrobacter frundii ATCC 8090	Unknown	−	ATCC

[a] + or − indicates that the relative fluorescent intensity (ΔR_n value) after 40 cycles was greater than the threshold value.

[b] 1, Dr. N. Strockbine, CDC, Atlanta, GA; 2, Dr. W. Yanko, LASCD, Whittier, CA.

levels of *Salmonella* species (25) and viruses (36), our goal in this paper was to develop a MB-based real-time PCR assay for the rapid, automated, sensitive, and specific detection of *E. coli* O157:H7.

The MB-based PCR assay provides the possibility of real-time quantitative detection of EHEC O157:H7 directly in the PCR tube. Our results demonstrated that the MB-based real-time PCR assay, when integrated with the IGm purification kit, is sensitive for the detection of EHEC O157:H7 to 2×10^2 CFU/ml. This detection level is very similar to that reported by a recent study using MB targeting the *stx2* gene (23). Because the threshold cycle (C_t) is inversely proportional to the logarithm of the initial number of target molecules, these data were used to formulate a standard quantification curve. The reported assay covers a wide dynamic range, with linear quantification up to 10^8 CFU/ml. One additional benefit of using the C_t values for quantification is that a much larger assay range is permitted than directly using total fluorescent emission, which has a dynamic range of approximately 1000.

Detection of EHEC O157:H7 using MB was also very specific. The *rfbE* gene, encoding for an enzyme necessary for O-antigen biosynthesis highly conserved among the *E. coli* O157 serovar, was used as the target for the real-time PCR assay (14). As demonstrated by our results, all *E. coli* O157:H7, O157:H$^-$, and O157:NM isolates tested were positively identified. No other *E. coli* serotype tested produced a positive result. Even two closely related *E. coli* O55:H7 and O55:NM strains, which have been falsely detected by other fluorogenic PCR assays, were not detected (21, 22).

Various immunological assays targeting the O-antigen also cross-react with bacterial species that share the same epitope as O157 (26, 27). False positive diagnostics were indeed reported for *C. freundii* (28), *Y. enterocolitica* O:9 (29), *Salmonella* group N (30), *V. cholerae* O1 (31), *E. hermannii* (32), and *E. coli* O7 and O116 (33). By targeting the unique *rfbE* gene sequence in the real-time PCR assay, none of the strains previously reported with cross-reactivity was positively detected, demonstrating that this is a more reliable molecular diagnostic assay than its serologic counterpart. This is due to the highly specific nature of MB, which has the capability to discriminate even a single base mismatch in the target sequence. Although similar *rfb* gene clusters have been cloned from *Salmonella*, *V. cholera*, and *Y. enterocolitica*, none of these species were detected by our assay.

Comparing to other fluorogenic PCR assays developed based on the detection of the *eaeA*, *stxI*, and *stx2* gene (21, 22), our approach is more specific. The *eaeA* gene is conserved among different STEC and could

TABLE 3

Detection of *Escherichia coli* O157:H7 in Raw Milk and Apple Juice Samples by Real-Time PCR Assays

	C_t values after enrichment[a]							
	Raw milk				Apple juice			
CFU/ml	4 h	6 h	8 h	10 h	8 h	11 h	14 h	17 h
3500	19.4	17.1	14.2	NT	14.3	NT	NT	NT
350	24.2	19.0	14.9	13.8	17.8	14.3	13.8	NT
35	27.9	21.4	16.1	13.9	ND	15.5	13.9	NT
3.5	ND	20.5	21.4	14.5	ND	15.8	14.7	13.9
0.35	ND	30.8	24.9	15.9	ND	ND	16.7	17.3
0.035	ND	30.2	25.8	NT	ND	ND	ND	ND

[a] ND, not detected even after 40 cycles; NT, not tested.

produce false-positive results with certain *E. coli* O55 and O111 serotypes (34). Similarly, SLT related genes are shared by many *E. coli* and non-*E. coli* STEC strains and do not provide serospecific discrimination. Although some SLT-producing, non-O157:H7 serotypes have been isolated from symptomatic patients, the implication of these serotypes in food-borne infections is not as well established (22).

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