GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

A duplex SYBR Green I real-time quantitative PCR assay for detecting *Escherichia coli* O157:H7

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Genet. Mol. Res. 12 (4): 4836-4845 (2013)
Received March 26, 2013
Accepted August 15, 2013
Published October 22, 2013
DOI http://dx.doi.org/10.4238/2013.October.22.3

ABSTRACT. PCR and hybridization assays are widely used for the detection and identification of *Escherichia coli* serogroups and serotypes. We used these techniques for the detection of *E. coli* O157:H7, a dominant serogroup among *E. coli* strains that are considered major public health problems worldwide. We developed a quantitative PCR assay using SYBR Green I, based on the published sequences of the *rfbE* and *fliC* genes from *E. coli* O157:H7. This method detected the *E. coli* O157:H7 O somatic antigen gene and the flagellar antigen gene simultaneously, with good specificity, sensitivity, and repeatability. The sensitivity of the assay was 2.95 x 10 copies/μL, which is 10^3 times more sensitive than obtained with a conventional PCR. The intra-assay and inter-assay coefficients of variation were less than 2%. We concluded that this duplex quantitative PCR assay is adequate for the identification and quantitative analysis of *E. coli* O157:H7. This provides a new identification method for clinical diagnosis of *E. coli* O157:H7 and for food safety analysis, as well as for molecular epidemiological studies of foodborne diseases.

Key words: Duplex; SYBR Green; Quantitative PCR; *Escherichia coli* O157:H7

INTRODUCTION

The bacterium *Escherichia coli* O157:H7 is a worldwide threat to public health and has been implicated in many outbreaks of hemorrhagic colitis. It was conclusively identified as a pathogen in 1982 following its association with 2 food-related outbreaks of an unusual gastrointestinal illness. The organism is now recognized as an important cause of foodborne disease, with outbreaks reported in the United States, Canada, United Kingdom, Japan, and so on. Illness is generally quite severe, and can include 3 different syndromes, i.e., hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (Orskov and Orskov, 1992; Nataro and Kaper, 1998; Paton and Paton, 1998b; Perna et al., 2001; Corrigan Jr. and Boineau, 2001). Most outbreaks have been associated with eating undercooked ground beef, unpasteurized milk and dairy products, vegetables or water, and contact with animal carriers or the environment (Gyles, 2007; Sánchez et al., 2010). In 1986, the organism was found in China and outbreaks of *E. coli* O157:H7 infections occurred in Anhui, Jiangsu, Henan, and Hubei Provinces of China from 1999 to 2000, in total, 20,000 cases were reported, including 177 deaths (Li et al., 2002; Zhang et al., 2007). Obviously, the disease has become a serious public health problem threatening people's welfare, and *E. coli* O157:H7 has a low infectious dose, with the average infectious dose being estimated at fewer than 50 organisms (Paton and Paton, 1998a; Chen et al., 2006; Sun, 2007; Sun et al., 2008), so it is very important to detect low levels of *E. coli* O157:H7.

Various diagnostic methods have been used for the identification of *E. coli* O157:H7, including phenotypic characterization, multiplex PCR, immunochromato-graphic strip, quartz crystal microbalance (QCM) piezoelectric immunobiosensor, and so on (Gannon et al., 1997; Paton and Paton, 1998a; Wang et al., 2007; Sun et al., 2008). However, these methods are not suitable for direct, quantitative detection of the organism in samples. Real-time PCR is a new method that allows the quantification of the target, and when combined with a rapid cycling platform, results can be generated in 30 min from the start of thermal cycling. Its simplicity, specificity, and sensitivity, together with its potential for high throughput and the ongoing introduction of new chemistries, more reliable instrumentation, and improved protocols, have made real-time PCR the benchmark technology for the detection of pathogenic organisms (Uhl et al., 2002).

The aim of the present study was the development of a duplex quantitative PCR (qPCR) assay using SYBR Green chemistry, which could not only quantify *E. coli* O157:H7 genomes in different samples, comparing its sensitivity with that of the conventional PCR, but also detect the *E. coli* O157:H7 O somatic antigen gene and the flagellar antigen gene simultaneously.

MATERIAL AND METHODS

Bacterial strains

A total of 34 strains were used in this study, including 7 *E. coli* O157:H7 and 27 other strains. The reference strain *E. coli* O157:H7 (CVCC248） was purchased from the China Control Institute of Veterinary Drug (CIVD). The other 6 *Escherichia coli* O157:H7 strains were isolated from domestic and free-ranging wild ruminants from Henan, Gansu, and Sichuan Provinces of China. Three strains of *E. coli* O157:H?, 6 strains of non-O157 *E. coli*, 1 strain of *Shigella flexneri*, and 1 strain of *Citrobacter* were included for comparison because they are closely related to *E. coli* O157:H7. In addition, 16 related strains representing the

families Enterobacteriaceae, Enterococcaceae, Streptococcaceae, and Pasteurellaceae, which could represent a differential diagnostic problem, were used for specificity checking (Table 1).

Table 1. Essential information of bacterial strains in the present study.

No.	Strain	Taxon	Origin	Host	Country
1	CVCC248	*Escherichia coli* O157:H7	Feces	Unknown	China
2	GSLT09	*E. coli* O157:H7	Feces	Camel	China
3	HNWH09	*E. coli* O157:H7	Feces	Cow	China
4	HNZZ09	*E. coli* O157:H7	Feces	Cow	China
5	HNDF11	*E. coli* O157:H7	Feces	Goat	China
6	GSLZ09	*E. coli* O157:H7	Feces	Yak	China
7	HNZZJ10	*E. coli* O157:H7	Feces	Chicken	China
8	HNZM11-1	*E. coli* O157:H?	Feces	Goat	China
9	HNZM11-2	*E. coli* O157:H?	Feces	Goat	China
10	HNZM11-3	*E. coli* O157:H?	Feces	Goat	China
11	XXHJ-1	*Streptococcus* spp	Palatine tonsil	Pig	China
12	XXHJ-2	*Streptococcus* spp	Palatine tonsil	Pig	China
13	XXHJ-3	*Streptococcus* spp	Palatine tonsil	Pig	China
14	XXHJ-4	*Streptococcus* spp	Palatine tonsil	Pig	China
15	XXHJ-5	*Streptococcus* spp	Palatine tonsil	Pig	China
16	XXHJ-6	*Streptococcus* spp	Palatine tonsil	Pig	China
17	S735	*Streptococcus suis* type 2	Palatine tonsil	Pig	China
18	SH28	*S. suis* type 1	Palatine tonsil	Pig	China
19	Y15	non-O157 *E. coli*	Feces	Goat	China
20	Y18	non-O157 *E. coli*	Feces	Goat	China
21	ZK10	non-O157 *E. coli*	Feces	Cow	China
22	HJ	non-O157 *E. coli*	Feces	Chicken	China
23	RZ	non-O157*E. coli*	Feces	Chicken	China
24	SQ09	non-O157 *E. coli*	Feces	Beef cattle	China
25	CVCC464	*Pasteurella multocida*	Unknown	Duck	China
26	CVCC458	*P. multocida*	Unknown	Chicken	China
27	ZD01	*Shigella flexneri*	Feces	Human	China
28	EYu-NY	*Enterococcus faecalis*	Feces	Pig	China
29	LH10	*Salmonella* spp	Liver	Chicken	China
30	CVCC 533	*S. pullorum*	Heart-blood	Chicken	China
31	C79	*S. pullorum*	Liver	Chicken	China
32	HF1	*Proteus mirabilis*	Liver	Chicken	China
33	HF2	*P. mirabilis*	Liver	Chicken	China
34	HY07	*Citrobacter*	Unknown	Unknown	China

All strains were stored in nutrient broth (Difco Laboratories, Detroit, MI, USA) containing 10% sterile glycerol at 70°C. Cultures were grown overnight (18 h) in Luria broth (Difco) at 37°C.

DNA preparation

Genomic DNA was extracted from bacterial strains using a boiling method. Briefly, l mL overnight bacterial culture was pelleted by centrifugation at 12,000 rpm for 5 min and resuspended in 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The suspension was placed in a boiling water bath for 5 min, placed in an ice bath for 2 min, and then centrifuged at 12,000 rpm for 5 min. The supernatants were stored at -20°C until they were used for the qPCR test.

qPCR

PCR primers were designed based on the *rfbE* (S83460) and *fliC* (NC_002655) gene sequences of *E. coli* O157:H7 (Table 2). To construct a standard curve, as well as to ascertain

the possible detection limits, traditional PCR was performed with the same primer pair. PCR products of *E. coli* O157:H7 were purified and cloned into the pMD 18-T vector (Takara) via TA cloning, according to manufacturer instructions. The copy number of the recombinant plasmids (pMD 18-T-*rfbE* and pMD 18-T-*fliC*) were adjusted to 2.95 x 10^{11} copies/μL as measured by a micro-ultraviolet/visible range spectrophotometer, respectively (NanoDrop ND1000). The recombinant plasmids were serially diluted 10-fold to 2.95 x 10^{10} copies/μL. Six dilutions, from 2.95 x 10^{8} to 2.95 x 10^{3} copies/μL, were used as standards to optimize the qPCR and to establish the standard curve.

Table 2. Target genes and primer sequences of the fluorescent quantitative PCR.

Genes	Sequence of primers	Size of product (bp)
rfbE	F: CTACAGGTGAAGGTGGAATGGT (22 bp) R: GTAGCCTATAACGTCATGCCAAT (23 bp)	119
fliC	F: CAAGTTGCCTGCATCGTCTA (20 bp) R: TCAGCTTCAAAACGTGATGC (20 bp)	168

The qPCR mixture included 10 μL SYBR Premix Ex Taq (TaKaRa Biotech), 0.4 μL of each primer (25 μM), and 2 μL of each plasmid template in a total volume of 20 μL. The qPCR program, which was run on a LightCycler 1.5 (Roche), consisted of 1 min of Taq polymerase activation at 95°C, followed by 40 cycles of 95°C for 4 s (denaturation), 55°C for 5 s (annealing), and 72°C for 12 s (extension), followed by a melting curve analysis. Each PCR was performed at least twice. Each PCR run included a positive control and no template control used as negative control to check for reagent contamination. After 40 amplification cycles, a melting analysis was carried out to verify the correct product by its specific melting temperature (Tm). The thermal profile for the melting curve analysis consisted of a denaturation for 0 min at 95°C, lowered to 61°C for 1 min and then increased to 95°C with continuous fluorescence readings. The standard curve and melting curve were established using the LightCycler software version 3.5.

Specificity, sensitivity, and reproducibility of qPCR

Thirty-four different bacterial strains (Table 1) and 1 control sample (deionized water) were utilized to assess the specificity of the qPCR assay. The qPCR detection limit was measured using 2.95 x 10^{0} to 2.95 x 10^{10} copies/μL of standard recombinant plasmid and was compared to traditional PCR using the same primer pair.

To evaluate the intra-assay variability, 3 different known concentrations of standard plasmid (2.95 x 10^{6}, 2.95 x 10^{7} and 2.95 x 10^{8}) were amplified by performing the assay described above in triplicate. To evaluate the inter-assay variability, these plasmids were amplified again 2, 4, and 6 days later. For each experiment, the crossing point and coefficient of variation for each assay was calculated.

RESULTS

Optimization of multiplex real-time PCR SYBR Green

Primer pairs were designed to obtain distinguishable Tm for *rfbE* and *fliC*. These

primers were highly specific for *rfbE* and *fliC* once submitted to *in silico* analysis by BLAST (data not shown). Traditional PCR produced the expected fragment of 119 bp (*rfbE*) and 168 bp (*fliC*). Sequence analysis showed that they were respectively identical to *E. coli* O157:H7 (S83460) and *E. coli* O157:H7 (NC_002655). Simplex real-time PCR SYBR Green amplification of DNA from *E. coli* O157:H7 strain CVCC248 yielded the same PCR products, respectively, and produced dissociation curves at Tm of 79.60°C (for *rfbE*) and 83.15°C (for *fliC*), The combination of the designed primer pairs in one duplex SYBR Green I real-time PCR produced, for the same templates, dissociation curves at Tm of 79.65°C and 83.20°C (Figure 1), using 10 µL SYBR Premix Ex Taq (TaKaRa Biotech), 10 pmol each primer, and thermal profile recommended by Taq polymerase manufacturer.

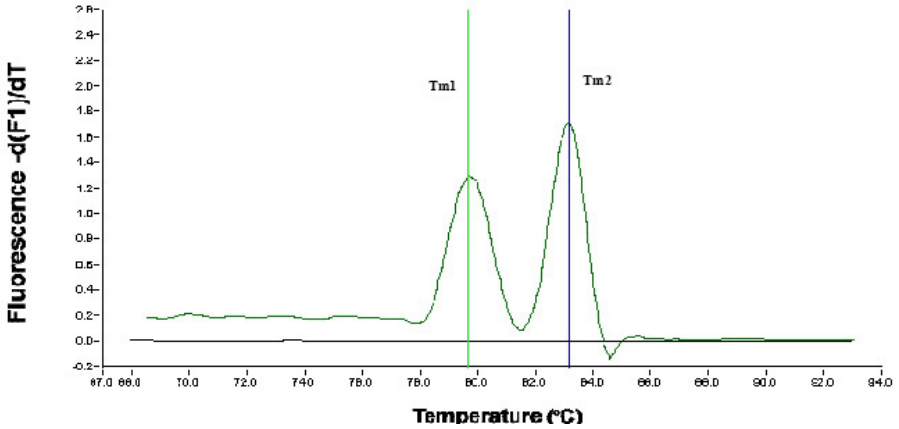

Figure 1. Melting curve of the SYBR Green I real-time PCR. Melting temperature: Tm1 = Tm for *rfbE*; Tm2 = Tm for *fliC*.

Performance parameters of multiplex real-time PCR SYBR Green

Serial dilutions of DNA from reference strains were used to test the response linearity and LOD of the method. The qPCR standard curve was plotted over a range of 2.95 x 10^8 to 2.95 x 10^3 copies/µL for both pMD 18-T-*rfbE* and pMD 18-T-*fliC* (Figure 2). The slope, intercept, and correlation coefficient were -3.352, 35.37, and 1.000, respectively. The efficiency was 98.76% according to the formula $E = 10^{-1/slope} - 1$.

The specificity test showed that all DNA samples from 7 *E. coli* O157:H7 strains produced the expected positive results, whereas no positive result was detected when using templates from 27 other strains (Figure 3). Although there was an amplification signal from *E. coli* O157:H? strains, the melting curve analysis showed that the Tm value of the products from *E. coli* O157:H? was only 79.65°C, but that from *E. coli* O157:H7 was 79.65°C and 83.20°C simultaneously (Figure 3). Other nonspecific amplifications and presence of primer dimers were not observed during the selectivity study. Thus, the specificity test showed 100% inclusivity and 100% exclusivity.

The detection limit of qPCR and traditional PCR was 2.95 x 10^1 copies/µL (Figures 4 and 5) and 2.95 x 10^4 copies/µL (Figure 6), respectively. The qPCR assay was 10^3 times higher compared to conventional PCR.

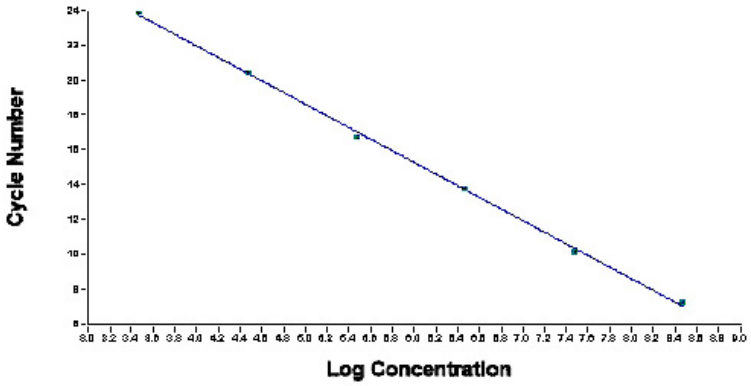

Figure 2. Standard curve of the SYBR Green I real-time PCR.

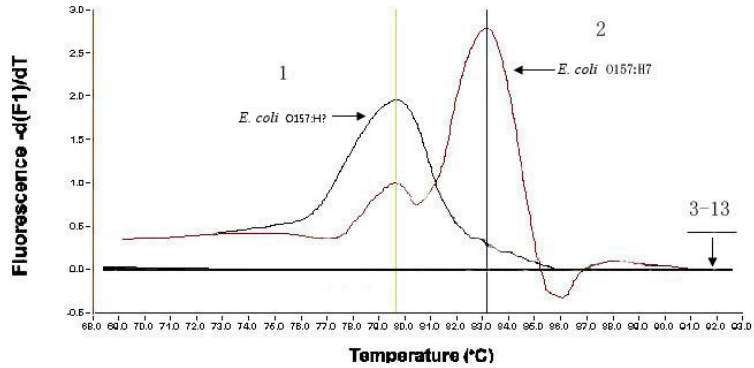

Figure 3. Specificity test of the SYBR Green I real-time PCR. *Lanes 1* to *13* = *Escherichia coli* O157:H? HNZM11-3, *E. coli* O157:H7 CVCC248, *Streptococcus* spp XXHJ-1, *S. suis* type 1 SH28, *S. suis* type 2 S735, non-O157 *E. coli Y15*, *Pasteurella multocida* CVCC464, *Shigella flexneri* ZD01, *Enterococcus faecalis E.Yu-NY*, *Salmonella pullorum* CVCC 533, *Proteus mirabilis* HF1, *Citrobacter* HY07, blank control, respectively.

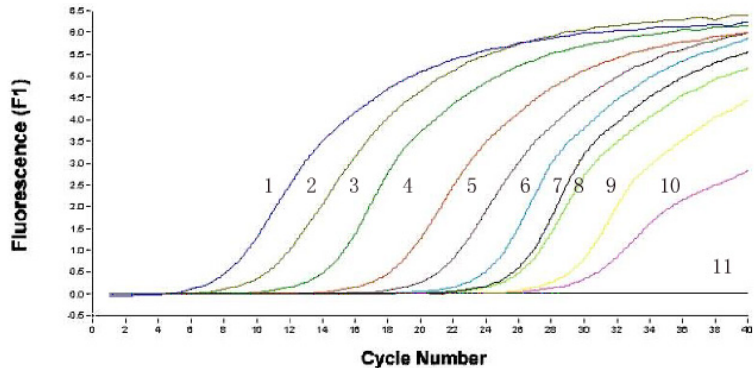

Figure 4. Amplification curves for detecting sensitivity by qPCR. *Lanes 1* to *10* = standard samples ranging from 2.95 x 10^{10} to 2.95 x 10^{1} copies/µL; *lane 11* = standard samples of 2.95 x 10^{0} copies/µL and blank control.

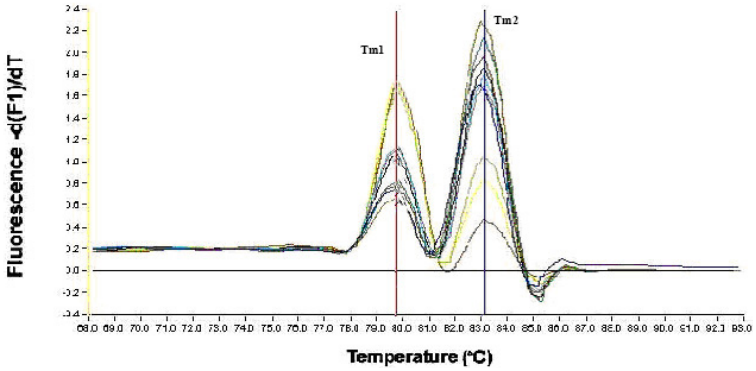

Figure 5. Melting curve for detecting sensitivity by qPCR. Melting temperature: Tm1 = Tm for *rfbE*; Tm2 = Tm for *fliC*.

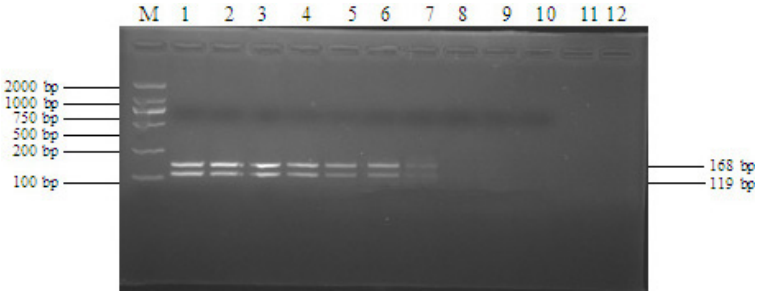

Figure 6. Sensitivity test of the amplification of common PCR. *Lane M* = DL2000 maker; *lanes 1* to *11* = mixture template diluted 2.95 x 10^{10} to 2.95 x 10^{0} copies/μL, respectively; *lane 12* = negative control.

Repeatability of the method was evaluated by testing 3 different concentrations of standard plasmids at different times, The intra- and inter-assay coefficients of variation were both less than 2.0% (Figure 7, Table 3).

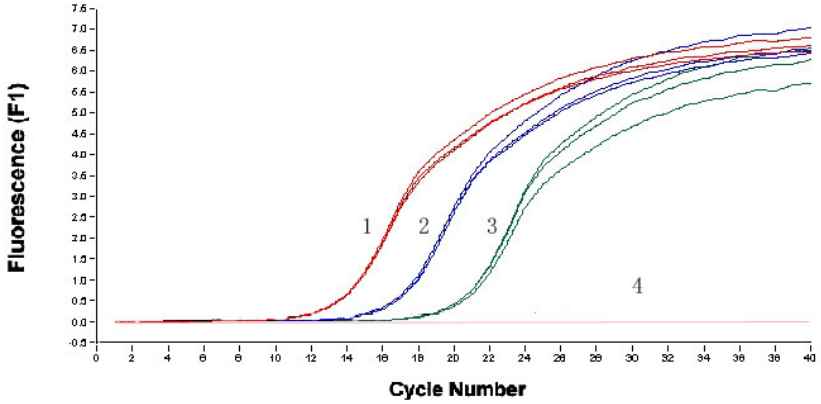

Figure 7. Reproducibility test of the SYBR Green I real-time PCR assay. *Lanes 1* to *3* = standard samples of 2.95 x 10^{8}, 2.95 x 10^{7} and 2.95 x 10^{6} copies/μL, respectively; *lane 4* = negative control.

Table 3. Reproducibility of intra- and inter-assay by SYBR Green I real-time PCR (N = 3).

Concentration of standard plasmid (copies/µL)	Intra-assay Ct value					Inter-assay Ct value				
	1	2	3	Mean ± SD	CV%	1	2	3	Mean ± SD	CV%
2.95 x 10^6	13.20	13.21	13.22	13.21 ± 0.01	0.08%	14.35	14.46	14.24	14.35 ± 0.09	0.63%
2.95 x 10^5	16.51	16.52	16.54	16.52 ± 0.01	0.06%	17.60	17.80	17.24	17.55 ± 0.23	1.31%
2.95 x 10^4	20.00	20.03	20.06	20.03 ± 0.03	0.15%	21.14	21.54	21.24	21.31 ± 0.17	0.80%

DISCUSSION

E. coli O157:H7 infection is a foodborne disease, and accumulating evidence indicates that it is related to consuming undercooked ground meat, unpasteurized milk and dairy products, fruit, vegetables, and juices, etc. Also, it is a worldwide problem for both public health and food safety. Therefore, it is absolutely necessary to develop a simple, rapid, sensitive, specific, inexpensive, and reproducible method to detect the pathogen, which is as important means to ensure food safety and prevent *E. coli* O157:H7 infection. The traditional methods for *E. coli* O157:H7 detection have limitations, such as time-consuming, lack of specificity and various interferences. Therefore, methods for rapid and specific detection are needed. PCR has been used to detect *E. coli* O157:H7. A number of *E. coli* O157:H7 genes have been targeted for diagnostic amplification by PCR, including those encoding the Shiga toxins (*stx1* and *stx2*), *eaeA*, *hlyA*, *fliC* (Gannon et al., 1997; Paton and Paton, 1998a; Osek, 2002; Sharma, 2002, 2006). However, quantification with PCR is still lacking in precision and further research is needed. Real-time PCR allows the quantification of the target, and when combined with a rapid cycling platform, results can be generated in 30 min from the start of thermal cycling. Because of the advantages of real-time PCR, many assays that perform better than the standard culture-based assays have been developed to detect pathogenic organisms (Uhl et al., 2002). At present, the most popular real-time PCR assay is based on the hybridization of a dual-labeled probe to the PCR product, and the development of a signal by loss of fluorescence quenching as PCR degrades the probe. Although this so-called "TaqMan" approach has proved to be easy to optimize in practice, the dual-labeled probes are relatively expensive (Maeda et al., 2003; Gubala, 2006; Kenar et al., 2007). In this study, the SYBR-Green I real-time PCR assay was successfully developed, which was based on the binding of the fluorescent dye SYBR Green I to the PCR product (Giglio et al., 2003; Gibellini et al., 2006). The qPCR assay could detect *E. coli* O157:H7 in various types of specimens, with a lower detection limit of 2.95 x 10^1 DNA copies/µL. Thus, qPCR is more sensitive than traditional PCR (2.95 x 10^4 copies/µL). Of the 34 samples from different genera of bacteria, only the 7 known *E. coli* O157:H7 samples produced the expected positive results using this assay. Moreover, the qPCR assay also showed excellent reproducibility, as demonstrated by the relatively fixed Ct value produced by the same concentration of plasmid template, and by the intra- and inter-assay coefficients of variation (both <2.0%). In addition, the results showed that the regression coefficient was -1.00 and the PCR amplification efficiency was 98.76%. These results further confirmed the usefulness of this method.

In summary, a rapid, sensitive, specific, and reproducible duplex SYBR Green I real-time PCR method was developed and in-house validated, which could detect the *E. coli* O157:H7 O somatic antigen gene and the flagellar antigen gene simultaneously. Considering the perfor-

mance parameters and the low cost of this SYBR Green approach, the reported method may be considered a suitable tool for laboratory diagnosis of *E. coli* O157:H7 and food safety determination. In particular, it represents an inexpensive method for testing a large number of samples, collected especially during the molecular epidemiological study of foodborne diseases.

ACKNOWLEDGMENTS

Research supported by the Henan Province Major Commonwealth Project (grant #81100912300). We thank Mr. Yangke Liu and Dr. Heping Huangfu for their skillful technical assistance.

REFERENCES

Chen S, Huang KL, Xu WT, Li Y, et al. (2006). Real-time quantitative PCR detection of *Escherichia coli* O157:H7. *J. Agric. Biotechnol.* 14: 779-782.

Corrigan Jr JJ and Boineau FG (2001). Hemolytic-uremic syndrome. *Pediatr. Rev.* 22: 365-369.

Gannon VP, D'Souza S, Graham T, King RK, et al. (1997). Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. *J. Clin. Microbiol.* 35: 656-662.

Gibellini D, Gardini F, Vitone F, Schiavone P, et al. (2006). Simultaneous detection of HCV and HIV-1 by SYBR Green real time multiplex RT-PCR technique in plasma samples. *Mol. Cell Probes* 20: 223-229.

Giglio S, Monis PT and Saint CP (2003). Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR. *Nucleic Acids Res.* 31: e136.

Gubala AJ (2006). Multiplex real-time PCR detection of Vibrio cholerae. *J. Microbiol. Methods* 65: 278-293.

Gyles CL (2007). Shiga toxin-producing *Escherichia coli*: an overview. *J. Anim. Sci.* 85: E45-E62.

Kenar L, Ortatatli M, Karayilanoglu T, Yaren H, et al. (2007). Comparison of real-time polymerase chain reaction and conventional polymerase chain reaction methods for the rapid identification of *Bacillus anthracis*. *Mil. Med.* 172: 773-776.

Li H, Jing H, Pang B, Zhao G, et al. (2002). Study on diarrhea disease caused by enterohemorrhagic *Escherichia coli* O157:H7 in Xuzhou city, Jiangsu province in 2000. *Zhonghua Liu Xing. Bing. Xue. Za Zhi.* 23: 119-122.

Maeda H, Fujimoto C, Haruki Y, Maeda T, et al. (2003). Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, tetQ gene and total bacteria. *FEMS Immunol. Med. Microbiol.* 39: 81-86.

Nataro JP and Kaper JB (1998). Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* 11: 142-201.

Orskov F and Orskov I (1992). *Escherichia coli* serotyping and disease in man and animals. *Can. J. Microbiol.* 38: 699-704.

Osek J (2002). Rapid and specific identification of Shiga toxin-producing *Escherichia coli* in faeces by multiplex PCR. *Lett. Appl. Microbiol.* 34: 304-310.

Paton AW and Paton JC (1998a). Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic *E. coli* hlyA, rfbO111, and rfbO157. *J. Clin. Microbiol.* 36: 598-602.

Paton JC and Paton AW (1998b). Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin. Microbiol. Rev.* 11: 450-479.

Perna NT, Plunkett G, III, Burland V, Mau B, et al. (2001). Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* 409: 529-533.

Sánchez S, Martinez R, Rey J, Garcia A, et al. (2010). Pheno-genotypic characterisation of *Escherichia coli* O157:H7 isolates from domestic and wild ruminants. *Vet. Microbiol.* 142: 445-449.

Sharma VK (2002). Detection and quantitation of enterohemorrhagic *Escherichia coli* O157, O111, and O26 in beef and bovine feces by real-time polymerase chain reaction. *J. Food Prot.* 65: 1371-1380.

Sharma VK (2006). Real-time reverse transcription-multiplex PCR for simultaneous and specific detection of *rfbE* and eae genes of *Escherichia coli* O157:H7. *Mol. Cell Probes* 20: 298-306.

Sun Y (2007). Rapid Detection of Enterohemorrhagic *Escherichia coli* O157. The doctoral dissertation of Jilin University.

Sun Y, Feng S, Guo XJ, Zhu Lw, et al. (2008). Development of immunochromatographic strip for detection of enterohemorrhagic *Escherichia coli* O157. *Chin. J. Zoonoses* 24: 356-359.

Uhl JR, Bell CA, Sloan LM, Espy MJ, et al. (2002). Application of rapid-cycle real-time polymerase chain reaction for the detection of microbial pathogens: the Mayo-Roche Rapid Anthrax Test. *Mayo Clin. Proc.* 77: 673-680.

Wang LJ, Wu CS and Wang P (2007). Research and characterization of QCM immunobiosensor for *Escherichia coli* O157:H7 detection. *Micronanoelectronic Fechnol.* 44: 358-359,382.

Zhang JH, Lu QY, Ceng SY, Lu YY, et al. (2007). Rapid detection of *Escherichia coli* O157:H7 by using real-time fluorescence PCR assays. *Chin. J. Zoonoses* 23: 839-842.

