Real-time PCR for Identification of Shiga Toxin-producing Escherichia Coli Serotypes

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) is a significant public health hazard. Rapid identification and serotyping of STEC is crucial for tracing the sources of infections. In this study, we describe a singleplex PCR assays for identification of STEC serotypes O26, O55, O111, O113 and O157. The primers targeted genes for O-antigen biosynthesis, namely, *wzy, wbgN* and *wdbI*. When the real-time PCR assays were tested against the five STEC serotypes, a single melting peak was observed for each serotype. The analytical specificity was found to be 100%. The melting temperatures (T_m) among strains of the same serotypes were highly consistent. The stable T_m showed that the PCR assay was reproducible. In summary, we have developed five real-time PCR assays for identification of five important STEC serotypes, the assays have great potential for application in clinical sectors for rapid diagnosis and surveillance of STEC.

Introduction

Since the first identification of Shiga toxin-producing *Escherichia coli* (STEC) in 1982, the enteric pathogen has become a significant public health hazard. Diseases associated with STECs range from uncomplicated diarrhea to the life-threatening hemolytic uremic syndrome. The major virulence factor of STEC is Shiga toxin, which is a potent cytotoxin causing damage to the human intestinal microvasculature,[1,2] tubular epithelium and the glomeruli.[3] STEC is transmitted by contaminated food, beverages or contacting with animals or their excreta.[4,5] O157:H7 is the most well-known serotype reported in human infections and was associated with a number of major outbreaks in different countries.[6-8]

Other than STEC O157:H7, certain non-O157 STEC serotypes, including O26, O55, O111 and O113 are also isolated from

food poisoning outbreaks worldwide and associated with severe human diseases.[9-13]

STEC serotype is defined by the presence of specific O (cell wall) and H (flagella) antigens on the cell surface. Conventional serological typing uses antibodies specific to O- and H-antigens of STEC. This approach is culture-based and performed on bacterial colonies, the procedures are hence time-consuming. There may be cross-reaction between antibodies leading to false positive results.[14] Besides, the limited types of antibodies available in the clinical laboratory also restricted the use of this typing method.[15] In view these pitfalls, new nucleic acid-based methods are introduced for typing of STEC.

Molecular methods have been developed for typing of STEC strains. These include plasmid fingerprinting, ribotyping, olymerase chain reaction (PCR)-based methods and amplified fragment length polymorphism.[16] Among various methods, PCR-based methods are widely used due to its simplicity and lower cost.

The genes encoding O-antigen biosynthesis are unique for a particular serotype. Examples of these genes are *wzy, wbdI* and *wbgN*, which encode the O-antigen polymerase, GDP-mannose mannosyl-hydrolase and fucosyltransferase, respectively.[12,14,17] These genes are located within the *rfb* gene cluster and are desirable targets for molecular typing of STEC.[13,18-20] In this study, real-time PCR assays targeting the *rfb* gene clusters were developed for typing of STEC serotypes O26, O55, O111, O113 and O157.

Materials and Methods

Bacterial strains and culture conditions

Thirty-five bacterial strains were used in this study, properties of these strains are summarized in Table 1. All of the bacterial strains were human clinical isolates. Identities of the strains have been confirmed previously by biochemical tests and serotyping. The bacterial strains were inoculated onto nutrient agar and incubated at 37°C for 18-24 hours.

Preparation of DNA extract

Ten bacterial colonies were suspended in 1 ml double-deionized water. The suspension was heated at 80°C for 15 minutes, followed by centrifugation at 11,000 ×*g* for 5 minutes. The supernatant containing DNA was transferred to a sterile Eppendorf tube. The extracted DNA samples were used immediately or stored at -20^{o}C until being used.

Primer design

O-antigen cluster gene sequences used in this project were retrieved from GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Primers specific to O26 *wzy*, O55 *wbgN*, O111 *wdbI*, O113 *wzy* and O157 *wzy* were designed using software Oligo 6.57 (Molecular Biology Insights, USA) and were checked for specificity using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences are listed in Table 2.

Table 1. Bacterial strains used in this study.

Bacterial strains	n	Bacterial strains	n
STEC serotypes[a]		Non-*E. coli* strains	
O26	5	*Enterococcus faecalis*	1
O55	3	*Staphylococcus aureus*	1
O111	5	*Klebsiella pneumoniae*	1
O113	5	*Proteus vulgaris*	1
O157	5	*Pseudomonas aeruginosa*	1
		Salmonella typhimurium	1
Other *E. coli* strains		*Salmonella enterica*	1
Enteropathogenic *E. coli* O127	1	*Shigella boydii*	1
Enteropathogenic *E. coli* O128	1	*Shigella flexneri*	1
		Vibrio cholerae	1

[a] One strain from each serotype was used for optimization of the real-time PCR assays.

Table 2. Primers designed for this study.

Primers	Sero-types	Target gene (Gen-Bank Accession no.)	Sequences (5' to 3')	Amplicon size (bp)
O26f	O26	*wzy* (NC_013361)	GCTGATGGCATTAAGGTTCAT	172
O26r			GTAGTGGTGAAAATACGGAA	
O55f	O55	*wbgN* (AF461121)	GCAGCATGAAAGGAAGTGTG	158
O55r			GGAGACTGAATTGGTGCATC	
O111f	O111	*wdbI* (NC_013364)	CCAGGTGGTAGGATTCGC	223
O111r			GATCATCTGGGAGATTCAATT	
O113f	O113	*wzy* (AF172324)	GCGTATTGGTGCAGGAAAGA	206
O113r			ATTGGTATGGCGAAGCTATG	
O113F[a]		*wzy* (AF172324)	GGGTGGTTAGCGAACGAT	141
O113R[b]			CCCATATCAGAACAGCAGTT	
O157f	O157	*wzy* (AF061251)	TAGGGGTTGTATGCTCGTTGT	174
O157r			CTAACTCTGGTGTCGGAAAG	

[a, b] O113F and O113R were alternative primer pair designed to confirm the results of the O113-specific PCR assay.

Real-time PCR assay

PCR for each serotype was performed in a 25-µl reaction mix containing 1x Quantifast SYBR green PCR reaction buffer (Qiagen, Valencia, CA), 200 nM of forward and reverse primers for O55 and O157, or 600 nM forward and reverse primers for O26, O111 and O113, and 1 µl of DNA extract. The PCR was run in an ABI7500 real-time PCR System (Applied Biosystems Inc., Foster City, CA) with initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 15 sec, 57°C for 30 sec, 72°C for 30 sec. For alternative primer pairs O113F and O113R, 50°C was used as the annealing temperature.

To confirm for the presence of specific amplicons, agarose electrophoresis was performed. After real-time PCR assay, 5 µl of real-time PCR product was electrophoresed in a 1.5% agarose gel (USB Corporation, USA) in 0.5x Tris-borate-EDTA buffer for 1 hour. The agarose gel was stained with 0.5 µg/ml ethidium bromide and was visualized on an ultra-violet transilluminator.

Assessment of analytical specificity of the real-time PCR assays

For assessment of analytical specificity, the real-time PCR assays were tested against 18 more STEC strains, 2 enteropathogenic *E. coli* and 10 other bacterial species (Table 1).

Results and Discussion

In this study, we used five pairs of primers specific to the O-antigen biosynthesis genes of STEC serotypes O26, O55, O111, O113 and O157. When each primer pair was tested against the respective serotypes, a single sharp melting peak with characteristic melting temperatures (T_m) were observed for each of the positive controls but not in the no-template control. Agarose gel electrophoresis was performed for the real-time PCR products. The product sizes matched with the expected sizes indicated in Table 3. Non-specific band was not observed.

When tested against 18 more STEC, 2 enteropathogenic *E. coli* and 10 unrelated bacterial species, primer for O26, O55, O111 and O157 detected 100% of their respective serotypes. No cross-reaction was observed between the STEC serotypes. None of the unrelated bacterial species produce false positive result (Table 3). On the other hand, two STEC O113 strains had no amplification product. The same result patterns were obtained in the subsequent PCR using new lots of DNA templates and alternative O113-specific primers (O113F and O113R). Hence, it was possible that these strains belonged to serotypes other than O113.

For the STEC strains, the melting temperatures among strains of the same serotypes were found to be very close with coefficient of variation (cv) ranged from 0.08-0.4% (Table 4). The stable T_m showed that the PCR assay was reproducible.

Although the PCR assays developed were singleplex, the problem of low throughput can be offset by the availability of high throughput real-time PCR systems. On the other hand, singleplex assay contains less

Table 3. Detection results of the serotype-specific real-time PCR assays.

Strains (n)	Percentage of strains detected by the real-time PCR assays				
	Primers designed for:				
	O26	O55	O111	O113	O157
STEC O26 (5)	100	0	0	0	0
STEC O55 (3)	0	100	0	0	0
STEC O111 (5)	0	0	100	0	0
STEC O113 (5)	0	0	0	60*	0
STEC O157 (5)	0	0	0	0	100
Enteropathogenic *E. coli* O127 (1)	0	0	0	0	0
Enteropathogenic *E. coli* O128 (1)	0	0	0	0	0
Enterococcus faecalis (1)	0	0	0	0	0
Klebsiella pneumoniae (1)	0	0	0	0	0
Proteus vulgaris (1)	0	0	0	0	0
Pseudomonas aeruginosa (1)	0	0	0	0	0
Salmonella typhimurium (1)	0	0	0	0	0
Salmonella enterica (1)	0	0	0	0	0
Staphylococcus aureus (1)	0	0	0	0	0
Shigella boydii (1)	0	0	0	0	0
Shigella flexneri (1)	0	0	0	0	0
Vibrio cholerae (1)	0	0	0	0	0

* Two of the STEC O113 strains may not belong to that serotype. The percentage will be 100% if these two strains are being excluded.

Table 4. Average Tm and cv of various STEC serotypes.

Strains (n)	T_m±SD	cv
STEC O26 (5)	75.74±0.25°C	0.33%
STEC O55 (3)	76.43±0.05°C	0.08%
STEC O111 (5)	77.96±0.31°C	0.40%
STEC O113 (5)	77.46±0.11°C	0.15%
STEC O157 (5)	76.82±0.29°C	0.38%

primer pairs, which reduces the chance of non-specific interactions between primers. Moreover, optimized singleplex assay does not require fluorogenic probe, which can reduce the running cost.

There are two aspects regarding the PCR assays that need further development. First, the assays were designed for identification of common STEC serotypes, however, there are other STECs causing diseases and deaths such as O103, O121 and O145.[4,14] Second, in this study, the assays were tested against DNA prepared from pure bacterial culture. In order to assess the applicability of the assays for direct detection of STEC serotypes in clinical samples, the assays should be tested against faecal samples spiked with known amount of STEC.

In summary, five singleplex real-time PCR assays were developed for identification of common STEC serotypes. The assays were found to be specific and have great potential for application in clinical sector for rapid diagnosis and surveillance of STEC.

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