Application Note

High-throughput detection of Shiga toxin-producing *Escherichia coli* using multiplex PCR and the QIAxcel® Advanced system

Jianfa Bai, Ph.D., Director of Molecular Research and Development

Kansas State Veterinary Diagnostic Laboratory, Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, 1800 Denison Avenue, Manhattan, KS 66506-5606, USA

Using the QIAxcel Advanced system after a multiplex PCR allowed us to perform efficient and reliable high-throughput screening of cattle feces for the presence of four major *E. coli* virulence genes and the seven major Shiga toxin-producing serogroups that give rise to infection in humans.

Introduction

Shiga toxin-producing *E. coli* (STEC) causes serious gastrointestinal disease and death in humans. The seven major *E. coli* serogroups that give rise to infections in humans are O26, O45, O103, O111, O121, O145 and O157 (1, 2). Cattle are considered one of the major asymptomatic STEC carriers, and the bacteria propagate in their gut and are shed in the feces, which is a major source of food contamination and human infection (3). Early detection of STEC is crucial for prevention and intervention strategies.

Nowadays most tests for STEC in veterinary laboratories are performed using real-time PCR, but this method is limited by the number of targets it can detect. Most real-time cyclers are limited to five channels, and in addition, developing multiplex qPCR using more than three channels is technically challenging. Detecting more targets by real-time PCR would require multiple reactions and result in significant cost increases.

We have developed an 11-gene multiplex PCR assay that detects four major *E. coli* virulence genes (*stx*1, *stx*2, *eae*, *ehx*A) along with the seven major STEC serogroups mentioned above. The level of throughput and the interpretation accuracy of this assay have been dramatically improved by using the QIAxcel Advanced, a fast capillary electrophoresis system with high resolution and reproducibility. ▷

After PCR amplification, samples in either strip tubes or 96-well plates can be analyzed directly using the QIAxcel Advanced instrument without further manipulation. Multiple steps of traditional agarose gel separation, including gel casting, PCR product loading, gel electrophoresis, ethidium bromide staining and gel imaging are not necessary when QIAxcel is used. The QIAxcel Advanced system can also perform automated data interpretation by recognizing predefined electrophoretic patterns. After a run, the positive or negative value for each of the eleven possible amplicons in every sample is presented in a report.

Materials and methods

Primer design

The *wzx* gene, which encodes a flippase required for O-polysaccharide export, was used to design specific primers for serogroups O26, O45, O103, O111 and O145. The *wbqE* gene, which encodes a putative glycosyl transferase, and *wbqF*, which encodes a putative acetyl transferase (6), were used to design primers for the detection of serogroup O121. The primers for O157 were designed from the *rfb*E gene, and primers used for virulence genes were designed and validated in our previous study (4). The 11 pairs of primers were specifically designed to match the majority of available target sequences and to amplify different amplicon sizes that could be easily separated on a gel or on the QIAxcel Advanced system (Table 1).

Table 1. The molecular targets and their amplicon sizes used in the 11-gene multiplex PCR. Specificity and amplicon sizes were confirmed by sequencing.

Target gene	Amplicon size (bp)	Target gene	Amplicon size (bp)
wzx_{O45}	890	wzx_{O26}	417
wzx_{O103}	740	*eae*	375
*stx*1	655	$rfbE_{O157}$	296
$wbqE_{O121}$ $wbqF_{O121}$	587	wzx_{O111}	230
wzx_{O145}	523	*ehxA*	168
*stx*2	477		

DNA templates

Field isolates obtained from cattle fecal samples were stored in CryoCare beads (Key Scientific Products, Stamford, TX) at –80°C. Single colonies were streaked onto blood agar plates (BAP, Remel, Lenexa, KS) and were incubated overnight at 37°C. One or two colonies of each strain were suspended in 1 ml of distilled water and boiled for 10 min. After a short centrifugation, 1 µl of the supernatant was used as the DNA template (approximate amount ranging from 1–100 ng DNA per 25 µl reaction). For cattle fecal sample preparation, approximately 1 g of feces was added to a tube of 9 mL Escherichia coli broth (Difco, ThermoFisher), and cultured at 40°C for 6 hr. One milliliter of the enrichment was transferred to a 1.5 ml tube and centrifuged. The supernatant was

discarded and the pellet was used for DNA extraction with QIAamp DNA Stool Mini Kit. The MagAttract 96 *cador* Pathogen Kit can be used for high-throughput DNA extractions. One microliter of extracted DNA was used for each PCR reaction (data not shown).

Multiplex PCR

The primer mix was prepared in 1x TE buffer, and each primer had a final concentration of 0.11 µM. The PCR reaction mix (Table 2) was prepared by mixing 12.5 µl of 2x QIAGEN Multiplex PCR Master Mix, 2.0 µl of 25 mM $MgCl_2$, 8.5 µl RNase-free water and 1 µl template DNA (1–100 ng per 25 µl reaction; 10 ng DNA template is recommended).

A modified PCR protocol described by Bai et al (4, 5) was used in this study. A 15 min denaturation step at 95° C was followed by a 35 cycles of amplification at 95° C for 20 s, 63° C for 30 s and 72° C for 90 s, with a final extension at 68° C for 10 min (Table 3).

Table 2. Preparation of reaction mix.

Component	Volume/reaction	Final concentration
RNase-free water	8.5 µl	
2x QIAGEN Multiplex PCR Master Mix	12.5 µl	1x
E. coli 11-plex Primer Mix	1.0 µl	0.11 µM
25 mM $MgCl_2$	2.0 µl	5 mM*
Total volume	**24.0 µl**	

* Final concentration includes $MgCl_2$ in the QIAGEN Multiplex PCR Master Mix

Table 3. PCR cycling conditions.

Initial activation step	**15 min**	**95°C**
3-step cycling: Denaturation	20 s	95°C
Annealing	30 s	63°C
Extension	90 s	72°C
Number of cycles	35	
Final extension	10 min	68°C
Hold		8°C

The following thermal cyclers have been tested and are compatible with this procedure and the described cycling conditions: TP600 thermal cycler (TaKaRa Bio Inc.), Eppendorf® Mastercycler® ep Gradient, Arktik Thermal cycler, Labnet PCR thermal cycler, Bio-Rad® C1000, Bio-Rad T 100 Thermal Cycler, MJ Research PTC-200, GeneAmp® PCR System 9700.

QIAxcel Advanced protocol

After amplification, PCR products were directly placed into the QIAxcel Advanced capillary electrophoresis system and separated using the QIAxcel DNA Screening Kit with the AM700 method, which includes the following electrophoresis parameters: alignment marker injection at ▷

4 kV for 10 s, sample injection at 2 kV for 10 s and separation at 3.5 kV for 700 seconds. The QX Alignment Marker 15 bp/3 kb was run simultaneously with the samples, and the sizes were estimated by comparison with a control sample containing all eleven possible amplicons (Table 1).

Following analysis with the QIAxcel ScreenGel® software, the operating and analysis software for the QIAxcel Advanced system, the results are displayed along with comprehensive reports including gel images, electropherograms and the result table. It is possible to customize the report according to different laboratory requirements.

Results and discussion

The analysis showed that the samples tested were positive for various combinations of O groups and virulence genes, as shown in Table 4 and Figures 1 and 2.

Table 4. Result table showing the presence (+) or absence (–) of four virulence genes in seven *E. coli* serogroups.

Lane	O-type	*eaeA*	*stx*1	*stx*2	*ehxA*
1	O26	–	+	–	+
2	O26	–	+	+	+
3	O45	–	+	–	+
4	O103	–	+	–	+
5	O103	–	+	+	+
6	O111	+	+	+	+
7	O111	+	+	–	+
8	O121	–	–	+	+
9	O145	+	+	–	+
10	O145	+	+	+	+
11	O157	+	+	+	+

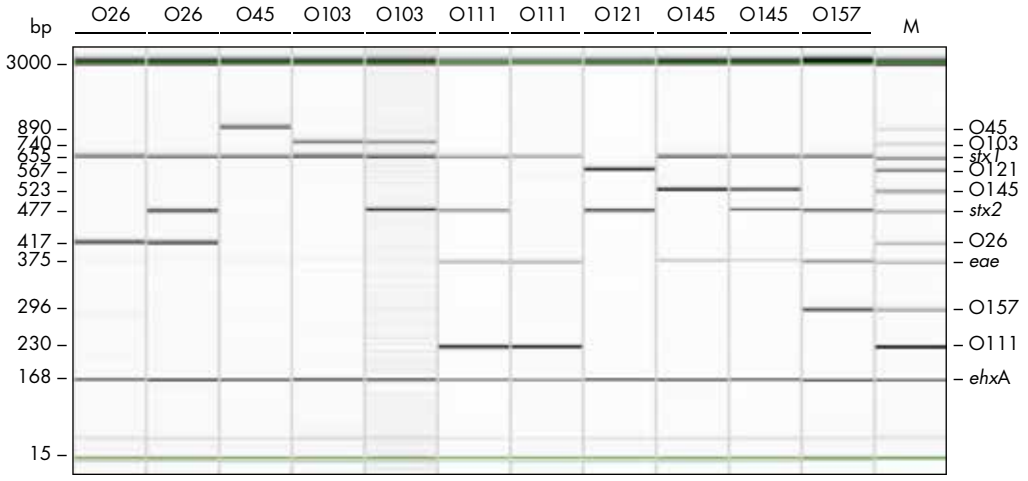

Figure 1. QIAxcel Advanced analysis of 11 isolates tested with 11-gene multiplex PCR. The PCR included the major *E. coli* virulence genes *stx*1, *stx*2, *eae* and *ehx*A, along with *E. coli* O-antigens from O26, O45, O103, O111, O121, O145 and O157 serogroups. Lane A12 contains a DNA ladder, which includes all 11 possible amplicons sizes and serves as a size marker. Amplicon sizes were verified by sequencing.

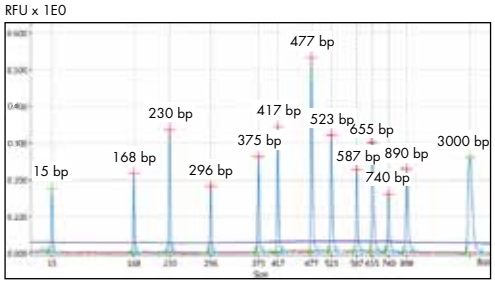

Figure 2. Electropherogram of the control sample from lane 12. All 11 amplicons used for identification are clearly visible and well separated. Peaks at 15 bp and 3000 bp correspond to alignment markers.

The strains were selected to represent all seven major *E. coli* serogroups and the four virulence genes. All of the samples carried the *ehx*A gene, which is present in most cattle fecal samples. However, the assay is only qualitative, and the ratios of the presence of the virulence genes in the table do not relate to STEC relative abundance or gene copy numbers within the cattle fecal samples. The Shiga toxin genes, especially *stx*2, are present in a much lower ratio in cattle samples, as described in previous publications (4, 5).

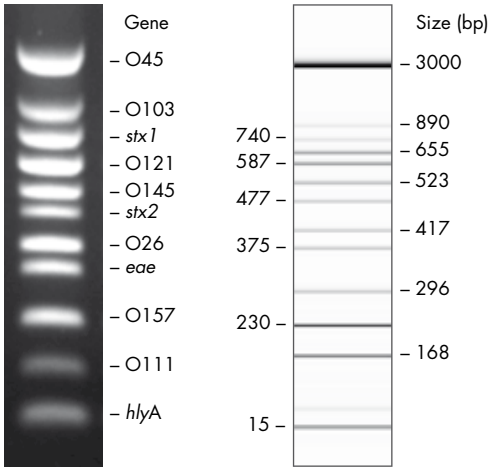

Figure 3. Comparison of the analysis results obtained with agarose gel electrophoresis and the QIAxcel Advanced. Sample containing all 11 amplicons was used as a positive control (5).

QIAxcel Advanced is a high sensitivity detection platform. When dealing with samples such as cattle feces, concentration of an *E. coli* strain can vary significantly. With the QIAxcel ScreenGel software, weak peaks can be observed in samples containing low concentrations of *E. coli* strains and may be difficult to interpret (Figure 4). After analyzing several thousands of field samples, we have established the best level of confidence in data analysis by using a 5% cutoff in the QIAxcel threshold setting. This correlates best with manual interpretations.

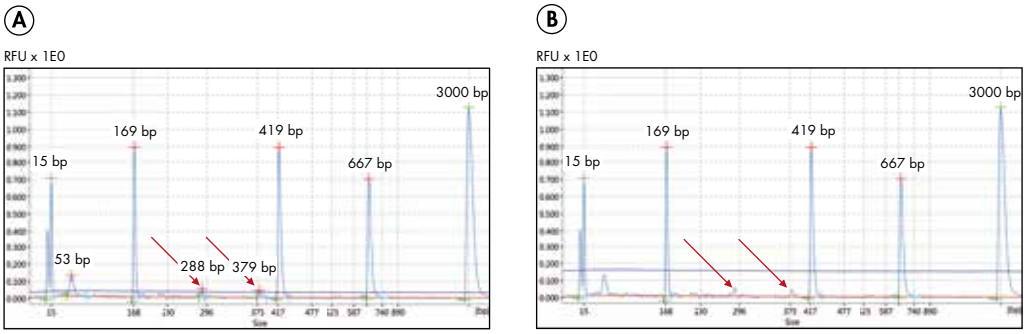

Figure 4. A **The threshold is used during peak detection.** Signals that exceed the threshold value (dark blue line) are detected as peaks. **B** If non-relevant peaks are identified, the threshold can be increased (e.g., to 5 %) to exclude the non-relevant or unspecific data from the analysis.

Automated sample loading, processing and analysis prevent human errors that can occur with manual sample handling, thus ensuring reliability and reproducibility of the measurements. Since no hazardous compounds are handled manually, this analysis method affords both convenience and safety. Using the recommended method, up to 96 samples per run can be analyzed unattended in about 90 minutes, or 12 samples in as little as 13 minutes. Digital data collection and management of experiments ensure traceability and standardized results.

Automated identification via peak calling

QIAxcel ScreenGel software automatically identifies DNA fragments from unknown samples using its peak calling function (peak calling table). This enables accurate detection and identification of the amplicons present in each sample. The peak calling table contains the sizes of all 11 possible amplicons generated by multiplex PCR and the tolerance in % for automated peak detection (Table 5).

Table 5. Peak calling instructions for 11-multiplex PCR.

Peak Calling Instruction *E.coli* 11-multiplex PCR					
Name	**Position**	**Tolerance**	**Name**	**Position**	**Tolerance**
ehxA	168 bp	18.000 %	wzx_{O145}	523 bp	4.400 %
wzx_{O111}	230 bp	13.500 %	$wbqE_{O121}$ $wbqF_{O121}$	587 bp	5.500 %
rfb_{O157}	296 bp	11.100 %	*stx*1	655 bp	5.200 %
eae	375 bp	5.600 %	wzx_{O103}	740 bp	5.700 %
wzx_{O26}	417 bp	5.000 %	wzx_{O45}	890 bp	8.400 %
*stx*2	477 bp	4.800 %			

The QIAxcel ScreenGel software compares the electrophoretic pattern obtained for each sample with the peak calling table and reports detected peaks in a Peak Calling Result Table that summarizes the results of the experiment (Table 6). This automated primary analysis of the sample makes results interpretation faster and more reliable for screening of a large number of samples.

Table 6. Based on the data presented in Figure 1and the peak calling instructions, a peak calling result table was generated. The table summarizes the experiment by reporting for each sample presence or absence of amplification products and their size estimates.

Peak Calling Result Table E.coli 11-multiplex PCR

Plate |R0|E0

Pos	Sample Info	*ehxA*	wzx_{O111}	rfb_{O157}	*eae*	wzx_{O26}	*stx*2	wzx_{O145}	wbq_{EO121} wbq_{FO121}	*stx*1	wzx_{O103}	wzx_{O45}
A1	*E.coli*-A-01	169	n/a	n/a	n/a	419	n/a	n/a	n/a	667	n/a	n/a
A2	*E.coli*-A-02	168	n/a	n/a	n/a	418	479	n/a	n/a	664	n/a	n/a
A3	*E.coli*-A-03	169	n/a	n/a	n/a	n/a	n/a	n/a	n/a	666	n/a	947
A4	*E.coli*-A-04	169	n/a	n/a	n/a	n/a	n/a	n/a	n/a	667	759	n/a
A5	*E.coli*-A-05	169	n/a	n/a	n/a	n/a	481	n/a	n/a	666	757	n/a
A6	*E.coli*-A-06	169	231	n/a	376	n/a	479	n/a	n/a	664	n/a	n/a
A7	*E.coli*-A-07	169	231	n/a	376	n/a	n/a	n/a	n/a	666	n/a	n/a
A8	*E.coli*-A-08	169	n/a	n/a	n/a	n/a	479	n/a	595	n/a	n/a	n/a
A9	*E.coli*-A-09	169	n/a	n/a	380	n/a	n/a	529	n/a	667	n/a	n/a
A10	*E.coli*-A-010	169	n/a	n/a	380	n/a	482	529	n/a	667	n/a	n/a
A11	*E.coli*-A-011	168	n/a	297	378	n/a	480	n/a	n/a	667	n/a	n/a

Conclusions

- The method presented here can identify the seven major *E. coli* serogroups and the four main virulence factors in one reaction.
- Separating multiplex PCR products with the QIAxcel Advanced system allows detailed, reliable and rapid analyses of complex samples containing many amplicons within a wide size range.
- Due to its fast turnaround time and automatic data interpretation, the QIAxcel Advanced system is highly suited for post-PCR separation, visualization and data presentation, especially for multiplex PCR runs in a high-throughput setting.

References

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3. Ferens, W.A. and Hovde, C.J. (2011) *Escherichia coli* O157:H7: Animal reservoir and sources of human infection. Foodborne Pathog. Dis. **8**, 465–487.
4. Bai, J., Shi, X., and Nagaraja, T.G. (2010) A multiplex procedure for the detection of six major virulence genes in *Escherichia coli* O157:H7. J. Microbiol. Meth. **82**, 85–89.
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6. Fratamico, P.M., Briggs, C.E., Needle, D., Chen, C.Y., and DebRoy, C. (2003) Sequence of the *Escherichia coli* O121 O-antigen gene cluster and detection of enterohemorrhagic *E. coli* O121 by PCR amplification of the wzx and wzy genes. J Clin. Microbiol. **7**, 3379–3383.

Ordering Information

Product	Contents	Cat. no.
E. coli 11-plex primer mix (200)	Mixture of 11 pairs of primers for 200 reactions	WBQX-001-200
E. coli 11-plex primer mix (1000)	Mixture of 11 pairs of primers for 1000 reactions	WBQX-001-1000
QIAGEN Multiplex PCR Kit (100)	For 100 x 50 µl multiplex PCR reactions: 2x QIAGEN Multiplex PCR Master Mix (providing a final concentration of 3 mM $MgCl_2$, 3 x 0.85 ml), 5x Q-Solution (1 x 2.0 ml), RNase-Free Water (2 x 1.7 ml)	206143
QIAGEN Multiplex PCR Kit (1000)	For 1000 x 50 µl multiplex PCR reactions: 2x QIAGEN Multiplex PCR Master Mix (providing a final concentration of 3 mM $MgCl_2$, 1 x 25 ml), 5x Q-Solution (1 x 10 ml), RNase-Free Water (1 x 20 ml)	206145
QIAxcel Advanced system	Capillary electrophoresis device: includes computer, QIAxcel ScreenGel software, and 1-year warranty on parts and labor	9001941
QIAxcel DNA Screening Kit (2400)	QIAxcel DNA Screening Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929004
QX Alignment Marker 15 bp/3 kb	Alignment marker with 15 bp and 3 kb fragments	929552
QX Nitrogen Cylinder (6)	6 QIAxcel Nitrogen Cylinders	929705
QIAamp DNA Stool Mini Kit (50)	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, InhibitEX® tablets, Buffers, Collection Tubes (2 ml)	51504

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