Detection of Shiga toxin-producing *Escherichia coli* in meat swabs by TaqMan real-time PCR targeting *stx* genes

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SUMMARY

Reliable methods for the detection and characterization of Shiga toxin-producing *Escherichia coli* (STEC) are essential for food safety. In this study, two pairs of PCR primers with respective TaqMan probes were used in real-time PCR for this purpose. The method efficiently amplified all variants of the Shiga toxin genes *stx1* or *stx2*. A detection limit of 10^1 CFU per sample was determined for the complete method with meat swab samples artificially contaminated with reference *E. coli* strains positive for Stx1 or Stx2 production. The optimized complete detection system was suitable for rapid and sensitive detection of Shiga toxin-producing *E. coli* strains in meat swabs.

Keywords

Escherichia coli; real-time PCR; shiga toxin; enterohemorrhagic; meat swabs

Although the bacterium *Escherichia coli* is a member of the normal flora of the gastrointestinal tract of humans and animals, several pathogenic types of *E. coli* may cause human diseases. *E. coli* O157:H7 and other enterohemorrhagic *E. coli* (EHEC) strains have emerged in recent years as important human pathogens associated with a spectrum of diseases ranging from diarrhea to hemorrhagic colitis and hemolytic-uremic syndrome. A subgroup of EHEC strains which produce Shiga toxins Stx1 and Stx2 are designated STEC. The production of Shiga toxins by STEC strains has a major role in pathogenesis, particularly in the pathogenesis of hemolytic-uremic syndrome [1-3].

Shiga toxin-producing *E. coli* often infect the cattle and Stx1-producing STEC predominate in diarrheal calves, whereas Stx2-producing STEC have been detected mainly in healthy animals [4-6]. Among the most important sources of human infection are direct contact with cattle and other ruminants, contaminated beef products, unpasteurized milk, as well as contaminated vegetables, fruits and drinking water [7].

The ability to control diseases associated with STEC and to limit outbreaks depends upon the rapid detection of these pathogens. The method based on sorbitol MacConkey agar culture coupled to the specific detection of the O157 antigen is used in most laboratories but this approach neglects other STEC serotypes [8]. In recent years, the detection of pathogenic *E. coli* strains has been greatly improved by using conventional PCR in the multiplex format [9, 10] or by real-time PCR [11, 12].

However, conventional PCR suffers from several disadvantages, such as the need for agarose gel electrophoresis and for confirmation of the amplified DNA fragments by nested PCR or Southern blot, which are time-consuming and labour-intensive and hence hardly acceptable for routine use.

In this study, the *stx1* and *stx2* genes of STEC were used as targets for 5'-nuclease (TaqMan) real-time PCR detection of STEC and the method was applied to the analysis of model meat swabs.

MATERIALS AND METHODS

Bacterial strains

Reference strains positive for s*tx1* (P17) and s*tx2* (SaI 4/LXIV/1) genes, respectively, were ob-

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tained from Institute of Medical Microbiology and Clinical Microbiology, Košice, Slovakia and from Institute of Veterinary Food Sciences, Justus-Liebig-University, Giessen, Germany. Commensal *E. coli* veterinary isolates NVI 41 and NVI 45 were obtained from National Veterinary and Food Institute, Bratislava, Slovakia. Cultures were grown in Nutrient Broth (Merck, Darmstadt, Germany) overnight at 37 °C. Cell concentrations of reference strains for the construction of standard curves were determined by the plate-count technique on Nutrient Agar plates (Merck) incubated at 37 °C for 24 ± 2 h.

Artificial inoculation

Swabs from 10 cm^2 of beef meat were artificially inoculated with STEC P17 and SaI 4/LXIV/1 (10^0, 10^1, 10^2, 10^3 CFU per sample), respectively.

Preparation of DNA

DNA from bacteria was extracted by cell lysis using boiling. A volume of 1 ml of the bacterial suspension was centrifuged at 10 000 *g*, the sediment was resuspended in 100 μl of 0.1 % v/v Triton X-100 (Serva, Heidelberg, Germany), incubated at 95 °C for 20 min, centrifuged at 12 000 *g* for 10 min and the supernatant was used as a DNA sample [13].

Real-time PCR

Each reaction of 25 μl contained 300 nmol.l^{-1} of the vt1-F and vt1-F primers, 300 nmol.l^{-1} of the primer vt2-F and vt2-R; 200 nmol.l^{-1} of the TaqMan probe vt1-P labelled with 6-carboxyfluorescein (FAM) and vt2-P labelled with tetrachloro-6-carboxyfluorescein (TET) (designed by NIELSEN et al. [14]; all oligonucleotides from Merck), 200 μmol.l^{-1} of each dNTP (Invitrogen, Gaithersburg, Maryland, USA), 1 U PlatinumTaq DNA polymerase (Invitrogen), 2.5 μl of 10× concentrated PCR buffer supplied with the polymerase, and 1 μl of the DNA sample.

Reactions were carried out in Mx3000P real-time PCR cycler (Stratagene, La Jolla, California, USA) using 40 cycles of 94 °C for 15 s and 63 °C for 60 s. Kinetics of the fluorescence signal in the FAM channel and hexachloro-6-carboxyfluorescine (HEX) channel were recorded and the threshold cycle values were calculated using the internal instrument software. Threshold cycle values of triplicate analyses of individual samples were averaged, plotted against the decadic logarithm of culture concentrations and calibration lines were constructed.

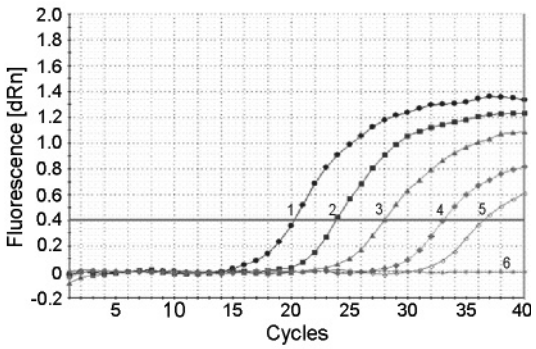

Fig. 1. Record of a representative TaqMan real-time PCR with lysates of decimally diluted STEC P17 culture of 2.5 × 10^6 CFU.ml^{-1} (1), 2.5 × 10^5 CFU.ml^{-1} (2), 2.5 × 10^4 CFU.ml^{-1} (3), 2.5 × 10^3 CFU.ml^{-1} (4), 2.5 × 10^2 CFU.ml^{-1} (5) and negative control (6).

RESULTS AND DISCUSSION

Real-time PCR detection of *stx1* and *stx2*

In this study, the optimized and evaluated method that could be applicable to screen for s*tx*-positive samples by real-time PCR is described. The advantage is the use of real-time PCR and TaqMan fluorescent probes, which produce a final result within 2 h after the strain isolation or culture enrichment [15].

For decimally diluted bacterial cultures of known concentrations, amplification curves with proportionally increasing threshold cycle values were recorded (Fig. 1). Threshold cycle values of parallel PCR reactions were plotted against bacterial concentrations and calibration lines were constructed. These were linear over the range of concentrations used (10^1 to 10^5 CFU.ml^{-1}) with

Tab. 1. Parameters of the calibration lines [threshold cycle *vs* log *c* (CFU.ml^{-1})] calculated from 5'-nuclease real-time PCR with decimal dilutions of *E. coli* strains.

Strain (toxin)	Parameter (average ± *SD*, *n* = 3)	
	Slope	*x*-intercept
E. coli P17 (Stx1)	-3.32 ± 0.10	44.59 ± 0.09
E. coli Sal4/LXIV/1 (Stx2)	-3.33 ± 0.18	44.81 ± 0.12

SD - standard deviation.

Tab. 2. Detection limit of 5'-nuclease real-time PCR.

Beef meat swabs inoculated with STEC P17 or Sal4/LXIV/1 [CFU per samples]	Quantity [average CFU per samples ± *SD*]
E. coli P17 (3.0×10^0)	n.d.
E. coli P17 (3.0×10^1)	$7{,}232 \times 10^1 \pm 0{,}21 \times 10^1$
E. coli P17 (3.0×10^2)	$1{,}484 \times 10^2 \pm 0{,}15 \times 10^2$
E. coli P17 (3.0×10^3)	$1{,}113 \times 10^3 \pm 0{,}05 \times 10^3$
E. coli Sal4/LXIV/1 (4.5×10^0)	n.d.
E. coli Sal4/LXIV/1 (4.5×10^1)	$5{,}132 \times 10^1 \pm 0{,}11 \times 10^1$
E. coli Sal4/LXIV/1 (4.5×10^2)	$2{,}494 \times 10^2 \pm 0{,}15 \times 10^2$
E. coli Sal4/LXIV/1 (4.5×10^3)	$3{,}312 \times 10^3 \pm 0{,}03 \times 10^3$

n.d. - not detectable, SD - standard deviation.

correlation coefficients $r^2 \geq 0.993$ and with practically identical slopes and *x*-intercepts (Tab. 1). Commensal *E. coli* isolates as a simulated background *E. coli* microflora at a concentration level of 10^6 CFU.ml^{-1} had no effect on the analytical parameters (data not shown).

Detection limit with meat swabs

To determine the detection limit of the method, the swabs from beef meat were inoculated with STEC strains P17 and SaI 4/LXIV/1 at levels of 10^0, 10^1, 10^2 and 10^3 CFU per sample respectively. After the DNA preparation, the detection limit of the method of 10^1 CFU per swab sample was determined (Table 2).

This detection limit was comparable with other published methods. STEFAN et al. [12] compared real-time PCR assay targeting *stx* genes with the enzyme-linked fluorescent assay (ELFA) VIDAS ECOLI. After overnight enrichment, 10 CFU STEC per sample could be detected using real-time PCR, while the detection limit of ELFA was 50 CFU per sample. Similar detection limits were determined for real-time PCR also by HEIJNEN et al. [11].

Microbiological criteria for *E. coli* counts on bovine carcasses are set to 10^2 CFU.cm^{-2} by the American Food Safety and Inspection Service, while e. g. Belgian legislation is stricter, stating the limit of 20 CFU.cm^{-2} [16]. From this point of view, the detection limit of 10 CFU per 10 cm^2 for the swabbed meat surface achieved by the presented method fulfilled even the stricter hygienic requirements.

CONCLUSIONS

Generally, the development of real-time PCR-based methods to detect pathogenic bacteria in food is leading to certain improvement in food safety and, consequently, in public health. The real-time PCR system described and evaluated in this study is suitable for the rapid and reliable identification of STEC strains in food and swab samples. This method is suitable for food safety and technological hygiene applications.

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