Rapid detection of *Escherichia coli* O157:H7 in food using enrichment and real-time polymerase chain reaction

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SUMMARY

Escherichia coli O157:H7 may contaminate various types of meat products and cause diarrhea and vomiting, and also more serious complications such as haemorrhagic colitis and haemolytic-uremic syndrome (HUS) in humans. Traditional microbiological analyses to detect this pathogen are labour-intensive and time-consuming. The objective of this study was to evaluate a real-time polymerase chain reaction (PCR) for detection of *E. coli* O157:H7 in raw and ready-to-eat meat products. The detection limit of real-time PCR determined on pure culture was 1.1×10^2 CFU·ml^{-1} when DNA was obtained by lysing cells and 30.6 pg·μl^{-1} when DNA was isolated and purified. Following a 20 h enrichment of a food sample in universal enrichment broth (UPB), the real-time PCR assay could detect 1.6 CFU·per 10 g of *E. coli* O157:H7 in chicken juice, raw beef, minced beef, beefsteak tartare, brunch beef and beefburger with background flora in the range of $< 10^2$ CFU·g^{-1} to 2.1×10^6 CFU·g^{-1}. The applied method could be a useful tool for rapid detection of *E. coli* O157:H7 in raw meat and ready-to-eat meat products.

Keywords

Escherichia coli O157:H7; detection; real-time PCR; meat; ready-to-eat meat products

Escherichia coli are normal inhabitants of the human intestines where these bacteria have useful functions, such as suppressing the growth of harmful bacterial species and synthesizing vitamins. A minority of *E. coli* strains are capable of causing human illness. Among them are verocytotoxigenic *E. coli* (VTEC) that are characterized by the production of a shiga-like toxin and have the potential to cause VTEC-associated illness. The predominant serogroups of *E. coli* in clinical cases are O45, O26, O91, O103, O111, O121, O145 and O157 [1]. *E. coli* O157:H7 was recognized as an important human pathogen in 1982 after an outbreak of haemorrhagic colitis due to consumption of hamburgers. Afterwards, this pathogen was implicated in serious foodborne outbreaks all over the world. *E. coli* O157:H7 is a pathogenic type of enterohaemorrhagic *E. coli* (EHEC) strain associated with food and water-borne infections [1, 2]. Undercooked or raw beef products like hamburgers have been implicated in many of the documented outbreaks, together with other foods such as milk, yoghurt, meat pies, sprouts or lettuce [1–3]. Nowadays, *E. coli* O157:H7 is known to be one of the most important food-borne pathogens threatening public health. The primary reservoir is considered to be cattle. Undercooked ground beef and beef products have often played a significant role in food-borne infections. *E. coli* O157:H7 produce large quantities of one or more related, potent toxins that cause severe damage to the lining of the intestine. The illness is associated with diarrhea and abdominal pain, and also more serious complications such as haemorrhagic colitis and haemolytic-uremic syndrome (HUS) may occur.

Standardized diagnostic and traditional procedures to detect the presence of *E. coli* O157:H7 in food samples are based on microbiological culturing methods requiring up to 5 days to obtain results [3, 4]. They are typically labour intensive and time consuming. In order to reduce the time required for analysis, alternative techniques such as different DNA-based methods have been applied

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to detect different strains of *E. coli* from various samples [5, 6]. Among them, polymerase chain reaction (PCR) has become a useful technique for detection of pathogens because it is quick, specific and sensitive. In recent years, a number of PCR-based assays have been developed for detection of *E. coli* O157:H7 in food, water and other samples. The detection has been improved by using multiplex PCR or by real-time PCR. Some of these assays have targeted only one gene [7, 8], while others have included several *E. coli* O157:H7-specific genetic markers [9-14].

There are also several reports on simultaneous detection of two or three pathogens including *E. coli* O157:H7 by multiplex PCR or multiplex real-time PCR [15-19]. Multiplex PCR involves the simultaneous amplification of two or more target genes per reaction by mixing multiple primer pairs with different specificities. PCR amplicons of different molecular weight can be separated by agarose gel electrophoresis, while real-time PCR uses precise measurement of fluorescence as a result of binding a fluorescent dye (i.e. SYBR Green I) to the minor grooves of the amplified double stranded DNA during the primer annealing and extension steps of each PCR cycle. Alternatively the hydrolysis probe (TaqMan system) may be used, where PCR products are detected by the exonuclease activity of *Taq* polymerase that releases a reporter dye from the 5' end of the labelled probe in each cycle of amplification [6].

PCR-based assays may be inhibited by components of the food sample matrix. Another problem is that PCR cannot differentiate viable and non-viable cells. Many PCR-based methods use centrifugation, filtration, DNA affinity columns and/or immunomagnetic separation (IMS) to remove inhibitory substances that are present in complex matrices and PCR can subsequently be run on recovered captured bacteria [7–9, 13, 14]. The enrichment step may eliminate possible inhibition of the enzymatic reaction because food components are diluted, and also cell viability may be determined by the enrichment step. Universal enrichment broth (UPB) and brain heart infusion broth (BHI) are examples of media used for concurrent growth of different bacteria [15, 17].

In the present work, we report on evaluation of a real-time PCR method for detection of *E. coli* O157:H7 and its application for the detection of this food-borne pathogen in complex food matrices. In parallel, a universal enrichment broth was applied, enabling simultaneous enrichment of *Listeria* and *Salmonella* strains besides *E. coli*, thus facilitating detection of multiple pathogens by real-time PCR.

MATERIALS AND METHODS

Bacterial strains

Fourteen strains of *Escherichia coli* were used in the study (Tab. 1). Strains of *E. coli* were obtained from the Institute of Public Health, Nova Gorica, Slovenia; Institute for Microbiology and Immunology, Ljubljana, Slovenia; and Cantonal Public Health Institution of Zenica, Laboratory for Sanitary and Clinical Microbiology, Zenica, Bosnia and Herzegovina. Among them, only strain *E. coli* ŽMJ 129 was O157:H7. *Listeria monocytogenes* ŽM 58 (Institute for Hygiene and Microbiology, Würzburg, Germany) and *Salmonella* Enteritidis ŽM 2 (our food isolate) were used in experiments where artificial contamination of food was performed. All cultures were maintained on Tryptic Soy Agar (TSA, Oxoid, Basingstoke, United Kingdom) plates at 4 °C. Cultures for assays and viable counts were grown in Tryptic Soy Broth (TSB, Oxoid) at 37 °C for 18–20 h and serially diluted in sterile Butterfield's Phosphate Buffer (pH 7.2, BPB) for use in experiments. To determine the number of cells for inoculation accurately, 100 μl of appropriate dilutions of each culture were spread-plated on TSA and incubated at 37 °C for 24 h.

DNA preparation

Bacterial DNA was extracted from pure cultures or enriched food samples in Universal Enrichment Broth (UPB; Becton Dickinson, Sparks, Maryland, USA), with PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster

Tab. 1. Detection of *E. coli* O157:H7 by real-time PCR.

Strain	Type	Result
E. coli ŽMJ 129	O157:H7	+
E. coli ŽMJ 131	O17	–
E. coli ŽMJ 132	O26	–
E. coli ŽMJ 134	O111	–
E. coli ŽMJ 135	O113	–
E. coli ŽMJ 128	O157	+
E. coli ŽMJ 130	O157	–
E. coli ŽMJ 137	O157	–
E. coli ŽMJ 138	O157	–
E. coli ŽMJ 139	O157	–
E. coli ŽMJ 140	O157	–
E. coli ŽMJ 141	O157	–
E. coli ŽMJ 142	O157	–
E. coli ŽMJ 143	O157	–

City, California, USA). One millilitre of overnight culture or 1 ml of UPB was centrifuged at 13 000 *g* for 3 min to sediment the bacterial cells. The sediment was suspended in 100 μl of PrepMan Ultra sample preparation reagent, mixed for 30 s and heated in a water bath at 95 °C for 10 min. The suspension was again centrifuged at 13 000 *g* for 3 min and the supernatant was transferred to a new tube. All lysates were stored at 4 °C until used (within 24 h).

For detection limit assays, DNA purification was done as follows. The starting material was 50 μl of DNA prepared with PrepMan Ultra, to which 400 μl of TE buffer (Tris 0.05 mol·l^{-1}; Promega, Madison, Wisconsin, USA; Na_2EDTA 0.02 mol·l^{-1}; Sigma, St. Louis, Missouri, USA; pH 8) and 50 μl of 3 mol·l^{-1} sodium acetate (Sigma) were added and mixed. Then, sample was mixed with 500 μl of isopropanol (Merck, Darmstadt, Germany) and incubated at room temperature for 15 min. The suspension was centrifuged at 13 000 *g* for 10 min, then the pellet was air-dried and resuspended in 50 μl of sterile distilled water. These DNA preparations were stored at –20 °C. The concentration and purity of the extracted DNA was evaluated by UV–VIS analysis with a Lambda Bio Plus Spectrophotometer (Perkin-Elmer, Norwalk, Connecticut, USA). Extraction and purification efficacy were evaluated by means of spectrophotometric analysis as UV absorption at 260 nm (A_{260}) and the A_{260}/A_{280} ratio.

Real-time PCR

PCR was performed on a total volume of 20 μl containing 2.0 μl of extracted DNA, 17.6 μl TaqMan *E. coli* 0157:H7 reaction mixture (TaqMan *E. coli* 0157:H7 Detection Kit; Applied Biosystems, Foster City, California, USA) mixed with an internal positive control (IPC) and 2 U per reaction of AmpliTaq Gold DNA polymerase (PE Applied Biosystems). Positive and negative controls consisted of the same chemicals, only instead of target DNA, 2.0 μl TaqMan *E. coli* 0157:H7 positive control and negative control TaqMan *E. coli* 0157:H7 was added. Amplification and detection were carried out in Prism 7500 sequence detection system (Applied Biosystems) with an initial incubation at 94 °C for 9 min followed by 40 cycles of 95 °C for 20 s, 60 °C for 1 min, and 72 °C for 35 s with fluorescence data collection. After real-time PCR completion, the cycle threshold (C_t) value was calculated by determining the point at which fluorescence exceeded the threshold signal. The automatic baseline function was used. The threshold was manually set to 0.1 for the *E. coli* TaqMan probe and 0.02 for the IPC probe.

Food samples spiked with *E. coli* O157:H7

The ability to detect *E. coli* O157:H7 in foods was evaluated by spiking food samples with different numbers of *E. coli* ŽMJ 129 and then recovering DNA from each spiked sample and subjecting it to real-time PCR. Chicken juice, the liquid that was produced when frozen chickens were thawed and could resemble the composition of the food, was used as a sterile food model and was prepared as described by Riedel et al. [20]. Raw meats (ground beef, minced beef) and ready-to-eat meat products (beef burgers, beefsteak tartare, brunch beef) were purchased from local supermarkets. Samples of 10 g of each food product were inoculated with 1 ml of a bacterial suspension containing *E. coli* O157:H7 at concentrations of 10^5 CFU·ml^{-1}, 10^4 CFU·ml^{-1}, 10^3 CFU·ml^{-1}, 10^2 CFU·ml^{-1} and 10^1 CFU·ml^{-1}. The food samples were put into bags and mixed with 90 ml of UPB using a Stomacher 400 Laboratory Blender (Seward, London, United Kingdom) for 1 min at normal speed. An un-spiked sample designated as a negative control was prepared by seeding 1 ml of sterile water into the corresponding sample in UPB. Amounts of 100 μl of appropriate dilutions of each food-UPB suspension without added *E. coli* ŽMJ 129 were spread-plated on TSA to determine the total viable counts. Food-UPB suspensions were incubated for 20 h at 37 °C. After 6 h and 20 h, DNA was prepared from 1 ml samples as described above.

Real-time PCR detection of *E. coli* O157:H7, *L. monocytogenes* and *S.* Enteritidis in beefsteak tartare

Aliquots of 10 g of beefsteak tartare were spiked with *E. coli* ŽMJ 129, *L. monocytogenes* ŽM 58 and *S.* Enteritidis ŽM 2 to achieve concentrations of each of 10^4 CFU·per 10 g, 10^3 CFU·per 10 g and 10^2 CFU per 10 g. Food samples were treated as described above. *E. coli* O157:H7 was detected as in the other samples, while *L. monocytogenes* and *Salmonella* were detected with a TaqMan *Listeria monocytogenes* Detection Kit (Applied Biosystems) and a TaqMan *Salmonella enterica* Detection Kit (Applied Biosystems), respectively.

RESULTS AND DISCUSSION

Real-time PCR for detection of *E. coli* O157:H7

Genomic DNA samples from different *E. coli* strains were tested initially to establish that the primers and probes used in the real-time PCR assay resulted in amplification of amplicons specific

to *E. coli* O157:H7. Positive results were obtained with the *E. coli* ŽMJ 129 strain (previously determined as the O157:H7) and with the *E. coli* ŽMJ 128 strain (previously determined only as O157; Tab. 1). No cross-reactivity was detected when other serotypes of *E. coli* (O17, O26, O111, and O113) were tested. Other strains of *E. coli* O157 gave negative results.

Detection limit

The detection limit of the real-time PCR assay was tested using serial dilutions of *E. coli* O157:H7 ŽMJ 129. Serial dilutions (10^6, 10^5, 10^4, 10^3, 10^2, 10, < 10 cells/ml) of the overnight culture were prepared. DNA was then extracted with PrepMan Ultra and subsequently tested using the real-time PCR method. The results obtained showed that

Tab. 2. Parameters of standard curves calculated from real-time PCR with decimal dilutions of *E. coli* ŽMJ 129 or decimal dilutions of purified DNA from *E. coli* ŽMJ 129.

DNA preparation	Detection range	Efficiency ± SD [%]	Slope ± SD	R^2
PrepMan Ultra extraction	$1.1 \times 10^2 - 1.1 \times 10^7$ CFU·ml^{-1}	106.4 ± 24.8	3.26 ± 0.48	0.99
DNA purification	0.03–306.30 ng·μl^{-1}	93.6 ± 5.4	3.49 ± 0.14	0.99

First standard curve was calculated from C_t versus N [log (CFU·ml^{-1})] and the second standard curve was calculated from C [ng·μl^{-1}] versus N [log (CFU·ml^{-1})]. C_t – threshold cycle, N – numbers of *E. coli* ŽMJ 129 [log (CFU·ml^{-1})], C – concentration of DNA isolated from *E. coli* ŽMJ 129 and decimally diluted.
SD – standard deviation.

Tab. 3. Real-time PCR detection of *E. coli* ŽMJ 129 in artificially contaminated food samples after 6 h and 20 h enrichment in UPB.

Food sample	Background bacteria N [CFU·g^{-1}]	N_0 of *E. coli* ŽMJ 129 (CFU per 10 g)	Real-time PCR result after enrichment in UPB	
			6 h	20 h
Chicken juice	$< 1.0 \times 10^2$	2.1×10^4	+	+
		2.1×10^3	+	+
		2.1×10^2	+	+
		2.1×10^1	+	+
Raw beef	2.1×10^6	1.6×10^5	+	+
		1.6×10^4	+	+
		1.6×10^3	+	+
		1.6×10^2	+	+
		1.6×10^1	–	+
Minced beef	9.0×10^5	1.2×10^5	+	+
		1.2×10^4	+	+
		1.2×10^3	+	+
		1.2×10^2	–	+
		1.2×10^1	–	+
Beefsteak tartare	2.3×10^5	7.2×10^4	+	+
		7.2×10^3	+	+
		7.2×10^2	+	+
		7.2×10^1	–	+
		7.2×10^0	–	+
Beefburger	$< 1.0 \times 10^2$	1.2×10^5	+	+
		1.2×10^4	+	+
		1.2×10^3	+	+
		1.2×10^2	+	+
		1.2×10^1	+	+
Brunch beef	1.2×10^2	1.6×10^4	+	+
		1.6×10^3	+	+
		1.6×10^2	+	+
		1.6×10^1	–	+
		1.6×10^0	–	+

N – numbers of background bacteria, N_0 – numbers of *E. coli* ŽMJ 129 used for spiking food samples.

reliable amplification could be observed for the samples with 1.1×10^2 cells per 1 ml. The numbers of CFU·ml^{-1} were confirmed by plating on TSA. The concentration of the extracted and purified DNA was 306 ng·μl^{-1} and decimal dilutions were tested using the real-time PCR method. After DNA purification, the detection limit of the real-time PCR was 30.6 pg·μl^{-1}. C_t values of real-time PCR reactions were plotted against bacterial concentrations (CFU·ml^{-1}) and against DNA concentrations (ng·μl^{-1}) and calibration curves were constructed. The efficiencies of amplification were calculated (Tab. 2) according to [21]. The standard deviation was higher using extracted DNA as there were more impurities than in samples where DNA was further purified. The results are comparable to results obtained by BUJŇÁKOVÁ et al. [22] who applied real-time PCR to detection of STEC (Shiga toxin-producing *E. coli*).

The detection limit of 1.1×10^2 CFU·ml^{-1} for *E. coli* O 157:H7 obtained by real-time PCR after preparing cell lysates was better than reported by OBERST et al. ($\geq 10^3$ CFU·ml^{-1}) [9] and in the same range as reported by FU et al. ($\leq 10^2$ CFU·ml^{-1}) [8].

Detection of *E. coli* O157:H7 in meat samples

Food samples were spiked with various concentrations of *E. coli* ŽMJ 129 and enriched in UPB (Tab. 3). The detection limit of real-time PCR was determined as 2.1–12 CFU per 10 g after 6 h enrichment when food had a low concentration of naturally present bacteria (chicken juice, beefburger). A lower detection limit (1.6 CFU per 10 g) was determined with overnight enrichment (20 h) also for foods that had higher concentrations of other bacteria. The detection limit of real-time PCR was comparable to that reported for other real-time PCR-based assays, i.e. SHARMA et al. [12]. Those authors found that by incorporating an enrichment period of 4 h and 16 h, real-time PCR allowed detection of 22 CFU·g^{-1} and 2 CFU·g^{-1} of faeces using *eaeA* and *stx* probes, respectively. The detection limit for *E. coli* O157:H7 in ground beef obtained using the combination of IMS and real-time PCR was 1.3×10^4 CFU·g^{-1} without enrichment of the sample [13]. OBERST et al. [9] reported the detection limit of real-time PCR for *E. coli* O157:H7 of approximately 10^4 CFU·ml^{-1} in ground beef-mTSB mixtures, and 10^2 CFU·g^{-1} after IMS and an 18 h enrichment of beef in TSB. The meat products tested in our study had microbial background levels from non-detectable in chicken juice and beefburger, to 9.0×10^5 CFU·g^{-1} in beef, minced beef and beefsteak tartare. Enrichment of meat samples in UPB could increase the numbers of target bacteria to the level detectable by PCR in 6 h or 20 h, dependent on the background bacterial level.

Beefsteak tartare tested in another set of experiments of this study had microbial background levels of 4.6×10^4 CFU·g^{-1}. After artificial contamination of samples with *E. coli* ŽMJ 129, *S.* Enteritidis ŽM 2 and *L. monocytogenes* ŽM 58, samples were enriched in UPB. Bacterial DNA was prepared after 6 h and 20 h of enrichment and subjected to real-time PCR (Tab. 4). Using this procedure, detection of all three pathogens was possible after using one enrichment medium and after simultaneous bacterial DNA preparation. Useful applications of UPB as an enrichment

Tab. 4. Real-time PCR detection of *E. coli* ŽMJ 129, *S.* Enteritidis ŽM 2 and *L. monocytogenes* ŽM 58 in artificially contaminated beefsteak tartare.

Sample No.	Bacteria	N_0 (CFU per 10 g)	Real-time PCR result
1	*E. coli*	1.4×10^4	+
	S. Enteritidis	4.5×10^5	+
	L. monocytogenes	4.6×10^4	+
2	*E. coli*	1.4×10^3	+
	S. Enteritidis	4.5×10^4	+
	L. monocytogenes	4.6×10^3	+
3	*E. coli*	1.4×10^2	+
	S. Enteritidis	4.5×10^3	+
	L. monocytogenes	4.6×10^2	+
4	*E. coli*	0	–
	S. Enteritidis	0	–
	L. monocytogenes	0	–

N_0 – number of *E. coli* ŽMJ 129 used for spiking food samples.

broth for these three pathogens and other bacteria have been previously documented [15, 17].

In the present study, detection limit comparable to other studies was achieved without the use of time-consuming and laborious DNA extraction. Use of immunomagnetic beads may further improve the detection limit by 100- to 1 000-fold by concentrating cells from enrichment of food sample [7–9, 12–14]. Further work is needed to improve the detection limit after a shorter period of enrichment and also to test naturally contaminated food samples.

We propose that the real-time PCR method is used for the detection of *E. coli* O157:H7 in food samples after a 6 h enrichment of food in UPB, while food samples which give negative results should be examined by real-time PCR after 20 h enrichment in UPB. This method will reduce the total time and labour required for the inspection of *E. coli* O157:H7 in food products.

CONCLUSIONS

We found that recovery of *E. coli* O157:H7 from meat and meat products using a 6 h and 20 h enrichment in UPB may be somewhat slower than using other methods, e. g. IMS, but it provided a good detection limit of 1.6 CFU per 10 g. Thus, the results of this study indicate that it is possible to detect and identify *E. coli* O157:H7 in enrichment broth by real-time PCR within 1 day.

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