VERSITA

Biologia **66**/3: 401—405, 2011
Section Cellular and Molecular Biology
DOI: 10.2478/s11756-011-0052-z

Multiplex PCR for detection of Escherichia coli O157:H7 in foods

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Abstract: *Escherichia coli* O157:H7 is well known enterohemorrhagic pathogen responsible for infections among animals including a man. The main source of this bacterium is cattle, that is mostly asymptomatic and through that *E. coli* O157:H7 can simple transfer to food products. Therefore, there is a need for rapid, sensitive and specific detection method. The present work is focused on its detection by a heptaplex polymerase chain reaction, which targets genes from known virulent regions of *E. coli* O157:H7. According to obtained results this approach is able to reach the detection sensitivity of 4 colony-forming units (CFU) from a culture and 6 and 8 CFU from milk and meat samples, respectively, independently of tested sample volume.

Key words: *Escherichia coli* O157:H7; multiplex PCR; detection; dairy and meat products.

Abbreviations: CFU, colony-forming units; EHEC, enterohemorrhagic *E. coli*; LB, Luria-Bertani broth; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SMAC, sorbitol MacConkey agar; Stx, shiga-like toxin.

Introduction

Escherichia coli is a rod-shaped (around 2–3 μm long and 0.6 μm wide), Gram-negative, facultative anaerobic commensal bacterium found in the gut of warm-blooded animals including humans. Although the most of strains are harmless, some strains have come into pathogenic state by acquiring virulence factors through pathogenicity islands and/or mobile genetic elements, e.g. plasmids, transposons and bacteriophages (Kaper et al. 2004). Enterohaemorrhagic *E. coli* (EHEC) belongs to pathogenic *E. coli* strains that produce toxins known as shiga-like toxins (Stxs) or verotoxins (Li et al. 2010). The infection may cause severe food-borne diseases and may lead to hemorrhagic colitis and a life-threatening diseases, such as haemolytic uraemic syndrome (Lim et al. 2010).

The focus of this study is on the *E. coli* O157:H7, the most frequently isolated serotype of EHEC. The "O" in the name points out the expressed somatic (cell wall) antigen number, whereas the "H" means flagella antigen number. This serotype is easily differentiated from other *E. coli* strains biochemically in cultural procedures, because it is sorbitol-negative and it lacks the ability to cleave 4-methylumbelliferyl-β-D-glucuronide as well (Thompson et al. 1990). The reservoir of this pathogen appears to be mainly cattle and other ruminants (e.g. sheep and goats). Unfortunately, carrying host is mostly asymptomatic, with no clinical signs of disease, but is capable of infecting others (Lim et al. 2010). Careful hygienic slaughtering practice and the use of antibiotics to prevent spreading of pathogen are the most common recommendations for reducing the incidence of *E. coli* O157:H7 in cattle on farm. The latter can also lead to the development of multiresistant strains, which is contraindicated. In addition, there is a variety of foods-associated incidences of *E. coli* O157:H7 including undercooked hamburgers, dried salami, unpasteurized fresh-pressed apple cider, milk, yogurt and cheese (Michino et al. 1999). Many incidences are also associated with the consumption of fruits and vegetables (sprouts, lettuce, coleslaw, salad), whereby contamination may be due to contact with faeces from domestic or wild animals during cultivation or handling (Rangel et al. 2005).

In this regard, prevention of infection requires rapid and specific control measures at all stages of the food chain, i.e. from agricultural production on the farm to processing, manufacturing and preparation of foods. The aim of the present work was to develop a simple and rapid heptaplex polymerase-chain reaction (PCR) assay for specific identification of EHEC O157:H7 in dairy and meat products. Identification was based on positive amplification of *E. coli* specific 23S rDNA region simultaneously with six virulence genes distinctive for the serotype O157:H7.

Table 1. Oligonucleotides used in this study.

Primer	Sequence 5' to 3'	Targeted gene	GenBank	Location within the gene	Length
STX1 (Fwd) STX1 (Rev)	CAGAGGAAGGGCGGTTTAAT TTCACGATTTTCACGTTCCA	*stx1* genes for shiga toxin 1	AB083043	242–261 1239–1220	998 bp
ESP (Fwd) ESP (Rev)	GCCTCTCAGGGTGACAGCGGT TGTGTCCACGCCCCCATCAA	serine protease (*espP*) gene	EU878759	229–249 1113–1094	885 bp
STX2 (Fwd) STX2 (Rev)	ACCAGGCTCGCTTTTGCGGG CACGTCTCCCGGCGTCATCG	shiga-like toxin II gene	AP000422	21422–21441 22156–22137	735 bp
Ecoli2 (Fwd) Ecoli1 (Rev)	GCACTTATCTCTTCCGCATT GCTTGACACTGAACATTGAG	rDNA coding 23S ribosomal RNA	CP000970	3651268–3651287 3651930–3651911	663 bp
EAE (Fwd) EAE (Rev)	TGAGCGCCCAGCAAATGGCT TGTGCGCTTTGGCTTCCGCT	intimin (*eae*) gene	EF540941	938–957 1492–1473	555 bp
HLYA (Fwd) HLYA (Rev)	CCTGGGGGATGGGCTGGATGT AGCATGCTTATCGGGGCCCCT	pO157 hemolysin toxin protein (*hlyA*) gene	CP001369	17463–17483 17945–17925	483 bp
H7 (Fwd) H7 (Rev)	GCGCCAGCAGAAGTTAAATC GCTGTCCGAAATCAACAACA	*fliC* gene for flagellin	AM228903	587–568 243–262	345 bp

Material and methods

Bacterial strains and media

The *E. coli* strain (Collection of microorganisms, Serovar O:157. Stool; Slovakia. Production of Vero cytotoxins VT1 and VT2. Sorbitol and β-glucuronidase negative), donated from EL spol. s r.o., Slovakia, with all genes tested in this work was used for heptaplex PCR of *E. coli* O157:H7 virulence genes. Negative controls, *E. coli* BL21 and DH5α, were from Novagen (USA).

E. coli O157:H7 was enriched in Trypticase soy broth, incubated overnight at 37 °C, spread on plates with sorbitol MacConkey agar (SMAC), and incubated again overnight at 37 °C. Luria-Bertani (LB) broth was inoculated with single colony, grown overnight on rotary shaker at 37 °C. Also negative control *E. coli* strains were grown overnight in LB broth on a rotary shaker at 37 °C. If agar plates were used, medium was solidified with addition of 1.5% agar (Serva).

The number of colony-forming units (CFU) needed for detection of PCR product was determined by cultivation. LB (1 mL) was inoculated with a single colony isolated from SMAC plates and incubated overnight at 37 °C, and serial 10–fold dilutions in 1% peptone were prepared, giving counts in the range of 10–10^{10} CFU/mL. Viable counts were obtained by culturing each dilution (50 µL) on tryptose soy agar plates with overnight incubation at 37 °C.

Multiplex PCR conditions

Oligonucleotide sequences of primers were designed and specificity for *E. coli* O157:H7 was analyzed by Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer pairs are listed in Table 1. All oligonucleotide primers were synthesized by Microsynth (Switzerland). Briefly, PCR assay was carried out directly from *E. coli* O157:H7 cells prepared according to modified procedure described previously by Chotár et al. (2006). Modification concerned the treatment of the overnight culture before washing steps. The tested *E. coli* O157:H7 overnight culture prepared from single colony grown on SMAC was treated in two ways: by boiling and with NaClO solution.

In case of boiling preparation, 1 mL of culture was centrifuged and cell pellet was suspended in 500 µL of sterile distilled water in a tube. The tube was vortexed, incubated at 96 °C for 10, 20, 30 and 60 min, then chilled on ice for 5 min, and centrifuged at 10,000×g for 2 min to remove debris. The supernatant (5 µL) was added directly to the PCR mixture. In case of NaClO treatment, 1 mL of the *E. coli* O157:H7 overnight culture was treated for 10 min with NAClO solution in resulting concentration of 5×10^{-4}%. In both cases, the viability of the cells was verified by counting the number of CFU on an LB plate after overnight incubation at 37 °C.

With the aim to prepare the sample for PCR assay with milk matrix, the pellet from NaClO-treated overnight culture was first re-suspended in 1 mL of sterile UHT-treated cow's milk, incubated at room temperature for 30 min, and then washed with 1 mL of water and phosphate buffered saline (PBS, pH 8). Simultaneously, with the aim to prepare the sample with meat matrix, the washed cell sample was first mixed and incubated at room temperature for 30 min with 1 g of pork meat sample, and then washed with water and PBS.

Cell suspension (5 µL) was used as a template in a final volume of 20 µL of PCR mixture containing components of KAPA2G^{TM} Fast PCR Kit (Kapa Biosystems): 1× optimized 5× KAPA2G Buffer B with $MgCl_2$, KAPA dNTP Mix (10 mM), KAPA2G Fast DNA Polymerase (0.4 U), and primer mix (1.2 µM STX1 primers, 1 µM ESP primers, 0.8 µM STX2 primers, 0.6 µM Ecoli1 and Ecoli2 primers, 0.4 µM EAE primers and 0.16 µM HLYA primers). The amplification programme consisted of denaturation at 95 °C for 1 min, 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 5 s and extension at 72 °C for 8 s, followed by a final extension at 72 °C for 2 min. If monoplex PCR was used, the concentration of each primer in PCR reaction was 0.625 µM and quantity of KAPA2G Fast DNA Polymerase was reduced to 0.2 U. The whole PCR reactions were analyzed by electrophoresis in 0.9% agarose gel stained with 0.5 µg/mL ethidium bromide, which was carried out in 1× BBE buffer (0.65 M boric acid, 0.029 M sodium tetraborate, 0.25 M EDTA, pH 7.8). UV transillumination of the bands on the agarose gel ought to show different sizes of seven desired amplicons. All obtained PCR products were sequenced by Microsynth (Switzerland) to verify their identity.

Results

Preparation of E. coli O157:H7 cell suspensions suitable for PCR

Due to well known infectivity of this enterohemorrhagic pathogen, there was a need for using safety precautions before preparation of cell suspension for PCR. Two ways to prepare cell suspensions from overnight culture before washing steps were used; and samples (5 μL) after washing with sterile water were used directly to PCR. The rest of preparations were subsequently stored at 4 ℃ for a week. Both preparations, freshly prepared and stored ones, were undergone viability testing. In the case of boiling preparation, the 10 min of incubation at 96 ℃ was enough to achieve PCR products, but only with fresh preparation. There were no PCR products observed after week of storage, although boiling treatment was enough for killing all viable cells in sample as well. The NaClO treatment exhibited satisfactory results with both freshly prepared and stored preparations with the same results observed after more than three months stored preparations. Therefore, the NaClO treatment of overnight culture was used in further experiments. The additional advantage of this treatment was that there was no need for preparing other overnight cultures reducing thus the work with infective *E. coli* O157:H7 living cells.

Monoplex PCRs

All primer pairs designed for identification of *E. coli* O157:H7 virulence factors (STX1, ESP, STX2, EAE, HLYA and H7) (Table 1) generated single PCR products of appropriate sizes when washed cell suspension of this pathogen was directly used in PCR reactions. No PCR products were generated with these primers by using negative control strains *E. coli* BL21 and DH5α. Amplification of 663 bp PCR product was observed when species-specific primer pair (Ecoli1 and Ecoli2; Chotár et al. 2006) was used with *E. coli* O157:H7 as well as *E. coli* BL21 or DH5α (Fig. 1).

Detection limits, i.e. number of cells needed to obtain a visible PCR product, for every single primer pair were determined by cultivation (Table 2). The amount of 4 cells was enough to positive amplification of appropriate PCR products with primer pair STX1 and ESP or STX2. Two cells were enough for the amplification of PCR products with primer pair EAE, and even one single cell with primer pair HLYA or H7. The observation of detection limit for species-specific Ecoli primer pair was the same as stated previously by Chotár et al. (2006).

Multiplex PCRs

Testing of multiplex PCR in presented work comprised three experimental models depending on type of matrix, in which the presence of *E. coli* O157:H7 cells was tested. The prepared cells suspensions were then directly used in the heptaplex PCR.

In the first model overnight culture was treated for 10 min with NaClO solution (resulting concentration of 5×10^{-4}%), washed with water and PBS, and then directly used as template in PCR (Fig. 2). In the second model the overnight culture was treated with NaClO mixed with a sample of UHT-treated cow's milk, followed by washing with water and PBS, and then directly used as template in PCR (Fig. 3). Finally, in the third model the NaClO-treated overnight culture was mixed with a sample of meat, followed by washing with water and PBS, and then directly used as template in PCR (Fig. 4).

Because the sensitivity of multiplex PCR is linked with visibility of all amplicons, the results of testing suitability of cell suspension prepared only from the overnight culture for heptaplex PCR were comparable with that of monoplex PCR. The visibility of the upper PCR product for *stx1* gene was detectable for at least 4 cells in the heptaplex PCR. The presence of milk background and meat matrix decreased the sensitivity by

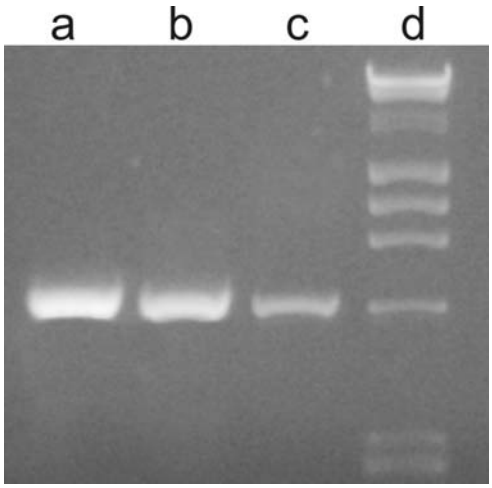

Fig. 1. PCR product amplified with species-specific primer pair Ecoli1 and Ecoli2 (Chotár et al. 2006). Washed cell suspension of *E. coli* BL21 (a), *E. coli* O157:H7 (b) and *E. coli* DH5α (c) was used directly to PCR; (d) DNA ladder λDNA – Hind*III* and ΦX174 DNA – Hae*III* (Finnzymes).

Table 2. Detection limits of PCR products amplification with primer pairs targeting studied virulence genes and species-specific 23S rDNA region from *E. coli* O157:H7 bacterial suspension.

Primer pair	Length	Number of CFU 32	16	8	4	2	1	0
STX1	998 bp							
ESP	885 bp							
STX2	735 bp							
Ecoli	663 bp							
EAE	555 bp							
HLYA	483 bp							
H7	345 bp							

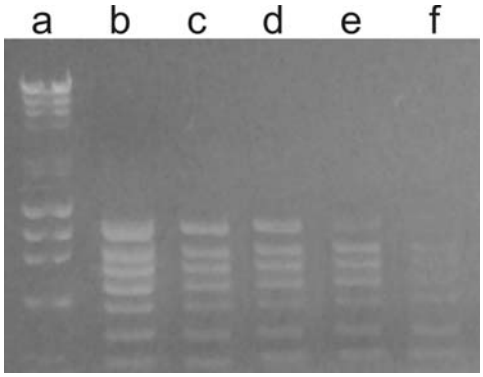

Fig. 2. Detection limit of heptaplex PCR assay from washed overnight culture *E. coli* O157:H7. (a) DNA ladder λDNA – Hind*III* and ΦX174 DNA – Hae*III* (Finnzymes). Amplification with 32 (b), 16 (c), 8 (d), 4 (e), and 2 (f) CFU of *E. coli* O157:H7.

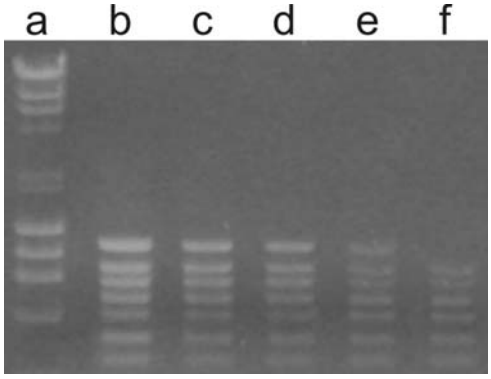

Fig. 3. Detection limit of heptaplex PCR assay from washed*E. coli* O157:H7 cell suspension mixed with milk. (a) DNA ladder λDNA – Hind*III* and ΦX174 DNA – Hae*III* (Finnzymes). Amplification with 48 (b), 24 (c), 12 (d) 6 (e) and 3 (f) CFU of *E. coli* O157:H7.

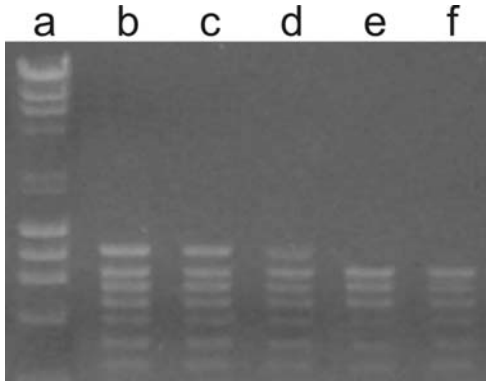

Fig. 4. Detection limit of heptaplex PCR assay from washed*E. coli* O157:H7 cell suspension mixed with meat. (a) DNA ladder λDNA – Hind*III* and ΦX174 DNA – Hae*III* (Finnzymes). Amplification with 32 (b), 16 (c), 8 (d), 4 (e), and 2 (f) CFU of *E. coli* O157:H7.

0.5-times (to 6 cells) and twice (to 8 cells), respectively.

Discussion

This work focuses on the serotype *E. coli* O157:H7, which causes severe disease worldwide. Three major virulence factors include: (i) Shiga toxins (Stx1 and Stx2); (ii) products of the pathogenicity island called the "locus of enterocyte effacement" (EaeA); and (iii) products of the F-like plasmid pO157 (HlyA, EspP). This pathogen survives well in diverse environments, from its silent reservoir in healthy cattle to the farm environment (Lim et al. 2010).

PCR assays in this work as mono- or heptaplex format were designed to cover the detection of genes from all the three above-mentioned virulence factors and detection of *hlyA* and *fliC* (H7) genes. The reason for this design was that every isolate of *E. coli* O157:H7 from veterinary, clinical or environmental samples could differ from each other in composition of its virulence genes. For example, Stx is a potent cytotoxin and is bacteriophage-encoded (Jacewicz et al. 1999). Stxs can be divided into two groups called Stx1 and Stx2. Stx1 is identical to Stx from *Shigella dysenteriae* I, except for a single amino acid residue. Virulent isolates of *E. coli* O157:H7 can express Stx1 only, Stx2 only, or both toxins. Strains with Stx2 are known to be more toxic than Stx1 strains (Boerlin et al. 1999). Moreover, these genes were used for detection of *E. coli* O157:H7 with mostly DNA used as template (Schmidt et al. 1999).

Detection limit of 10^2 CFU/mL pure culture was achieved (Si et al. 2007) by using one of the virulence genes from the locus of enterocyte effacement, *eaeA*, coding for the protein intimin (Beebakhee et al. 1992). Certain oligonucleotide primers exhibiting a similarity to the 3' end of the *E. coli* O157:H7 *eaeA* gene are quite specific for this organism in PCR assays (Gannon et al. 1993).

EspP is the pO157-encoded type V secreted serine protease and is known to cleave pepsin A (Brunder et al. 1997). Recently, Dziva et al. (2007) reported that EspP influences the intestinal colonization of calves and adherence to bovine primary intestinal epithelial cells. This gene together with *hlyA* was used in characterization of *E. coli* O157:H$^-$ strains (Schmidt et al. 1999).

To provide simultaneous identification of *E. coli* O157:H7, we have used also primers targeted to a portion of the *fliC* gene encoding the H7 flagellar antigen. The *fliC* gene of *E. coli* O55:H7 appears to be most closely related to that found in non-motile *E. coli* O157:H7 strains (Gannon et al. 1997).

It can be concluded that the PCR assays described in this study are appropriate for detecting *E. coli* O157:H7 right from food samples prepared only by simple washing steps, and the obtained bacterial cell suspension can be used directly to PCR. Multiplex PCR in this study was designed to cover typical genes from all known virulence factors classes of *E. coli* serotype O157:H7. Sensitivity of this method was found to be high. Jothikumar & Griffiths (2002) published a rapid multiplex PCR method, which allowed to detect $\geq 10^3$ CFU/mL in pure culture. However, in our work a higher sensitivity to detect *E. coli* O157:H7 was achieved not only in pure culture, but also in samples with milk and meat matrix. Since the preparation of sample for this assay is based on centrifugation, assay is independent from volume of the starting sample, i.e. the needed

amount of cells could be obtained from larger volume of milk or homogenized meat sample. Moreover, PCR is less labour-intensive and more rapid than cultivation and conventional methods of bacterial identification. Finally, the PCR assays presented here are cheaper in comparison with quantitative PCR assays.

Acknowledgements

This work was supported by funding from the Slovak Research and Development Agency (grant No. VMSP-P-0111-09). We are grateful to Mária Šuleková for her expert technical assistance.

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Received December 10, 2010
Accepted March 1, 2011