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Multiplex real-time PCRs for detection of *Salmonella*, *Listeria monocytogenes*, and verotoxigenic *Escherichia coli* in carcasses of slaughtered animals

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Abstract

Introduction: The study objective was to develop and evaluate a new TaqMan multiplex real-time PCR method for *Salmonella*, *L. monocytogenes*, and verotoxigenic *Escherichia coli* (VTEC) detection in slaughtered animal carcasses. **Material and Methods:** The procedure included an enrichment step, DNA extraction, and two multiplex real-time PCRs. The first PCR detected the *invA* and *hly* genes of *Salmonella* and *L. monocytogenes* respectively, the second the *vtx1*, *vtx2*, and *eae* genes of VTEC. **Results:** The validation of this method resulted in 100% relative sensitivity, specificity, and accuracy as compared to the reference ISO methods. The limit of detection per swab sample was established at 1 cfu for *Salmonella* and *L. monocytogenes* and 2 cfu for VTEC. The authors analysed 265 slaughterhouse-collected swabs from cattle, pig, and poultry carcasses. Among 125 from cattle, 51 were positive for VTEC, 29 for *Salmonella*, and 1 for *L. monocytogenes*. Among swabs from pig carcasses (n = 95), three, two, and one sample were positive for these pathogens respectively. None of the microorganisms tested for was identified in 45 samples of poultry origin. **Conclusion:** The obtained results showed that the method developed can rapidly identify the main bacterial pathogens that may contaminate carcasses of food-producing animals.

Keywords: carcasses, foodborne pathogens, multiplex real-time PCRs, identification, food safety.

Introduction

Salmonella, *Listeria monocytogenes*, and verotoxigenic *Escherichia coli* (VTEC) have emerged as significant foodborne microorganisms and continue to be dangerous pathogens for human health (4, 22). In 2014, a total of 96,831 confirmed cases of *Salmonella*, *L. monocytogenes*, and VTEC human infections were reported in the European Union, with incidences of 23.4, 0.52, and 1.56 cases per 100,000 population, respectively (4). Contamination of meat which occurs during slaughter and processing exposes consumers to a major risk of foodborne diseases (23). Thus, microbiological control at the slaughterhouse stage is highly important. Consequently, availability of rapid and specific methods for detection of foodborne microorganisms is essential. The ISO standards for *Salmonella*, *L. monocytogenes*, and VTEC O157 detection involve sample culture in pre-enrichment and selective media, followed by confirmation of bacterial isolates according to their morphological, biochemical, and immunological characteristics (10–13). These methods, although reliable and relatively inexpensive, are laborious and require several days before the analysis is completed (5). Furthermore, the obtained results often are not available before the food has been either released for trading or consumed, which may result in the spread of pathogens (15). To overcome these limitations of the ISO standards, several alternative detection methods have been developed, including those based on real-time PCR due to its sensitivity and specificity (18). Moreover, real-time PCR based on the use of specific primers and differently labelled probes allows for detection of more than one microorganism in a single reaction (30). Several studies have reported the application of

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multiplex real-time PCR for detection of *Salmonella*, *L. monocytogenes*, and *E. coli* O157, but to our knowledge, there have been no investigations including VTEC as a group of pathogenic *E. coli* (6, 7, 16, 24, 27).

The aim of the present study was to develop and evaluate a new, effective TaqMan multiplex real-time PCR method for detection of *Salmonella*, *L. monocytogenes*, and VTEC in carcasses of slaughtered animals. The method consists of an initial enrichment step in non selective medium followed by DNA extraction and two real-time PCRs targeting the genes *invA* for *Salmonella*, *hly* for *L. monocytogenes* and *stx1*, *stx2* and *eae* for VTEC An internal amplification control (IAC) was also included to monitor the possible presence of PCR inhibitors.

Material and Methods

Bacterial strains. Bacterial strains from the collection of the Department of Hygiene of Food of Animal Origin or obtained from the American Type Culture Collection were used (Tables 1 and 2). The bacteria were stored at −80°C, and for the study *Salmonella* and VTEC were grown on Lab-Agar (Biocorp, Poland), while *L. monocytogenes* was cultured on Tryptone Soya Yeast Extract Agar (Oxoid, UK) at 37ºC for 18–24 h. All other bacteria were grown according to their individual requirements as described by the supplier.

DNA extraction. Bacterial DNA from the strains and swab samples was extracted with a Genomic Mini kit (A&A Biotechnology, Poland) under the manufacturer's protocol. The original instruction was modified regarding the incubation time with lysostaphin (Sigma-Aldrich, USA) which was increased to 30 min. In the initial step, 1 mL of the bacterial suspension was centrifuged at 13,000 g for 3 min and the cell pellet was used for DNA extraction.

Primers and probes for multiplex real-time PCRs. The following genes were selected for identification of the target microorganisms: *invA* (*Salmonella*), *hly* (*L. monocytogenes*), and *stx1*, *stx2*, and *eae* (VTEC). The primer and probe sequences (Table 3) were commercially designed by Blirt S.A. (Poland) using the NCBI Gene, CLC DNA Workbench 6, Beacon Designer Online Edition, NCBI Nucleotide Blast, and NCBI Primer Blast software applications. The oligonucleotides were provided by Metabion International AG (Germany).

Specificity of primers and probes. The reference bacterial strains (Tables 1 and 2) were used to test the specificity of the designed primers and probes in two real-time PCRs: one for *Salmonella* and *L. monocytogenes* and the other for VTEC. A total of 57 strains, including 41 target and 16 non-target bacteria, were used for validation of the real-time PCR for *Salmonella* and *L. monocytogenes*, and for VTEC amplifications of DNA from 53 strains (34 VTEC and 19 non-VTEC) were performed. In both reactions IAC was included to monitor a possible inhibition of PCR (Table 3).

Multiplex real-time PCRs. PCR amplification of the *Salmonella*- and *L. monocytogenes*-specific genes was performed in a final volume of 25 µL, containing 12.5 µL of iQ Multiplex Powermix (Bio-Rad, USA), 300 nM of each specific primer, 200 nM of Ssinv and LMhly probes, 160 nM and 80 nM of IAC primers and probe respectively, 7×10^3 copies of IAC DNA, and 2.5 µL of target DNA. In the PCR for VTEC the same primer and probe concentrations were used, with the IAC primers and probe at 240 nM and 120 nM respectively and 14×10^3 copies of IAC DNA. All amplifications were carried out in a CFX 96 Real-Time PCR Detection System (Bio-Rad) using the following thermal profile: 10 min at 95°C, followed by 35 cycles of denaturation at 95°C for 15 s and 1 min at 60°C for the annealing/extension step.

Estimation of relative sensitivity, specificity, and accuracy. To estimate the relative sensitivity (SE), specificity (SP), and accuracy (AC) of the method, 10 blind and 20 spiked swab samples were analysed using multiplex real-time PCRs and the standard culture methods for *Salmonella*, *L. monocytogenes*, and *E. coli* O157 (10–13). The inoculation of sponges (Whirl-Pak Speci-Sponge bag, Nasco, USA) was done with low (10 cfu/swab, 10 samples) and high (100 cfu/swab, 10 samples) concentrations of the pathogens and in the presence of background microflora (*E. coli*, *L. welshimerii*, and *P. aeruginosa*, 50 cfu per sample of each). Then 100 mL of tryptone soya broth (TSB) (Oxoid) was added to each sample, which was subsequently stomached and incubated at 37ºC for 24 h. DNA was extracted and used in real-time PCRs. The parameters (SE, SP, AC) were calculated for each individual microorganism using the formulas specified in the ISO 16140:2003 standard (14).

Limit of detection. To determine the limit of detection (LOD), 30 sponges were inoculated with a low level of *Salmonella*, *L. monocytogenes*, and VTEC (10 samples with one cfu, 10 samples with two cfu, and 10 samples with three cfu) as well as spiked with background microflora (*E. coli*, *L. welshimerii*, and *P. aeruginosa*, 50 cfu per sample of each). Then 100 mL of TSB was added, and the samples were homogenised, and incubated at 37ºC for 24 h. After DNA extraction, two multiplex real-time PCRs for *Salmonella*, *L. monocytogenes*, and VTEC were performed. The limit of detection was determined for each individual microorganism and it was assumed as the level at which 70% of positive results were achieved.

Analysis of natural samples. Slaughterhouse visits collected 265 swabs from cattle (n = 125), pig (n = 95) and poultry (n = 45) carcasses and these were examined. Cattle and pig carcasses were swabbed with four sterile sponges in the brisket area (400 cm^2) after

evisceration of the animals. In the case of poultry carcasses, the skin surface was wiped with sterile swabs directly before chilling. All samples were transported to the laboratory in an ice-pack container and analysed within 24 h. The cattle and pig sponges received 160 mL of TBS and 10 mL was added to the poultry samples, all then being stomached and incubated at 37°C for 24 h. DNA was extracted and used in real-time PCRs as described above.

Table 1. Bacterial strains used in development and validation of real-time PCR for *Salmonella* and *L. monocytogenes*

Strain	Presence of genes		Strain	Presence of genes	
	invA	*hly*		*invA*	*hly*
Salmonella Anatum ATCC 9270	+		*L. monocytogenes* 3c (id. 04CEB717LM)	−	+
Salmonella Arizonae ATCC 13314	+	−	*Listeria ivanovii* ATCC 19119	−	−
Salmonella Enteritidis ATCC 13076	+	−	*Listeria innocua* ATCC 33090	−	−
Salmonella Paratyphi A ATCC 9150	+	−	*Listeria seeligeri* ATCC 35967	−	−
Salmonella Typhimurium ATCC 14028	+	−	*Listeria welshimeri* ATCC 35897	−	−
Salmonella Schleissheim (id. 1, 2, 3, 4, 5, 6)	+	−	*Campylobacter coli* ATCC 43478	−	−
Salmonella Typhimurium (id. 7)	+	−	*Campylobacter jejuni* ATCC 33560	−	−
Salmonella Enteritidis (id. 8, 9, 12, 13, 14, 15)	+	−	*Clostridium perfringens* ATCC 13124	−	−
Salmonella Dublin (id. 10, 11, 16, 17)	+	−	*Escherichia coli* ATCC 43888	−	−
Salmonella London (id. 18)	+	−	*Escherichia coli* ATCC 8739	−	−
Listeria monocytogenes ATCC 19118	−	+	*Escherichia coli* ATCC 43889	−	−
L. monocytogenes 1/2a (id. 2, 3, 1M, 05CEB424LM)	−	+	*Escherichia coli* ATCC 25922	−	−
L. monocytogenes 1/2b (id. 9, 34M, 42M, 06CEB406LM)	−	+	*Kocuria rhizophila* ATCC 9341	−	−
L. monocytogenes 1/2c (id. 1, 7M, 06CEB405LM)	−	+	*Pseudomonas aeruginosa* ATCC 27853	−	−
L. monocytogenes 4b (id. 22, 76M, 06CEB422LM)	−	+	*Rhodococcus equi* ATCC 6939	−	−
L. monocytogenes 3a (id. 06CEB36LM)	−	+	*Staphylococcus aureus* ATCC 25923	−	−
L. monocytogenes 3b (id. 06CEB712LM)	−	+	*Vibrio parahaemolyticus* ATCC 17802	−	−

Table 2. Bacterial strains used in development and validation of real-time PCR for VTEC

Strain	Presence of genes			Strain	Presence of genes		
	stx1	*stx2*	*eae*		*stx1*	*stx2*	*eae*
VTEC O26 (id. G08)	−	−	+	VTEC O146 (id. 560)	+	+	−
VTEC O91 (id. F08)	+	−	−	VTEC O157:H7 ATCC 43888	−	+	−
VTEC O103 (id. E08, 697, 3160)	+	−	+	VTEC O157:H7 ATCC 43889	−	+	+
VTEC O111 (id. C08)	+	+	+	*Escherichia coli* ATCC 8739	−	−	−
VTEC O121 (id. B08, 5833, 9900)	−	+	+	*Escherichia coli* ATCC 25922	−	−	−
VTEC O145 (id. A08)	+	−	+	*Campylobacter coli* ATCC 43478	−	−	−
VTEC O157 (id. D08, 251, 1098, 5346)	−	+	+	*Campylobacter jejuni* ATCC 33560	−	−	−
VTEC O84:H28 (id. 13)	+	−	+	*Clostridium perfringens* ATCC 13124	−	−	−
VTEC O2:H32 (id. 17)	−	+	−	*Listeria innocua* ATCC 33090	−	−	−
VTEC O153:H2 (id. 21)	−	+	−	*Listeria ivanovii* ATCC 19119	−	−	−
VTEC O21:H25 (id. 23, 27)	−	+	−	*Listeria seeligeri* ATCC 35967	−	−	−
VTEC O153:H25 (id. 26)	−	+	−	*Listeria welshimeri* ATCC 35897	−	−	−
VTEC O117:H4 (id. 42)	−	+	−	*Kocuria rhizophila* ATCC 9341	−	−	−
VTEC O139:H19 (id. 44)	−	+	−	*Pseudomonas aeruginosa* ATCC 27853	−	−	−
VTEC O174:H2 (id. 45, 46)	−	+	−	*Rhodococcus equi* ATCC 6939	−	−	−
VTEC O185:H7 (id. 47)	−	+	−	*Staphylococcus aureus* ATCC 25923	−	−	−
VTEC O2:H6 (id. 50)	−	+	−	*Vibrio parahaemolyticus* ATCC 17802	−	−	−
VTEC O113:H21 (id. 57)	−	+	−	*Salmonella* Anatum ATCC 9270	−	−	−
VTEC O8:H16 (id. 60)	−	+	−	*Salmonella* Arizonae ATCC 13314	−	−	−
VTEC O152:H8 (id. 61)	+	−	−	*Salmonella* Enteritidis ATCC 13076	−	−	−
VTEC O26 (id. 9440)	−	+	+	*Salmonella* Paratyphi A ATCC 9150	−	−	−
VTEC O111 (id. 6030)	+	−	+	*Salmonella* Typhimurium ATCC 14028	−	−	−

Table 3. Primers, probes, and IAC for *Salmonella*, *L. monocytogenes*, and VTEC used for multiplex real-time PCRs

Microorganism	Target gene	Primers and probes	Sequence (5'→3')
		Ssinv-probe	6-FAM-CCTGAATTACTGATTCTGGTACTAATGGTGATG-BHQ1
Salmonella	*invA*	SS-F	GCTTTCTCTACTTAACAGTGCTC
		SS-R	CAGTGCGATCAGGAAATCAAC
		LMhly-probe	HEX-CACTCTGGAGGATACGTTGCTCAATTCAAC-BHQ1
L. monocytogenes	*hly*	LM-F	CAACTCAGAATATATTGAAACAACTTC
		LM-R	ACAATTTCGTTACCTTCAGGATC
		stx1-probe	6-FAM-CGCATAGTGGAACCTCACTGACGCA-BHQ1
	stx1	stx1-F	CATTCGTTGACTACTTCTTATCTGG
		stx1-R	ACGTAAAGCTTCAGCTGTCAC
		stx2-probe	HEX-ACCGTTACTGCAAAGTGCTCAGTTGACAG-BHQ1
VTEC	*stx2*	stx2-F	GACACATTTACAGTGAAGGTTGAC
		stx2-R	CAGGTACTGGATTTGATTGTGAC
		eae-probe	TXR-AGTCAGTTTATTTGTATGACCAGCGACACC-BHQ2
	eae	eae-F	GTTCGGCACCTCTTGTTGC
		eae-R	CCTTGTCATCGGTCATGTTGC
		IAC-probe	Cy5-CATCATGCGATGTCTGTTGCTTCTAATCCA-BHQ3
IAC	-	IAC-F	GGAGACCACAACGGTTTCC
		IAC-R	TAAGAAATCAGATGGATTTGGACC

Results

All *Salmonella* (n = 23) reference strains were positive for the *invA* gene, as were those of *L. monocytogenes* (n = 18) for *hly*, and of VTEC (n = 34) for *stx1*, *stx2*, and *eae*, showing the specificity of the primers and probes used. The obtained threshold cycle (Ct) values in multiplex reactions ranged from 15.5 to 20.1 for *Salmonella*, from 19.5 to 22.5 for *L. monocytogenes*, and from 16.4 to 26.0 for VTEC. All non-target strains used were negative for all these gene markers. The validation of the developed method involved determination of the SE, SP, and AC. The parameters were calculated for each individual pathogen based on the results of the analysis of 10 blind and 20 artificially inoculated swab samples with *Salmonella, L. monocytogenes*, and *E. coli* O157 by multiplex real-time PCRs and reference methods. The obtained PCR results showed SE, SP, and AC at 100% levels compared to the ISO standards. The LOD was established at one cfu per swab for *Salmonella* (90% positive results with Ct range from 23.75 to 27.37) and *L. monocytogenes* (70% positive results with Ct from 27.91 to 31.10), and two cfu for VTEC (80% positive results, Ct from 20.72 to 23.35). The method developed was used to analyse 265 swabs collected at slaughterhouses from cattle, pig, and poultry carcasses. Among 125 samples from cattle, 51 were positive for VTEC (Ct 20.06–32.17), 29 for *Salmonella* (Ct 22.28–32.03), and 1 for *L. monocytogenes* (Ct 29.89). Three (Ct 30.40–30.94), two (Ct 31.07–32.07), and one (Ct 29.46) swab from pigs (n = 95) were also positive for these pathogens respectively. None of the microorganisms tested for was detected in 45 samples from poultry carcasses.

Discussion

Salmonella, *L. monocytogenes*, and VTEC are still recognised as the most dangerous foodborne bacterial pathogens (4). In the present study, multiplex real-time PCRs for simultaneous detection of these microorganisms were developed and evaluated. The method involved an initial enrichment step in TSB followed by DNA extraction, and two TaqMan based real-time PCRs for the *invA*, *hly*, *stx1*, *stx2*, and *eae* target genes. In the first stage of the study the nonselective medium was used to allow simultaneous growth of the pathogens and thereby to improve the efficiency of the detection process. Such media as TSB, buffered peptone water (BWP), No. 17 medium, and universal pre-enrichment broth (UPB) were also used in multiplex PCR based methods by other authors (1, 3, 8, 16). However, some studies reported that nonselective media may cause false negative results, especially by interference with complex background flora present in analysed samples (19). Therefore, the use of selective enrichment broth for simultaneous cultivation of multiple pathogens was also described (28), but it should be underlined that such media may inhibit or delay the growth of target microorganisms (31). Moreover, in the present study, one enrichment step was used for Gram-positive and Gram-negative microorganisms, whereas some authors have reported that detection of *L. monocytogenes* with PCR method is less sensitive and have recommended two enrichment steps (21). The obtained results demonstrated that the use of TSB was suitable for simultaneous growth of *Salmonella*, VTEC, and *L. monocytogenes*. The LOD was established at one cfu per swab for *Salmonella* and *L. monocytogenes* and two cfu for VTEC. Background

microflora present in the analysed samples (*E. coli*, *L. welshimerii*, and *P. aeruginosa)* did not significantly interfere with the LOD, even when the samples were inoculated with the target pathogens at low levels. It was reported that the DNA isolation procedure had a strong effect on the efficiency of the detection process in real-time multiplex PCR (16). It is very important that the protocol used successfully eliminate inhibitors that can affect the PCR results by lowering or completely preventing the amplification (20). The effectiveness of this process could be verified by the Ct values obtained for the IAC. In the current study, no remarkable alterations were detected when natural or contaminated samples were analysed.

The most important stage of the present study was to design the primers and probes used in two real-time PCRs, which determine the reliability of the developed method. This step is especially important during the development of multiplex assays, where the presence of more than one primer pair and probe increases the probability of nonspecific interactions (26). The pathogen-specific oligonucleotides were selected to target the genes *invA* for *Salmonella*, *hly* for *L. monocytogenes*, and *stx1*, *stx2*, and *eae* for VTEC, which were previously described as optimal markers for these bacteria. The *invA* marker contains a highly conserved sequence unique for *Salmonella* DNA and its amplification is considered the international gold standard for detection of this pathogen (9). Several virulence-associated markers have been proposed for *L. monocytogenes* identification, but the *hly* gene, which encodes listeriolysin O, is one of the most frequently used (17, 29). Regarding VTEC, the virulence markers *stx1*, *stx2*, and *eae* targeted in the present study encode Shiga toxins and adhesion protein intimin respectively (2). VTEC detection based on these genes has previously been documented well (25). The specificity of primers and probes used in the present study was confirmed with 110 bacterial strains including 75 target and 35 non-target strains. Moreover the results of validation of the method developed showed a 100% SE, SP, and AC as compared to the reference methods. The usefulness of the protocol developed was determined with natural samples from 265 slaughterhouse carcasses. Among them 54 were positive for VTEC, 31 for *Salmonella*, and 2 for *L. monocytogenes*. IAC gave positive results for all analysed samples, proving that there was no matrix inhibition.

In conclusion, a new multiplex real-time PCR method was developed for detection of *Salmonella*, *L. monocytogenes*, and VTEC in carcasses of slaughtered animals. The assay showed a high SE, SP, and AC, and achieved a low LOD. The method may be a valuable alternative to the ISO standard investigations and allows rapid and specific detection of foodborne pathogens in animal carcasses before their commercial distribution. In the future, the evaluation of this assay for other food matrices may also extend its application in food safety analyses.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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