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Real-time PCR methods for detecting *Salmonella* spp. in food after different DNA extraction procedures

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Abstract. The aim of this paper was to evaluate two real-time PCR (qPCR) protocols for the detection of *Salmonella* spp. in minced meat and chicken neck skin, after DNA extraction using the *InstaTM Gene matrix* (BioRad, USA) and DNA extraction based on thermal cell lysis. The applied molecular methods were sensitive and specific for the rapid detection of *Salmonella* spp. in minced meat and chicken neck skin. The qualitative results were identical regardless of the applied DNA extraction or qPCR protocols. Lower Cq values were achieved after DNA extraction using the *InstaTM Gene matrix*.

1. Introduction

Salmonella species are one of the main foodborne pathogens [12]. The most common sources of human infections are food products of animal origin, especially pork and poultry meat [3,4,5,6,7]. In the European Union, 91,662 cases of salmonellosis were confirmed during 2017 [14]. In Serbia, 1,850 cases of salmonellosis were diagnosed during 2017, which is 16.4% more cases than in 2016 [13]. The standard method requires at least four days for the detection of *Salmonella* spp. in food. Modern food microbiology demands the implementation of faster methods for the detection of *Salmonella* spp. [2,8]. The qPCR method meets this requirement, but it is still relatively more expensive than the cultural method. The aims of this study were to:

1. Evaluate a modified qPCR protocol for the detection of the *invasion gene* (*inv A*) [9] and the *tetrathionate respiration gene* (*ttr*) [11] *Salmonella* spp. in minced meat and chicken neck skin samples and to compare results with the reference method [17].
2. Compare two DNA extraction procedures and determine the effect of using different volumes of BPW pre-enrichments for the DNA extractions.

2. Materials and methods

2.1. Type of samples

A total of 154 samples (Table 1 and 2) were examined for the presence of *Salmonella* spp. using qPCR methods for detecting the inv A and *ttr* genes of *Salmonella* spp. with parallel testing using the reference method [17].

Table 1. Examined food samples

Food category	Natural samples	Artificially contaminated samples
Chicken neck skin	50	30
Minced meat	74	0
Total	**124**	**30**

Chicken neck skin samples were artificially contaminated with a reference strain of *S.* Typhimurium (ATCC 14028) at two contamination levels (1-10 and 10-100 cfu per 25 g of sample). Uninoculated samples were used as negative controls.

2.2. Isolation of Salmonella spp.
The cultural detection of *Salmonella* spp. was conducted using the reference method [17].

2.3. DNA extraction
After the sample pre-enrichment in Buffered Peptone Water (Oxoid, UK) for 16-20 h at 34-38 °C, two DNA extraction procedures were applied: DNA extraction based on thermal cell lysis (TL) and DNA extraction using the *InstaTM Gene matrix* (IGM) (BioRad, USA) as we described in our previously published paper [1].

The detection of *Salmonella* spp. was also performed after DNA extraction of pooled pre-enriched test portions obtained by mixing 200 and 300 µL of pre-enrichment of naturally contaminated samples with 800 µL and 1200 µL of pre-enrichment in which *Salmonella* spp. was not detected. The PCR was performed with the addition of 2 or 4 µL of extracted DNA.

2.4. Real-time PCR methods
The detection of *inv A* (Protocol *invA*) [9] and *ttr* genes in *Salmonella* spp. (Protocol *ttr*) [11] was performed with the modifications described in our previously published study [1].

2.5. Terms and Statistical Analysis
The obtained Cq values were analysed by t-test in *Excel* (Microsoft Corporation, USA). The comparison and interpretation of the results (Table 2) between the reference and alternative methods were conducted in accordance with the ISO 16140 [10].

3. Results and discussion

In the presented study, two non-patented qPCR protocols after two different DNA extraction procedures were compared with the reference method [17] for the detection of *Salmonella* spp. Additionally, genomic DNA of *Salmonella* spp. was detected after DNA extraction of pooled pre-enriched test portions. The qualitative results of this study were identical regardless of the applied DNA extraction procedure or the qPCR protocol for the detection *Salmonella* spp. in chicken neck skin and minced meat samples (Table 1). No false negative results were detected. The relative trueness and the sensitivity for both the alternative and reference methods are summarized in Table 2. The results were compared to those of the reference method for a total of 154 naturally or artificially contaminated chicken neck skin and minced meat samples [17].

Table 2. Comparison of gene detection results between the reference and alternative methods

Protocol	No of samples	Alternative method	Reference method		SE_{alt}	SE_{ref}	RT
			R+	R-			
invA	154	**A+**	PA = 35	PD = 0	100 %	100 %	100 %

ttr	154	**A -**	ND = 0	NA = 119	100 %	100 %	100 %
		A+	PA = 35	PD = 0			
		A -	ND = 0	NA = 119			

Legend: Alternative method: positive (A+) / negative (A -); Reference method: positive (R+) / negative (R-); Positive Agreement (PA); Positive Deviation (PD); Negative Deviation (ND); Negative Agreement (NA); Sensitivity - alternative method (SE_{alt}); Sensitivity - reference method (SE_{alt}); Relative trueness (RT).

PCR detection of *Salmonella* genes in the artificially inoculated chicken neck skin demonstrated that the best Cq values (the lowest Cq) were obtained using the qPCR protocol for the detection of *ttr* gene, after DNA extraction by IGM (Table 3).

Table 3. Cq values obtained after testing the artificially inoculated chicken neck skin samples

No of samples	**Contamination level CFU/25 g**	**qPCR**			
		Protocol *invA*		**Protocol *ttr***	
		IGM	**TL**	**IGM**	**TL**
5	10-100	17.86	22.25	15.21	19.26
20	1-10	19.14	22.39	17.07	20.18
5	0	No C_q	No C_q	No C_q	No C_q

The detection of *Salmonella* spp. genes in 10 minced meat (pork) samples after DNA extraction in pooled pre-enriched test portions (Table 4) showed an expected impact on the Cq values. By comparing the Cq values after the IGM extraction from 1 ml of BPW with the extraction from 200 and 300 µl of BPW, with the addition of 2 µl of the template, or from 300 µl of BPW with the addition of 4 µl template, the following p values were obtained: 0.004, 0.0185 and 0.4884, respectively. By comparing the Cq values after TL extraction from 1 ml of BPW with the extraction from 200 and 300 µl of BPW, with the addition of 2 µl template, or from 300 µl of BPW with the addition of 4 µl template, the following p values were obtained: 0.0075, 0.0673 and 0.2380, respectively.

Table 4. Cq values obtained after testing the naturally contaminated minced meat (pork, n=10) using the *ttr* protocol, after IGM or TL extraction from different pre-enrichment volumes

Volume of the DNA used as template (µl)		**2**	**2**	**2**	**4**
Volume of the BPW used for DNA Extraction (µl)		**1000**	**200**	**300**	**300**
Mean Cq ± SD	**IGM**	24.52 ± 1.26	26.49 ± 1.40	25.98 ± 1.27	24.93 ± 1.33
	TL	25.14 ± 1.54	27.46 ± 1.89	26.63 ± 1.87	26.06 ± 1.82

Statistical analysis of the Cq values obtained after both extraction procedures showed that after extraction from 300 µl of pre-enrichment, using 4 µl DNA as a template, the results were identical to those obtained after extraction from 1 ml of pre-enrichment with the addition of 2 µl of DNA. Extraction from pooled samples could reduce the cost of a PCR method several times, but for routine application, it is necessary to carry out a validation study in accordance with some of the internationally accepted protocols [10] or implement the procedure defined by the standard for sample preparation [15,16].

The applied molecular methods are confirmed as being sensitive and specific for the rapid detection of *Salmonella* spp. in minced meat and chicken neck skin. The duration of analysis for the qPCR methods is approximately 24 h, in contrast to 4-5 days for the reference method [17]. These methods could be used as screening methods, but the reference method remains irreplaceable for confirmatory purposes.

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