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Development of a novel *invA* gene-based real-time PCR assay for the detection of *Salmonella* in food

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Abstract: In this study, the primers and the probe were designed, and a completely new real-time polymerase chain reaction (PCR) protocol for detecting the *invA* gene of *Salmonella* in food was optimised and validated in-house. The inclusivity test used 76 different *Salmonella* isolates with no false-negative results. The exclusivity was tested using 45 non-*Salmonella* microorganisms with no false-positive results. The method was also successfully applied while examining five different artificially contaminated food categories. The results were compared to the standard method (ISO 6579-1) and two previously validated real-time PCR methods. The developed assay is sensitive and specific for rapidly detecting *Salmonella* in food.

Keywords: molecular detection; food safety; validation; pathogen; artificial contamination

Despite numerous eradication programs, *Salmonella* is one of the leading foodborne pathogens (EFSA 2019). The standard method for *Salmonella* detection in food is time-consuming, so the modern food industry and public healthcare demand the development of rapid methods for detecting this pathogen (Law et al. 2014; Dmitric et al. 2019). Several alternative methods, including real-time polymerase chain reaction (qPCR) protocols, have been developed to detect *Salmonella* in food (Law et al. 2014). For routine testing, these methods must be validated by ISO 16140 or other internationally accepted similar protocols. The real-time

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PCR protocols applied in this study are based on the specific detection of the *invA* and *ttr* genes in *Salmonella*. The invasion gene, *invA*, is located within the *Salmonella* pathogenicity island 1, and *Salmonella* needs to enter the host's intestinal epithelial cells, which is a crucial step in the pathogenesis (Garrido et al. 2013). The *ttr* locus is located in pathogenicity island 2 and encodes the tetrathionate reductase involved in tetrathionate respiration. This ability is important under anaerobic growth conditions since it confers opportunities to outgrow the fermenting commensal competitors (Fàbrega and Vila 2013).

The aims of this study were: *i*) designing, optimising, and validating in-house a new real-time PCR protocol for detecting the *invA* gene and comparing its efficiency with the standard method (ISO 2017) for *Salmonella* detection in food; *ii*) comparison of the novel real-time PCR protocol with validated real-time PCR protocols for detecting the *invA* (Anderson et al. 2011) and *ttr* genes in *Salmonella* (Malorny et al. 2004).

MATERIAL AND METHODS

Type of samples. A total of 150 samples divided into 5 categories (ćevapi – minced meat preparation, poultry meat, raspberries, milk, chicken neck skin) were tested.

Bacterial strains used for inclusivity and exclusivity tests. While validating the new protocol for detecting the *invA* gene in *Salmonella* in food, inclusivity checks were performed using 76 serologically or genotypically distinct *Salmonella* isolates. The genomic DNA of 45 non-*Salmonella* microorganisms was used during the exclusivity check.

Proficiency test (PT) material. After optimisation of the real-time PCR method for the detection of *Salmonella*, verification through participation in Proficiency testing schemes organised by internationally recognised institutions accredited for this activity was performed [Provider 1: Poultry samples – Vetqas, APHA Scientific, UK; Provider 2: Oatmeal sample – Food Microbiology (QMS), LGC Standards, UK].

Artificial contamination. The artificial contamination was based on a previously described procedure (Dmitric et al. 2018). Briefly, the reference strain of *S. Enteritidis* (ATCC 13076) was used for artificial contamination of the samples, except for chicken neck skin samples contaminated with *S. Typhimurium* (ATCC 14028). The contamination level was 1–10 (20 samples) or 10–100 CFU (colony forming unit) in 25 g of the sample (5 samples). Five samples were used as negative controls.

Reference culture method. Microbiological isolation of *Salmonella* was performed by ISO 6579-1 (ISO 2017).

DNA extraction. After the pre-enrichment step in Buffered Peptone Water (Oxoid, UK) at 37 °C ± 1 °C for 18 h ± 2 h, DNA was extracted using the Chelex resin (Insta™ Gene matrix, BioRad, USA).

Real-time PCR methods. TaqMan real-time PCR methods targeting the *invA* (Protocol A) and *ttr* (Protocol M) genes for the detection of *Salmonella*, based on previously described procedures, were used (Dmitric et al. 2018). The newly developed assay (Protocol MD) for the detection of *Salmonella* using real-time PCR was conducted in a final volume of 25 µL containing 2× Brilliant III Ultra-Fast qPCR Master Mix (Agilent Technologies, USA), 500 nM of each primer, 200 nM probe, and 2 µL of DNA template. *Salmonella* probe (Probe – *invA* MD2) was labelled at the 5' end with reporter dye, 6-carboxyfluorescein (FAM), and with Dark Quencher at the 3' end (Table 1). Internal amplification control (IAC; DNA Extraction Control mix 610, Bioline, UK), primers and a probe for IAC were added to the reaction according to the manufacturer's instructions. Amplification conditions were: hot start at 95 °C for 3 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s. The results of fluorescence measurements were analysed using the real-time PCR instrumentation software (AriaMx Real-Time PCR System and Stratagene Mx3005P PCR System, Agilent Technologies, USA).

Interpretation of data and statistical analysis. The results of quantification cycle value (Cq values) were interpreted and statistically analysed in accord-

Table 1. Sequences of primers and TaqMan probe

Target	Description	Sequence (5'–3')	PCR product
invA gene	*invA* MD2.1_f (forward primer)	GTTCCTTTGACGGTGCGATG	179 bp
	invA MD2_r (reverse primer)	GATCTGGGCGACAAGACCAT	
	invA MD2_Pro (probe)	TCGGTGGGGATGACYCGCCA	

PCR – polymerase chain reaction; MD – Protocol MD (assay developed for this study)

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ance with the previously described procedure (Dmitric et al. 2018). Briefly, when an assay showed a Cq ≤ 39, the result was interpreted as positive. When an assay showed a Cq value ≥ 39 with IAC Cq ≤ 33, the result was interpreted as negative. When an assay showed a Cq value ≥ 39 with IAC Cq ≥ 33, the reaction was considered to have failed. To compare obtained Cq values, *t*-tests were performed in Microsoft Office 2010.

Primer and TaqMan probe design. Using the Primer 3 software (Rozen and Skaletsky 2000), primers and probe were designed to amplify the 179 bp long sequence of *invA* gene of *Salmonella* (Table 1).

Determination of the limit of detection (LOD) and amplification efficiency. The LOD was determined using the genomic DNA of *S. Enteritidis* (ATCC 13076), which was serially diluted to different concentrations after DNA extraction. The amplification efficiency was calculated using Equation 1:

$$e = \frac{10^{-1}}{s - 1} \quad (1)$$

where: s – the curve standard curve slope, using the real-time PCR instrument software (Agilent AriaMx Software v1.1).

RESULTS AND DISCUSSION

During this study, a new real-time PCR assay for detecting *Salmonella* in food was developed and optimised. The design of primers and the TaqMan probe was based on the published DNA sequence of the *invA* gene of *Salmonella*. The specificity of the oligonucleotide set must be verified during the development of the method, thus confirming that the method exclusively detects the target sequence (Broeders et al. 2014).

Theoretical test for specificity (in-silico test). The designed primers and probe were tested for specificity using the BLAST (Altschul et al. 1990) in the National Center for Biotechnology Information (NCBI) nucleotide database. The specificity of the primers and probe was compared to other published target gene sequences, and the absence of homology with the sequences of other microorganisms was confirmed.

Analytical specificity. The International Organization for Standardization (ISO) has published a standard that defines the performance characteristics of molecular methods, including verification of specificity, during which the method should be assessed against 50 isolates of the target microorganism (inclusivity). In addition, the method must be verified against non-target microorganisms, with taxonomically related and non-closely related microorganisms included (exclusivity). For this purpose, a minimum of 30 isolates should be tested (ISO 2011). The analytical specificity of the newly designed primers and probe was performed by DNA testing of 76 *Salmonella* isolates (Table 2) and DNA testing of 45 isolates of other genera (Table 3). The novel method successfully detected all *Salmonella* isolates. On the other hand, no DNA amplification from isolates not belonging to the genus *Salmonella* was registered, which experimentally confirmed this novel method's specificity.

The LOD and amplification efficiency. The analytical sensitivity of the PCR method refers to the minimum number of copies of the target DNA that can be reliably detected in a sample. It is usually expressed as the LOD and represents the concentration of target DNA that can be seen with a stated probability (95% probability is commonly used). The lowest LOD in ideal conditions, theoretically possible with a confidence level of 95%, is 3 copies per PCR (Bustin et al. 2009; Forootan et al. 2017). The sensitivity of the real-time PCR method for detecting the *invA* gene of *Salmonella* cell suspension was 10^3 CFU·mL^{-1} of pre-enrichment, corresponding to a concentration of 10 DNA copies per PCR reaction (Anderson et al. 2011). The sensitivity was comparable to the real-time PCR assay for detecting the *ttr* gene (Anderson et al. 2011; Dmitric et al. 2018). The LOD of the real-time PCR method (Protocol MD) was 10 copies per PCR, which met the requirement that $LOD_{95\%}$ of a qualitative real-time PCR method should not exceed 20 copies of the target sequence (Grohmann et al. 2016).

The characteristics of PCR can be determined from a standard curve based on a series of ten-fold DNA dilutions within the dynamic range of the method (Raymaekers et al. 2009). The dynamic range is the range over which an increase in starting material concentration results in a corresponding increase in the amplification product (TFS 2016). The standard curve determines the reaction characteristics, including slope and correlation coefficient. The correlation coefficient (R^2) is a measure of how well the data fit the standard curve. The value of R^2 actually reflects the linearity of the standard curve. Ideally, $R^2 = 1$, although 0.999 is generally the maximum value. The slope of the log-linear amplification phase measures the reaction efficiency. To obtain accurate and reproducible results, reactions must have efficiency as close to 100% (TFS 2016). The slope of linear regression

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Table 2. *Salmonella* isolates used for inclusivity test

Salmonella (origin/accession number)	Group/antigenic formula	Number of tested isolates	Results	
			positive	negative
S. Enteritidis (Lab)	O:9 (D1)	10	10	0
S. Typhimurium (Lab)	O:4 (B)	10	10	0
S. Enteritidis (ATCC 13076)	O:9 (D1)	1	1	0
S. Typhimurium (ATCC 14028)	O:4 (B)	1	1	0
S. Kentucky (Lab)	O:8 (C2-C3)	10	10	0
S. Montevideo (Lab)	O:54	1	1	0
S. Choleraesuis (Lab)	O:7 (C1)	1	1	0
S. Hadar (Lab)	O:8 (C2-C3)	1	1	0
S. Gallinarum (Lab)	O:9 (D1)	1	1	0
S. Kiel (Lab)	O:2 (A)	1	1	0
S. Nitra (Lab)	O:2 (A)	1	1	0
S. Eastbourne (Lab)	O:9 (D1)	1	1	0
S. Finkenwerder (Lab)	O:6,14 (H)	1	1	0
S. Glostrup (Lab)	O:8 (C2-C3)	1	1	0
S. Ahuza (Lab)	O:43 (U)	1	1	0
S. Bispebjerg (Lab)	O:4 (B)	1	1	0
S. Potsdam (Lab)	O:7 (C1)	1	1	0
S. Wagenia (Lab)	O:4 (B)	1	1	0
S. Brandenburg (Lab)	O:4 (B)	1	1	0
S. Bracknell (Lab)	O:13 (G)	1	1	0
S. Senftenberg (Lab)	O:1,3,19 (E4)	1	1	0
S. Agona (Lab)	O:4 (B)	1	1	0
S. Braenderup (Lab)	O:7 (C1)	1	1	0
S. Infantis (Lab)	O:7 (C1)	5	5	0
S. enterica subsp. *diarizonae* (Lab)	IIIb 50 : z_{10} : –	1	1	0
S. enterica subsp. *diarizonae* (Lab)	IIIb 17 : z_{10} : e, n, x, z_{15}	2	2	0
S. enterica subsp. *diarizonae* (Lab)	IIIb 17 : l, v : z	1	1	0
S. enterica subsp. *diarizonae* (Lab)	IIIb 50 : i : z	1	1	0
S. enterica subsp. *diarizonae* (Lab)	IIIb (6)14 : z_{10} : z	1	1	0
S. enterica subsp. *diarizonae* (Lab)	IIIb 14 : l, v : z53	1	1	0
S. spp. (Lab)	–	14	14	0
Total	–	76	76	0

ATCC – American Type Culture Collection, United States; Lab – Veterinary Specialized Institute Kraljevo

lines under ideal conditions is –3.3219 with a reaction efficiency of 100%, which means that the number of target molecules (template) is doubled after each PCR cycle (Raymaekers et al. 2009). The reaction must have an efficiency between 90 and 110%, corresponding to a slope between –3.58 and –3.10 (TFS 2016). When verifying the newly designed oligonucleotides, the average PCR efficiency calculated from the slope of the standard curve was 96.86%. The average correlation coefficient (R^2) value was 0.998, and the slope was 3.4. The results show that the method has an ideal performance.

Robustness determination. A very important requirement the assay must meet is robustness, enabling its implementation in other laboratories. Testing this characteristic involves subjecting the proposed method to small procedural changes to determine their effect on the method characteristics (ISO 2011).

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Table 3. Non-*Salmonella* genomic DNA used for exclusivity test

Microorganism	Result
Escherichia coli (ATCC 25922)	no Cq
Pseudomonas aeruginosa (ATCC 27853)	no Cq
Enterococcus faecalis (ATCC 29212)	no Cq
Geobacillus stearothermophilus (ATCC 7953)	no Cq
Proteus mirabilis (ATCC 25933)	no Cq
Enterobacter aerogenes (ATCC 13048)	no Cq
Sarcina lutea (ATCC 9341)	no Cq
Clostridium perfrigens (ATCC 13124)	no Cq
Escherichia coli (NCTC 13216)	no Cq
Escherichia coli (ATCC 8739)	no Cq
Listeria monocytogenes (ATCC 13932)	no Cq
Listeria innocua (ATCC 33090)	no Cq
Rhodococcus equi (ATCC 6939)	no Cq
Listeria ivanovii (ATCC 19119)	no Cq
Citrobacter freundii (ATCC 43864)	no Cq
Bacillus cereus (ATCC 14579)	no Cq
Bacillus subtilis subsp. *spizizeni* (ATCC 6633)	no Cq
Candida albicans (ATCC 10231)	no Cq
Saccharomyces cerevisiae (ATCC 9763)	no Cq
Aspergillus brasiliensis (ATCC 16404)	no Cq
Wallemia sebi (ATCC 42694)	no Cq
Staphylococcus aureus (ATCC 25923)	no Cq
Staphylococcus saprophyticus (ATCC 15305)	no Cq
Leptospira interrogans ser. Pomona (RTI)	no Cq
L. interrogans ser. icterohaemorrhagiae (RTI)	no Cq
L. interrogans ser. Bataviae (RTI)	no Cq
L. interrogans ser. Bratislava (RTI)	no Cq
L. interrogans ser. Canicola (RTI)	no Cq
L. grippotyphosa (RTI)	no Cq
L. borgpeterseni ser. Serjoe (RTI)	no Cq
L. borgpeterseni ser. Hardjo type bovis (RTI)	no Cq
L. borgpeterseni ser. Perepelitsin (RTI)	no Cq
Staphylococcus epidermidis (Lab)	no Cq
Staphylococcus intermedius (Lab)	no Cq
Bacillus cereus (Lab)	no Cq
Mycoplasma gallisepticum (ATCC 15302)	no Cq
Mycoplasma synoviae (ATCC 25204)	no Cq
Brucella suis (Lab)	no Cq
B. abortus biotype 1 (strain 544, Lab)	no Cq
B. melitensis biotype 1 (strain 16M, Lab)	no Cq
Coxiella burnetii (Strain Nine Mile, ANSES)	no Cq
Chlamydophila psittaci (Strain 02DC15, FLI)	no Cq
Chlamydophila abortus (Strain 13DC98, FLI)	no Cq
Citrobacter youngae (PT)	no Cq
Staphylococcus cohnii (PT)	no Cq

FLI – Friedrich Loeffler Institute, Germany; ANSES – French Agency for Food, Environmental and Occupational Health & Safety; ATCC – American Type Culture Collection, United States; NCTC – The National Collection of Type Cultures, England; RTI – KIT Biomedical Research, Royal Tropical Institute, Netherlands; Lab – Veterinary Specialized Institute Kraljevo; Cq – quantification cycle

The novel real-time PCR method successfully detected *Salmonella* DNA in the modified conditions, meaning the new method was suitably robust (Table 4).

Testing artificially contaminated samples. After the optimisation of the real-time PCR method (Protocol MD), 150 artificially contaminated samples were tested, with parallel testing using the ISO 6579-1 standard method (Table 5). In two samples in which, at the contamination level of 1–10 CFU·(25 g)$^{-1}$, *Salmonella* was not detected by the standard method, the Cq values obtained were close to the limit (39.00). In one sample of raspberries, the Cq value was 40.80 (negative agreement), while in one milk sample, the Cq value was 37.48 (positive deviation). There were no false-negative results. Internal Amplification Control (IAC) was applied during the test (DNA Extraction Control mix 610, Bioline, UK) at the optimum concentration and did not adversely affect the detection sensitivity of the target microorganism (IAC Cq in the range of 30–32). Test results from artificially contaminated samples showed the applied real-time PCR method (Protocol MD) is sensitive and can detect 1–10 CFU *Salmonella* in 25 g of ćevapi, poultry meat, raspberries, milk, and chicken neck skin. The minimum requirement related to the method sensitivity of qualitative methods for the detection of foodborne pathogens is the ability to detect 1–10 bacterial cells in a defined amount of the food matrix under investigation (ISO 2011).

Testing artificially contaminated poultry samples. Comparison of the characteristics of the new method (Protocol MD) with Protocol A and Protocol M was performed by testing 30 artificially contaminated poultry meat samples (Table 5). Test results from artificially contaminated samples showed that the applied real-time PCR methods were sensitive and were able to detect 1–10 CFU in 25 g of poultry

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Table 4. Standard procedure modifications

Factors	Procedure	Modified procedure 1	Modified procedure 2	Modified procedure 3	Modified procedure 4
qPCR equipment	AriaMx	Mx3005P	AriaMx	AriaMx	Mx3005P
Master mix	AT[a]	AB[b]	AT[a]	AB[b]	AT[a]
DNA template (µL)	2	5	5	2	2

[a] Brilliant III Ultra-Fast qPCR Master Mix (Agilent Technologies, SAD); [b] Path-ID™ qPCR Master Mix, Applied Biosystems

meat. Excellent agreement (100%) among the tested methods was achieved, with all three methods successfully detecting the *Salmonella* genome in all contaminated samples.

Differences in the achieved Cq values were observed (Table 6). The lowest Cq values were obtained using Protocol MD. Quantification cycle (Cq) is the cycle number in which the fluorescence signal cuts the threshold line, and this value is inversely proportional to the initial amount of the target sequence. For example, if we compare two samples, a sample containing twice as many copies of the target sequence will achieve Cq one cycle earlier (TFS 2016). During this experiment, the method comparison was performed under identical conditions (same device, same master mix, same operator), which means that the novel Protocol MD compared to Protocol A and Protocol M produced better results, i.e. detected lower DNA concentrations. The mean Cq values of Protocols A and MD were statistically significantly different ($P < 0.05$) after the examination of artificially contaminated samples, while no statistical difference was found between Protocols M and MD ($P > 0.05$).

Participation in PT schemes. External quality assessment through participation in PT is of great importance for verifying the methods used in the laboratory (Raymaekers et al. 2009). Following optimisation of the new protocol for detection of the

Table 5. Results obtained after testing artificially contaminated samples with the ISO 6579-1 and the qPCR methods (Protocol MD, M and A)

Type of sample	Level of contamination CFU·(25 g)$^{-1}$	Positive/total samples		
		ISO 6579-1	Protocol MD	Protocol M and A
Ćevapi	10–100	5/5	5/5	–
	1–10	20/20	20/20	–
	0	0/5	0/5	–
Raspberries	10–100	5/5	5/5	–
	1–10	19/20	19/20[a]	–
	0	0/5	0/5	–
Milk	10–100	5/5	5/5	–
	1–10	19/20	20/20[b]	–
	0	0/5	0/5	–
Chicken meat	10–100	5/5	5/5	5/5
	1–10	20/20	20/20	20/20
	0	0/5	0/5	0/5
Chicken neck skin	10–100	5/5	5/5	–
	1–10	20/20	20/20	–
	0	0/5	0/5	–

[a] raspberry sample negative after testing by the standard method achieved a Cq value of 40.80; [b] milk sample negative after testing by the standard method achieved a Cq value of 37.48; Cq – quantification cycle; CFU – colony forming unit; Protocol MD – assay developed for this study; Protocol M – Malorny et al. 2004; Protocol A – Anderson et al. 2011

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Table 6. Obtained Cq values after testing artificially contaminated chicken meat

Sample mark	Level of contamination CFU·(25 g)$^{-1}$	Protocol M	Protocol A	Protocol MD
1	1–10	19.76	21.72	18.61
2		18.89	21.13	18.22
3		18.49	20.85	17.81
4		18.61	20.85	17.77
5		18.40	20.90	17.75
6		18.93	21.56	18.35
7		18.91	21.00	18.27
8		19.87	22.25	18.99
9		19.04	21.05	18.02
10		19.33	21.76	18.53
11		18.28	20.33	17.50
12		14.72	16.98	13.74
13		18.47	20.83	17.61
14		17.43	19.38	16.41
15		18.12	20.28	17.38
16		18.90	21.46	18.08
17		17.33	19.00	16.36
18		21.20	24.25	20.77
19		19.41	21.73	18.80
20		19.52	21.97	18.67
21	10–100	17.38	19.21	16.34
22		16.72	18.50	15.64
23		17.52	19.48	16.93
24		16.60	18.34	16.44
25		17.74	19.43	17.49
Average Cq	1–10	18.68	20.96	17.88
	10–100	17.19	18.99	16.57

CFU – colony forming unit; Protocol M – Malorny et al. 2004; Protocol A – Anderson et al. 2011; Protocol MD – assay developed for this study; Cq – quantification cycle

Salmonella genome by real-time PCR, verification was performed by participating in PT (Table 7). The method successfully detected *Salmonella* genomic DNA in all contaminated samples, including the contamination level of < 10 CFU of *S. Senftenberg* per sample.

Table 7. Proficiency testing

Provider	Sample	Microorganism	Level of contamination	Genome detected (yes/no)
1	Poultry	*S. Typhimurium* (+ *E. coli*)	~ 50 000 CFU/sample	yes (Cq = 20.95)
		Citrobacter youngae	unknown	no (no Cq)
		S. Typhimurium	~ 500 CFU/sample	yes (Cq = 18.96)
		S. Senftenberg	< 10 CFU/sample	yes (Cq = 36.42)
		S. Agona	~ 22 CFU/sample	yes (Cq = 18.33)
2	Oatmeal	*S. Bracknell*	7 CFU·g^{-1}	yes

CFU – colony forming unit; Cq – quantification cycle

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CONCLUSION

The results of this study indicate a new, high-performance, optimised, and in-house validated assay was developed, which after validation between different laboratories, could be acceptable as a screening real-time PCR assay for the detection of *Salmonella* in food. This novel real-time PCR is very likely to be a suitable method for *Salmonella* detection in other types of samples.

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