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Original Scientific Article

DETECTION OF *SALMONELLA* IN POULTRY FAECES BY MOLECULAR MEANS IN COMPARISON TO TRADITIONAL BACTERIOLOGICAL METHODS

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Summary: Comparison of traditional (cultivation-dependent) and molecular (nucleic acid-based) bacteriological methods was performed to detect *Salmonella* in reference capsules containing quantified amounts of *Salmonella enterica* subsp. *enterica* serovars Panama, Typhimurium or Enteritidis and in poultry faeces that was naturally contaminated with *Salmonella* or *Salmonella*-negative but spiked with reference materials. Traditional techniques were performed according to ISO 6579 using different enrichment (MSRV, MKTTn and RVS, respectively) and isolation plating media (XLD, BGA and Rambach agar, respectively). Molecular detection was preceded by the pre-enrichment step. Detection efficiency of two DNA isolation kits, namely High Pure foodproof I Kit (Roche Diagnostics, Germany) and QIAamp DNA Stool Mini Kit (Qiagen, Germany), in combination with classical and real-time PCR assay was compared. Results showed that traditional and molecular detection of *Salmonella* was unambiguous for reference control capsules, but was hindered for faecal samples. RVS medium was less appropriate than MSRV and MKTTn. Combination of MKTTn with Rambach agar plates generated the highest number of positive results with traditional approach. However, recommendation of using the semi-solid MSRV medium was confirmed as it enabled detection of *Salmonella* in high proportion of samples, which was the least variable depending on the selection of isolation plating media. In contrast to culture-based methods, the molecular approach, especially a combination of High Pure foodproof I Kit and real-time PCR assay, enabled successful detection in all *Salmonella*-positive samples and should therefore be considered an important supplement to traditional protocol for *Salmonella* detection in foodstuffs.

Key words: *Salmonella*; foodstuffs; faeces; detection; cultivation; molecular; PCR

Introduction

The routine microbiology laboratories for detection of different bacterial pathogens are complementing traditional diagnostic assays with continually evolving molecular techniques as they are not negatively affected by the presence of growth inhibitory compounds and enable rapid detection (1-3). Surveillance of alimentary zoonoses, diseases that are transmitted from animals to humans through food, and early detection of their causative agents in food producing animals and their environment are very important for the assurance of safe food. Food safety is a growing public health issue, since it was estimated that up to 30 % of the population in industrialized countries is suffering from foodborne illnesses (4). Salmonelloses are the second most frequently reported human zoonoses in the European Union and can cause relatively vast economic damage due to chronic effects of the infections (5). The common reservoir of *Salmonella* is the intestinal tract of animals, however they can be detected in a wide variety of foodstuffs and food ingredients (5). Animal-to-human transmission occurs when bacteria are introduced into the food preparation process or through direct contact with infected animals and faecally contaminated environments.

In-country laboratory-based monitoring of foodborne pathogens is being promoted (4). Traditional microbiological methods offer standardized proce-

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dures for their detection (*e.g.* ISO standards), but are time consuming and not always compatible with short-time-to-result demand. Therefore, food microbiology aims for supplementation of classical methods with molecular techniques based on detection of microbial nucleic acids in foodstuffs, which shorten the analysis time and lower the limit of detection. It was shown previously that the polymerase chain reaction (PCR) has a great potential to speed-up the detection of *Salmonella* in food (6) and can be performed in a manner equivalent to the standard ISO 6579 culture method, which is set as the golden standard for *Salmonella* detection in food and feedstuffs (7,8).

The main objective of our study was to evaluate the detection efficiency for different contamination levels of *Salmonella* spp. in the presence of competitive microorganisms. As a complex matrix, poultry faeces was selected for the starting material. The use of molecular methods polymerase chain reaction (PCR) and real-time PCR was compared to traditional, cultivation-dependent bacteriological methods.

Materials and methods

Reference materials (RMs) and poultry faeces were used. The RMs consisted of gelatin capsules containing a quantified amount of sub-lethally injured *Salmonella* strains of serovars Panama (SPan), Typhimurium (STM) or Enteritidis (SE) as spiked spray dried milk prepared by the Community Reference Laboratory (CRL) for *Salmonella* (9). The levels of contamination were SPan 5 (5 colony forming particles per capsule [cfp/caps]), STM 10 (10 cfp/caps), STM 100 (100 cfp/caps), SE 100 (100 cfp/caps) and SE 500 (500 cfp/caps). Faeces, negative or positive for *Salmonella* spp., and reference capsules were stored at –20° C till use. We examined (*i*) 24 poultry faecal samples (numbered FC-1 to FC-24; 10 g each, negative for *Salmonella* spp.) in combination with a blank capsule (five samples) or a capsule containing STM (five samples STM 10 and four samples STM 100) or SE (five samples SE 100 and five samples SE 500), (*ii*) 20 faecal samples which were naturally contaminated with *Salmonella* and not spiked with capsules (numbered F-1 to F-20; 10 g each), and (*iii*) 10 control samples (numbered C-1 to C-10; no faeces added) consisting of two blank, two SPan 5, three STM 10 and three SE 100 capsules, respectively. In addition, two negative control samples were examined: procedure control (*i.e.* C-11; no faeces or capsule added) and negative faeces control (*i.e.* C-12; 10 g of faeces negative for *Salmonella* spp.). The sample outline is summarized in Table 1.

Traditional bacteriological methods

Cultivation-dependent detection of *Salmonella* was performed according to ISO 6579:2002 (10), including Amendment 1:2007 (11), and the instructions provided by CRL for *Salmonella* (9). In brief, detection involved the following stages: (*i*) overnight sample pre-enrichment in a nonselective broth medium BPW (Buffered Peptone Water), (*ii*) 24- (for the first isolation) and 48-hour (for the second isolation) enrichment in selective broth media MKTTn (Muller Kauffmann TetraThionate-novobiocin broth), RVS (Rappaport Vassiliadis medium with Soya) and MSRV (Modified Semi-solid Rappaport Vassiliadis medium; 11), (*iii*) isolation of colonies presumed to be *Salmonellae* on solid selective and differential plating media BGA (phenol red/Brilliant Green Agar), XLD (Xylose-Lysine-Deoxycholate agar) and R (Rambach agar; 12), and (*iv*) biochemical screening of *Salmonella* isolates on the confirmation media TSI (Triple Sugar/Iron agar), UA (Urea Agar) and LDC (1-Lysine decarboxylation medium). If colonies grown on the isolation media were not well separated, single colony isolation was performed on NA (Nutrient Agar) plates after 24-hour incubation at 37° C and followed by the aforementioned confirmation. For each of the samples from the three selective enrichment media, at least one individual colony, considered to be typical or suspect for *Salmonella*, was examined biochemically. If the selected colonies were not confirmed as *Salmonella*, maximum of five additional typical colonies were tested from the original isolation medium stored at 5° C. Sample was denoted with positive result if growth of *Salmonella* spp. was present at least on one of the isolation media. If not stated otherwise, media and reagents were prepared according to Annex B of ISO 6579:2002.

Molecular methods

Molecular detection of *Salmonella* involved the isolation of microbial DNA that was followed by *Salmonella*-specific PCR and real-time PCR assays. DNA was extracted from 1 mL of the pre-enrichment broths using two different commercial kits, namely the High Pure foodproof I Kit (Roche Diagnostics, Germany) and QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturers' instructions. The latter was not applied for samples devoid of faeces. Microbial DNA was subjected to PCR amplification using *Salmonella* genus-specific primers ST11 and ST15 (13) that were proved as appropriate

Table 1: Outline of the samples used for the study: control samples (C), naturally contaminated faecal samples (F), and faecal samples supplemented with capsules (FC).

Sample name	Faeces	Capsule	Sample name	Faeces	Capsule	Sample name	Faeces	Capsule
C-1	/	blank	F-1	pos	/	FC-1	neg	SE 100
C-2	/	SPan 5	F-2	pos	/	FC-2	neg	blank
C-3	/	blank	F-3	pos	/	FC-3	neg	STM 100
C-4	/	SE 100	F-4	pos	/	FC-4	neg	SE 100
C-5	/	STM 10	F-5	pos	/	FC-5	neg	STM 100
C-6	/	STM 10	F-6	pos	/	FC-6	neg	SE 500
C-7	/	STM 10	F-7	pos	/	FC-7	neg	SE 500
C-8	/	SE 100	F-8	pos	/	FC-8	neg	blank
C-9	/	SE 100	F-9	pos	/	FC-9	neg	SE 500
C-10	/	SPan 5	F-10	pos	/	FC-10	neg	SE 100
C-11	/	/	F-11	pos	/	FC-11	neg	STM 10
C-12	neg	/	F-12	pos	/	FC-12	neg	blank
			F-13	pos	/	FC-13	neg	SE 100
			F-14	pos	/	FC-14	neg	STM 10
			F-15	pos	/	FC-15	neg	STM 100
			F-16	pos	/	FC-16	neg	blank
			F-17	pos	/	FC-17	neg	STM 10
			F-18	pos	/	FC-18	neg	SE 500
			F-19	pos	/	FC-19	neg	STM 10
			F-20	pos	/	FC-20	neg	blank
						FC-21	neg	STM 10
						FC-22	neg	SE 100
						FC-23	neg	SE 500
						FC-24	neg	STM 100

Legend: neg: faeces negative for *Salmonella*, pos: faeces positive for *Salmonella*, SPan: *Salmonella* Panama (SPan 5: 5 cfp/caps), STM: *Salmonella* Typhimurium (STM 10 and STM 100: 10 and 100 cfp/caps, respectively), SE: *Salmonella* Enteritidis (SE 100 and SE 500: 100 and 500 cfp/caps, respectively), blank: no cfp/caps

for the confirmation of *Salmonella*-colonies obtained by the standard ISO 6579 culture method (14). Amplification was performed according to the optimized touchdown protocol as described previously (15). Real-time PCR was performed using the LightCycler foodproof *Salmonella* Detection Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Briefly, a 20-µl reaction mixture was composed of foodproof *Salmonella* enzyme solution containing FastStart Taq DNA polymerase, internal amplification control (IC), master mix containing primers and hybridization probes specific for *Salmonella* DNA and *Salmonella*-specific IC, and 5 µl of sample DNA, foodproof *Salmonella* positive control template or PCR-grade water as negative control. Amplification was performed by LightCycler 1.2 Real-Time PCR System (Roche Diagnostics, Germany). The inclusivity of foodproof *Salmonella* master mix for the *Salmonella* genus and exclusivity for other genera was extensively tested by the manufacturer.

Results

Reference materials vs. faecal samples

Detection of *Salmonella* in reference materials was unambiguous with no false negative or positive results regardless of the employed method (samples C in Tables 2 and 3). Both the traditional and the molecular methods in all the tested combinations were equally appropriate with detection limit of 5 cfp/sample or lower. On the other hand, detection of *Salmonella* in samples containing poultry faeces was limited as it depended on the method type and the level of *Salmonella* contamination. Detection limit was impaired for traditional methods (above 10 cfp/sample) in comparison to molecular methods (10

cfp/sample or lower), since one sample of naturally contaminated faeces (F-2) and eight faecal samples containing reference capsules (all five samples supplemented with STM 10 [FC-11, FC-14, FC-17, FC-19 and FC-21], two samples with STM 100 [FC-3 and FC-5], and one sample with SE 100 [FC-1]) were denoted falsely negative but tested positive when molecular detection was performed (Table 3). To detail, *Salmonellae* from capsules STM 10 were detected by traditional methods only from the control samples, but not in samples containing faecal material. However, they were detected in all samples FC (100 %) when applying the molecular methods (particularly, real-time PCR in combination with High Pure foodproof I Kit) (Table 4). *Salmonellae* from samples FC supplied with higher cfp number of *Salmonella* Typhimurium (STM 100) or with *Salmonella* Enteritidis in equivalent cfp number (SE 100) were detected in marked proportions of samples (50 % or 80 %, respectively) by traditional bacteriological methods, but in all cases (100 %) when molecular approach was employed (Table 4). *Salmonellae* from SE 500 capsules with the highest *Salmonella*-contamination level were detected in all samples FC by both the traditional (with MSRV enrichment only) and the molecular approach (Table 4). *Salmonellae* from faecal samples F were detected in 95 % and in 100 % of cases applying traditional (particularly, MKTTn in combination with Rambach agar) and molecular (all combinations) methods, respectively (Table 4).

To summarize, detection of *Salmonella* spp. in reference materials succeeded over the entire experimental range of contamination levels and did not depend on the method type, but was impaired in faecal samples when traditional approach was employed, enabling detection in 30 out of 39 *Salmonella*-positive faecal samples (19/20 for samples F and 11/19 for samples FC, respectively) (Table 3). No samples supplied with blank capsule and negative control samples tested falsely positive.

Traditional bacteriological methods vs. molecular methods

Results of cultivation-dependent detection of *Salmonella* in faecal samples after 24 and/or 48-hour incubation showed that MKTTn and MSRV selective enrichment media generated less falsely negative results than RVS (Tables 2 and 3). MKTTn enabled detection in 19 out of 20 *Salmonella*-positive samples F (19/20) and 10 out of 19 *Salmonella*-positive samples FC (10/19), MSRV in 17/20 and 9/19, and RVS in 14/20 and 3/19 samples, respectively. By traditional approach, 13 of 20 *Salmonella*-positive samples F and only 2 of 19 *Salmonella*-positive FC samples (FC-15 and FC-23) tested positive from all three enrichment media. All the rest tested positive from two (5/20 for samples F and 7/19 for samples FC) or only one (1/20 and 2/19, respectively) selective enrichment medium (Table 3). In the majority of faecal samples, detection failed from RVS enrichment regardless of the isolation medium, in particular with faecal samples FC that were contaminated with serovar Enteritidis (Tables 2 and 3). Although serovar Typhimurium was detected after RVS enrichment in 50 % of FC samples supplied with STM 100, detection of serovar Enteritidis of the same cfp number (SE 100) was completely absent (0 %) or markedly impaired (detection in 20 %) when supplied in higher cfp number SE 500 (Table 4).

The highest number of true positive results (19/20 samples F and 9/19 samples FC, Table 2) was attributed to MKTTn when in combination with Rambach agar. However, the lowest number of positive results was also attributed to MKTTn, namely when it was combined with XLD or BGA isolation plating medium (6/20 or 0/20, respectively for faecal samples F; Table 2). Isolation of *Salmonella* colonies originating from different selective enrichment media was not affected by the selection of plating media with the above mentioned exception of MKTTn, which was likewise the only selective medium that generated higher number of positive cases when results obtained from all the three isolation plating media were combined in comparison to results obtained from individual isolation media (4 positive out of 5 samples FC supplied with SE 100 [4/5] for combined results vs. 3/5 for individual combinations of isolation media with MKTTn, respectively; Table 2). MKTTn and MSRV enrichments were comparably effective regarding the detection level, however only MSRV enabled detection of *Salmonella* in all samples FC supplied with SE 500 (Tables 2 and 3). That was the only case for faecal samples where detection by traditional approach was not limited.

Results of molecular detection of *Salmonella* showed that it was successful, since the presence of *Salmonella* was confirmed not only in control samples but also in all faecal samples (detection level of 100 %; Table 4). However, differences were observed for samples FC regarding the procedure for DNA isolation (using the High Pure foodproof I Kit [protocol HP] or QIAamp DNA Stool Mini Kit [protocol S]) and

the type of PCR reaction (classical PCR or real-time PCR) (Tables 3 and 4). Protocol S enabled detection in more FC samples in comparison to protocol HP when using classical PCR detection (15/19 for S vs. 10/19 for HP), but protocol HP in more samples when performing real-time PCR (19/19 for HP vs. 15/19 for S) (Table 3). Protocol HP enabled detection of *Salmonella* in all samples FC, while protocol S generated false negative result for one sample supplemented with STM 10 capsule (FC-19) and for one with SE 100 (FC-1) (Table 3). Real-time PCR enabled detection in all samples FC, while classical PCR failed for three samples (FC-1, FC-19 and FC-22) (Table 3).

The highest number of positive cases using the molecular approach for samples FC, where detection efficiency depended on the method type in contrast to samples F, was obtained by protocol HP in combination with real-time PCR (19/19), followed by protocol S in combination with either classical or real-time PCR (15/15, however different samples were denoted as positive in four cases [FC-10, FC-11, FC-13 and FC-22] depending on PCR reaction type) (Table 3). The highest number of false negative results was obtained by protocol HP in combination with classical PCR that generated 10 positive cases out of 19 *Salmonella*-positive samples FC (10/19) (Table 3). When classical PCR reaction tested negative, in all but two such cases (samples FC-1 and FC-19 that failed to test positive when applying protocol S) real-time PCR tested positive (Table 3). When applying protocol S, two samples (FC-11 and FC-13) tested positive solely by classical PCR (Table 3).

Table 2: Results of traditional detection of *Salmonella*: number of positive samples after 24 and/or 48-hour incubation in selective enrichment media MKTTn, RVS and MSRV as detected on individual isolation media BGA, XLD and R, respectively

Sample name	Faeces	Capsule	No. of samples	MKTTn				RVS				MSRV			
				BGA	XLD	R	No. of pos. samples	BGA	XLD	R	No. of pos. samples	BGA	XLD	R	No. of pos. samples
C	/	blank	2	0	0	0	0	0	0	0	0	0	0	0	0
	/	SPan 5	2	2	2	2	2	2	2	2	2	2	2	2	2
	/	STM 10	3	3	3	3	3	3	3	3	3	3	3	3	3
	/	SE 100	3	3	3	3	3	3	3	3	3	3	3	3	3
C-11	/	/	1	0	0	0	0	0	0	0	0	0	0	0	0
C-12	neg	/	1	0	0	0	0	0	0	0	0	0	0	0	0
F	pos	/	20	0	6	19	19	14	14	14	14	17	17	17	17
FC	neg	blank	5	0	0	0	0	0	0	0	0	0	0	0	0
	neg	STM 10	5	0	0	0	0	0	0	0	0	0	0	0	0
	neg	STM 100	4	1	1	2	2	2	2	2	2	1	1	1	1
	neg	SE 100	5	3	3	3	4	0	0	0	0	3	3	3	3
	neg	SE 500	5	2	4	4	4	1	1	1	1	5	5	5	5

Note: See Table 1 for details on samples and Table 3 for details on positive samples.

Legend: MKTTn: Muller Kauffmann TetraThionate-novobiocin broth, RVS: Rappaport Vassiliadis medium with Soya, MSRV: Modified Semi-solid Rappaport Vassiliadis medium, BGA: phenol red/Brilliant Green Agar, XLD: Xylose-Lysine-Deoxycholate agar, R: Rambach agar, no. of samples: number of samples used for the study, no. of pos. samples: number of samples positive for the growth of *Salmonella*

Table 3: Results of traditional and molecular detection of *Salmonella*

Sample name	Faeces	Capsule	Traditional			Molecular			
			MKTTn	RVS	MSRV	HP		S	
						PCR	rtPCR	PCR	rtPCR
C-1	/	blank	–	–	–	–	–	/	/
C-3	/	blank	–	–	–	–	–	/	/
C-2	/	SPan 5	+	+	+	+	+	/	/
C-10	/	SPan 5	+	+	+	+	+	/	/
C-5	/	STM 10	+	+	+	+	+	/	/
C-6	/	STM 10	+	+	+	+	+	/	/
C-7	/	STM 10	+	+	+	+	+	/	/
C-4	/	SE 100	+	+	+	+	+	/	/
C-8	/	SE 100	+	+	+	+	+	/	/
C-9	/	SE 100	+	+	+	+	+	/	/
C-11	/	/	–	–	–	–	–	/	/
C-12	neg	/	–	–	–	–	–	–	–
F-2	pos	/	–	–	–	+	+	+	+
F-16	pos	/	+	–	–	+	+	+	+
F-1	pos	/	+	–	+	+	+	+	+
F-3	pos	/	+	–	+	+	+	+	+
F-4	pos	/	+	–	+	+	+	+	+
F-20	pos	/	+	–	+	+	+	+	+
F-9	pos	/	+	+	–	+	+	+	+
F-5	pos	/	+	+	+	+	+	+	+
F-6	pos	/	+	+	+	+	+	+	+
F-7	pos	/	+	+	+	+	+	+	+
F-8	pos	/	+	+	+	+	+	+	+
F-10	pos	/	+	+	+	+	+	+	+
F-11	pos	/	+	+	+	+	+	+	+
F-12	pos	/	+	+	+	+	+	+	+
F-13	pos	/	+	+	+	+	+	+	+
F-14	pos	/	+	+	+	+	+	+	+
F-15	pos	/	+	+	+	+	+	+	+
F-17	pos	/	+	+	+	+	+	+	+
F-18	pos	/	+	+	+	+	+	+	+
F-19	pos	/	+	+	+	+	+	+	+
FC-2	neg	blank	–	–	–	–	–	–	–
FC-8	neg	blank	–	–	–	–	–	–	–
FC-12	neg	blank	–	–	–	–	–	–	–
FC-16	neg	blank	–	–	–	–	–	–	–
FC-20	neg	blank	–	–	–	–	–	–	–
FC-11	neg	STM 10	–	–	–	+	+	+	–
FC-14	neg	STM 10	–	–	–	+	+	+	+
FC-17	neg	STM 10	–	–	–	+	+	+	+
FC-19	neg	STM 10	–	–	–	–	+	–	–
FC-21	neg	STM 10	–	–	–	–	+	+	+
FC-3	neg	STM 100	–	–	–	–	+	+	+
FC-5	neg	STM 100	–	–	–	–	+	+	+
FC-15	neg	STM 100	+	+	+	+	+	+	+
FC-24	neg	STM 100	+	+	–	–	+	+	+
FC-1	neg	SE 100	–	–	–	–	+	–	–
FC-4	neg	SE 100	+	–	+	–	+	+	+
FC-10	neg	SE 100	+	–	+	+	+	–	+
FC-13	neg	SE 100	+	–	+	+	+	+	–
FC-22	neg	SE 100	+	–	–	–	+	–	+
FC-6	neg	SE 500	+	–	+	+	+	+	+
FC-7	neg	SE 500	–	–	+	+	+	+	+
FC-9	neg	SE 500	+	–	+	+	+	+	+
FC-18	neg	SE 500	+	–	+	+	+	+	+
FC-23	neg	SE 500	+	+	+	–	+	+	+

Note: Sample was denoted with positive result if growth of *Salmonella* was detected at least on one isolation medium. Where no faeces was added to samples, QIAamp DNA Stool Mini Kit DNA extraction was not performed. See Table 1 and Table 2 for details.

Legend: HP: High Pure foodproof I Kit (Roche Diagnostics, Germany), S: QIAamp DNA Stool Mini Kit (Qiagen, Germany), PCR: polymerase chain reaction, rtPCR: real-time PCR, + positive result (*Salmonella* detected), – negative result (*Salmonella* not detected)

Table 4: Summary of the results of traditional and molecular detection of *Salmonella*

Sample name	Faeces	Capsule	Traditional			Molecular			
						HP		S	
			MKTTn	RVS	MSRV	PCR	rtPCR	PCR	rtPCR
C	/	SPan 5	**1**	**1**	**1**	**1**	**1**	/	/
	/	STM 10	**1**	**1**	**1**	**1**	**1**	/	/
	/	SE 100	**1**	**1**	**1**	**1**	**1**	/	/
F	pos	/	0.95	0.70	0.85	**1**	**1**	**1**	**1**
FC	neg	STM 10	0	0	0	0.6	**1**	0.8	0.6
	neg	STM 100	0.5	0.5	0.25	0.25	**1**	**1**	**1**
	neg	SE 100	0.8	0	0.6	0.4	**1**	0.4	0.6
	neg	SE 500	0.8	0.2	**1**	0.8	**1**	**1**	**1**

Note: Samples containing blank capsule and negative control samples are not shown. Numbers indicate proportions of samples that were correctly detected as positive (no. of positive samples vs. no. of tested samples). See Tables 1-3 for details.

Discussion

Three types of samples were analyzed within the scope of the present study: artificially prepared gelatin capsules with quantified amounts of *Salmonella* spp. strains of different serovars (samples C), samples containing poultry faeces supplemented with capsules (samples FC) and the least defined samples containing naturally *Salmonella*-contaminated poultry faeces (samples F). Prior to the interpretation of results, faeces used for the artificial contamination was confirmed as negative for *Salmonella* spp. as negative faeces control and faecal samples supplied with blank capsules tested negative by both the traditional and the molecular methods. The first type of samples (samples C) served as a reference material with defined sample parameters to enable determination of the experimental detection limit in optimal conditions. Faecal samples (samples FC and F) were more complex as they contained variable endogenous and dietary components in addition to the faecal microbiota that may interfere with the growth of *Salmonella* or selected bacterial species in *in vitro* conditions (16) and inhibit their detection or quantification by molecular means (17-20). Food samples harboring diverse microflora resemble faecal samples in a sense of exerting a negative effect on the detection of food pathogens, which was previously reported for *Salmonella* (21,22). Culture-based methods using selective agents are not always sufficiently effective in eliminating or suppressing contaminating microflora (23-25). Therefore, negative results have to be interpreted with caution when detection of selected microbial taxa in the environmental samples is being aimed for like foodborne pathogen detection or detection of infectious agents shedding into the environment.

Our results confirmed that the efficiency of detection markedly depends on the sample complexity, since the limit of *Salmonella* detection was lower for reference materials (5 cfp/sample or lower regardless of the method type) than for faecal samples (above 10 cfp/sample for traditional methods and 10 cfp/sample or lower for molecular methods). At the same time, it was confirmed that molecular approach improves detection in environmental samples as cultivation-dependent detection of *Salmonella* was unambiguous only when reference control capsules were considered but was hindered in faecal samples in comparison to molecular detection with markedly lower detection limit. These differences were less apparent for the naturally *Salmonella*-contaminated faecal samples. Therefore, the contamination level of these samples was generally higher than in faecal samples supplied with reference capsules, since it was reported that the recovery of *Salmonella* from environmental samples depends not only on the interference by competing flora but also on the contamination level (26).

Regarding traditional bacteriological methods, results of our study indicate that medium RVS might be considered less appropriate for cultivation-dependent detection of *Salmonella* in poultry faeces than media MKTTn and MSRV. Albeit the enrichment medium MKTTn in combination with Rambach agar plates generated the lowest number of false negative results by culture-based methods in general, the only case of unhindered detection

of *Salmonella* from faecal samples of certain type was supported by the MSRV enrichment, similarly to the efficient detection supported by molecular methods. Results favor the strong recommendation of using the semi-solid MSRV enrichment medium for detection of *Salmonella* contamination in animal samples and samples from food production chains or foodstuffs, since it generated high and consistent detection rate in comparison to MKTTn where detection rate was high but depended on the selection of isolation plating media. Our results are in accordance with the findings of Eriksson and Aspan (27) and with the collaborative studies of CRL for *Salmonella* (9), where it was suggested to replace a selective broth medium in the ISO procedure by a semi-solid medium to obtain a higher detection rate of *Salmonella* in faecal samples. However, the use of more than one enrichment and isolation medium proved important as detection of *Salmonella* in faecal samples was more successful when results from different isolation plating media were combined in one case and depended on the enrichment medium type in other cases.

When aiming for nonselective detection of foodborne pathogens of a certain kind, detection limit for different microbial variants applying a selected method should not differ considerably. However, medium RVS, which generated the highest number of false negative cases in our study, appeared to be selective with regard to *Salmonella* serotype. With RVS enrichment, detection of serovar Enteritidis was impaired in comparison to serovar Typhimurium. Our results were not surprising, since it was reported previously that various *Salmonella* serotypes may perform differently in a given culture medium or sample-medium ratio (28). Therefore, investigation of complex environmental, sanitary or clinical samples by traditional bacteriological methods requires standardized procedures introduced after comprehensive research learning from past deficiencies to introduce critical corrigenda.

Despite the improved detection limit of molecular methods for the selected foodborne pathogens, they can not distinguish between viable microorganisms capable of causing a zoonotic infection and their genetic material present in samples merely as a remnant of formerly live organisms. However, the preenrichment procedures in a non-selective medium enable efficient recovery of viable *Salmonella* from different food samples or matrices to be followed by PCR analysis (29,30). The additional step of bacterial enrichment is therefore strongly recommended to increase the numbers of *Salmonellae* originating from the investigated samples in order to improve the sensitivity of molecular diagnostic techniques (7,30-32). Despite the additional diagnostic procedures applied, isolation of bacteria remains the definitive step for the identification of foodborne pathogens. However, their early detection in breeding animals and their secreta/excreta can help decrease the number of faecal shedders of pathogens like *Salmonella* from animal and food production systems, therefore interrupting the infection-transmission cycle. Many methods for the rapid detection and serotype identification of *Salmonella* in foodstuffs have been developed to date, since rapid and reliable detection of pathogens is crucial for ensuring food safety (27,30,33,34). The specialized, chromogenic culture media and the immuno assays are being employed, but nucleic acid-based assays like PCR and its variants are becoming the most important rapid diagnostic techniques in clinical and food microbiology within the last two decades.

One of the chromogenic media developed to detect *Salmonella* is Rambach agar (12). It is based on a combination of biochemical characteristics and is reported to be highly specific. Among the isolation plating media that were tested in our study, Rambach agar plates proved to be more suitable in combination with the selective enrichment medium MKTTn than XLD plates and these more than BGA plates. Our results are in accordance with the previously reported evaluation of enrichment broths and plating media for the detection of *Salmonella* in poultry (35), where it was reported that the most effective isolation medium was the chromogenic medium CHROMagar (*Salmonellae* were identified in 79.3 % of the positive samples), followed by Rambach agar (48 %) and finally by classical media like XLD (34.5 %) and BGA (13.8 %).

In contrast to traditional cultivation, molecular approach enabled successful detection of *Salmonella* both in reference materials and in faecal samples. Although molecular detection is not dependent on the selection of growth media, its success depends on the selection and appropriate combination of nucleic acid-based methods. The Qiagen's QIAamp DNA Stool Mini Kit was applied for faecal samples as it included a commercial mixture of reagents to ensure targeted removal of faecal metabolites that can degrade the isolated DNA and inhibit the downstream enzymatic applications. On the other hand, all samples were processed by the High Pure foodproof I Kit by Roche, which is optimized to provide

DNA of high quality from *Salmonella*-enrichment cultures of various food samples.

In our study, differences were observed for faecal samples spiked with reference capsules regarding the efficiency of PCR detection of *Salmonella* after DNA isolation procedure using the two selected commercially available kits. In combination with classical PCR detection, QIAamp DNA Stool Mini Kit enabled detection in more samples, but in fewer with real-time PCR, for which the High Pure foodproof I Kit was confirmed to be the optimal choice as reported before (36). Although the latter is formulated to generate highly purified DNA from food samples, it also proved appropriate for faecal samples with similarly high potential for PCR inhibition, possibly due to supplementary enrichment step diluting the inhibitors. The High Pure foodproof I Kit enabled detection of *Salmonella* in all *Salmonella*-positive faecal samples, while the QIAamp DNA Stool Mini Kit surprisingly generated false negative results regardless of the following PCR reaction type.

Real-time PCR proved to exert higher sensitivity than classical PCR reaction since it enabled detection in all faecal samples, however when not preceded by DNA isolation using the QIAamp DNA Stool Mini Kit. One single combination of suitable DNA isolation protocol and PCR test, namely the High Pure foodproof I Kit in combination with *Salmonella*-adapted real-time PCR detection kit (both from Roche Diagnostics, Germany), was sufficient for detection in all *Salmonella*-positive samples.

We can conclude that traditional bacteriological methods, being asserted as the procedure of a choice for the detection of *Salmonella* in foodstuffs for most laboratories around the globe, ought to be supplemented with molecular methods to generate results in shorter time and with lower detection limit. Inconsistency of cultivation-dependent, and to a lesser extent of molecular detection, indicates the importance of using more than one method for confirmation or exclusion of possible contamination of biological materials analyzed in the process of food-safety monitoring. Molecular approach, possibly the combination of real-time PCR assay with the High Pure foodproof I Kit or alternative provided by the manufacturer, can enable detection of *Salmonella* without generating false negative results. Therefore, it should be considered as an important supplement to the traditional protocol. Efforts targeted to early detection of *Salmonella* will contribute to successful control and reduction of foodborne salmonelloses in the future.

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References

1. van Belkum A, Renders NHM, Smith S, Overbeek SE, Verbrugh HA. Comparison of conventional and molecular methods for the detection of bacterial pathogens in sputum samples from cystic fibrosis patients. FEMS Immunol Med Microbiol 2000; 27: 51-7.

2. Houpikian P, Raoult D. Traditional and molecular techniques for the study of emerging bacterial diseases: One laboratory's perspective. Emerg Infect Dis 2002; 8: 122-31.

3. Jannes G, De Vos D. A review of current and future molecular diagnostic tests for use in the microbiology laboratory. In: O'Connor L, ed. Methods in molecular biology. diagnostic bacteriology protocols. Totowa: Humana Press, 2006: 1-21.

4. WHO. Food safety and foodborne illness. Geneva: World Health Organization, 2007. Fact Sheet no. 237.

5. EFSA. The community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007. EFSA Journal 2009; 223: 1-313.

6. Olsen JE. DNA-based methods for detection of food-borne bacterial pathogens. Food Res Int 2000; 33: 257-66.

7. Piknová L, Štefanovičová A, Drahovská H, Sásik M, Kuchta T. Detection of *Salmonella* in food, equivalent to ISO 6579, by a three-days polymerase chain reaction-based method. Food Control 2002; 13: 191-4.

8. Tomás D, Rodrigo A, Hernández M, Ferrús MA. Validation of real-time PCR and enzyme-linked fluorescent assay-based methods for detection of *Salmonella* spp. in chicken feces samples. Food Anal Methods 2009; 2: 180-9.

9. CRL-*Salmonella*. SOP Bacteriological Collaborative Study VII (2003) on the detection of *Salmonella* spp. Bilthoven: CRL-*Salmonella*, 2003: 1-9.

10. ISO. International standard ISO 6579:2002. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. Geneva: International Organization for Standardization, 2002.

11. ISO. International standard ISO 6579:2002/ Amd 1:2007. Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage. Geneva: International Organization for Standardization, 2007.

12. Rambach A. New plate medium for facilitated differentiation of *Salmonella* spp. from *Proteus* spp. and other enteric bacteria. Appl Environ Microbiol 1990; 56: 301-3.

13. Aabo S, Rasmussen OF, Rossen L, Sorensen PD, Olsen JE. *Salmonella* identification by polymerase chain reaction. Mol Cell Probes 1993; 7: 171-8.

14. Štefanovicová A, Reháková H, Škarková A, Rijpens N, Kuchta T. Confirmation of presumptive *Salmonella* colonies by the polymerase chain reaction. J Food Prot 1998; 61: 1381-3.

15. Ocepek M, Pate M, Mićunović J, Bole-Hribovšek V. Comparison and optimization of two PCR tests for identification of *Salmonella* in poultry feedstuffs, liver and faeces. Slo Vet Res 2006; 43: 61-6.

16. Chander Y, Kumar K, Gupta SC, Goyal SM. Evaluation of CHROMagar salmonella medium for the isolation of *Salmonella* from animal manure. Int J Appl Res Vet Med 2005; 3: 35-9.

17. Widjojoatmodjo MN, Fluit AC, Torensma R, Verdonk GPHT, Verhoef J. The magnetic immunopolymerase chain-reaction assay for direct detection of salmonellae in fecal samples. J Clin Microbiol 1992; 30: 3195-9.

18. Monteiro L, Bonnemaison D, Vekris A, et al. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. J Clin Microbiol 1997; 35: 995-8.

19. Wilson IG. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 1997; 63: 3741-51.

20. Zhang L, Xu Z, Patel BKC. An improved method for purifying genomic DNA from forest leaf litters and soil suitable for PCR. J Soils Sediments 2009; 9: 261-6.

21. Beckers HJ, van Leusden FM, Meijssen MJ, Kampelmacher EH. Reference material for the evaluation of a standard method for the detection of salmonellas in foods and feeding stuffs. J Appl Bacteriol 1985; 59: 507-12.

22. Beckers HJ, van der Heide J, Fenigsen-Narucka U, Peters R. Fate of salmonellas and competing flora in meat sample enrichments in buffered peptone water and in Muller-Kauffmann's tetrathionate medium. J Appl Bacteriol 1987; 62: 97-104.

23. Arroyo G, Arroyo JA. Selective action of inhibitors used in different culture media on the competitive microflora of *Salmonella*. J Appl Bacteriol 1995; 78: 281-9.

24. Struijk CB. Guidelines for method validation techniques used in the microbiological examination of food samples. Food Control 1996; 7: 53-8.

25. Delcenserie V, Bechoux N, China B, Daube G, Gavini F. A PCR method for detection of bifidobacteria in raw milk and raw milk cheese: comparison with culture-based methods. J Microbiol Methods 2005; 61: 55-67.

26. Arroyo G, Arroyo JA. Efficiency of different enrichment and isolation procedures for the detection of *Salmonella* serotypes in edible offal. J Appl Bacteriol 1995; 79: 360-7.

27. Eriksson E, Aspan A. Comparison of culture, ELISA and PCR techniques for salmonella detection in faecal samples for cattle, pig and poultry. BMC Vet Res 2007; 3: 1-19.

28. Feder I, Nietfeld JC, Kelly B, Butine MD, McNamara P, Chengappa MM. Evaluation of enrichment techniques for the isolation of *Salmonella choleraesuis* from swine feces. J Microbiol Methods 1998; 33: 143-51.

29. Malorny B, Anderson A, Huber I. *Salmonella* real-time PCR-Nachweis. J Verbr Lebensm 2007; 2: 149-56.

30. Malorny B, Huehn S, Dieckmann R, Krämer N, Helmuth R. Polymerase chain reaction for the rapid detection and serovar identification of *Salmonella* in food and feeding stuff. Food Anal Methods 2009; 2: 81-95.

31. Chiu C, Ou JT. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. J Clin Microbiol 1996; 34: 2619-22.

32. Trkov M, Majeríková I, Jerašek B, Štefanovičová A, Rijpens N, Kuchta T. Detection of *Salmonella* in food over 30 h using enrichment and polymerase chain reaction. Food Microbiol 1999; 16: 393-9.

33. Eijkelkamp JM, Aarts HJM, van der Fels-Klerx HJ. Suitability of rapid detection methods for *Salmonella* in poultry slaughterhouses. Food Anal Methods 2008; 2: 1-13.

34. Saroj SD, Shashidhar R, Karani M, Bandekar JR. Rapid, sensitive, and validated method for detection of *Salmonella* in food by an enrichment broth culture – Nested PCR combination assay. Mol Cell Probes 2008; 22: 201-6.

35. Rall VLM, Rall R, Aragon LC, da Silva MG. Evaluation of three enrichment broths and five plating media for *Salmonella* detection in poultry. Braz J Microbiol 2005; 36: 147-50.

36. Schneider A, Grönewald C, Fandke M, Kurth B, Barkowski S, Berghof-Jäger K. Real-time detection of the genus *Salmonella* with the LightCycler system. Biochemica 2002; 4: 19-21.

PRIMERJAVA MOLEKULARNIH IN KLASIČNIH BAKTERIOLOŠKIH METOD ZA UGOTAVLJANJE SALMONEL V KOKOŠJEM BLATU

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Povzetek: Primerjali smo klasične (gojiščne) in molekularne (genetske) bakteriološke metode za ugotavljanje salmonel v referenčnih kapsulah, ki so vsebovale znano število bakterij serovarov Panama, Typhimurium ali Enteritidis vrste *Salmonella enterica* subsp*. enterica*, ter v blatu kokoši, ki je bilo bodisi naravno okuženo s salmonelami bodisi negativno na salmonele in okuženo z referenčnim materialom. Pri klasičnih tehnikah smo glede na ISO 6579 uporabili različna gojišča za obogatitev (MSRV, MKTTn in RVS) in za izolacijo na ploščah (XLD, BGA in Rambach agar). Molekularno določanje smo začeli po koraku predobogatitve. Primerjali smo učinkovitost dveh kompletov za osamitev DNK, in sicer High Pure foodproof I (Roche Diagnostics, Germany) in QIAamp DNA Stool Mini (Qiagen, Germany) kompletov, v kombinaciji s klasično in s PCR reakcijo v realnem času. Rezultati so pokazali, da je bilo ugotavljanje salmonel s klasičnimi in z molekularnimi metodami v referenčnih kontrolnih kapsulah neovirano, v vzorcih blata pa omejeno. Gojišče RVS je bilo manj primerno kot gojišči MSRV in MKTTn. Gojišče MKTTn je v kombinaciji s ploščami Rambach dalo največ pozitivnih rezultatov pri klasičnem pristopu. Priporočeno poltrdno gojišče MSRV je omogočalo ugotovitev salmonel pri velikem deležu vzorcev. Ta je bila najmanj odvisna od izbire gojišč za izolacijo, zato smo potrdili njegovo primernost. V nasprotju od gojiščnih metod je molekularni pristop, še posebno kombinacija High Pure foodproof I kompleta s PCR reakcijo v realnem času, omogočal uspešno ugotavljanje salmonel v vseh pozitivnih vzorcih, zato bi ga morali upoštevati kot pomembno dopolnitev klasičnega protokola za ugotavljanje salmonel v živilih.

Ključne besede: *Salmonella*; živila; blato; ugotavljanje; gojenje; molekularno; PCR