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Original Research Paper

COMPARISON AND OPTIMIZATION OF TWO PCR TESTS FOR IDENTIFICATION OF *SALMONELLA* IN POULTRY FEED-STUFFS, LIVER AND FAECES

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Summary: Classical cultivation method for Salmonella detection is relatively slow, which can sometimes cause serious clinical and economic consequences. The aim of our study was to compare the efficiency of different methods for rapid Salmonella detection in different samples and to optimize the most appropriate method of detection.
With the comparison and optimization of two previously described methods we established a new effective method for the rapid detection of Salmonella in animal tissues, faeces and feedstuffs after the enrichment step. This method, including the initial incubation on the nutrient media, was used to detect Salmonellae in the feedstuff samples that contained as low as 2 CFU of Salmonella before the incubation. After DNA extraction with a commercially available DNA extraction kit and after amplification by a previously described nested PCR we were able to find 175 CFU of Salmonella in a tissue sample without pre-incubation.

Key words: microbiology; veterinary; Salmonella – diagnosis – genetics; comparative study; DNA, bacterial – isolation and purification; polymerase chain reaction; feces – analysis; liver – analysis; animal feed – analysis

Introduction

Salmonella infections are of considerable health and hygienic problem worldwide, as the majority of *Salmonellae* are potentially pathogenic for humans and animals. *Salmonella* contributes to great morbidity and also to mortality, particularly in the undeveloped parts of the world. Salmonellosis is a zoonosis that falls under appropriate regulations (1). The animal-to-human transmission is usually due to the consumption of the food of animal origin. Direct human-to-human, human-to-animal and animal-to-human modes of transmission are also possible (2).

Salmonella-caused diseases became widespread with the usage of new feedstuffs (fish, bone and meat flour), intensive farm breeding and frequent consumption of frozen half-prepared food. The investigations have shown that the animals and the food originating from the animals (poultry meat, eggs, milk) represent the most important source of infection for humans, even though human-to-human transmission is possible. The ability of *Salmonella* to multiply in food plays an important role (3). *Salmonellae* can survive in feedstuffs for several years (4); this represents a source of infection for animals (5, 6).

Salmonella enterica subsp. *enterica* serovars Enteritidis (antigenic formula is: $\underline{1}$, 9 12: g,m: -) and Typhimurium (antigenic formula is: $\underline{1}$, 4, [5], 12: i: 1,2) (7) are among the most frequent agents causing diarrhoea in domestic and wild animals and enteritis in humans and rodents. *S.* Enteritidis is often isolated from poultry meat and eggs and can be also transmitted vertically (8). *S.* Typhimurium is isolated mostly from pigs. Outbreaks caused by multidrug-resistant *Salmonella* strains have been reported (9, 10, 11). In order to control and treat *Salmonella* infections, effective diagnostic and epizootiological methods are needed (12).

The existing standard culture method for the detection of *Salmonella* (13) requires five working days to generate and confirm positive results. It involves pre-enrichment in the buffer peptone water, selective enrichment, plating on the selec-

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tive agar, and subsequent identification by biochemical and serological tests. In the recent years, more rapid and specific PCR methods, based on the DNA sequence of *Salmonella* genes, have been developed to identify or to characterize pure culture strains (14, 15, 16, 17, 18, 19, 20). The aim of this study was to evaluate two previously published PCR methods for the detection of *Salmonella* in food and field samples and, on this basis, to develop a simple PCR-based protocol suitable for routine analysis of viable *Salmonella* in feedstuffs, animal tissues and faeces.

Material and methods

Samples

Salmonella-free poultry feedstuffs ("NSK" for laying hens and "BRO-finišer" finisher for broilers - TMK Ljubljana, Slovenia), chicken liver and faeces, tested at the Institute of Microbiology and Parasitology, Veterinary Faculty of Ljubljana, were used for the present study.

Bacterial strains and preparation of the inoculum

Microorganisms used in this study were either isolates from the Internal Collection of Veterinary Faculty (ICVF) or reference strains: *S.* Enteritidis (CAPM 5439), *S.* Typhimurium (ATCC 14028), *Proteus mirabilis* (DSM 788), *Klebsiella pneumoniae* subsp. *pneumoniae* (ATCC 51503), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 10536), *Citrobacter* sp. (ICVF) and *Listeria monocytogenes* (Wurzburg 4A).

The cultures from the archive were inoculated on blood agar (BA) and incubated 20-24 hours at 37°C. Single colonies from the pure culture of *S.* Enteritidis and *S.* Typhimurium were then inoculated in tryptic soy broth (TSB) and incubated 20-24 hours at 37°C. Serial ten-fold dilutions of the culture were made in 0.9% saline; the number of the colony forming units (CFU) was evaluated on the colony counting plates (CCP) after 20-24 hours of incubation at 37°C. The number of the cells in the initial suspension was 7 x 10^9 CFU/ml. Further dilutions are shown in.

Table 1: Dilutions of Salmonella culture

No	Dilution ratio	CFU/ml	CFU/sample
1	undiluted	7,000,000,000	3,500,000,000
2	1:10 (10^{-1})	700,000,000	350,000,000
3	1:10 (10^{-2})	70,000,000	35,000,000
4	1:10 (10^{-3})	7,000,000	3,500,000
5	1:10 (10^{-4})	700,000	350,000
6	1:10 (10^{-5})	70,000	35,000
7	1:10 (10^{-6})	7,000	3,500
8	1:10 (10^{-7})	700	350
9	1:2 (0.5 x 10^{-7})	350	175
10	1:5 (10^{-7})	70	35
11	1:2 (0.5 x 10^{-7})	35	17.5
12	1:2 (0.25 x 10^{-7})	17.5	8.75
13	1:2 (0.13 x 10^{-7})	8.75	4.38
14	1:2 (0.63 x 10^{-8})	4.38	2.19
15	1:2 (0.31 x 10^{-8})	2.19	1.09
16	1:2 (0.16 x 10^{-8})	1.09	0.54

Inoculation and pre-enrichment

The preparation of the samples was done either with (i) direct extraction without prior incubation or (ii) with previous incubation on the nutrient medium specified below.

(i) Three parallels of 0.5 ml of dilutions 7 to 16 (Table 1) of *S.* Typhimurium culture and 0.5 g of liver were added to 4.5 ml of buffered peptone water (BPW). The mixture was homogenized by vortex for 30 seconds and incubated for 20 minutes at room temperature. 1 ml of the homogenate was used for DNA extraction.

(ii) Feedstuffs and faecal samples were homogenized and divided into 25 g portions. Each portion was mixed with 225 ml BPW and homogenized. Three parallels of 0.5 ml of dilutions 10 to 16 (Table 1) of *S.* Typhimurium culture were added to each sample of faeces and feedstuff. The prepared samples of feedstuffs and faeces and the rest of the previously homogenized liver were incubated in BPW for 18 hours at 37°C, inoculated on tetrathionate broth (TTB) and incubated again for 18 hours at 37°C. 1 ml of TTB was used for DNA extraction.

DNA extraction

DNA extraction from the bacterial cultures. DNA was extracted using the simplified boiling method. A loop full of pure culture was suspended in 50 ml of PCR-grade water (Invitrogen, Carlsbad, CA, USA), heated at 100°C for 15 minutes and centrifuged at 12000 rpm for 2 minutes. The supernatant was used as a source of DNA for PCR.

DNA extraction from liver, feedstuffs and faeces. DNA extraction was done using three different

methods: (Ex-A) extraction with saccharose (Sigma-Aldrich, St. Louis, MO, USA), Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and proteinase K (Sigma-Aldrich, St. Louis, MO, USA) (21), (Ex-B) immunomagnetic separation with anti-*Salmonella* Dynabeads (Dynal, Oslo, Norway) and extraction with a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions (22), and (Ex-C) extraction with a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions.

DNA amplification

PCR specificity test. The primers were selected according to the available data in the literature (14, 21). Two different PCR tests were used:

(A-1)*:* The amplification mixture and the protocol described by Rychlik et al. (21) were followed without any modifications.

(A-2): A combined test, using the primers described by Aabo et al. (14) and the amplification protocol described by Trkov et al. (22). A 50 ml amplification mixture was used: 25 µl Taq PCR Master Mix (Qiagen, Hilden, Germany), 22 ml of PCR-grade water (Invitrogen, Carlsbad, CA, USA), 50 pmol of each primer (ST11 and ST15, Invitrogen, Carlsbad, CA, USA) and 2 ml of template DNA.

Detection of *Salmonella* in liver, feedstuffs and faeces with PCR. At first, DNA was amplified as described for the specificity test. Later, the reaction mixtures and the protocols were modified as follows.

(A-1m) The protocol of A-1 was modified using touch down PCR. The first 6 cycles consisted of initial denaturation for 1 minute at 94°C, annealing for 1 minute at 62°C (with the subtraction of 1°C in every cycle) and elongation for 1 minute at 72°C. The next 30 cycles consisted of initial denaturation for 30 seconds at 94°C, annealing for 30 seconds at 56°C and elongation for 30 seconds at 72°C. The final elongation was at 72°C for 7 minutes.

(A-2m) Similarly, the protocol of A-2 was also modified. The first 6 cycles consisted of initial denaturation for 1 minute at 94°C, annealing for 1 minute at 63°C (with the subtraction of 1°C in every cycle) and elongation for 1 minute at 72°C. The next 30 cycles consisted of initial denaturation for 30 seconds at 94°C, annealing for 30 seconds at 57.5°C and elongation for 30 seconds at 72°C. The final elongation was at 72°C for 7 minutes.

(A-2mm) In order to improve the specificity of the amplification we further modified A-2m using hot start PCR with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and increased annealing temperatures (for 1°C) were used. The 50 µl amplification mixture consisted of 1.25 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 5 µl 10x PCR buffer with 1.5 µl $MgCl_2$ (1.5 mmol), 0.2 mM each of dNTP (Invitrogen, Carlsbad, CA, USA), 50 pmol of each primer (Invitrogen, Carlsbad, CA, USA), 33.25 ml PCR-grade water (Invitrogen, Carlsbad, CA, USA) and 5 ml template DNA.

The amplification products were separated on 2% agarose gel and analyzed using a visualization system combining transilluminator and camera (Gel Doc 1000, Bio-Rad, Hercules, CA, USA).

Inoculation of the samples to the solid media

One loop (approx. 0.01 ml) of the dilution was inoculated on Rambach (RA) and xylose-lysine-deoxycholate (XLD) agars. Suspicious colonies were subcultured on Drigalski agar (DA) in order to obtain pure culture. The cultures from BPW, TTB, BA and CCP were inoculated to selective media (XLD, RA, DA). The colonies were determined serologically with slide agglutination and commercial biochemical test API (bioMerieux, Marcy-l'Etoile, France).

Results

PCR specificity test

All *Salmonella* strains used in our study showed a specific amplification product with the primers described by Rychlik et al. (21) and Aabo et al. (14). The other 6 different bacterial species used to test the specificity of the primers gave negative results.

Direct detection of Salmonella in liver

After DNA extraction by Ex-A and amplification by A-1 negative results were obtained after the first amplification, while nested PCR gave positive results for the samples containing at least 3500 CFU/sample.

DNA extraction with the commercial extraction kit (Ex-C) and amplification by A-1m enabled the detection of 3500 CFU/sample after the first amplification, while nested PCR gave positive results for the samples containing at least 175 CFU/sample. The results are shown in Table 2.

Ex-B and A-2 were not used for direct detection of *Salmonella* in liver.

Detection of Salmonella in liver, feedstuffs and faeces after enrichment

Detection results of *Salmonella* in liver and faeces of poultry are presented in Table 2.

Due to non-specific amplification products, obtained with all PCR methods, we additionally optimized the PCR tests for the feedstuff samples. An in-house method, including DNA extraction with the commercial kit (Ex-C) and amplification according to the A-2 using Platinum Taq DNA polymerase, was optimized. The obtained PCR results were then compared with the results of the culture method (Table 3).

According to the results of the colony counting the detection limit of the PCR test was 2.19 CFU/sample (Table 3).

Table 2: Detection of *Salmonella* Typhimurium in poultry liver and faeces

			Number of positive samples (3 parallels)										Total number of samples
Dilutions - CFU/sample			*3500*	*350*	*175*	*35*	*17.5*	*8.75*	*4.38*	*2.19*	*1.09*	*0.54*	
Detection in liver without pre-enrichment	Ex-A A-1	1st amp.	0	0	0	0	0	0	0	0	0	0	30
		2nd amp.	2	0	0	0	0	0	0	0	0	0	
	Ex-C A-1	1st amp.	2	1	0	0	0	0	0	0	0	0	30
		2nd amp.	3	3	3	0	0	0	0	0	0	0	
Detection in liver and faeces after pre-enrichment	Ex-A and A-1m		-	-	-	2	3	3	3	3	0	0	21
	Ex-B and A-2m		-	-	-	2	3	3	3	3	0	0	21
	Ex-C and A-2m		-	-	-	3	3	3	3	3	0	0	21

Legend: 1st amp. = first amplification; 2nd amp. = second amplification; - = not performed

Table 3: Comparison of the culture method and PCR for detection of *Salmonella* Typhimurium in feedstuffs

Sample	CFU/sample	Cultivation	PCR
1	35	+	+
2		+	+
3		+	+
4	17.5	+	+
5		+	-*
6		+	+
7	8.75	+	+
8		+	+
9		+	+
10	4.38	+	+
11		+	+
12		+	+
13	2.19	+	+
14		+	+
15		+	+
16	1.09	-	-
17		-	-
18		-	-
19	0.50	-	-
20		-	-
21		-	-
22	negative control	-	-

Discussion

Classical cultivation method for *Salmonella* detection is relatively slow, which can sometimes cause serious clinical and economic consequences. The aim of our study was to compare the efficiency of different methods for rapid *Salmonella* detection in different samples and to optimize the most appropriate method of detection.

In our study previously described methods of cultivation, DNA extraction and amplification (21, 22,) were used and compared. The methods that best suited our needs were optimized with the aim of choosing the method that would be comparable to the cultivation method in the terms of quality and reliability. Considering the fact that the costs of the novel diagnostic methods still exceed the cost of the classical methods, its main advantage was supposed to be the rapidity.

At first, the specificity of the primers described by Czech (21) and Danish (14) authors was tested with different bacterial species: *Escherischia coli*, *Proteus* sp., *Citrobacter* sp., *Klebsiella* sp., *Listeria* sp. and *Salmonella enterica* subsp. *enterica*. The results were comparable with the findings of Lin and Tsen (23). Because of the increasing clinical importance of the two *Salmonella* serovars, *S.* Enteritidis and *S.* Typhimurium, respectively, they were selected for the testing. A variety of different bacteria were used in order to check the methods' specificity for the genus *Salmonella,* while the two different serovars were used to detect any possible differences in the sensitivity between the serovars. All the three primer sets tested in our study were specific for the genus *Salmonella.* The PCR test performed by serial dilutions of the two most common *Salmonella* serovars, isolated in our laboratory, showed no differences in the sensitivity between the serovars. It was concluded that both serovars could be effectively detected by the same PCR method. So, for further studies only *S.* Typhimu-rium were used.

On the basis of these results the PCR test for the detection of *Salmonella* in different samples (liver, feedstuffs, faeces) was assessed and optimized.

For the direct detection of *Salmonella* in liver, nested PCR was inevitable, although the risk of cross contamination with the amplicons of the previous amplification plays a considerable role. Our results generated with the Ex-A and A-1 (detection of 3.500 CFU/ml) were in general comparable with the results of the Czech study (21), but isolation of DNA with Ex-C proved to be slightly more effective (detection of 175 CFU/ml). Since we still wanted to improve the detection limit, we performed the PCR method after the initial incubation also for liver samples.

After initial incubation of the samples (liver, faeces, feedstuffs), the amplification signal was clearly visible after the first run of amplification, therefore nested PCR was not necessary. These methods proved to be highly sensitive, as they were capable of detecting 2 cells in the sample. They are comparable with the method described by Croci et al. (24) who detected 1 to 10 CFU/25 grams. They also proved to be better than the method described by Cheung et al. (25), which detected 1.5×10^3 CFU.

The modified DNA extraction method (Ex-B) is time consuming and expensive but equally effective as the other two methods. Ex-A is the cheapest, but demanding and less suitable for standardization. Ex-C proved to be the most suitable for all kinds of samples. It is commercially available, reasonably priced, and easy to perform and standardize. Regardless of the extraction method it was necessary to incubate the samples for 18-24 hours at 37°C in order to get sufficient DNA yield. Amplification of the feedstuffs samples often resulted in non-specific amplification products, regardless of the extraction and amplification method. The problem was solved by using hot start PCR and by increasing annealing temperatures for 1°C.

Probably the most important achievement of the study is the optimized amplification protocol, combined with the usage of Platinum Taq DNA polymerase (A-2mm). This is a highly specific and effective method for the amplification of the DNA, extracted with the commercial kit from any sample, after the initial incubation on the nutrient media. This method was used to detect *Salmonella* in the feedstuff samples that contained as low as 2 CFU of *Salmonella* before the incubation. The results completely matched the results of the culture method and were even slightly better than the results of the similar studies (21, 22). Thus our goal was achieved: an effective and cost-friendly method for the rapid detection of *Salmonella* in different samples was optimized.

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PRIMERJAVA IN OPTIMIZACIJA DVEH TESTOV S POLIMERAZNO VERIŽNO REAKCIJO ZA UGOTAVLJANJE SALMONEL V ŽIVALSKIH TKIVIH IN VZORCIH IZ OKOLJA

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Povzetek: Klasična gojiščna preiskava za ugotavljanje salmonel je relativno dolgotrajna, kar ima včasih lahko resne klinične in ekonomske posledice. Cilj naše raziskave je bila primerjava uporabnosti različnih metod za hitro dokazovanje salmonel v različnih vzorcih in optimizacija najprimernejše metode. S primerjavo in optimizacijo dveh že poprej opisanih metod smo razvili novo učinkovito metodo za hitro ugotavljanje salmonel v živalskih tkivih, iztrebkih in živilih po prejšnji inkubaciji na obogatitvenem gojišču. S to metodo smo ugotovili salmonele v vzorcih krmil, ki so pred inkubacijo vsebovali samo 2 CFU salmonel. S kombinacijo izolacije DNK s komercialnim kitom in poprej opisane nested PCR pa nam je uspelo ugotoviti 175 CFU salmonel v vzorcu tkiva brez poprejšnje inkubacije.

Ključne besede: mikrobiologija – veterinarska; Salmonella – diagnostika-genetika; primerjalna študija; DNA, bakterijska – izolacija in čiščenje; polimerazna verižna reakcija; feces – analize; jetra – analize; krmila – analize