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SHORT
COMMUNICATION



A semi-nested PCR method with increased sensitivity for the specific, direct detection of *Salmonella enterica* strains in poultry ectoparasites

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ABSTRACT

The darkling beetle, *Alphitobius diaperinus*, and the poultry red mite, *Dermanyssus gallinae* are among the most common pests of poultry farms. Both pests can be carriers and reservoirs of various pathogens including zoonotic ones like *Salmonella*. Salmonellosis is one of the most common foodborne diseases reported in the EU. We developed a semi-nested PCR method for the direct detection of *Salmonella enterica*. When testing the specificity of the novel PCR, we successfully detected various *S. enterica* strains, whereas *Escherichia coli* and *Citrobacter* strains gave negative results. The authenticity of the PCR products was confirmed by DNA sequencing. The sensitivity of the semi-nested PCR was tested on serial dilution of bacterial cultures and extracted DNA. We found our new method more sensitive than the previous PCRs. We also screened ectoparasite samples, collected from a poultry farm in Hungary, and three out of the eight samples were positive for *S. Enteritidis*. This novel PCR seems suitable for the detection of *S. enterica* strains in poultry ectoparasites without the need of sample pre-enrichment.

KEYWORDS

salmonellosis, red mite, darkling beetle, poultry pathogens, PCR detection

The darkling beetle (*Alphitobius diaperinus*) and the poultry red mite (*Dermanyssus gallinae*) are among the most common pests in poultry farms (Axtell, 1994). The darkling beetles and their larvae are scavengers; they feed on a wide variety of organic matter, from poultry feed to droppings or carcasses. The red mites are ectoparasites and feed on the blood of poultry species, in particular the blood of domestic fowl (Leschen and Steelman, 1988; Axtell and Arends, 1990; Strother et al., 2005; Pritchard et al., 2016; Oh et al., 2020). Whereas the darkling beetle can have a negative effect on the poultry production mostly when an overgrowth beetle population is present (Axtell, 1994), the red mite infections may raise also animal welfare issues besides the obvious economical loss (Sigognault Flochlay et al., 2017). Moreover, both pests are possible carriers and reservoirs of different pathogens (from viruses to protozoans), including bacteria: *Campylobacter* spp., *Escherichia coli*, and *Salmonella* spp. (McAllister et al., 1996; Skov et al., 2004; Strother et al., 2005; Hazeleger et al., 2008; Poole and Crippen, 2009; Agabou and Alloui, 2010; Zheng et al., 2012; Crippen et al., 2018; Schiavone et al., 2022; Tamburro et al., 2022). It has been experimentally demonstrated that the poultry red mite is an effective vector of *Salmonella enterica* serovar Gallinarum biovar Gallinarum, the causative agent of fowl typhoid disease (Coccioleto et al., 2020). In addition, other findings have suggested that the poultry red mite may act as a reservoir for

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S. Gallinarum, allowing the pathogen to persist in poultry farms (Pugliese, et al., 2019). On the other hand, in a survey of red mites collected in poultry farms in Japan, Huong et al. (2014) have not found *S. enterica* in any of the samples. Salmonellosis is one of the most common foodborne disease, and it is the second most reported zoonotic disease in the EU according to the European Union One Health 2021 Zoonoses Report (European Food Safety Authority, 2022). PCR-based molecular assays are already available for the detection of *Salmonella* infection (Aabo et al., 1993; Szmolka et al., 2006). However, these assays were developed for the detection of *Salmonella* spp. after pre-enrichment or isolation from the poultry intestine, not for direct detection in non-host samples.

Therefore, the aim of our study was to provide a specific and sensitive molecular diagnostic tool for the direct detection of *S. enterica* in poultry ectoparasites and insects such as the red mite and the darkling beetle.

We collected four *A. diaperinus* and four *D. gallinae* samples, respectively, from a poultry farm in Hungary (Table 1). After collection, samples were stored at -20°C prior to molecular biological analyses. For nucleic acid extraction, samples were homogenised in 500 μL $1 \times \text{PBS}$ buffer using micropestle (Eppendorf) in 1.5 mL microtube, then centrifuged in an Eppendorf 5424R tabletop centrifuge (Eppendorf) at $9,400 \times g$ for 5 min. The nucleic acid extraction was performed using 200 μL supernatant with the IndiSpin Pathogen kit (Indical Bioscience) following the manufacturer's manual.

For the detection of *Salmonella* spp. in ectoparasites, we developed a semi-nested PCR method. Round 1 was a known PCR, published by Aabo et al. (1993) and later tested by Szmolka et al. (2006). The name and sequence of the primers are ST11F (5'-AGC CAA CCA TTG CTA AAT TGG CGC A-3') and ST15R (5'-GGT AGA AAT TCC CAG CGG GTA CTG-3'). These primers amplify a 429-bp fragment from the gene of the bacterial DNA/RNA non-specific endonuclease. To increase the sensitivity, we designed a new, internal forward primer ST23F3 (5'-GCA CAA CCT TCG ACA CAG ACG-3') and used it in the second round of the semi-nested system along with the reverse primer ST15R. The product of the second PCR round was a 407-bp DNA fragment.

A Labcycler Basic (SensoQuest) thermocycler was used with the following thermal cycling profile. The initial

denaturation at 94°C for 3 min, was followed by 35 cycles consisting of denaturation (94°C for 30 s), annealing (61°C for 60 s) and extension (72°C for 30 s) steps. After the final cycle of both rounds, an additional final extension was applied at 72°C for 10 min as recommended by Szmolka et al. (2006). The reaction mixture of round 1 consisted of 12.5 μL of $2 \times \text{DreamTaq Hot Start Green PCR Master Mix}$ (Thermo Fisher Scientific), 0.5 μL of primer ST11F (10 μM), 0.5 μL of primer ST15R (10 μM), 2.5 μL of the template DNA (100–300 ng in average) and 9.5 μL double-distilled, ultrapure water (ddH_2O) to complete the volume to 25 μL . The components of the reaction mixture of round 2, were almost the same, except that ST23F3 (10 μM) was used as forward primer and 1 μL of the round 1 reaction mixture was used as target DNA.

To test the sensitivity of the semi-nested PCR system, two sets of 10-fold serial dilutions were prepared. The first serial dilution was made from an overnight (37°C , 18 h) broth culture of *S. enterica* strain (ATCC 13076). The concentration of the bacterium suspension was $4.2 \times 10^8 \text{ CFU mL}^{-1}$ (colony-forming unit millilitre $^{-1}$), from which eight-step dilutions were prepared. After homogenisation in an Elmasonic P30H ultrasonic bath (Elma) at room temperature for 15 min, DNA was extracted from each dilution using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research Corporation) following the manufacturer's instructions. Subsequently, an additional 10-fold serial dilution was prepared from the extracted DNA of the stock *S. enterica* suspension.

The DNA concentration of the first dilution series was measured with Qubit dsDNA High Sensitivity Assay Kit with Qubit 3 Fluorometer (Thermo Fisher Scientific). Only the first three dilutions had measurable amount of nucleic acids. The DNA concentration of the stock *S. enterica* suspension was $7.3 \text{ ng } \mu\text{L}^{-1}$, in the ten-fold and hundred-fold dilutions, it was $1.6 \text{ ng } \mu\text{L}^{-1}$ and $0.3 \text{ ng } \mu\text{L}^{-1}$, respectively. The DNA concentration of extracted DNA dilution series was $7.7 \text{ ng } \mu\text{L}^{-1}$ for the stock, $1.4 \text{ ng } \mu\text{L}^{-1}$ for the ten-fold dilution, and $0.1 \text{ ng } \mu\text{L}^{-1}$ for the hundred-fold dilution.

To determine the specificity of the developed semi-nested PCR assay, both reference (ref) and wild type (wt) strains of two *E. coli*, a *Citrobacter rodentium* and four *Salmonella* spp. strains were included. The two *E. coli* strains examined were: EC1 (ATCC 25922, ref); ECM (VB1/Ec1, wt, isolated from broiler caecum). The *Salmonella* spp. strains represented the main serovars including SE1 (*S. Enteritidis*

Table 1. Summary of poultry ectoparasite/insect, *A. diaperinus* and *D. gallinae* samples collected in the present study, and the outcome of *Salmonella*-specific, semi-nested PCR and subsequent DNA sequencing

Sample ID	Ectoparasite	Date of sampling	PCR result	BLAST result (NCBI Acc.No) & identity
A-23-01	<i>Alphitobius diaperinus</i>	13. 03. 2023	positive	<i>S. Enteritidis</i> (CP125220) – 100%
A-23-02	<i>Alphitobius diaperinus</i>	13. 03. 2023	negative	–
A-23-03	<i>Alphitobius diaperinus</i>	13. 03. 2023	negative	–
A-23-04	<i>Alphitobius diaperinus</i>	13. 03. 2023	negative	–
M-23-02	<i>Dermanyssus gallinae</i>	31. 05. 2023	negative	–
M-23-03	<i>Dermanyssus gallinae</i>	04. 07. 2023	negative	–
M-23-04	<i>Dermanyssus gallinae</i>	04. 07. 2023	positive	<i>S. Enteritidis</i> (CP125220) – 100%
M-23-05	<i>Dermanyssus gallinae</i>	04. 07. 2023	positive	<i>S. Enteritidis</i> (CP125220) – 99.4%



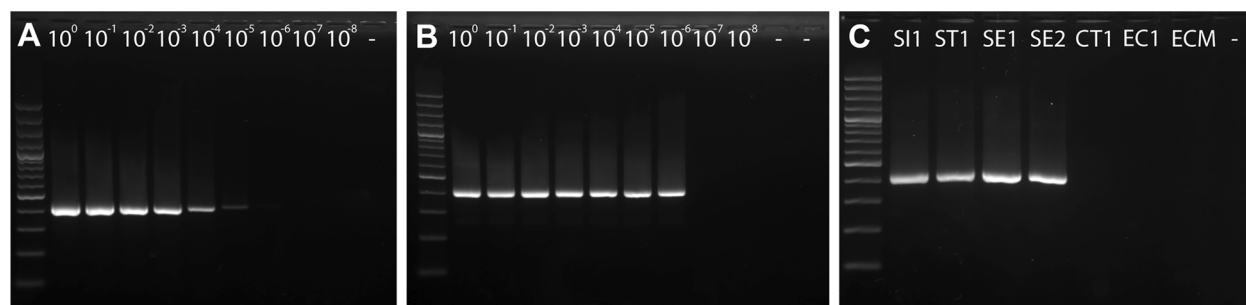


Fig. 1. The sensitivity and the specificity of the *Salmonella enterica*-specific, semi-nested PCR assay developed in the present study. A: the sensitivity test of PCR round 1 on the 10-fold serial dilution of the *S. Enteritidis* stock suspension (DNA concentration $7.7 \text{ ng } \mu\text{L}^{-1}$). B: PCR round 2 of the 10-fold serial dilution. C: the specificity test of PCR round 2; SI1: *S. Infantis*, ST1: *S. Typhimurium*, SE1: *S. Enteritidis* reference strain (ATCC 13076), SE2: *S. Enteritidis* wild strain, CT1: *Citrobacter rodentium* strain, EC1: *E. coli* strain ATCC 25922, ECM: *E. coli* strain VB1/Ec1 (isolated from broiler caecum). DNA fragment size marker: GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific) on 1.5% agarose gel.

ATCC 13076, ref); SE2 (*S. Enteritidis* SE46, wt, isolated from human faeces); ST1 (*S. Typhimurium* ATCC 14028, ref); and SI1 (*S. Infantis* VB1/S1, wt, isolated from broiler caecum). The PCR products were purified with ExoSAP IT PCR product Cleanup Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Sanger DNA sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies), and detected on Applied Biosystems Genetic Analyzer 3,500 (Thermo Fisher Scientific). The sequences were submitted to BLAST search using the Megablast algorithm on the NCBI website.

The detection limit of round 1 of the PCR was $4.2 \text{ CFU } \mu\text{L}^{-1}$, which corresponded to $7.7 \times 10^{-5} \text{ ng } \mu\text{L}^{-1}$ extracted DNA (Fig. 1A). The full, semi-nested PCR performed better with an as low detection limit as $0.42 \text{ CFU } \mu\text{L}^{-1}$, i.e. $7.7 \times 10^{-6} \text{ ng } \mu\text{L}^{-1}$ DNA concentration (Fig. 1B). Identical detection limits were obtained with both dilution series.

All four *Salmonella* spp. strains were found positive with the developed semi-nested PCR, whereas none of the *E. coli* and *Citrobacter* strains were detectable (Fig. 1C). BLAST search with the DNA sequences of the PCR products confirmed the specific amplification of all the reference and wild type strains of *Salmonella* spp. (i.e. strains SI1, ST1, SE1 and SE2).

When testing the field samples of mites and insects from a poultry farm, three samples were found positive using the new semi-nested PCR (Table 1), although none of them were positive after round 1. Based on BLAST search, all three DNA sequences belong to strain *S. Enteritidis*. The closest DNA sequence (with 99.4–100% nucleotide identity) was *S. enterica* subsp. *enterica* serovar Enteritidis strains m140 (NCBI Acc. No CP125220). Based on a 328-bp alignment, the pairwise sequence identities among the three positive samples were 100% between samples A23-01 and M23-04, and 99.4% between M23-05 and A23-01/M23-04.

Our goal was to develop and test a PCR that is more sensitive than those described and used for the detection of *S. enterica* strains previously. In a previous study, 144 of 146 *Salmonella* serovars have been identified using the outer

primer pair (Aabo et al., 1993). Adding a second round to this PCR helped to increase the sensitivity of the detection by one order of magnitude, i.e. the detection limit our semi-nested PCR method is ten times lower than that of the original PCR used by Szmolka et al. (2006).

Additional molecular methods have been developed to detect *Salmonella* from multiple sample types (Almeida et al., 2013; Mooijman et al., 2019). Mooijman et al. (2019) have performed a study in the frame of Mandate M/381 to validate the European and International Standard method (EN ISO 6579:2002/Amd.1:2007) for the detection of *Salmonella* spp. in samples from the primary production stage. The samples have been pre-enriched in non-selective broth and selective agar medium. The detection rate was 100% for test samples from the high concentration group, but only 67.4% for samples contaminated with low concentration *Salmonella* Enteritidis (Mooijman et al., 2019). Valiente Moro et al. (2007) developed a molecular detection tool using a simple, filter-based DNA preparation in combination with a *Salmonella* spp.-specific, 16S rDNA-based PCR, which resulted in a detection limit of $2 \times 10^4 \text{ CFU}$ per mite (Valiente Moro et al., 2007). Almeida et al. (2013) have used *in situ* hybridization and real-time PCR to detect *S. Enteritidis* in artificially contaminated, pre-enriched samples. The sensitivity of their method was as low as 1 CFU per 25 g or mL of sample (Almeida et al., 2013). Our result show that a similar low detection limit can be achieved even without pre-enrichment procedure.

Based on the outcome of the specificity test, the developed semi-nested PCR reliably detected *Salmonella* strains without any false positive detection of possible contaminant *E. coli* strains (which are common co-infections of *Salmonella* spp. in poultry). Thus, our results demonstrate that this novel semi-nested PCR is sufficiently specific, whereas it is more sensitive, than the previous *Salmonella*-specific PCRs. We expect that it will be useful in the direct detection of *Salmonella* spp. in various samples of ectoparasites and insects from poultry houses.

Conflict of interest: The corresponding author, Edit Eszterbauer is a section editor of the Acta Veterinaria Hungarica.



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