Medical and Veterinary Entomology (2007), **21**, 148–152

Detection of *Salmonella* sp. in *Dermanyssus gallinae* using an FTA® filter-based polymerase chain reaction

C. VALIENTE MORO, S. DESLOIRE, C. CHAUVE and L. ZENNER
UMR 958 ENVL/INRA Protozoaires Entéricoles et Parasites des Volailles, École Nationale Vétérinaire de Lyon, Marcy l'Étoile, France

Abstract. *Salmonella* spp. bacteria are responsible for some of the most important zoonoses worldwide. Because *Dermanyssus gallinae* (DeGeer) (Acari: Dermanyssidae) has been recently reported to be an experimental vector of *Salmonella* Enteritidis, it would be of benefit to evaluate the presence of this bacterium in mites. A molecular detection tool associating a simple filter-based DNA preparation with a specific 16S rDNA *Salmonella* sp. polymerase chain reaction (PCR) amplification was described. The limit of detection with this method was 2×10^4 bacteria per mite. To adapt this technique for large-scale studies, two sizes of mite pools were tested and a preliminary investigation was carried out on mites from 16 currently or previously contaminated farms. Mites sampled from one farm of each type were positive for *Salmonella*, suggesting that *Dermanyssus* could act as a reservoir between flocks. In further investigations, it will be necessary to carry out a large-scale study to assess the role of *D. gallinae* in the epidemiology of avian salmonellosis.

Key words. *Dermanyssus gallinae*, *Salmonella*, filter paper DNA preparation, PCR detection.

Introduction

Poultry products are among the most important sources of *Salmonella* that can be transmitted through the food chain to humans (Lacey, 1993). These bacteria coexist within the chicken and, although not necessarily harmful, they are responsible for some of the most widespread zoonoses in the world. *Salmonella* Enteritidis is the serovar most often incriminated in human *Salmonella* infection and many food safety laws and hygiene controls are aimed at preventing its transmission. These bacteria survive in various wildlife reservoirs, and their presence in arthropods has been recorded (Davies & Breslin, 2003). *Dermanyssus gallinae* is an important and widespread haematophagous ectoparasite of layer hens, which causes significant economic losses, decreased egg production and anaemia (Chauve, 1998). Moreover, this parasite has been suspected of being or demonstrated to be a vector of several major pathogenic agents, including *Salmonella gallinarum*, which has been isolated from mites collected from infested poultry farms (Zeman *et al.*, 1982; Valiente Moro *et al.*, 2005). Recently, this mite has been shown to be an experimental vector of this bacterium: it can become infected with *S.* Enteritidis through cuticular contact or via a contaminated bloodmeal. The bacteria can multiply, be transmitted to the next generation and transtadial transmission from protonymphs to deutonymphs is also possible. Finally, it has been demonstrated that *D. gallinae* mites can retransmit the pathogen by bite and after their ingestion by birds (Valiente Moro *et al.*, 2007a, 2007b). To confirm the role of *D. gallinae* as a natural vector of *Salmonella* and/or as a reservoir in poultry farms necessitates the detection of bacteria from mites collected in contaminated farms, for which a suitable tool is required.

The object of this study was to develop a method for detecting *Salmonella* sp. in mites and to test this method in a preliminary investigation using mites collected from contaminated farms.

Correspondence: Sophie Desloire, UMR 958 ENVL/INRA Protozoaires Entéricoles et Parasites des Volailles, École Nationale Vétérinaire de Lyon, 1 Avenue Bourgelat, 69280 Marcy l'Étoile, France. Tel.: + 33 4 78 87 25 75; Fax: + 33 4 78 87 25 77; E-mail: s.desloire@vet-lyon.fr

Materials and methods

Determination of assay sensitivity

Mites were collected from laying hen breeding facilities in the Rhône-Alpes region (France). The absence of *Salmonella* in the mites was previously confirmed in three groups of 15 individuals by culture on *Salmonella* Medium Identification Detection (SMID) (Biomérieux, Craponne, France). In order to estimate the sensitivity of the assay, a culture of *Salmonella enterica* ssp. Enterica serotype Enteritidis (*S.* Enteritidis) was added to a single uninfected mite. An *S.* Enteritidis culture was diluted to an approximate number of 1 McFarland standard (3×10^8 colony-forming units (CFU)/mL) and then 10-fold serial dilutions were prepared in Peptone buffered water (PBW) (Biomérieux) to obtain 1×10^8 to 1×10^2 CFU/mL cultures. From each dilution, four aliquots of 20 μL were prepared and a single blood-fed *D. gallinae* was added to two of them and crushed with a sterile plastic pestle. The four samples were then subjected to the DNA screening method described below. The four lowest bacterial dilutions were used as a control by streaking on SMID. Each experiment was carried out three times.

Salmonella detection in artificially infected D. gallinae *adult mites and detection limits in a pool of mites*

An *in vitro* feeding device (Bruneau *et al.*, 2001) was used to artificially feed and infect mites. They were fed on *S.* Enteritidis infected blood at the rate of 10^8 CFU/mL, for 4–5 h in 25° C at 60–70% relative humidity (RH) and total darkness. Mites were collected the day after the meal, then crushed in 20 μL of PBW and the resulting homogenate was subjected to DNA extraction. To determine the most suitable size of the pool to be analysed, a single infected mite was artificially mixed and crushed together with non-infected mites and the resulting homogenate was tested by polymerase chain reaction (PCR) for the presence of *S.* Enteritidis. A total of 22 infected mites were crushed individually in 20 μL of PBW. Five μL of each homogenate was subjected to the DNA preparation method and tested by PCR for the presence of *S.* Enteritidis. The remaining 15 μL PBW was made up to 40 μL. For 10 pools, 14 engorged but non-infected *D. gallinae* mites were added and crushed in these 40 μL; for 12 pools, 29 mites were added. Finally, 10 μL of this homogenate was treated as described below for DNA preparation and tested by PCR amplification.

DNA preparation by FTA Cards® and molecular detection of Salmonella

DNA templates for PCR reactions were prepared using FTA® technology as described by the manufacturer (Whatman, Brentford, U.K.), using the protocol recommended for samples with blood. The homogenates from a single crushed mite or from a pool of mites were left for 30 min and 2 h, respectively, to allow blood debris to settle, after which 5 μL and 10 μL, respectively, were removed and applied to the FTA® filter card. Discs of 1.2 mm diameter (corresponding to about a tenth of the total homogenates application area) were punched from the card, rinsed as described and used directly as templates for PCR amplification.

A primer pair targeting the 16S rDNA gene of every known serovar of *Salmonella enterica* sp. except subgroups VI and VII, and of *S. bongori* spp., was used to amplify a product of 574 bp (Lin & Tsen, 1996; Ziemer & Steadham, 2003). Each 25-μL PCR reaction contained 0.5 μM of each oligonucleotide primer (5′-TGTTGTGGTTAATAACCGCA-3′ and 5′-CACAAATCCA-TCTCTGGA-3′), 200 μM of each dNTP (Amersham BioScience, Chalfont St. Giles, U.K.), 1 mM of $MgCl_2$ (Invitrogen, Carlsbad, CA, U.S.A.), 2.5 μL 10X buffer (Invitrogen), 1 U of recombinant Taq Polymerase (Invitrogen), and 2 μL DNA extract, or the FTA® filter paper disc. In each PCR experiment, positive and negative controls were added, respectively, a DNA from a colony of *S.* Enteritidis, extracted by a Qiamp DNA extraction kit (Qiagen, Courtaboeuf, France), and a punch where only PBW was applied. The thermocycler (MWG Biotech, Ebersberg, Germany) conditions were 10 min of denaturation at 95° C, followed by 37 cycles of denaturation at 94° C (1 min), annealing with decreasing temperature (45 s, 65−58° C for the first 12 cycles and 58° C for the remaining 25 cycles), an extension at 72° C (1 min), and a final extension step of 5 min at 72° C. Electrophoresis of the amplification reactions was carried out in 2% agarose gels in 1X Tris-acetate/ethylenediaminetetraacetic acid (EDTA).

Analysis of mites sampled from Salmonella-contaminated farms

The presence of *Salmonella* was tested in mite samples collected at two types of laying farms: six farms were currently declared positive for *Salmonella* by the DSV (Direction des Services Vétérinaires) and 10 had been declared positive previously. For each farm, six to 20 pools of 15 mites (a total of 249 pools) were analysed for the presence of *S.* Enteritidis as described above. Mites were previously washed twice in 100 μL 4% paraformaldehyde for 7 min while being constantly agitated and once in 100 μL sterile water following the protocol already described (Valiente Moro *et al.*, 2007a).

Results and Discussion

As *D. gallinae* has been demonstrated to be an experimental vector of *S.* Enteritidis, a rapid and simple method for detecting *Salmonella* within mites is required to investigate the role of this mite as a natural vector of *Salmonella* in poultry farms. For this reason, PCR, already demonstrated as specific and sensitive for *Salmonella* detection (Kwang *et al.*, 1996; Baümler *et al.*, 1997) was used in association with FTA® technology, which is a simple and less time-consuming method of DNA preparation. This filter paper preparation was successfully used to detect foodborne bacteria such as *Salmonella* (Lampel *et al.*, 2000) and pathogenic agents in arthropods (Higgins *et al.*, 2000; Snowden *et al.*, 2002).

When sample discs corresponding to the serial dilutions of pure culture were used as templates, an amplification signal was observed from as few as 50 CFU (Fig. 1A). This sensitivity level is similar to that reported for the detection of *Salmonella enterica* serotype Typhimurium with FTA® technology (Lampel *et al.*, 2000). When a mite was added to the *S.* Enteritidis culture, the level of detection became 5×10^2 CFU (Fig. 1B). As the sample paper disc subjected to the amplification corresponds to a tenth of the total area upon which the homogenate was applied and a quarter of the total homogenate was deposited on the filter paper, this assay was able to detect 2×10^4 bacteria per mite. The 10-fold decrease in sensitivity could be explained by the presence of potential PCR inhibitors within mite tissues and residues resulting from previous bloodmeals, as demonstrated previously (Desloire *et al.*, 2006). In a study describing the detection of *Borrelia burgdorferi* DNA inside blood-fed ticks, Schwartz *et al.* (1997) demonstrated that inhibitors of PCR amplification were present in engorged ticks. However, Peter *et al.* (1995) obtained a better sensitivity with 200 *Cowdria ruminantium* rickettsiae per tick when amplifying a part of the pCS20 sequence of *C. ruminantium*, but the authors determined this detection limit by adding a pure DNA sample extracted from the tick to the bacterial culture, rather than the whole arthropod.

Salmonella Enteritidis was amplified from 43 of 70 mites (61%) artificially fed on infected blood. The other mites probably contained a lower number of bacteria than the limit of detection of 2×10^4 CFU. Although each mite is able to ingest 2×10^4 CFU of bacteria during a bloodmeal under our conditions (0.2 μL from an inoculum with 10^8 CFU/mL), in practice, there are many individual variations in the process of blood ingestion, which could explain why not all the mites were found to be positive on PCR (Valiente Moro *et al.*, 2007a). To facilitate the monitoring of *Salmonella* through *D. gallinae* in breeding hens, two sizes of pools, of 15 and 30 individuals, respectively, were tested. No positive PCR signal was obtained from the 30-mite pools but seven of 10 of the 15-mite pools gave a positive signal even though each of the 22 infected mites used in pools had been previously confirmed as positive by PCR (Fig. 2). This method was therefore used subsequently with 15 mites per pool.

Preliminary field studies were then undertaken using this methodology. Of six currently infected farms, only one farm had four positive pools (out of six) for *Salmonella*. Of the 10 farms that had previously been declared infected, only one farm had one positive pool (out of six). It is unlikely that this low number of positive cases was a result of a low level of infection within the mites, as we have already demonstrated that *Salmonella* can multiply inside the mites and that a third of experimentally infected mites carry more than 2×10^4 CFU of *Salmonella* after 14 days (Valiente Moro *et al.*, 2007a). We assume that in contaminated poultry, some mites contain a similar number of *Salmonella* after bacterial multiplication.

This preliminary study validates the use of this methodology in poultry farms. Moreover, despite limited sampling, we demonstrate that *D. gallinae* can be naturally infected in a contaminated poultry environment. Another interesting result is that *Salmonella* was detected in mites collected from a farm that was not currently contaminated. This suggests that mites infected during a previous outbreak survived the sanitation periods as well as the cleaning and disinfection programmes and may be a source of infection for replacement birds. *Dermanyssus gallinae*

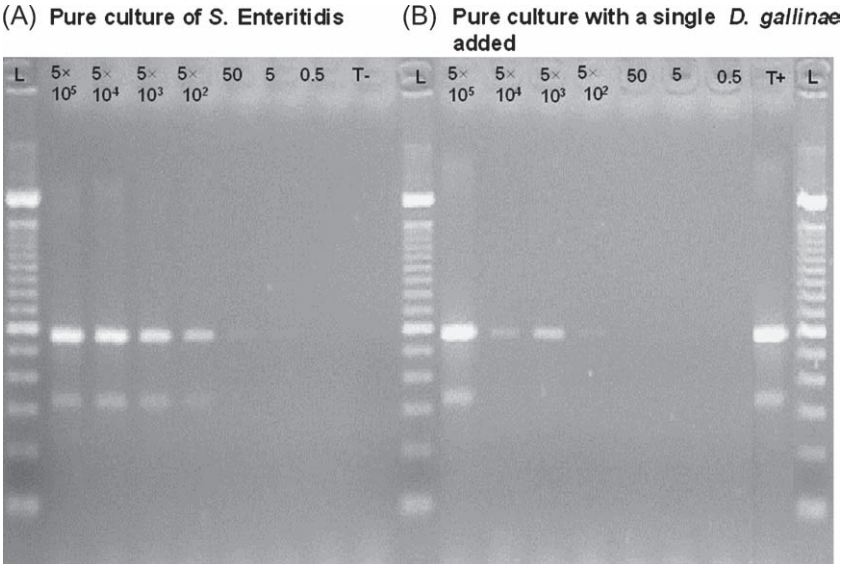

Fig. 1. Detection limit of polymerase chain reaction (PCR). (A) Pure culture of *Salmonella* Enteritidis. Products of PCR from reactions performed from a punched FTA® disc on which an *S.* Enteritidis culture, ranging from 5×10^5 to 0.5 CFU, was applied. The 100-bp DNA ladder, and negative and positive reaction control PCR products, are in lanes L, T−, and T+, respectively. (B) Pure culture with a single *Dermanyssus gallinae* added. Products of PCR from reactions performed from a punched FTA® disc on which an *S.* Enteritidis culture, ranging from 5×10^5 to 0.5 CFU, with a *D. gallinae*, was applied.

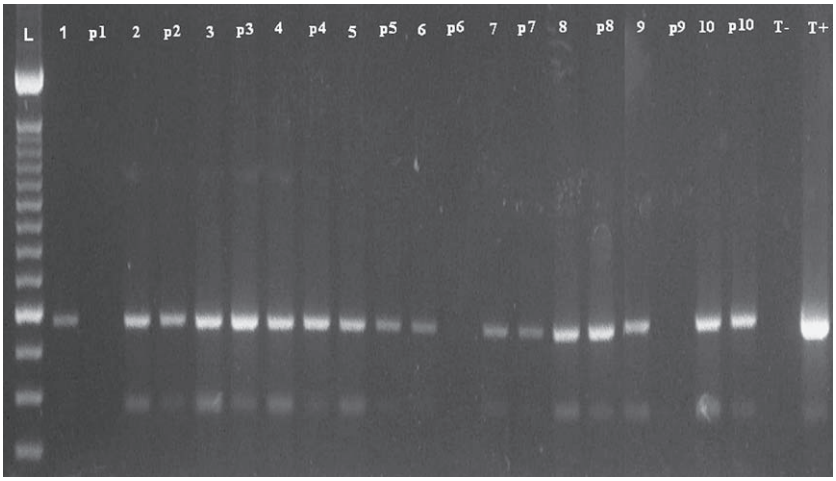

Fig. 2. Detection limits in pools of 15 mites. Polymerase chain reaction products from reaction performed from a punched FTA® disc on which an homogenate containing 15 *Dermanyssus gallinae (*one infected and 14 uninfected) was applied. For each of the 10 pools, PCR product from the single infected mite of the pool is shown in a lane, with PCR product corresponding to this pool in the following lane. The 100-bp DNA ladder, and negative and positive reaction control PCR products are, respectively, in lanes L, T− and T+.

could therefore act as a potential reservoir and will certainly play a role in the epidemiology of avian salmonellosis, as previously suggested by Zeman *et al.* (1982).

Methodological tools are now available to assess the role of *D. gallinae* in avian salmonellosis. Moreover, this preliminary study shows that this mite can naturally harbour the pathogen and allow its persistence between outbreaks. In order to accurately evaluate the prevalence of mites carrying *Salmonella* in poultry farms, it will be necessary to carry out a national study in partnership with DSV (Direction des Services Vétérinaires) in France.

Acknowledgements

C. Valiente Moro and S. Desloire contributed equally to this work. We thank Cuog Dang Quoc for technical assistance. We also thank the Laboratoire Central de Recherche Avicoles et Porcines of AFSSA (Agence Française de Sécurité Sanitaire des Aliments), Ploufragan (France) for providing the *Salmonella* strain, and Gerald Landrier, of the ITAVI (Institut Technique de l'AVIculture) organization, and avian veterinarians for collecting mite samples at infected poultry farms.

References

Baümler, A.J., Heffron, F. & Reissbrodt, R. (1997) Rapid detection of *Salmonella enterica* with primers specific to iroB. *Journal of Clinical Microbiology*, **35**, 1224–1230.

Bruneau, A., Dernburg, A., Chauve, C. & Zenner, L. (2001) First *in vitro* cycle of the chicken mite, *Dermanyssus gallinae* (De Geer, 1778), utilizing an artificial feeding device. *Parasitology*, **123**, 583–589.

Chauve, C. (1998) The poultry red mite *Dermanyssus gallinae*: current situation and future prospects. *Veterinary Parasitology*, **8**, 364–376.

Davies, R.H. & Breslin, M. (2003) Persistence of *Salmonella* Enteritidis Phage Type 4 in the environment and arthropod vectors on an empty free-range chicken farm. *Environmental Microbiology*, **5**, 79–84.

Desloire, S., Valiente Moro, C., Chauve, C. & Zenner, L. (2006) Comparison of four methods of extracting DNA from *D. gallinae* (Acari: Dermanyssidae). *Veterinary Research*, **37**, 725–732.

Higgins, J.A., Hubalek, Z., Halouzka, J., Elkins, K.L., Sjostedt, A., Shipley, A.M. & Ibrahim, M.S. (2000) Detection of *Francisella tularensis* in infected mammals and vectors using a probe-based polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene*, **62**, 310–318.

Kwang, J., Littledike, E.T. & Keen, J.E. (1996) Use of polymerase chain reaction for *salmonella* detection. *Letters in Applied Microbiology*, **22**, 46–51.

Lacey, R.W. (1993) Food-borne bacterial infections. *Parasitology*, **107** (Suppl), 75–93.

Lampel, K.A., Orlandi, P.A. & Kornegay, L. (2000) Improved template preparation for PCR-based assays for detection of food-borne bacterial pathogens. *Applied and Environmental Microbiology*, **66**, 4539–4542.

Lin, C. & Tsen, H. (1996) Use of two 16S DNA targeted oligonucleotides as PCR primers for the specific detection of *Salmonella* in foods. *Journal of Applied Bacteriology*, **80**, 659–666.

Peter, T.F., Deem, S.L., Barbet, A.F., Norval, R.A., Simbi, B.H., Kelly, P.J. & Mahan, S.M. (1995) Development and evaluation of PCR assay for detection of low levels of *Cowdria ruminantium* infection in *Amblyomma* ticks not detected by DNA probe. *Journal of Clinical Microbiology*, **33**, 166–172.

Schwartz, I., Varde, S., Nadelman, S.R.B., Wormser, G.P. & Fish, D. (1997) Inhibition of efficient polymerase chain reaction amplification of *Borrelia burgdorferi* DNA in blood-fed ticks. *American Journal of Tropical Medicine and Hygiene*, **56**, 339–342.

Snowden, K.F., Logan, K.S. & Vinson, S.B. (2002) Simple, filter-based PCR detection of *Thelohania solenopsae* (Microspora) in fire ants (*Solenopsis invicta*). *Journal of Eukaryotic Microbiology*, **49**, 447–448.

Valiente Moro, C., Chauve, C. & Zenner, L. (2005) Vectorial role of some dermanyssoid mites, (Acari, Mesostigmata, Dermanyssoidea). *Parasite*, **12**, 99–109.

Valiente Moro, C., Chauve, C. & Zenner, L. (2007a) Experimental infection of *Salmonella* Enteritidis by the Poultry Red Mite, *Dermanyssus gallinae*. *Veterinary Parasitology*, **146**, 329–336.

Valiente Moro, C., Fravalo, P., Amelot, C., Chauve, C., Salvat, G. & Zenner, L. (2007b) Colonization and organ invasion in chicks experimentally infected with *Dermanyssus gallinae* contaminated by *Salmonella* Enteritidis. *Avian Pathology*, in press.

Zeman, P., Stika, V., Shalka, V.B., Bartik, M., Dusbabeck, F. & Lavickova, M. (1982) Potential role of *Dermanyssus gallinae* (De Geer 1778) in the circulation of the agent of pullurosis-typhus in hens. *Folia Parasitologica*, **29**, 371–374.

Ziemer, C.J. & Steadham, S. (2003) Evaluation of the specificity of *Salmonella* PCR primers using various intestinal bacterial species. *Letters in Applied Microbiology*, **37**, 463–469.

Accepted 10 April 2007

