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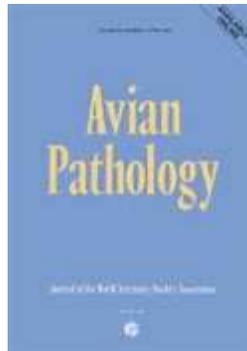
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Colonization and organ invasion in chicks experimentally infected with *Dermanyssus gallinae* contaminated by *Salmonella* Enteritidis

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Short title : *S. Enteritidis* infection in chicks via *D. gallinae*

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Colonization and organ invasion in chicks experimentally infected with *Dermanyssus gallinae* contaminated by *Salmonella* Enteritidis

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Abstract.

The poultry red mite, *Dermanyssus gallinae* is the most important and common ectoparasite of laying hens in Europe. This haematophagous mite has been experimentally demonstrated to be a vector of *Salmonella* Enteritidis by acquiring bacteria through the blood meal or cuticular contact. We have evaluated another route of infection by orally inoculating chicks with mites previously infected by *S. Enteritidis*. Two methods of infecting the mites were tested: mites contaminated by cuticular contact or during the blood meal. After the washing of mites with Paraformaldehyde, groups of 10 *Salmonella*-contaminated mites were inoculated individually into 1-day-old chicks. The titre of the inoculum suspension was evaluated by crushing mites and followed by bacteriological counting. It was 3×10^4 colony-forming units (CFU) and 2.7×10^6 CFU/chick, respectively, for cuticular contact and orally mediated contamination of mites. Each bird was found to be positive 12 days postinoculation. *Salmonella* colonized the intestinal tracts and invaded livers and spleens. Caecal content concentration reached a mean number of *S. Enteritidis* of 8.5×10^4 MPN *Salmonella* g⁻¹. This experiment demonstrated the ability of mites to orally infect one-day-old chicks with subsequent colonization and multiplication of *Salmonella*. Consequently, mites infected by *S. Enteritidis* constitute potential reservoir hosts of this bacterium, allowing it to persist in the poultry house as a source of infection for newly introduced animals. If contaminated mites are found in poultry facilities, effective red mite control should be performed before new batches are introduced into the facility.

Introduction

Salmonella is associated with many of the most important outbreaks of food-borne diseases worldwide (Lacey, 1993). Eggs or broiler meat contaminated with *S. enterica* have been described as sources of infection for consumers. As poultry is one of the most important reservoirs of *Salmonella*, its transmission to humans through the food chain is a major public health concern. Since the 1980s, the serovar most often responsible for *Salmonella* intoxication in humans is *S. Enteritidis*, strongly associated with egg production. *S. Typhimurium* human cases are more commonly associated with consumption of contaminated meat (poultry, pig and bovine) (European Food Safety Authority, 2005). Poultry facilities have identified risk factors associated with primary production contamination. Birds can be infected from contaminated food, water, litter, and rodents and the persistence properties of *Salmonella* in the birds' environment still represents an important characteristic of *Salmonella* epidemiology in poultry (Davies & Breslin, 2003). Many investigations have emphasized the role of wildlife species such as litter beetles, nematodes or houseflies in the transmission or the persistence of *S. Enteritidis* in poultry (Noreen & Greenberg, 1980; Chadfield *et al.* 2001; Olsen & Hammack, 2000; Rose *et al.* 2000; Skov *et al.* 2004). The role of *Dermanyssus gallinae* as a causative agent in the transmission of *Salmonella* between laying hens in successive flocks is still under question.

The poultry red mite, *Dermanyssus. gallinae*, is a common haematophagous arthropod found in laying hen facilities where it can cause bird anaemia, decreased egg production and in extreme cases, death of its bird hosts (Kirkwood, 1967). This mite parasites its host only during blood meals and the rest of time it hides in the darkness to digest and/or reproduce. It can also bite humans and is recognized as a real problem for people working on poultry farms (Chauve, 1998). Moreover, its population can rapidly reach great number of individuals since its life cycle is particularly short (about 10 days), and the current methods of control having

for most of them low efficiency, it has become a very difficult pest to eradicate (Beugnet *et al.* 1997). *D. gallinae* is also suspected to be a possible vector of various pathogens (Valiente Moro *et al.* 2005) and has been demonstrated to be an experimental vector of *Salmonella* Enteritidis (Valiente Moro *et al.* 2006). It has been shown that the mite can be infected by *S.* Enteritidis after both cuticular contact and during a contaminated blood meal, that the bacteria can multiply, can be transmitted to the next generation and that transtadial transmission from protonymphs to deutonymphs is also possible. Finally, it has been shown that previously infected mites are also able to recontaminate the blood in an *in vitro* feeding device. Since birds commonly peck and even ingest red mites in breeding hen facilities, the ingestion of contaminated mites by chickens as a potential route for poultry infection is of particular interest and merits further investigation.

Therefore, we have orally contaminated chicks with mites previously infected by *S.* Enteritidis. The ability of *Salmonella* to colonize the intestinal tract of the birds and invade their internal organs after inoculation of mites was then analysed.

Materials and Methods

Mites and bacterial strain. Mites were collected from broiler chickens in the Rhône-Alpes region (Bresse, France) and were manipulated with a paint brush and a suction pump connected to a collector. The absence of *Salmonella* in the mites was confirmed on 20 groups of 10 mites by culture of crushed mites onto SMID. The *Salmonella* Enteritidis strain was provided by the “Laboratoire Centrale de Recherche Avicole et Porcine” (AFSSA, Ploufragan, France) which had previously isolated it from a poultry hatchery sample. It was maintained by regular plating on *Salmonella* Medium Identification (SM ID), (Biomérieux, Craponne, France).

Chicks. One-day-old Isabrown chicks were used for the experiments. Chicks came from a layer hatchery free of *Salmonella*. Prior to the test, 10 chicks were confirmed to be *S. Enteritidis*-free by analysing their intestinal tract. The bottoms of the boxes in which the chicks had been transported as well as any droppings in the boxes were also checked for *Salmonella* contamination with negative results. All experimental birds received a commercial broiler finisher diet which was confirmed as *S. Enteritidis*-free. All experimental procedures were performed according to French law concerning animal experimentation (decree 87-848 modified by 2001-464 of 29/5/2001).

Infection of mites. As described in our experimental model, two methods of infecting the mites with *Salmonella* were used (Valiente Moro *et al.* 2006). The infection during the blood meal consisted of in an *in vitro* feeding device using chicken skin over a pipette tip used as a blood reservoir. Adult chickens (*Gallus gallus*) were killed, their blood defibrinized by vigorous shaking and their skin carefully dissected from the corpses and scraped free of subcutaneous tissue. The absence of *Salmonella* in the blood used was previously checked by culture on specific medium SM ID. Blood was infected with *S. Enteritidis* at the rate of 10^8 CFU/mL. Using a pipette the mites were then placed in contact with the chicken skin overlying the infected blood reservoir and allowed to feed for 4 hours in an incubator under standardized conditions (temperature: $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$, relative humidity: 75% and total darkness). This resulted in an average contamination of 2×10^4 *Salmonella*/mite. Cuticular infection of the mites was achieved by leaving them on a dry *Salmonella* coating. Briefly, 500 μ l of a *S. Enteritidis* concentrated solution in Trypton Salt (TS) (BioMérieux, Craponne, France) was streaked onto SM ID agar and incubated at 37°C for 18-24 h. Sterile Whatman paper was placed in contact with the bacterial coating and then transferred to a sterile petri dish. This allowed the mites to walk on the paper without suffering from the humidity

produced by the agar. They remained in contact with *Salmonella* for 48 h at room temperature and were finally collected and stored in sterile plastic tubes until inoculation of chicks.

Inoculum preparation. Forty pools of 10 mites were selected by randomly taking engorged mites which had been artificially infected during bloodmeals 6 to 13 days before. The same sampling was performed for cuticle-infected mites which had been infected 6 to 10 days earlier. To ensure that any bacteria subsequently detected were those located inside the mites, we used the external decontamination and washing protocol previously described by Zeman *et al.*, (1982); each set of 10 mites was washed twice in 100 µl of 4% paraformaldehyde for 7 min, once in 100 µl of sterile water followed by a final washing in 100 µl of TS. The final washing solutions were pooled and analysed for the presence of *Salmonella* to check the efficiency of the washing protocol. Each group of mites was finally suspended in 100 µl of TS and stored at 4°C. These mite suspensions were used as the inoculum for the chicks. Control mites, namely non contaminated mites, were also washed to check that the Paraformaldehyde solution was not toxic for chicks. Six pools of 10 mites were kept for each route of infection to assess the titre of the feeding suspension. Mites were crushed in 200 µl of TS and a ten-fold serial dilution was made. Then, 100 µl of each dilution were streaked onto Rambach agar and incubated at 37°C for 18-24 h. When bacterial levels were undetectable, the presence of *Salmonella* in the remaining 80 µl of the initial solution was checked by pre-enrichment in Peptone Buffer Water (PBW), (BioMérieux, Craponne, France) and enrichment on MSRV agar.

Experimental infection of chicks. Ninety eight one-day-old chicks were inoculated orally: 34 received mites contaminated during the blood meal, 34 were infected with mites contaminated by the cuticular route and 30 chicks, the negative control, were inoculated with

uncontaminated mites. Using a 200 µl pipette, each chick received orally 10 mites suspended in 100 µl of TS. Each group of birds was housed in separate isolator units and kept 12 days after inoculation.

Fecal samples. At 6 days postinoculation (Figure 1), fecal samples were randomly collected from each incubator (about 25g per incubator). These samples were transferred to 225 ml of BPW (BioMérieux, Craponne, France) and mixed by stomaching for 30 sec. After preenrichment for 16-20 h at 37°C, an MSR/V (Modified Semi-solid Rappaport Vassiliadis agar) plate was spot-inoculated with three drops of the pre-enriched medium and incubated at 41.5°C for 24 h and if necessary for 48 h. On MSR/V agar, *Salmonella* strains migrated from the inoculation spot. The margin of this migratory zone was streaked onto a Rambach agar plate. If no migration was noted after 48 h of incubation, the MSR/V result was considered as negative. After 24 h incubation of the Rambach agar plate, typical colonies were biochemically confirmed using API20E gallery (BioMérieux, Craponne, France).

Internal organ samples. At 12 days after inoculation in each trial (Figure 1), all chicks were sacrificed to allow bacteriological culture of their internal organs. For chicks inoculated with uncontaminated mites, livers, spleens and caeca were aseptically removed, pooled in groups of 5, transferred to BPW (1/10 dilution w/w) and homogenized for 30 sec in a Stomacher Model. For chicks inoculated with contaminated mites, their livers and spleens were pooled in groups of 5 and mixed by stomaching for 30 sec in BPW. *Salmonella* detection was performed individually on their caeca. All samples were incubated for 16-20 h at 37°C, and a MSR/V agar plate was spot-inoculated and incubated for 24 and 48 h at 41.5°C. Finally, a loopful was streaked onto Rambach and Xylose Lysine Desoxycholate (XLD) agar and incubated for 18-24 h at 37°C. The enumeration of *Salmonella* was also performed by direct

plating of 100 µl from the liver and spleen pool suspensions onto Rambach agar and cultured overnight at 37°C. For caeca individually isolated from contaminated chicks, the *Salmonella* count was performed using a MPN approach based on miniaturisation of MSR/V enrichment. The method consisted in 3 repeat successive dilutions using the mini MSR/V method (Fravalo *et al.* 2003). Any presumed colonies of *S. Enteritidis* were confirmed biochemically and serotyped.

Results

Determination of the feeding suspension titres. The number of *Salmonella* associated with the pool of 10 *Dermanyssus* inoculated per bird was determined by crushing mites, homogenizing the suspension and subsequent plating of convenient ten-fold serial dilutions in TS onto Rambach agar. The mean infectious titres were respectively 3.0×10^4 CFU/chick and 2.7×10^6 CFU/chick for birds inoculated with cuticular contaminated mites and those contaminated by blood-engorged mites (Figure 2A et 2B). Before the chicks were inoculated, the final washing solutions of the mites were also controlled. The residual contamination measured for the 4 ml pools of the final washing solutions from the 340 cuticle-contaminated mites was 10 CFU/100 µl and 7.5×10^4 CFU/ 100 µl for the blood-contaminated mites.

Isolation of *S. Enteritidis* from fecal samples and tissue. At 6 days after inoculation, faecal samples from both sets of infected chicks were positive for *Salmonella* while the control corresponding to chicks inoculated with uncontaminated mites remained negative for *Salmonella*. On day 12 post-inoculation, *S. Enteritidis* was isolated from the caeca of all birds which had received contaminated mites with an average number of *S. Enteritidis* above 8.5×10^4 MPN *Salmonella* g⁻¹ (Table 1). Using direct plating, *S. Enteritidis* was counted from

the spleen and the liver of all birds. Chi² square analysis did not show any significant difference ($P < 0.5$) between the infection routes.

Discussion

It has recently been demonstrated that *D. gallinae* is an experimental vector of *S. Enteritidis* (Valiente Moro *et al.* 2006). It has been shown that the mite can be infected through the blood meal or by cuticular contact. This infection is followed by bacterial survival of up to 14 days inside the mite as well as multiplication, transovarial and transtadial transmission and contamination of host blood after a blood meal. However, the contamination of hens through eating previously infected mites has not yet been demonstrated. Therefore, in the present study, we inoculated chicks with mites experimentally infected by *S. Enteritidis* in order to assess the potential of *D. gallinae* to transmit *Salmonella* in this way.

The mean infectious titre of the inoculum given to chicks was equal to 3×10^4 CFU and 2.7×10^6 CFU for chicks respectively inoculated with cuticle-infected and engorged mites. Other experimental studies performed with young chicks at 1-day-old or 1-week-old showed that low dosages of *S. Enteritidis* ranging from less than 10 organisms to 5×10^4 CFU could induce infection in chicks (Cooper *et al.*, 1994; Duchet-Suchaux *et al.* 1995; Van Immerseel *et al.* 2004). It is important to note that this dose is dependant on the age of the host, the number of bacteria in the organs and the serotype of *Salmonella* used. For example, it is about 10^{12} CFU for older chickens infected with *S. Typhimurium* considering the fact that they can trigger their host's defence mechanisms after the colonization of the gastrointestinal tract (Muir *et al.* 1998). In our experiment, mites used as the infecting inoculum contained sufficient infectious levels to cause infection in one-day-old chicks.

After the chicks had been orally inoculated with contaminated mites, excretion of *S. Enteritidis* was observed from 6 days post-inoculation in both infected groups. When the birds were sacrificed on day 12, all those which had received contaminated mites showed increased *Salmonella* levels in their livers, spleens and caeca (higher than $8,5 \times 10^4$ MPN *Salmonella* g⁻¹ tissue) indicating colonisation and multiplication of the bacteria. The results of our tests on the final washing solutions were respectively 10 CFU/100 µl and 7.5×10^4 CFU/100 µl, respectively, for cuticule-infected and engorged mites. It suggests that the washing procedure was not performed adequately. However, it would be expected that the mites that were infected by walking on a *Salmonella* coating would have more *Salmonella* on the outside than the mites that have been infected by a blood meal. But the results show that the washing solution of the engorged infected mites is more contaminated than the other group. So, another explanation for this contamination could be the accidental crushing of a mite during the washing process. This hypothesis is supported by the fact that the level of contamination in the solution could be compatible with the concentration found in a single infected mite 7 or 14 days after the engorgement considering the fact that bacterial multiplication has been observed in some mites (Valiente Moro *et al.* 2006).

Previous studies on *S. Enteritidis* infections in poultry have been primarily carried out by inoculating chickens with a suspension of bacteria to demonstrate the invasive character of these bacteria when administered orally to chickens (Desmidt *et al.* 1997). In a model using 1-week-old chicks, CFU levels were highest during the first week(s) after oral inoculation with 5×10^4 *S. Enteritidis* and then decreased progressively (Duchet-Suchaux *et al.* 1995). Another study involving *S. Enteritidis* as an inoculum of 2×10^2 CFU administered orally to 1-day-old chicks showed that the clearance of *Salmonella* was fast with 60% of caecal samples which were found to be *S. Enteritidis* positive at 18 h pi (Ashge *et al.* 2001). Hinton *et al.* (1989) further demonstrated that birds may readily become infected by eating feed contaminated with

S. Enteritidis. This latter experiment is closer to our study because *Salmonella* transmitted via feed given to birds, is similar to *Salmonella* transmitted via mites ingested by birds.

In spite of the *Salmonella*-positive results in the washing solutions, the level of infection obtained in both the infection models tested shows that previously in- and outside infected mites, when eaten by one-day-old chicks, could represent a new source of *Salmonella* infection. The level of infection obtained in both the infection models tested shows that previously infected mites, when eaten by one-day-old chicks, could represent a source of *Salmonella* infection. The invasion of organs such as the liver and spleen is an indication of systemic infection. So, the reproductive organs could be also contaminated and it could introduce a risk for humans when eggs are consumed. However, we can not say how many mites are ingested each day by hens and it is difficult to assess the real impact of the mite in breeding facilities. However, we can state that this mode of infection is possible in one-day-old chicks. Moreover, the challenge model we have used has been performed with chicks, so, in future investigations, it would be interesting to confirm if such infection takes place in older birds. To give a ruling on the possibility of this type of contamination in the field, it would also necessary to study the levels of *Salmonella* in facilities which have already had problems with *Salmonella* contamination or acarian infestation both before and after the arrival of new flocks. Finally, carriage of *Salmonella* by arthropods has already been reported in previous studies. Most recorded examples refer to litter beetles (*Alphitobius diaperinus*) or cockroaches but their role in the transmission of infection remains unproven (McAllister *et al.* 1994; Davies & Wray, 1995; Gray *et al.* 1999). Ash & Greenberg (1980) showed that the German cockroach, *Blattella germanica*, is an effective mechanical transmitter of *S. typhimurium* via faeces, although the bacteria were recoverable from its gut for about 10 days longer than from the faeces. In addition, the role of litter beetles as a potential reservoir for *Salmonella enterica* between broiler flocks has also been observed (Skov *et al.* 2004). Davies

& Wray (1993) demonstrated that the feeding of contaminated *Lucilia sericata* larvae to chickens is a potent means of establishing *S. Enteritidis* infection.

In conclusion, this study has demonstrated an experimental model of *S. Enteritidis* infection which could occur in poultry farms: natural consumption of contaminated mites leading to a lasting infection. It is the first time that *Salmonella* infection has been induced via the ingestion of an arthropod vector. Infected *D. gallinae* could represent a new source of contamination when ingested by chickens.

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Table 1. Isolation and enumeration of *S. Enteritidis* in spleen, liver and caecum on day 12 post-inoculation (^aPercentage of positive birds, ^bwith the number of CFU per gram of tissue, ^cCaecal walls and contents estimated using MPN method)

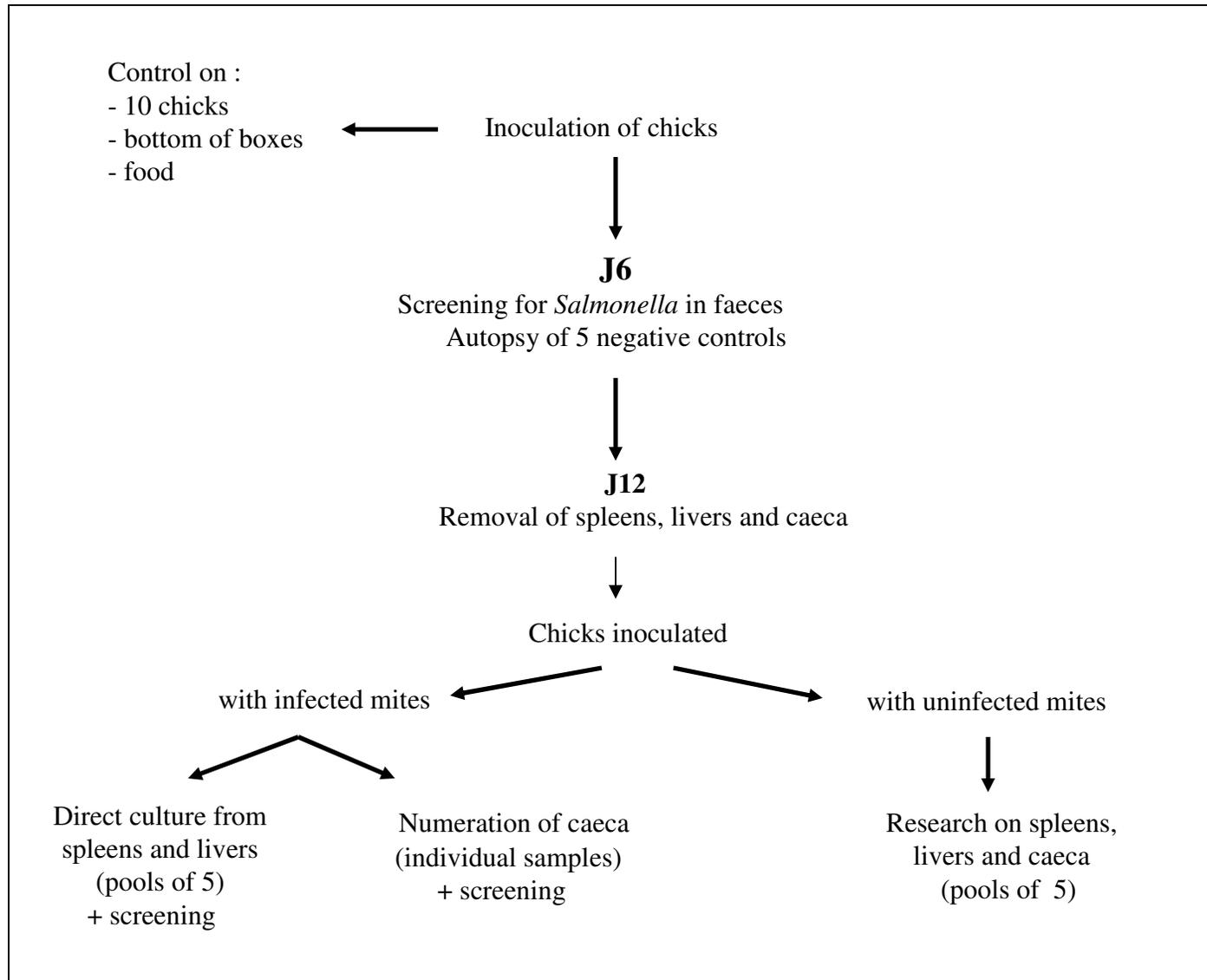
	Spleen		Liver		Caecum	
	(CFU/g tissue) (x5) %positive ^a	CFU/g ^b	(CFU/g tissue) (x5) %positive ^a	CFU/g ^b	(CFU/g tissue) (x1) %positive ^a	MPN <i>Salmonella</i> /g ^b
engorged mites	100	2.7 _{+/-1.8} x10 ³	100	1.9 _{+/-1.9} x10 ²	100	> 8.6x10 ⁴
cuticular mites	100	3.6 _{+/-3.5} x10 ³	100	1.8 _{+/-2.9} x 10 ³	100	> 8.6x10 ⁴

Figure legends

Figure 1. *Experimental protocol.*

Figure 2. *Determination of the feeding suspension titres calculated from cuticular contaminated mites (A) and from blood-meal contaminated mites (B). The number of salmonella associated with the pool of 10 Dermanyssus inoculated per bird was determined by crushing mites, homogenizing the suspension and plating convenient ten-fold serial dilutions in TS onto Rambach agar. The experiment was repeated six times.*

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**Figure 1**

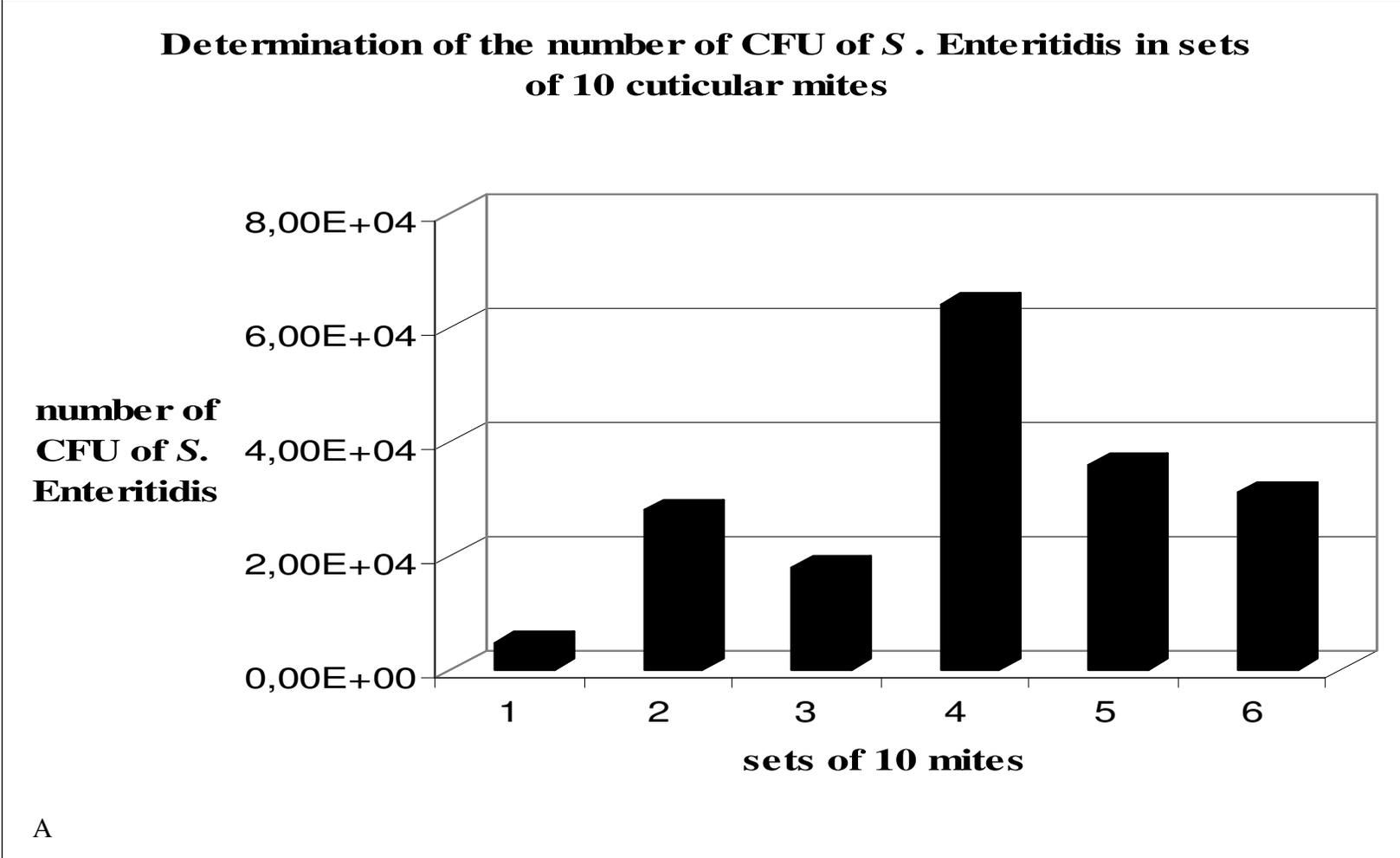


Figure 2A

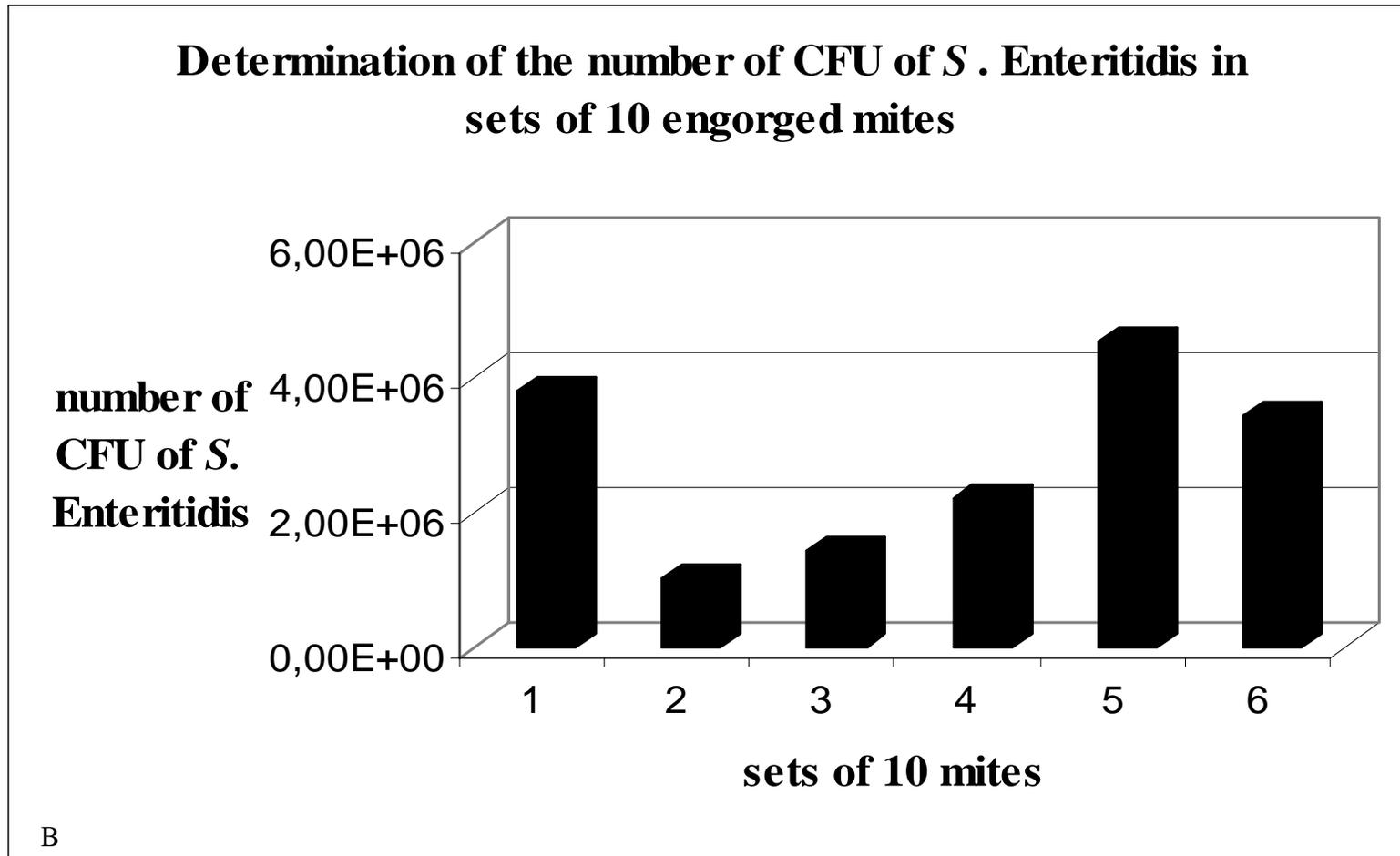


Figure 2B