Preliminary Study: Natural and experimental infection of the Lesser Mealworm *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) with *Histomonas meleagridis* (Protozoa: Sarcocystidae)

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Complete List of Authors:	Huber, Karine; INRA/ENVL, UMR958 Protozoaires Entériques des Volailles Gouilloud, Linda; INRA/ENVL, UMR958 Protozoaires Entériques des Volailles Zenner, Lionel; INRA/ENVL, UMR958 Protozoaires Entériques des Volailles
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A preliminary study of natural and experimental infection of the lesser mealworm *Alphitobius diaperinus* (Coleoptera: *Tenebrionidae*) with *Histomonas meleagridis* (Protozoa: *Sarcomastigophora*)

Karine Huber*, Linda Gouilloud & Lionel Zenner

UMR 958 Protozoaires Entériques des Volailles, INRA-ENVL, 1, avenue Bourgelat – 69280 Marcy L'Etoile - France.

Short title: Infection of *A. diaperinus* with *H. meleagridis*

*To whom correspondence should be addressed. Tel : +33 (0)4.78.87.27.63. Fax : +33 (0)4.78.87.25.77. Email: k.huber@vet-lyon.fr

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A preliminary study of natural and experimental infection of the lesser mealworm *Alphitobius diaperinus* (Coleoptera: *Tenebrionidae*) with *Histomonas meleagridis* (Protozoa: *Sarcomastigophora*)

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Histomonas meleagridis is a protozoan parasite causing histomoniasis (histomonosis), a disease of gallinaceous fowl. In order to determine whether the lesser mealworm, *Alphitobius diaperinus*, is capable of harbouring *H. meleagridis*, the presence of the parasite was tested by polymerase chain reaction (PCR) in lesser mealworms collected in the field. Parasite DNA was detected in two larvae from two farms undergoing an outbreak of histomoniasis. Insects were also artificially infected, killed after incubation and analyzed by PCR for the presence of parasite DNA. After 4 days, two larvae (out of 20) remained positive. In another experimental infection to investigate the viability of histomonads in the larvae, living parasites were detected in five of 20 larvae 4 days after infection. These results indicate that although *A. diaperinus* can become infected with *H. meleagridis* it appears to have a low susceptibility to infection and would probably not be a major route of contamination between flocks.

Introduction

Histomonas meleagridis is a protozoan parasite causing histomoniasis (histomonosis), a disease of poultry leading to high mortality especially in turkeys. Although the infection results mainly from ingestion of eggs of caecal worms (*Heterakis gallinarum*), it has been shown that *H. meleagridis* was present in some farms in which its presence was dissociated from that of the nematode (Chossat, 2002) suggesting an alternative source of contamination. Lateral transmission was demonstrated in turkeys by Hu and McDougald (2003) in the absence of worms and arthropods and the authors speculated that 'The only opportunity for the birds to become infected was from usual litter pecking and coprophagy and possibly from contamination of the feed by infected pen mates'. However alternative means of contamination should also be explored.

The lesser mealworm (also called the darkling beetle), *Alphitobus diaperinus* (Panzer) is often present in very large numbers in poultry units. It readily feeds on diverse sources of nutrients ranging from chicken feed to poultry faeces and dead chickens (Kumar, 1986). During this feeding, the beetle may become contaminated with several pathogenic agents. It has been reported to harbour viruses (McAllister *et al.*, 1995; De Las Casas *et al.*, 1976), bacteria (De Las Casas *et al.*, 1972; McAllister *et al.*, 1996), fungi (De Las Casas *et al.*, 1972) and also parasite tapeworms, in which a true host relationship is established (Elowni *et al.*, 1979; Espaine *et al.*, 1971). It has been shown that it could play a role in the transmission of protozoa, i.e. *Eimeria* spp. (Reyna *et al.*, 1983; Goodwin & Waltman, 1996).

In order to determine if *A. diaperinus* is able to harbour *H. meleagridis*, adults and larvae collected in the field were examined for the presence of the parasite. Insects were also artificially infected and analyzed by the polymerase chain reaction (PCR) for the presence of

parasite DNA. Another experimental infection was performed to investigate the viability of histomonads in the insects.

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Materials and Methods

DNA amplification. An individual insect was crushed in 200 µl of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM TRIS-HCl pH 8.0, 0.2% 2-b mercaptoethanol) and incubated at 65°C for 1 h. Proteins were removed with one volume chloroform. DNA was precipitated with one volume of isopropanol, the pellet was washed with ethanol (70% v/v) and resuspended in 50 µl of distilled water. The PCR amplified a DNA target of 209 bp of the small subunit ribosomal DNA sequence and was performed as described by Huber *et al.* (2005a). Two µl of DNA were used as template in the PCR which had a final volume of 12.5 µl and contained 1X buffer (Invitrogen, Carlsbad, CA), 1.5 mM MgCl₂, 60 µM of each dNTP (Amersham Biosciences, Buckinghamshire, UK), 5 pmol of each primer [HIS5F (5'-CCTTTAGATGCTCTGGGCTG-3') and HIS5R (5'-CAGGGACGTATTCAACGTG-3')] and 0.25 units of Taq polymerase (Invitrogen). They were performed in a TGradient thermocycler (Biometra, Goettingen, Germany). The reaction conditions were 5 min at 96°C, 40 cycles each of 1 min at 95°C, 1 min at 59°C and 1 min at 72°C, with a final extension period of 5 min at 72°C. DNA fragments were separated on 3% (w/v) agarose gels and visualized by UV illumination of ethidium bromide stained DNA.

The sensitivity of the PCR for adult and larval beetles was assessed using artificially-infected materials. A known number of *H. meleagridis* obtained by serial dilutions in order to have concentrations ranging from 1×10^3 to 1×10^{-1} parasites per beetle were added to single insects (adults and larvae) coming from an uninfected farm. These samples were subjected to DNA extraction and PCR as described above.

Field samples. Adults and larvae were collected from 14 farms from France between years 2003 and 2005 (Table 1) representing three types of infection status: (1) four farms free of

histomoniasis; (2) five farms having undergone an outbreak of histomoniasis in previous flocks; (3) five farms undergoing an outbreak of histomoniasis. Samples were stored at -20°C.

Experimental infections. The suspension of *H. meleagridis* used to prepare the inocula was obtained from the HmP-12 stain (Callait *et al.*, 2002). Lesser mealworms were obtained from a laboratory colony started in 2004 from individuals originating on a farm that was free of histomoniasis. They were maintained in plastic boxes at room temperature and fed with commercial chicken feed (Agri Sud Est, Vienne, France). Water-saturated cotton was placed into the boxes to provide humidity and water.

Six groups of 10 adults and six groups of 10 larvae ranging in length from 3 to 12 mm from the 7th and 8th generation were placed in Petri dishes. The beetles were starved for 24 h, after which time absorbent cotton soaked with 7 ml of cultured *H. meleagridis* (6×10^5 parasites per ml, HmP-12 strain) was placed in the dishes of 4 groups of adults and 4 groups of larvae. The cotton for the remaining larvae and adult groups was soaked with Stepkowski media (Stepkowski & Klimont, 1979) as a negative control. After 24 h, two infected groups and one control group of adults and two infected groups and one control group of larvae exposed to *H. meleagridis* were removed and frozen at -20°C. The remaining lesser mealworms were removed from the Petri dishes and placed in other sterile dishes that contained sterile chicken feed and cotton soaked with sterile deionized water. Four days after the infection, they too were frozen at -20°C.

One group of larvae and one group of adults frozen after 24 h and after 4 days were individually placed in 1 ml of paraformaldehyde 4%, agitated and incubated for 7 min. This step was repeated. Then they were rinsed in 1 ml of distilled water. All samples were subjected to DNA extraction and PCR as described above.

In order to test the parasite viability in the larvae, six groups of 10 larvae were infected as described previously. After 24 h, two infected groups and one control group of larvae exposed to *H. meleagridis* were removed and treated. Individual larvae were crushed aseptically in 50 µl of Stepkowski media (Stepkowski & Klimont, 1979) at 39°C and then transferred in a flat bottom glass tube containing 500 µl of Stepkowski media. Tubes were vortexed and then incubated at 39°C. Samples of 15 µl were taken daily for 8 days from the bottom of the tube, and examined microscopically (magnification: x400) to detect live histomonads. Every day 300 µl of supernatant were removed and replaced by fresh media and then the tubes were vortexed.

The remaining larvae were removed from the Petri dishes and placed in sterile dishes that contained sterile chicken feed and cotton soaked with sterile deionized water. Four days after the infection, they were treated as described above.

One group of larvae treated at 24 h and one group treated at 4 days were washed with paraformaldehyde as described above.

Results and Discussion

The sensitivity of the PCR reaction evaluated was found to be one parasite per adult beetle and 10 per larvae (Figure 1). Field samples from farms free of histomoniasis or having undergone an outbreak of histomoniasis in previous flocks were all negative. Adult beetles coming from farms actually undergoing an outbreak of histomoniasis were also all negative, but parasite DNA was detected in two larvae from two of these farms (Table 1).

In order to examine the ability of the *A. diaperinus* to become infected, an experimental infection was performed (Table 2). All the control groups were negative and the

parasite DNA was never detected in the adults. However, 24 h after infection the parasite DNA was detected in five larvae (out of 20), one from the unwashed group and four from the washed group. After 4 days two larvae remained positive (out of 20), one in the unwashed group and one in the washed group.

To investigate the viability of histomonads in the larvae, another experimental infection was performed (Table 2). Twenty four hours after infection, living parasites were detected in four larvae (out of 20), two from the unwashed group and two from the washed group. After 4 days five larvae remained positive (out of 20), four in the unwashed group and one in the washed group.

These results show that *A. diaperinus* has a low susceptibility to infection. The histomonas DNA was not detected in adult beetles whereas it was detected in larvae. Adult and larvae have different feeding habits. Adults are essentially mycophagous but larvae tend to feed more frequently on dead chicken and poultry faeces (Huber *et al.*, 2005b). In *H. meleagridis* infection of turkeys parasites are excreted in caecal droppings during the acute phase of the disease (Huber *et al.*, 2006) allowing the larvae to become contaminated. However, since histomonas DNA was not detected in adult beetles, the chance of this insect spreading the parasite within and among farms is probably low under normal circumstances.

Living parasites were observed in larvae 4 days after infection demonstrating the ability of this insect to maintain the contamination for several days. It is necessary to determine exactly how long the larvae can remain contaminated in order to assess the relative importance of *A. diaperinus* as a biological source of contamination.

A. diaperinus may be only a mechanical vector of the parasite. Mechanical transmission of a pathogen in insects may occur in two forms. The agent may be transported on the cuticle of the insect, or may be ingested and retained in a viable state as it passes through the insect gut. After the treatment of the external teguments of *A. diaperinus*, the PCR

and the culture of some individuals remained positive, indicating that the parasite is not on the tegument but inside the larvae but the next step will be to verify whether contaminated lesser mealworms are able to infect turkeys.

This study has shown that *A. diaperinus* is unlikely to be an important vector for *H. meleagridis* but that it could provide an accidental means of residual environmental contamination. An increase in *A. diaperinus* density could also increase the chance of contact between pathogen and host so a proper poultry management system to control beetle infestation is essential to reduce the risk of accidental transmission.

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Table 1. *PCR analysis of A. diaperinus collected from poultry farms between January 2003 and May 2005.*

Farms	Reared species	Number of individuals tested	Number of positive individuals detected by PCR
<i>Farms free of histomoniasis</i>			
1	chickens	14 adults	Negative
2	chickens	12 adults	Negative
3	chickens	11 adults	Negative
4	chickens	12 adults	Negative
<i>Farm with histomoniasis on a previous flock</i>			
5	turkeys	40 adults	Negative
		3 larvae	Negative
6	turkeys	86 adults	Negative
		4 larvae	Negative
7	turkeys	90 adults	Negative
8	turkeys	10 adults	Negative
		20 larvae	Negative
9	turkeys	31 adults	Negative
		19 larvae	Negative
<i>Samples collected during an outbreak of histomoniasis</i>			
10	turkeys	13 adults	Negative
		15 larvae	1 positive
11	chickens	25 adults	Negative
		38 larvae	1 positive
12	turkeys	15 adults	Negative
		15 larvae	Negative

13	turkeys	27 adults	Negative
		3 larvae	Negative
14	Guinea-fowl	30 adults	Negative

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Table 2. Number of positive *A. diaperinus* (adults and larvae) detected by PCR and number of *A. diaperinus* larvae carrying viable histomonads, 24 h and 4 days after experimental infection with *H. meleagridis*.

	Number of positive individuals detected by PCR		Number of individuals carrying viable histomonads ^a
	Adults	Larvae	Larvae
<i>24 h after infection^b</i>			
Control group	0/10	0/10	0/10
Unwashed	0/10	1/10	2/10
Washed with paraformaldehyde	0/10	4/10	2/10
<i>4 days after infection^a</i>			
Control group	0/10	0/10	0/10
Unwashed	0/10	1/10	4/10
Washed with paraformaldehyde	0/10	1/10	1/10

^aLarvae were crushed in Stepkowski media and incubated at 39°C. A daily microscopic examination was performed to detect live histomonads.

^bStarved insects were placed for 24h in an absorbent cotton wool, soaked with 7 ml of cultured *H. meleagridis* (6x10⁵ parasites per ml, HmP-12 strain).

Figure 1. *Sensitivity of the PCR for the detection of Histomonas meleagridis in Alphitobius diaperinus samples. Adult (A) and larvae (B) were artificially infected to obtain concentrations of 10^3 H. meleagridis per beetle (lane 1), 10^2 parasites per beetle (lane 2), 10 parasites per beetle (lane 3), 1 parasite per beetle (lane 4), 10^{-1} parasites per beetle (lane 5), positive control for H. meleagridis (lane 6), negative control (lane 7) and a 100 bp DNA ladder (lane 8).*

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