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RESEARCH ARTICLE

Pigeons are Not Susceptible to Intracloacal Infection with *Histomonas meleagridis*

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ABSTRACT

Histomonas meleagridis is a trichomonad parasite, which is capable to cause severe inflammations of ceca and livers in gallinaceous birds. It rarely can be satisfactorily explained, how the parasite was introduced into the flock. The role of wild birds as possible reservoir or as vectors has not been explored sufficiently. In the present study two experiments were done to determine if pigeons are susceptible to intracloacal infections with *H. meleagridis* and have the potential to act as vectors. In a first experiment nine racing pigeons (*Columba livia* forma *domestica*) were infected intracloacally with 400,000 living histomonads. Three further pigeons were kept as contact birds. Histomonal DNA was detected in cloacal swabs until one week after infection, but reisolation was not possible. In a second experiment 24 racing pigeons were either infected intracloacally with 250,000 viable histomonads or with the same culture, which had been inactivated. Histomonal DNA was detected in cloacal swabs of both groups until five days after infection, but reisolation was not possible. In both experiments neither clinical signs nor gross lesions were observed in any bird and in the ceca no histomonal DNA was detected. It was concluded that pigeons do not act as vectors for *H. meleagridis* after intracloacal infection.

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INTRODUCTION

Histomonas meleagridis is a trichomonad parasite, which occurs worldwide in gallinaceous birds (McDougald, 2005). Especially in turkeys it causes a severe disease that is characterized by a diphteroid inflammation of the ceca and by necrosis in the liver (Sentíes-Cué *et al.*, 2009; Popp *et al.*, 2011). Mortality in affected turkeys often exceeds 50% (Callait-Cardinal *et al.*, 2007). In the European Union outbreaks of histomonosis can necessitate the culling of whole flocks, because since 2003 no prophylactic or therapeutic drugs are licensed for prophylaxis or therapy of histomonosis in food producing animals (Popp *et al.*, 2012).

The disease is more prevalent during warmer seasons (Callait-Cardinal *et al.*, 2007; Hauck *et al.*, 2010a) and has been observed in all types of rearing systems, from free ranging birds (Esquenet *et al.*, 2003; Popp *et al.*, 2011) to breeder flocks with the highest levels of bio security (Lister, 2010; Aka *et al.*, 2011).

Within an affected flock the parasite spreads by the uptake of the trophozoites in freshly shed feces either by cloacal drinking (Hu *et al.*, 2004) or orally (Liebhart and Hess, 2009). However, it rarely can be satisfactorily explained, how the parasite was introduced into the flock. The trophozoites of *H. meleagridis* possess only a low tenacity (Lotfi *et al.*, 2012), thus eggs of the nematode *Heterakis gallinarum* containing stages of *H. meleagridis* are regarded as a very important vector (McDougald, 2005). However, *H. gallinarum* rarely is found in turkey flocks with histomonosis. Insects, especially the lesser mealworm (*Alphitobius diaperinus*) or flies, may serve as vectors (Huber *et al.*, 2007; Hauck *et al.*, 2010a), and the existence of resistant cyst stages has been proposed (Zaragatzki *et al.*, 2010).

The role wild birds can play as possible reservoir or as vectors have not been explored sufficiently, even though they have been implicated as possible vectors (Lister, 2010). *H. meleagridis* can infect a variety of gallinaceous wild birds like pheasants, peafowl, or partridges (Lund and Chute, 1974), but ducks and geese can also be infected and harbor the parasite (Lund *et al.*, 1974; Callait-Cardinal *et al.*, 2006). After intramuscular and subcutaneous injection with livers of histomonas-

infected turkeys, pigeons, like turkeys and chickens, developed abscesses containing the parasite at the site of the injection (Tyzzer and Fabyan, 1920).

In the present study two experiments were done to determine if pigeons are susceptible to intracloacal infections with *H. meleagridis* and have the potential to act as vectors.

MATERIALS AND METHODS

Inocula: *H. meleagridis* strain Turkey/ Germany/ GB542/ 04 was isolated from an outbreak in a commercial turkey flock in Germany. The strain was shown to be free from *Tetratrichomonas gallinarum* and *Blastocytis* sp. using PCR (Grabensteiner and Hess, 2006). Until being used it was stored in liquid nitrogen. After thawing it was passaged in cell culture medium M199 (Biochrom, Berlin, Germany) containing 10 % inactivated horse serum and 2 g/l rice powder. For the first trial the 6th passage was used, for the second trial the 7th passage.

For inactivation metronidazole was added to a concentration of 2000 µg/ml (Sigma-Aldrich, Munich, Germany) and the culture was subjected to three freeze-thawing-cycles. No histomonads could be isolated from this culture by further passaging.

Design of first experiment: Nine racing pigeons (*Columba livia* forma *domestica)* were infected intracloacally with 400,000 histomonads. Three further pigeons were left uninfected in the same aviary as contact birds. One cloacal swab was taken from each bird directly before infection and two swabs were taken from each bird one and three days after infection, as well as one, two, three, and four weeks after infection. After the first and second week after infection three infected birds were euthanized and samples of the ceca were taken. Four weeks after infection the remaining birds including the not infected birds were euthanized and samples of the ceca were taken.

Design of second experiment: Twenty four racing pigeons were divided in two groups with twelve birds each. The two groups were kept in two different aviaries in the same room. The birds in group 1 were infected intracloacally with 250,000 viable histomonads in 1 ml medium. The birds in group 2 were inoculated with 1 ml of the same culture, which had been inactivated as described above. One cloacal swab was taken from each bird directly before infection and two cloacal swabs were taken from each bird one hour after infection and one, three, and five days after infection. One hour after infection and one, three, and five days after infection three birds of each group were euthanized and samples of the ceca were taken.

Investigation of samples: The swab taken before infection and one of the swabs taken at every time point after infection as well as the ceca were investigated for the presence of histomonal DNA by qPCR with an internal amplification control. DNA was extracted from samples as described by Hafez *et al.* (2005). Multiplex qPCR was done on the MRX 3005P (Agilent Technologies, Santa Clara, CA, USA) using the QuantiTect Multiplex PCR Master Mix (Qiagen, Hilden, Germany). To 12.5 µl of the master mix 1 µl primer mix, 10 µl template DNA from swabs or 1 template DNA from ceca, and nuclease-free water to a final volume of 25 µl were added. The primer mix contained primers HmqF2 (ccgtgatgtcctttagatgc) and HmqR (gatcttttcaaattagctttaaattattc), which amplify a 87 base pair fragment of the histomonal ribosomal RNA gene, with a concentration of 10 pmol/µl, primers EGFP-12-F and EGFP-10-R, which amplify part of the green fluorescent protein (gfp) gene, with a concentration of 1 pmol/µl, the corresponding probes HmQs (ctgcacgcgcg ctacaatgttaaa) modified with 6–FAM and Black Hole Quencher with a concentration of 5 pmol/µl and EGFP-Hex modified with Cyan 5 and Black Hole Quencher 2 with a concentration of 0.5 pmol/µl as well as a plasmid containing the gfp gene. The sequence of HmqR was modified from primer Hmr (Grabensteiner and Hess, 2006). Primers and probe for the gfp gene were taken from Hoffmann *et al.* (2006). The thermal profile consisted of an initial denaturation step of 15 min at 95°C and 45 cycles of denaturation for 60 s at 94°C, annealing for 30 s at 50°C and extension for 30 s at 60°C. Fluorescent data were collected after annealing.

The second of the two swabs taken at every time point after infection was immediately placed in medium for reisolation of *H. meleagridis*. In the first trial M199 (Biochrom, Berlin, Germany) containing 10 % horse serum and 2 g/l rice powder was used. In the second trial PBS containing 10 % horse serum and 2 g/l rice powder was used (Hauck *et al.*, 2010b).

Comparison of pH in cultures from cloacal swabs from pigeons and chickens: Cloacal swabs were taken from 15 not infected pigeons and 15 not infected chickens. Ten swabs of each species were immediately placed in M199 based medium and the five remaining swabs in PBS based medium. pH in cultures was measured after 24 hours.

RESULTS

First experiment: Before the infection no histomonal DNA was detected in the cloacal swabs. One day after infection qPCR of all swabs taken from infected pigeons was positive. Three days after infection DNA of *H. meleagridis* was detected in cloacal swabs of seven infected birds. One week after infection cloacal swabs of four birds contained *H. meleagridis* DNA. In the swabs taken two, three, and four weeks after infection no histomonal DNA was detected. Swabs taken from the not infected birds remained negative throughout the trial (Table 1). Reisolation of the parasite was not successful.

During the entire observation period neither clinical signs nor gross lesions were observed in any bird. In the ceca no histomonal DNA was detected.

Second experiment: Before the infection no histomonal DNA was detected in the cloacal swabs. One hour after the infection histomonal DNA was present in all cloacal swabs, regardless if the birds had received living or inactivated histomonads. One day later qPCR was positive for five of the nine remaining birds that had been infected with living histomonads and for seven of the nine remaining birds that had received inactivated

histomonads. Three days after infection two of six birds infected with living *H. meleagridis* and four of six birds inoculated with inactivated histomonads were tested positive for *H. meleagridis* DNA. Five days after infection at the end of the study this was the case for two of three pigeons infected with the living parasite and one of three pigeons infected with the inactivated culture (Table 2). Reisolation of the parasite was not successful in any case.

During the entire observation period neither clinical signs nor gross lesions were observed in any bird. In the ceca no histomonal DNA was detected.

Table 1: Investigation of cloacal swabs taken from pigeons infected intracloacally with 400,000 histomonads and from contact birds

		Time after infection					
		1 d	3 d	1 wk	2 wk	3 wk	4 wk
Infected birds	PCR	9/9*	7/9	4/9	0/6	0/3	0/3
	Isolation	0/9	0/9	0/9	0/6	0/3	0/3
Contact birds	PCR	0/3	0/3	0/3	0/3	0/3	0/3
	Isolation	0/3	0/3	0/3	0/3	0/3	0/3

*Positive birds / investigated birds.

Table 2: Investigation of cloacal swabs taken from pigeons infected intracloacally with 250,000 viable histomonads or with 250,000 inactivated histomonads

		Time after infection			
		1 h	1 d	3 d	5 d
Pigeons infected with viable histomonads	PCR	12/12*	5/9	2/6	2/3
	Isolation	0/12	0/9	0/6	0/3
Pigeons infected with inactivated histomonads	PCR	12/12	7/9	4/6	1/3
	Isolation	0/12	0/9	0/6	0/3

*Positive birds / investigated birds.

Comparison of pH in cultures from cloacal swabs from pigeons and chickens: 24 hours after inoculation the mean pH in M199 based medium was 4.6 in cultures with swabs from pigeons and 5.4 in cultures with swabs from chickens. In PBS based medium it was 6.6 in cultures with swabs from pigeons and 6.8 in cultures with swabs from chickens.

DISCUSSION

The present study explored if pigeons can act as vectors for the trichomonad parasite *H. meleagridis* after intracloacal infection. The results of the first experiment showed that histomonal DNA could be detected for up to one week after intracloacal infection in cloacal swabs from pigeons. However, reisolation was not possible. The conflicting results might have been due to the acidic pH in cultures inoculated with cloacal swabs from pigeons, or the PCR might have detected DNA of non-viable histomonads.

So for the second experiment a lower infection dose was chosen, a control group, which was inoculated with inactivated histomonads, was included and reisolation was tried in PBS based medium without glucose, which is more stable in pH (Hauck *et al.*, 2010b). Histomonal DNA was detected in the birds inoculated with the inactivated parasite to the same extent as in the birds that had received the living trophozoites, and again reisolation was not possible. This indicated that the parasite does not survive in pigeons after intracloacal infection, even though pigeon tissue in principal supports histomonal growth (Tyzzer and Fabyan, 1920).

The failure to detect histomonal DNA in the ceca even one hour after the infection shows that the inoculum did not reach the ceca, where *H. meleagridis* survives in other species. In turkeys and chickens sucking movements of the vent may transport parasites and other particles from the lips of the vent into the cloaca and material in the cloacae can quickly reach the ceca by retrograde peristalsis of the large intestine (Sorvari *et al*., 1977; Hu *et al.*, 2004). The purpose of this mechanism might be the transport of urine into the ceca, where it is concentrated to save water (Akester *et al*., 1967). Comparable investigations about pigeons are lacking.

If histomonads had survived in the ceca, is a matter of speculation, since the ceca of pigeons are rudimentary and considerably smaller than those of birds susceptible to an infection with *H. meleagridis* (McLelland, 1989). Also there is no information about the bacterial flora in the ceca of pigeons, which has an influence on the development of infections with *H. gallinarum* (Springer *et al*., 1970).

Even though it was shown that pigeons do not act as vectors for *H. meleagridis* after intracloacal infection, it is possible that pigeons can be infected with *H. meleagridis* via eggs of *H. gallinarum*, since in the feces of pigeons eggs of *Heterakis* sp. were found (Sari *et al*., 2008).

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