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Oral infection of turkeys with *in vitro*-cultured *Histomonas meleagridis* results in high mortality

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Short title: Oral infection of turkeys with *H. meleagridis*

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Abstract

In the present study a well defined clonal culture of *Histomonas meleagridis* was used to investigate whether turkeys can be infected orally with *in vitro*-propagated parasites in the absence of any vector. Therefore, two subsequent experiments were arranged to study the morbidity and mortality of a virulent strain of histomonads in turkeys following crop instillation of protozoa and a feed restriction for 5 hours. Soon after infection, the parasites triggered total morbidity and mortality in a group of 14 turkeys of which 10 birds were orally infected and the others were kept as in-contact birds. Administration of the same number of histomonads into either the cloaca or the crop of individually housed birds was investigated subsequently, to avoid any bird to bird transmission. All eight cloacally and six out of eight orally infected turkeys contracted severe histomonosis, with only two birds surviving the infection expressing no clinical signs. During pathological investigations all birds showed pathognomonic lesions in livers and caeca indicative for histomonosis, except the two turkeys which survived the infection. The results of the experiments verify for the first time the effective oral infection of day-old turkeys with clonal *in vitro* cultured *Histomonas meleagridis*.

Introduction

Histomonas meleagridis was first mentioned by Tyzzer (1920) as a flagellated, highly pathogenic parasite in turkeys. The disease is named histomonosis (synonyms: blackhead disease, enzootic typhlohepatitis) and affects predominantly gallinaceous birds but also other avian species (McDougald, 2008). Following infection, the flagellate targets the caecum and may cause severe typhlitis. Furthermore, it could pass the portal vein system by which it is transported to the liver and could produce necrotic lesions in the organ. A broad dissemination of the parasite is possible, especially at the final stage of the disease (Grabensteiner *et al.*, 2006; Singh *et al.*, 2008).

The multiplication of histomonads takes place in the caeca of infected birds and is followed by the excretion of the flagellates through the faeces. However, the route of infection is still a matter of particular interest. Swales (1950) summarized several experiments in which cultures, faeces, organs or embryonated eggs of *Heterakis gallinarum* bearing *H. meleagridis* were used for the infection of host birds. Based on available data he concluded that the direct infection of the mentioned inocula without a vector would be possible, but not as effective as the indirect infection via *H. gallinarum* for inducing the disease in host birds. Later on, Lund (1956) and Gibbs (1962) underlined this estimation by performing additional infection studies.

In recent years the infection of histomonads was again restudied. Hu *et al.* (2004) showed that the oral application of *in vitro* cultured *H. meleagridis* cells did not cause any lesions in the liver and the caecum of turkeys, whereas the cloacal administration of the inoculum of the same origin triggered severe histomonosis in infected birds.

Lately, the high virulence and rapid transmission from infected to in-contact birds of a well-defined clonal culture of *H. meleagridis*/Turkey/Austria/2922-C6/04 was demonstrated in several *in vivo* experiments by infecting birds via the cloaca (Hess *et al.*, 2006a; Liebhart *et al.*, 2008; Hess *et al.*, 2008). This observation, together with the contradictory results present in the literature, raised the likelihood of a successful oral infection of turkeys through application of this type of culture in the absence of any vector. Therefore, two consecutive experiments were performed to investigate the potential of *in vitro* grown virulent histomonads to establish a successful infection in turkeys following oral application.

Material and Methods

Experimental animals. Day-old turkeys (B.U.T.⁹™, Aviagen Turkeys Ltd., Tattenhall, UK) were obtained from a commercial hatchery and subsequently housed in rooms under negative pressure. At the first day of life the animals were earmarked by tags, fixed subcutaneously (Swiftack™, Heartland Animal Health, Inc., Missouri, USA) for explicit identification. In every experiment, the groups consisted of an equal male and female ratio. Water and feed (unmedicated turkey starter feed) were provided *ad libitum*, except for a five-hour abstinence from feed after the infection of the birds. The animal experiments published in this work were discussed and approved by the institutional ethics committee and approved by Austrian law (licence number 68.205/0026-II/10b/2008).

Inocula. The infectious material consisted of the mono-eukaryotic culture *Histomonas meleagridis*/Turkey/Austria/2922-C6/04 established as described recently (Hess *et al.*, 2006b). In all experiments highly virulent short time cultivated (21 passages) parasites were used either for intra-crop or intra-cloacal infection of host birds. For obtaining the same

number of passages in every experiment, the cultures used for inoculation were thawed from liquid nitrogen and cultured for two passages prior to infection. The inocula were processed in a way that every infected or challenged bird received 10^4 parasitic cells in 0.3 ml of culture medium (Medium 199 with Earle's salts, L-Glutamine, 25 mM HEPES and l-amino acids (Gibco, Invitrogen, Lofer, Austria), 15% foetal calf serum (Gibco, Invitrogen, Lofer, Austria) and 0.66 mg rice starch (Sigma-Aldrich, Vienna, Austria).

Experimental design. *Experiment 1.* In this experiment the turkeys were kept in pens on deep litter (wood shavings) offering room of 0.5 m² for 4 birds and 2 m² for 14 birds. Four birds (numbers 1-4) representing group I were infected with the above mentioned histomonads intracloacally at the 14th day of life. At the same time 10 birds (numbers 5-14) of group II were infected orally with the same inoculum and 4 birds (numbers 15-18) were left as in-contact birds. Birds of both groups I and II were housed in two separated pens.

Experiment 2. In the second experiment every bird was housed individually in a separate cage on wire floor (22.5 cm² of floor space) provided with its own feeding bowl and water supply. One half of the birds of group I (numbers 1-8) were infected cloacally at the first day of life whereas the remaining 8 animals (numbers 9-16 (=group II)) obtained the inoculum via crop installation using a crop tube placed on a 1ml syringe (B. Braun Melsungen AG, Melsungen, Germany).

Physical examination and post mortem diagnosis. All animals were examined daily for any clinical signs indicative for histomonosis. Beside excretion of live histomonads, feed and water intake, changes in the behaviour and ruffling of feathers were controlled. Turkeys that died or which had to be euthanized due to severe clinical signs or which were killed at the end of the experiments were necropsied in order to look for relevant lesions in the liver and the caecum.

Sampling procedure. From day 0 post infection (p.i.) onwards, cloacal swabs were taken at intervals of 3 to 4 days for reisolation of *H. meleagridis* and placed directly into in a 2 ml microfuge tube containing 1.5 ml fresh culture medium as described above. After 48 and 96 hours of incubation at 40°C, the samples were investigated microscopically for the presence of live histomonads.

Results

Experiment 1. The first excretion of live histomonads was observed for the first time on day 2 p.i. in the cloacally infected bird number 2 and is listed in detail in Table 1. Reisolation from every turkey was positive at least once in this group. First clinical signs of histomonosis were recognized on day 6 p.i. in the intracloacally infected birds (group I). During the following days, the animals of this group crowded together and were in a poor condition until day 9 p.i. when birds 2 and 3 showed sulphur-coloured diarrhoea. Every bird of this group died between 10 and 15 days p.i.. In comparison to group I, the onset of morbidity was noticed with a delay of 5 days at 11th d.p.i. in group II. On the same day, the physical condition of the birds decreased and did not improve until the end of the experiment. The first sulphur-coloured droppings could be seen from 6 birds (numbers 7, 9, 14, 15, 16 and 18) on the same day. Numbers 12 and 15 died on day 13 p.i. and two more birds (13 and 17) showed typical diarrhoea. Three dead birds (9, 14 and 17) were noticed on the next day when bird no. 6 showed yellowish faeces. This bird died one day later. On day 16 p.i. three birds (8, 16 and

18) had to be euthanized due to their bad condition and three animals (5, 7 and 13) died of histomonosis which terminated the experiment. The notification of dead birds is summarized in Figure 1. At post mortem examination, all birds showed pathognomonic lesions for histomonosis in the caeca and livers.

Experiment 2. Except from one bird (number 10), histomonads were reisolated at least once from every turkey (Table 2). Neither the beginning of the first successful reisolation nor the frequency of positive swabs showed any difference between orally infected birds and turkeys inoculated via the cloacae. With the exception of one bird (15) all other orally infected turkeys survived the 16th day p.i., in contrast to the majority of animals infected via the cloaca. First clinical abnormalities were noticed on day 12 p.i. when some coagula of blood in the faeces of the cloacally infected birds 2, 13 and 15 occurred. Additionally bird number 15 was in a bad condition and excreted sulphur-coloured droppings. A total of 7 birds (1, 6, 7, 8, 11, 12 and 16) displayed haemorrhagic droppings on the following day. Furthermore, birds 5, 9 and 12 were drowsy and numbers 2, 6 and 8 displayed typical diarrhoea for histomonosis for the first time. On day 14 p.i. even more animals started with clinical symptoms (4 and 9 showed bloody faeces and 1, 4, 7, 11 and 16 had yellowish droppings). The first animals died at the same day. Moreover, numbers 9 and 12 started with sulphur-coloured excreta. The last birds died during the next few days which is comparatively shown in Figure 1.

Typical lesions for histomonosis could be detected in autopsies of all birds (Figure 2) except numbers 10 and 14 which did not show any pathological changes after they were killed at the termination of the experiment.

Discussion

Histomonosis caused by *H. meleagridis* is of important for both turkeys and chickens (McDougald, 2008). A crucial factor for understanding the pathogenesis of histomonosis is the way birds get infected. This is also helpful to create an efficient protection strategy. The indirect way of infection with histomonads via the embryonated eggs of the nematode *H. gallinarum* was first published by Graybill & Smith (1920). The efficacy of this infection route was demonstrated in numerous other studies performed since this initial observation. In 1925 Tyzzer and Collier (1925) described the successful direct infection of turkeys by oral or rectal application of fresh liver lesions. Later on, Allen (1941) noticed a high mortality rate of turkeys (92%) after oral infection. However, detailed information about the application is not mentioned. This single observation has to be seen in context with several other studies in which only limited success was reported of inducing histomonosis after oral infection of birds in the absence of any vector (McKay & Morehouse, 1948; Horton-Smith & Long, 1956; Lund, 1956).

A genetic difference between histomonads was recently demonstrated by van der Heijden *et al.* (2006). This could be a possible explanation for the divergent results reported in these studies. Furthermore, Lund *et al.* (1966) speculated about different biological features between histomonads propagated *in vitro*. The finding that clonal cultures of *H. meleagridis* of different origin express different susceptibilities towards the same plant compounds would strengthen the biological variation between certain histomonads (Grabensteiner *et al.*, 2007). However, genetic and biological differences are insufficient to explain the observation noticed by Hu *et al.* (2004) who demonstrated that turkeys did not contract the disease after oral infection with *in vitro*-cultured histomonads contrary to birds receiving the same inoculum intracloacally.

Feed restriction prior to the application of live histomonads is an additional parameter which should be addressed in the context of a successful oral infection. Parasites applied orally had to pass the crop, the proventriculus and the gizzard first, representing an acidic environment down to a pH of 2.2 in turkeys and 2.5 in chickens (Denbow, 2000). These conditions could severely limit the survival of histomonads as demonstrated by Horton-Smith & Long (1956). In their study, no lesions indicative for histomonosis were detected in the livers or the caeca of untreated chickens which were sacrificed 10 days after oral infection. Restriction of feed for 18 hours prior to the oral infection resulted in a higher pH in the crop, the gizzard and the duodenum. Furthermore, more severe lesions were noticed during sectioning of birds. To further enhance the effect of a higher pH may have within this experimental model, an alkali mixture was applied to the birds prior to infection. As a consequence, the number of birds showing lesions caused by *H. meleagridis* went up to 100%. Lund (1956) demonstrated that the oral inoculation of histomonads together with a solid digestible material is less favourable for successful infection in comparison to the application of a diluted suspension, as the feed is retained for a longer time within the gizzard. It seems very likely that the deprivation of feed for 5 hours p.i. performed in the present investigation contributes towards the successful oral infection with *in vitro*-cultivated protozoa, especially for the 14-day-old birds. For the day-old poult this parameter appears to be less relevant as feed intake in the first 24 hours of life is very moderate (Bigot *et al.*, 2003) and the pH is similar to the one recorded in older birds (Herpol, 1966).

In fact, the oral route of infection was very effective even after a single application and the exclusion of a possible reinfection, as demonstrated in the second experiment. Consequently, at least some of the histomonads were able to survive the gut passage and to reach the caecum, even though a delay of 2.5 days was noticed comparing both routes of infection. Obviously, the inoculum used in both experiments contained very resistant parasites. In this context it should be mentioned that the growth conditions varied considerably between the studies available in literature. For example, Hu *et al.* (2004) propagated the parasites in modified Dwyer's medium (McDougald & Galloway, 1973) whereas the clonal culture of *Histomonas meleagridis*/Turkey/Austria/2922-C6/04 was established and passaged in Medium 199 with Earle's salts, L-Glutamine, 25 mM HEPES and l-amino acids, foetal calf serum and rice starch. Most likely, the use of different media and incubation procedures has a severe influence on the parasites used for infection.

In this context it should be mentioned that the presence of a resistant stage was already suggested in the early observations reported by Tyzzer (1919). So far the existence of cysts of *H. meleagridis* could not be verified, even though Mielewiczik *et al.* (2008) found cyst stages in the faeces of experimentally infected chickens but failed with purification and enrichment for further analysis. Whether these are real cysts or pseudocysts as known from other flagellates needs further approval.

To date it can only be speculated whether the oral route of infection is of relevance for the transmission of *H. meleagridis* between birds and the spreading of the parasite on a poultry farm. Hu *et al.* (2004) demonstrated the infection of in-contact birds in a group of turkeys housed on deep litter and contributed this to the uptake via the cloaca, a phenomenon known as cloacal drinking. This transmission occurs very rapidly after infection as demonstrated by us recently (Hess *et al.*, 2006a). However, as birds continuously peck towards excreted faeces and the contaminated litter the oral infection has to be considered as a possible infection route, depending on the excreted stages within the droppings.

If resistant stages of the parasites are developed in the host bird similar to the ones present in the *in vitro* cultures used in our experiments, the likelihood of an oral infection of birds housed on deep litter would, according to our present investigations, seem to be high.

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Table 1. Experiment 1: Reisolation of *H. meleagridis* and time-of-death following infection of turkeys kept on deep litter

day p.i.	Group I Cloacally infected birds (numbers 1 – 4)				Group II Orally Infected birds (numbers 5 – 14)										In-contact birds (numbers. 15 – 18)			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0	- ^a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	x ^b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	x	x	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	x	x	-	x	-	-	-	-	-	-	-	-	-	-
9	-	x	x	x	-	-	-	-	-	-	-	-	x	-	x	x	-	x
12	-			x	-	-	x	-	-	-	-	x	-	x	-	x	-	-
14	x				x	-	x	x					x		-			-
	15 ^c	10 ^c	12 ^c	13 ^c	16 ^c	15 ^c	16 ^c	16 ^d	14 ^c	12 ^c	11 ^c	13 ^c	16 ^c	14 ^c	13 ^c	16 ^d	14 ^c	16 ^d

^a Reisolation of *H. meleagridis* was negative.
^b Reisolation of *H. meleagridis* was positive.
^c Bird died on day p.i. indicated.
^d Day p.i. bird had to be euthanized.

Table 2. Experiment 2. Reisolation of *H. meleagridis* and time of death following infection of individually housed turkeys

day p.i.	Group I Cloacally-infected birds (numbers 1 – 8)								Group II Orally-infected birds (numbers 9 – 16)							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
0	- ^a	-	-	nd ^b	nd	nd	nd	nd	-	-	-	-	nd	nd	nd	nd
2	x ^c	x	-	-	x	-	-	-	-	-	x	-	x	-	-	-
5	x	x	x	-	-	-	x	x	-	-	-	x	x	x	x	-
7	-	-	x	x	-	-	-	x	-	-	x	x	-	-	x	x
9	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-	x
12	x	x	-	-	-	x	x	-	x	-	x	-	-	-	-	-
14	x	-		x		x	x	x	x	-	-	-	-	-		x
16	-									-	-	x	-	-		x
19										-		x	-			
21												x	-			
28													-			
35													-			
	17 ^d	15 ^d	14 ^d	15 ^e	14 ^d	16 ^d	14 ^d	15 ^d	16 ^d	42 ^f	17 ^e	17 ^d	22 ^d	42 ^f	14 ^d	19 ^d

^a Reisolation of *H. meleagridis* was negative.

^b Sampling not done.

^c Reisolation of *H. meleagridis* was positive.

^d Bird died on day p.i. indicated.

^e Day p.i. bird had to be euthanized.

^f Bird killed at termination of the experiment.

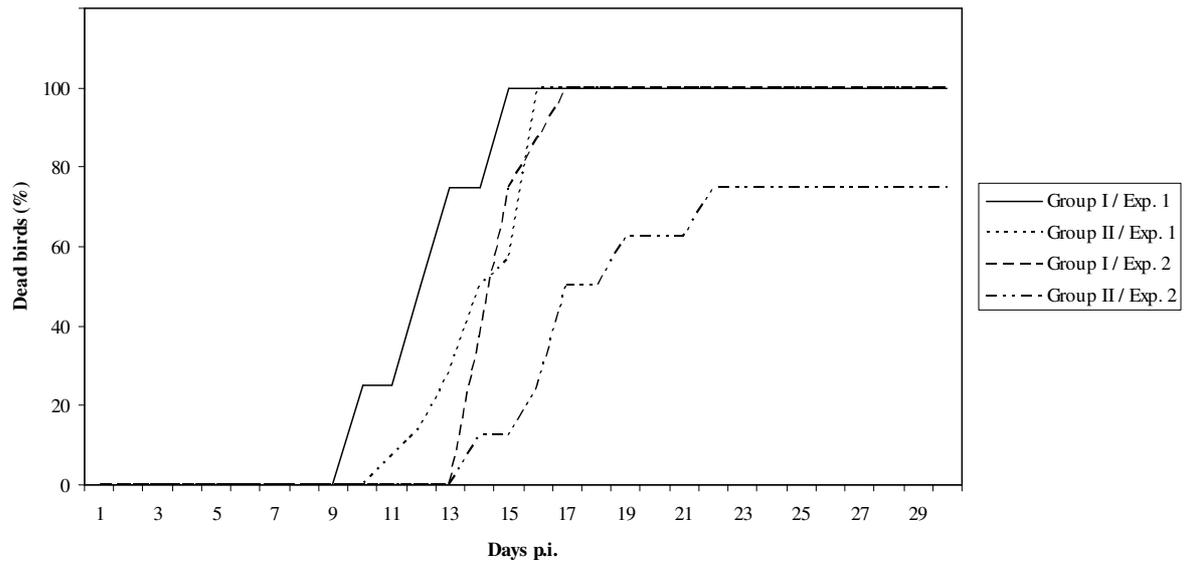


Figure 1. Cumulative death rates of turkeys housed in groups unseparated (Expt. 1) or individually (Expt. 2) after cloacal (groups I) versus oral (groups II) infection with *H. meleagridis*.



Figure 2. Livers and caeca of individually housed turkeys (Expt. 2) following infection with cultured *H. meleagridis* via the cloaca (A) (bird no. 5) or crop installation (B) (bird no. 9).



Figure 2. Livers and caeca of individually housed turkeys (experiment 2) following infection with cultured *H. meleagridis* via the cloaca (A) (bird no. 5) or crop installation (B) (bird no. 9).