Safety of avirulent histomonads to be used as a vaccine determined in turkeys and chickens

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ABSTRACT In the present work, chickens and turkeys were infected with virulent or attenuated Histomonas meleagridis to investigate and compare the effect of both isolates on birds. Thereby, histomonads of a clonal culture were propagated in vitro either for a short period of time (21 passages) to preserve virulence or for 295 passages to achieve attenuation. On the first day of life birds of each species were infected with either virulent or attenuated parasites. Throughout the experiment, all birds were examined daily for clinical signs attributable to the infection. Furthermore, the excretion of viable parasites was determined after in vitro reisolation from cloacal swabs. For the investigation of pathological changes of organs a defined number of infected birds were killed on d 4, 7, 10, 14, and 21 postinfection (PI) and necropsy was performed. By this routine, changes in livers and ceca were classified by a scoring system to evaluate the severity of lesions. Samples of cecum, liver, and lung were generated and screened for the presence of parasites by PCR and immunohistochemistry. Turkeys infected with virulent histomonads showed first clinical manifestation of histomonosis on d 10 PI, whereas the remaining birds did not express clinical signs. Positive reisolations of virulent and attenuated histomonads were obtained intermittently from individual chickens and turkeys from d 2 PI until the end of the experiment. Both species of birds displayed lesions in the ceca and the liver following infection with virulent parasites, whereas no changes occurred in birds inoculated with attenuated histomonads. The PCR revealed the dissemination of virulent histomonads in ceca, livers, and lungs of some chickens and turkeys in contrast to attenuated parasites, which were exclusively found in cecal samples. The attenuated isolate of H. meleagridis did not induce clinical signs or pathological changes and offers high safety after infection of chickens and turkeys. Therefore, the in vitro attenuation and the use of avirulent histomonads represent a viable tool for vaccination against histomonosis.

Key words: histomonosis, vaccination, chicken, turkey, pathogenicity

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INTRODUCTION

Histomonosis (synonyms include Blackhead disease and histomoniasis) is a parasitic disease of gallinaceous birds and is caused by the flagellated parasite Histomonas meleagridis. After a flock of chickens or turkeys gets infected by the protozoan, the disease can result in high losses of birds (McDougald, 2008). In turkeys, the parasite triggers severe inflammation of ceca and livers of affected birds whereas in chickens much less severe involvement was demonstrated (Beg and Clarkson, 1970), reflecting the course of the disease. It was reported that morbidity and mortality attributed to histomonosis were more severe in turkeys than in chickens (Tyzzer, 1934). However, histomonosis of laying hens can cause a permanent decrease in egg production, as was shown recently (Esquenet et al., 2003).

In recent years, except for an arsenic drug (Histostat, Alpharma Inc., Bristol, TN) in the United States, all substances formerly licensed in numerous countries were banned for usage in food-producing animals, as was the case for member states of the European Union (CEC, 1995, 2002). Consequently, prevention and therapy of the disease could no longer be performed. Hence, the need for new options for the treatment of the disease is evident.

Recently, it was shown that vaccination with in vitro attenuated histomonads against the disease resulted in full protection of turkeys against a severe challenge (Hess et al., 2008). However, data about the degree of...
attenuation were incomplete because no bird in this study was investigated for lesions without receiving a challenge infection. In a continuous study, orally vaccinated and nonchallenged turkeys were necropsied (Liebhart et al., 2010). However, in that work the time period between vaccination and killing of birds was 10 and 16 wk, respectively, after which regeneration of organs should already be completed (Lund, 1972).

The virulence of short-term in vitro propagated histomonads has already been shown in turkeys and chickens (Hess et al., 2006a; Windisch and Hess, 2010), whereas no data about the progressive pathology induced by long-term cultivated parasites are available. Therefore, the aim of the present study was to perform pathological examination of chickens and turkeys on predetermined time points following infection with attenuated or virulent histomonads. These investigations were essential for continuous work in establishing vaccination with attenuated histomonads because this prophylactic treatment must not cause explicit side effects in host birds.

**MATERIALS AND METHODS**

**Animals**

In the present experiment, 34 Lohmann Brown chickens (Lohmann Tierzucht GmbH, Cuxhaven, Germany) and 35 British United Turkeys Big 6 (Aviagen Turkeys Ltd., Tattenhall, UK) turkeys were kept in pens on deep litter from the first day of life. All birds were numbered (Swiftack, Heartland Animal Health Inc., Fair Play, MO) for individual identification. The chicks had unlimited access to feed (commercial starter feed for chickens or for turkeys) and water, except a feed restriction for 5 h directly after the infection. The experiment was discussed and approved by the institutional ethics committee and licensed by Austrian law (license numbers 68.205/0017-BrGT/2005 and 68.205/0168-BrGT/2005).

**Infection Cultures**

Protozoans of the clonal culture *H. meleagridis*/*Turkey/Austria/2922-C6/04 were established and propagated in vitro as described recently (Hess et al., 2006b). After storage of the parasites in liquid nitrogen, the cultures were thawed shortly before the experiment was started and propagated for infection after passages 21 or 295. The exact cell number was determined using a Neubauer cell counting chamber (Sigma-Aldrich, Vienna, Austria) followed by dilution in fresh medium up to the required concentration. Every dose of virulent or attenuated histomonads consisted of 10^4 parasites resuspended in 300 μL of medium (Gibco Medium 199, Invitrogen, Lofer, Austria), 15% fetal calf serum (Invitrogen), and 0.66 mg of starch of rice (Sigma-Aldrich).

**Experimental Setup**

Three groups of turkeys and 3 groups of chickens were placed on first day of life. On the same day, 14 birds of the chickens 1 (C1) group and 15 turkeys of the turkeys 1 (T1) group received virulent histomonads following short-time in vitro propagation (21 passages), whereas 15 birds of the chickens 2 (C2) group and the same number of birds of turkeys 2 (T2) group were inoculated with attenuated, long-time cultivated (295 passages) parasites. The administration of the isolates to turkeys was performed exclusively via the oral route using a crop tube placed on a 1-mL syringe (B. Braun Melsungen AG, Melsungen, Germany). The same infection routine was performed with chickens of both infected groups, which received an additional dose of parasites cloacally to ensure successful infection. In the process, a conventional 1-mL pipette was adjusted for the required amount of inoculum (300 μL). The chickens 3 (C3) and turkeys 3 (T3) groups consisted of 5 noninfected birds representing negative control birds. All groups were kept in separate rooms to prevent any cross-infection.

**Clinical Examination and Excretion of the Parasite**

During the experiment, all birds were examined daily for clinical signs. The excretion of histomonads was investigated by in vitro reisolations from cloacal swabs. Samples of the cloacal content of every bird were collected on d 0, 2, 5, 9, 12, 16, and 19 postinfection (PI) and brought in 2-mL tubes (Sarstedt, Nümbrecht, Germany) containing 1.5 mL of culture medium for *H. meleagridis* as described above. After incubation at 40°C for 96 h, the reisolations were controlled by microscope for the presence of viable cells of the parasite.

**Postmortem**

On d 4, 7, 10, and 14 PI, 3 birds of groups C1, C2, T1, and T2 and 1 bird of groups C3 and T3 were killed in a predetermined order, according to their identification number. The remaining birds were killed on d 21 PI when the experiment was completed. All birds that were killed or were euthanized because of severe histomonosis were anesthetized by intravenous application of thiopental (Sandoz, Kundl, Austria) and subsequently bled to death. After killing, the birds were necropsied and examined for pathological changes. The severity of lesions found in ceca and livers were differentiated using lesion scores (LS) as previously published (Windisch and Hess, 2010; Zahoor et al., 2010). Hence, changes in the mentioned organs were classified from LS 0 (normal) to LS 4 (maximum lesions), which are outlined in Tables 1 and 2. During necropsy, tissue samples of various organs were generated for continuous examination as described below.
Table 1. Lesions scores\(^1\) (LS) and the detection of the parasite by PCR and immunohistochemistry (IHC) of chickens infected with virulent (chickens 1 group) and attenuated (chickens 2 group) histomonads\(^2,3\)

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\(^1\)Cecum: 0 = no pathological changes; 1 = sporadic inflammation or mild thickening of the wall of one cecum; 2 = sporadic inflammation or mild thickening of the wall of both ceca; 3 = inflammation of both ceca and thickening of the intestinal wall with liquid fibrin or sporadic fibrinous coagula in the lumen (if only one cecum was affected, then lesion score 2 was applied); 4 = severe inflammation and necrosis in both ceca with compact fibrinous masses in the lumen of the ceca (if only one cecum was affected, then lesion score 3 was applied). Liver: 0 = no pathological changes; 1 = a few single punctiform necrosis, diameter ≤1 mm; 2 = single punctiform necrosis, disseminated throughout the organ, diameter ≤1 mm, or a few single punctiform necrosis, diameter ≥1 mm; 3 = single punctiform necrosis, disseminated throughout the organ, diameter ≥1 mm or some large-area necrosis; 4 = confluent necrosis throughout the organ.

\(^2\)C1 = chickens 1 group (n = 14); C2 = chickens 2 group (n = 15).

\(^3\)Minus sign (−) = negative result; plus sign (+) = positive result.

Table 2. Lesions scores\(^1\) (LS) and the detection of the parasite by PCR and immunohistochemistry (IHC) of turkeys infected with virulent (turkeys 1 group) and attenuated (turkeys 2 group) histomonads\(^2,3\)

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\(^2\)T1 = turkeys 1 group (n = 15); T2 = turkeys 2 group (n = 15).

\(^3\)Minus sign (−) = negative result; plus sign (+) = positive result; ND = not done.
Organ samples of cecum, liver, and lung of the birds were examined by PCR to identify parasitic DNA in tissues. In a first step, DNA of 25 mg of tissue was isolated of the latter organs using DNeasy Tissue Kit (Qiagen, Valencia, CA). Subsequently, amplification of DNA of \textit{H. meleagridis} was carried out applying the reagent of HotstarTaq Master Mix Kit (Qiagen). Specifications of the PCR protocol performed in the present work were reported recently (Grabensteiner and Hess, 2006).

**Immunohistochemistry**

Tissue samples of the same organs used for PCR were fixed for at least 24 h in 10% neutral buffered formalin and thereafter embedded in paraffin. Tissue slices of 4 µm thickness were prepared using the microtom Microm HM 360 (Microm Laborgeräte GmbH, Walldorf, Germany) and mounted on glass slides. Then, dewaxing and dehydration of the tissue slices was performed. Before the antigen was exposed to antibodies against \textit{H. meleagridis}, slides were heated in citrate buffer (pH 6.0) and endogenous peroxidase activity was blocked with 1.5% H$_2$O$_2$ in methanol. After short-time incubation with a dilution of normal goat serum (Vector Laboratories, Burlingame, CA), the primary antibody was directly applied on the tissues and left for adhesion with the antigen of histomonads overnight at 4°C. Accordingly, a secondary antibody (biotinylated anti-rabbit IgG antibody; Vector Laboratories) was used that was visualized by the DAB Substrate Kit for peroxidase (Vector Laboratories). By this method histomonads were stained prominently brown in contrast to surrounding tissue, which was counterstained with Mayer’s hemalum (Merck, Darmstadt, Germany) for the identification of host cells. The detailed protocol of the described immunohistochemistry (IHC) was published by Singh et al. (2008).

**RESULTS**

**Clinical Signs and Mortality**

Only turkeys of the T1 group infected with virulent histomonads developed clinical signs. Turkeys in this group first exhibited clinical signs on d 10 PI, when the whole group was crowded together and showed drowsiness. Two days later, the birds contracted sulfur-colored diarrhea and severe apathy. Both turkeys of the T1 that were supposed to be killed on d 14 PI died on the same day. The remaining birds of the T1 group died or had to be euthanized because of severe histomonosis before d 21 PI.

**Reisolation**

The number of positive reisolations per group on different sampling days is shown in Figure 1. First positive reisolations were obtained on d 2 PI from individual birds of all infected groups. Overall, 8/14 chickens and 6/15 turkeys infected with the virulent isolate were found positive by reisolation at least once during the experiment. Similar results were noticed in groups of birds (n = 15) infected with the attenuated inoculum: 6 positive results of chickens and 10 positive samples of turkeys.
Postmortem

Pathological findings of every infected bird that were quantified using LS are given in Tables 1 and 2. None of the infected chickens or the turkeys showed gross lesions indicative of histomonosis on d 4 PI. Three days later the ceca of all necropsied chickens and turkeys infected with the virulent isolate displayed signs of inflammation and the presence of fibrin within the lumen (LS 2 to 4) whereas the livers were found normal. On d 10 PI maximum LS were noticed in ceca of all killed birds of the same groups (C1 and T1) and, moreover, all livers were severely affected (LS 3 to 4; Figure 2). Similar lesions were found in livers and ceca of the T1 group on d 14 PI and during following necropsies. Three chickens of C1 displayed again maximum changes in the ceca on d 14 PI but only 1 bird showed lesions in the liver. On the last killing day, 1 of the 2 remaining chickens that were infected with virulent histomonads did not display any changes of ceca and livers (LS 0), in contrast with severely affected ceca of the other bird (LS 4). Pathological aberrances of organs other than cecum and liver were not observed during the study. None of the chickens and turkeys infected with attenuated histomonads and noninfected birds of the control groups displayed any lesions (Figure 2).

PCR

The DNA of *H. meleagridis* was detected in ceca, livers, and lungs of individual birds infected with the virulent isolate (Tables 1 and 2). In contrast, no positive

![Figure 2. Lesion in the ceca of chickens and turkeys infected with virulent histomonads (A) detected on d 7 postinfection (PI). Three days later, all birds displayed exceeding changes in the ceca and severe lesions in livers. Birds infected with attenuated parasites (B) did not show any inflammation or necrosis in the same organs on d 7 and 10 PI. Color version available in the online PDF.](https://academic.oup.com/ps/article-abstract/90/5/996/1503192)
signal was obtained by processing samples from livers and lungs taken from chickens and turkeys inoculated with attenuated parasites. Only cecal samples obtained from these birds resulted in positive findings.

**IHC**

Positive findings of *H. meleagridis* were detected in samples of ceca, livers, and lungs of birds infected with virulent parasites (Tables 1 and 2). On d 4 PI the parasites of the same isolate could be demonstrated within the cecal lumen of chickens whereas in turkeys this finding was first noticed 3 d later. In livers, only a low number of virulent parasites without the presence of leukocytes was noticed on d 4 and 7 PI. The same observation was found in positive lungs on d 7, 10, and 14 PI. Different leukocytes, mainly lymphocytes, were present because of severe infiltration of numerous virulent histomonads in infiltrated tissues of cecum between d 7 and 21 PI and liver starting on d 10 PI.

Chickens and turkeys receiving attenuated histomonads were found positive at first on d 4 PI, whereas no sample of liver or lung of the same birds displayed signals for the presence of the protozoan. Moreover, it was noticed that attenuated parasites were primarily localized within the lumen of the intestine and did not penetrate into the cecal wall like virulent histomonads (Figure 3). On the same slides, the hemalum stain of the surrounding cecal tissues of birds of C2 and T2 did not reveal distinct inflammation or necrosis against attenuated histomonads.

**DISCUSSION**

In the present study, the effect of attenuated *H. meleagridis* applied to chickens and turkeys was comparatively investigated with the progression of severe histomonosis of the same species of birds triggered by virulent parasites. Turkeys were infected orally because this route of application with the clonal culture *H. meleagridis*/Turkey/Austria/2922-C6/04 passage 21 was already proven to activate the disease (Liebhart and Hess, 2009). In chickens, data about the effective oral infection with the same kind of inoculum were limited because birds were inoculated either via the cloaca (Hess et al., 2006a; Windisch and Hess, 2010) or the cloaca and crop (Zahoor et al., 2010) in recent experiments. In addition, it was shown in a previous study that chickens had to undergo a special treatment prior to oral infection (starvation or the administration of an alkali mixture) with a suspension of histomonads to observe organ lesions of histomonosis (Horton-Smith and Long, 1956). Consequently, the dual mode of infection of chickens was applied to avoid unsuccessful infection.

The detection of histomonads by reisolation resulted in low numbers of positive samples because of intermittent excretion of the parasite as was observed recently (Liebhart et al., 2010). Furthermore, as a result of the experimental scheme some birds were sampled only once after infection, which also lowered the chance of positive findings. Despite this, the excretion of viable parasites from at least 40% of birds in every group was noticed, which demonstrated the successful multi-
plication of both virulent and attenuated histomonads. Substantial differences in the number of positive reisolations of virulent and attenuated histomonads were not observed.

The fact that turkeys infected with virulent parasites contracted clinical signs is in agreement with several recently performed experiments (Hess et al., 2006a, 2008). In the aforementioned studies the reisolation of viable parasites of infected or vaccinated birds confirmed infection. Pathological changes of inner organs were not observed during necropsy at the end of the above-mentioned experiments, which was not performed before the sixth week PI, in difference to this study. However, the presence of lesions in organs of surviving birds could be detected only during a short period of time PI because of rapid tissue recovery. The healing of lesions attributed to histomonosis was recently shown for chickens (Zahoor et al., 2010) and mentioned some time ago by Lund (1972) for birds in general.

The design of the experiment and the applied methodology in the present work were chosen to reveal potential pathological changes in the main target organs of the hosts following infection with virulent or attenuated parasites. Hence, it could be shown that virulent in vitro propagated histomonads induced gross lesion between d 5 and 7 PI whereas changes in the livers were detected for the first time at 10 d PI. Most of the affected organs displayed severe lesions (LS 3 or 4). During the experiment the severity of pathological changes in ceca and livers of turkeys did not regenerate compared with chickens, where only 1/5 livers from the birds killed on d 14 and 21 PI was affected. The progression of histomonosis in birds infected with virulent parasites is in agreement with previous studies (Farmer et al., 1951; Clarkson, 1962; McDougald and Hansen, 1969). The monitoring period of 21 d was therefore a good baseline to investigate the effect of attenuated histomonads in chickens and turkeys.

In vivo studies to investigate the degree of attenuation of in vitro propagated histomonads are very limited and reported only by Tyzzer (1934, 1936). He noticed disparities in the virulence of different strains of the parasite based on pathological lesions but birds of the respective groups were not killed on different time points PI. Later on, Lund et al. (1966) stated that in vitro passaging may not induce attenuation but the outgrowth of a certain strain in a mixture of isolates is favored. This postulation is contradicted by the usage of clonal cultures.

In addition to clinical signs and necropsy, PCR and IHC were performed in the present work to detect the parasite in certain tissues of birds because Tyzzer (1934) demonstrated that macroscopic lesions are not mandatory for the presence of the parasite. A similar observation was recently reported by Amin et al. (2011) who examined organs of chickens and turkeys following infection with the Histomonas-related flagellate Tetratrichomonas gallinarum.

Some inconsistency was noticed investigating the same organ by PCR and IHC, even though macroscopic lesions were present. This phenomenon can probably be explained by a low sensitivity of the used method. Furthermore, it cannot be precluded that a false-negative result occurred because of complete absence of DNA or antigen of histomonads in the processed piece of tissue (25 mg of tissue for PCR and 4-µm slice of organ for IHC).

The present investigation revealed data about the presence of attenuated and virulent histomonads soon after infection in turkeys and chickens. Whereas virulent parasites were detected in ceca, livers, and lungs of several chickens and turkeys, none of the liver and lung samples processed from the birds infected with attenuated parasites resulted in positive findings. Instead, attenuated histomonads were restricted to the ceca of the birds confirmed by IHC, with which high numbers of parasites could be located only in the cecal lumen, indicating that the ability to penetrate the tissue was highly reduced. This demonstrates that the pathogenicity of cultivated clonal parasites used in the present study is almost neutralized after long-term passaging.

In conclusion, detailed investigation on the pathogenicity of the isolate H. meleagridis/Turkey/Austria/2922-C6/04 passage 295 did not result in adverse effects on birds’ health, which completes results of existing studies. Furthermore, no pathomorphological or histological lesions caused by the attenuated isolate could be demonstrated. Therefore, the present work validated safety of in vitro attenuated H. meleagridis used for protection of turkeys against histomonosis.

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