IMMUNITY AND TRANSMISSION OF *HISTOMONAS MELEAGRIDIS* IN TURKEYS

by

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(Under the Direction of Larry R. McDougald)

ABSTRACT

The immune response to *Histomonas meleagridis* and dynamics of lateral transmission was investigated in turkeys. The inoculation of birds 2-16 weeks old produced no evidence of age differences in susceptibility. Commingling of uninoculated poults with infected poults in battery cages showed that litter type was not important in transmission, but infection rates were higher on bedding compared with wire floors. Repeated infection and treatment with dimetridazole produced birds resistant to reinfection, shown by reduced liver and cecal lesions. Immune protection was investigated by inoculation of poults with live or dead *Histomonas* from cultures. Vaccination by subcutaneous or intramuscular routes produced a protective response as shown by a reduction in the number of infected birds and the severity of lesions after commingling with infected birds. An ELISA test was devised to analyze the antibody response. Turkeys produced IgG antibodies in response to infection and after vaccination with killed histomonads.

INDEX WORDS: Histomonas, Immunity, Antibodies, Enzyme-linked Immunosorbent Assay, Transmission

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DEDICATION

To the birds, who fascinate and inspire me, fuel my passion and enrich my life

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CHAPTER 1

Introduction and Literature Review

Histomoniasis, also known as blackhead disease or entero-hepatitis, is an infectious disease in gallinaceous birds caused by the parasitic protozoan *Histomonas meleagridis.* Histomoniasis can cause 90-100% mortality in turkeys but is less severe in chickens. Only one compound is effective in prevention (nitarsone) and there is no effective treatment for the disease since regulatory authorities banned the use of nitromidazole products in the early 1990's in the U.S. and later in Europe.

Attempts to provide active immunity against histomoniasis have been considered unsuccessful or impractical overall. Studies have shown thart some degree of immune protection may occur after infection and treatment with antihistomonal drugs. Precipitating antibodies have been detected in recovered birds. Immunization of birds has not been adequately investigated, particularly with regard to the administration of cultured *Histomonas.*

The cecal worm (*Heterakis gallinarum)* plays an important role in the transmission of histomoniasis and *Histomonas* is known to survive for a long period of time in cecal worm eggs providing a reservoir of infection year after year. Previous research has shown that the disease can spread through a flock of turkeys in the absence of vectors by cloacal drinking. It is unclear whether the infection spreads by direct contact with infected birds or by contact with contaminated bedding.

The objectives were to investigate the immune response in turkeys to *H. meleagridis* and to determine what role bedding serves in the indirect transmission of histomoniasis within turkey flocks.

***Histomonas meleagridis* and histomoniasis**

Histomoniasis, also known as enterohepatitis or blackhead, is a severe disease of gallinaceous birds caused by the parasitic protozoan *Histomonas meleagridis* (8, 48). *Histomonas* is in the phylum Parabasala, the class Trichomonadae, the family Monoceromonadidae in Cavalier-Smith's six kingdom system (5).

Dwyer (13) determined *Histomonas* to be related to *Dientamoeba, Trichomonas* and *Entamoeba* based on antigenic analysis. *Dientamoeba* and *Histomonas* have been shown to cross-react strongly in fluorescent antibody studies, indicating many shared antigens. *Histomonas* contains a few antigens which cross-reacted with *E. invadens* and *E. histolytica.*

The nucleotide sequence analysis of a small subunit rRNA of the organism showed a close relationship between *Dientamoeba* and *Histomonas* (20). The rRNA subunit of both organisms have reduced G-C content and increased G-C chain length in relation to other members of the Parabasalidae family. A comparison of the nucleotide sequence among all identified parabasalid gene sequences and the phylogenetic trees constructed from the host genes showed 100% maximum likelihood, distance, and parsimony between *Histomonas* and *D. fragilis.*

In 1893, Cushman first described histomoniasis as the cause of the decline of turkey production in Rhode Island and called it blackhead disease (8). Two years later, Smith of the U.S.D.A. described the disease as infectious enterohepatitis and named the

responsible organism *Amoeba meleagridis* (48). The author found the protozoan in fresh cecal preparations and described the organism as round or oval and 8-15 μm in diameter. In tissues fixed and stained, they ranged from 6-10 um. in diameter. The organism was described as having a defined outline with a group of minute granules that probably represented a nuclear structure (48).

Liver lesions associated with histomoniasis were first described by Cushman in 1893 but the primary site of the infection is the ceca. The cecal lesions were described as a mass of yellow, hardened lumps which developed into thickened cecal tissue and contained a fibrinous or cheesy textured core. Necrosis of the ceca may occur and birds then succumb to peritonitis. Durant (12) showed that histomoniasis could be controlled in turkeys by cecal abligation. He surgically ligated the cecal necks to restrict passage of material into the ceca. Even though post-surgical losses were high, he found that ligated birds did not contract the disease after two years on contaminated soil.

Smith (48) described the diseased liver of infected birds as having circular elevations with a central crater-like depression. The appearance of lesions differed with the age of the lesion and virulence of the infection. Early lesions contained slightly depressed circular areas of mottled dark red color. Later, lesions became yellowish, enlarged and they had a firm texture compared to normal liver tissue. Smith also added that lesions due to histomoniasis could be found in other tissues such as the pancreas, kidneys, and spleen..

Tyzzer studied the morphology of blackhead organisms and found a variety of forms in stained preparations (53). The author stated "if it were not for certain constant features, it would be difficult to prove that we are dealing with one and not several

species of the parasite." Tyzzer described the protozoans found in early lesions and at the periphery of infected areas as irregular and amoeba-like, which he called the invasive stage. The author observed sluggish amoeboid organisms harvested from freshly killed turkeys. These organisms were 8-17 microns in size in fixed tissue and distinctly amoeboid; some long forms measured 30 μm. The organism contained an extranuclear body and particles within vacuoles.

A vegetative phase was also noted and thought to be a transitional phase. This form was larger, (12-21 μm,) was less mobile, and had basophilic staining cytoplasm. Inclusions seen in the invasive stage were not present but a small amount of reticular or granular material was observed around the nucleus. Tyzzer thought the vegetative phase produced great distention and destruction of host tissue through swelling of tightly packed parasites. The author described a resistant phase that was smaller than other forms, (5-11 μm,) with acidophilic stainingcytoplasm.

Tyzzer concluded that the parasite multiplied in tissues by binary fission, as no multinucleated organisms or evidence of other types of reproduction were found. He noted that other amoebas, known to reproduce by binary fission, increased in number very rapidly in culture medium, even though the dividing forms were difficult to demonstrate.

Under certain circumstances, the organism exhibited characteristic flagellated motility. To date, only one other species, *Histomonas wenrichi,* has been added to this genus. Others have described it's movements as amoeboid or rhythmic and pulsing. Tyzzer and Fabyan (52) observed one or two flagella on histomonads occurring in the

cecal lumen form and Tyzzer (55) later observed one beating flagellum that caused the parasite to rotate in a counter-clockwise fashion.

Curtice (7) identified the chicken as the natural host for *Histomonas meleagridis*. He described the disease to be less severe in hens, yet deadly in turkeys exposed to infected hens or yards where hens had been reared. Tyzzer and Fabyan (52) showed that the same organism which was responsible for causing histomoniasis in turkeys was also responsible for histomoniasis in chickens. By feeding turkeys *H. gallinarum* ova from chickens, liver tissues from infected chickens, or soil from contaminated hen yards.

Many gallinaceous species other than turkeys and chickens have been found to develop histomoniasis. However, the severity of the disease varies with the species of host. *Histomonas meleagridis* has been reported in bobwhite and japanese quail, ruffed grouse, guinea fowl, pheasant, chukar, peafowl and rhea (9, 10, 31-36, 38). After receiving sporadic reports of histomonads in ducks and geese, Lund attempted to infect both species (37). He found that ducks and geese were unsatisfactory hosts for *H. meleagridis* but they could develop mild infection after exposure via the cecal worm.

Curtice (7) found age to be a factor in susceptibilty to histomoniasis in turkeys, 90% of the poults raised on contaminated soil became infected while only 20% of the older turkeys became infected. Later, Kendall (27) found there was no significant difference in susceptibility to histomoniasis in turkeys 7-20 weeks old when infected *per os* with *H. gallinarum* ova or cloacally with *H. meleagridis.*

Clinical diagnosis

In turkeys, signs of histomoniasis include huddling birds with ruffled feathers and drooping wings. Cecal cores are passed in feces, feed intake decreases and water intake

increases. Watery, sulfur-yellow colored droppings develop indicating liver involvement. The birds lose or fail to gain body weight and heavy mortality begins approximately three days after signs develop. The disease is less severe in most chicken breeds with signs limited to diarrhea, cecal cores in feces and a general unhealthy appearance of the birds.

Diagnosis of the disease is usually made by gross examination and the observation of typical lesions in the liver and ceca (43). Enlarged ceca containing white, caseous cores can become necrotic resulting in death from peritonitis. Liver tissue becomes congested and lesions begin to develop. Liver lesions can be large or small, few or numerous as necrosis begins and birds succumb to liver failure.

Diagnosis may be confirmed by microscopic examination of cecal contents or by histopathology. Histomonads can be found in stained liver tissue and occur in clusters. *Histomonas* can also be grown in culture from the cecal tissue of infected birds *in vitro* (44).

Factors affecting pathogenicity of *Histomonas meleagridis*

Previous work demonstrated a distinct relationship between the pathogenicity of *H. meleagridis* and certain species of bacteria although the role of bacteria in the development of virulence is not well understood. Harrison (23) found that some, but not all, liver lesions caused by *H. meleagridis* contained bacteria, the most prevalent species being *Escherichia coli.* Birds killed early in the course of the disease had lower bacterial counts in liver lesions than birds allowed to die from the disease.

Franker and Doll (19) suggested there may be a synergistic relationship between host flora and *Histomonas* or *Heterakis*. Only 1 of 12 gnotobiotic turkeys developed lesions after *per os* inoculation with 1,000 germ-free *Heterakis* eggs, while 11 of 12

conventionally reared turkeys developed lesions of blackhead. Bradley (4) also worked with bacteria-free and monocontaminated poults and found that *H. meleagridis* in combination with *E. coli, Clostridium perfringens,* or *Bacillus subtilis* were capable of producing histomoniasis while various other combinations of bacteria did not. Gnotobiotic turkeys inoculated with bacteria alone or germ-free *Heterakis* eggs did not become ill. These results suggested that there must be a proper combination of bacteria present in the bird to produce the disease.

Springer (49) worked with germ-free chickens as well and found that the same combination of bacteria and *H. meleagridis* that caused histomoniasis in turkeys did not produce the disease in chickens. He concluded that the etiology of histomoniasis in chickens was much more complex than in turkeys, requiring two or more species of bacteria to produce the disease.

Host species and breed also may play a role in pathogenicity. Lund was able to infect many gallinaceous species with *Histomonas meleagridis* but the severity of the infection varied. The infections in chukar partridge, turkeys and peafowl were more severe than in ringneck pheasants and chickens (30, 32, 35). Histomoniasis in mature bobwhite quail produced late lesions in the ceca, infrequent liver lesions with almost no birds showing signs of morbidity and no mortality. Lund also compared chicken breeds for susceptibility to histomoniasis and found differences in susceptibility and severity (30).

Turkey cecal coccidium, *Eimeria adenoeides,* appears to interfere with histomoniasis in turkeys (45). Conversely, in chickens, the number of birds with liver

lesions and the severity of liver lesions increased significantly when birds were infected with *Eimeria tenella* and *Histomonas* (42).

Transmission

Cushman reported early in his investigation of *Histomonas* that there was a relationship between histomoniasis in turkey poults and poults being reared in yards previously occupied by chickens or being reared with chickens concurrently (8). The author suggested that turkeys and chickens should not be reared together as a way of managing the disease in turkeys.

In early experiments, Curtice demonstrated that *Histomonas* was not transmitted vertically from hen to poult via the egg, although poults died of histomoniasis at 12-14 days of age (7). It was determined that young poults were being infected after placement in contaminated ground pens which had been occupied earlier by chickens. He also concluded that chickens, guinea fowl and pheasants were carriers of the disease. Tyzzer and Fabyan demonstrated that the organism could be transfered from bird to bird via the cecal worm, *Heterakis gallinarum* (51) This was an important discovery because it showed how the chicken served as a reservoir of infection and led to recommendations for prevention of blackhead by segregation of turkeys from chickens.

Transmission via infected embyonated *Heterakis* ova is considered the most important source of histomoniasis in turkeys and chickens. Graybill (22) was the first to realize an undefined relationship between *Heterakis* and *Histomonas* after birds fed embryonated *Heterakis* eggs developed signs of histomoniasis. The disease could not be produced by giving the birds incubated feces or culture where *Heterakis* worms or ova had been removed. Tyzzer (54) showed that turkeys could become infected when housed

with chickens or occupy ground that chickens had been grown on. Later, Tyzzer (55) defined the relationship between *Histomonas* and *Heterakis,* only embryonated *Heterakis* ova could produce the disease, male worms and unembryonated worm eggs could not.

Gibbs (21) found *Histomonas* in the reproductive tract and ova of *Heterakis* by light microscopy. Histomonads were identified in all reproductive parts of the female heterakids and among the sperm in males. Springer (50) demonstrated that female *Heterakis* containing unembryonated ova could not produce histomoniasis when fed to birds but embryonated ova would produce the disease. He also showed that histomoniasis could be produced by feeding male *Heterakis* to birds although Tyzzer did not.

Larvae of *Heterakis* worms containing histomonads release the organism during the larvae's molting process. Fine (18) chickens were fed embryonated worm ova and author could recover similar numbers of adult heterakids between 10 and 20 days after injestion of the ova from birds that bacame infected with *Histomonas* (18). He also demonstrated that not all *heterakis* ova harbor *H. meleagridis.*

Many studies have shown that *Heterakis* ova are able to remain viable in contaminated soil for several years and once hatched can still cause histomoniasis (16, 17, 28, 47). *Heterakis* ova are resistant to environmental changes and therefore serve as a reservoir of infection from year to year.

The earthworm has been shown to be a carrier of *H. gallinarum* and to serve as a reservoir for *H. meleagridis.* Curtice (7) was the first to demonstrate the transmission of blackhead disease via earthworms but concluded that the earthworm was probably a carrier of infected soil. Ackert (1) determined that *Heterakis* was transmitted by the

earthworm *Helodrilus gieseleri*. Lund (39) observed heterakids emerging from body pores of the earthworm when they were warmed in the laboratory. As a follow-up, infected earthworms were fed to pheasants and poults, producing blackhead disease and cecal worm infections.

Direct transmission of *Histomonas* has been shown to be unreliable. Moore (46) reported that histomoniasis was produced in turkeys by feeding droppings or diseased tissue from infected birds. Farmer (15) reported that a low infection rate was achieved by feeding emulsified cecal lesions and no infection occurred in birds fed emulsified liver lesions. Lund (29) produced cecal lesions in 43 out of 109 poults at 6-9 weeks of age, by administering 10,000-50,000 histomonads orally. In the study, only two birds developed liver lesions, one of which died.

Hu (26) showed that blackhead disease could spread through a pen of turkeys on litter in the absence of cecal worms or other known vectors. A battery model was developed (24) where uninfected birds, comingled with infected birds on paper floors, became infected with *Histomonas*. The spread of infection was presumed to be due to cloacal drinking (25) where substances coming in contact with the cloaca are drawn into the bird by rhythmic muscle contractions of the cloaca.

Control of histomoniasis

Prevention and management are the only options for the poultry industry in controlling the disease. Dimetridazole and ipronidazole were the only effective drugs approved in the U.S. for treating histomoniasis (41). Both were nitroimidazoles and were able to cure birds with advanced stages of the disease. The U.S. Food and Drug Administration withdrew registration of these compounds in the early 1990's because

they were suspect carcinogens. Nitarsone, an arsenical compound, is effective in prevention of blackhead when used continually in the feed but is an expensive drug. Currently there is no effective product available for treatment of histomoniasis after an outbreak of the disease has begun.

Immunization

The first attempts of stimulating protective immunity against *H. meleagridis* consisted of cloacal inoculation with live cultures of attenuated strains. *Histomonas* loses viulence for turkeys upon repeated culture *in vitro* (56). Tyzzer inoculated both chickens and turkeys with a non-pathogenic strain of *Histomonas* and frozen pathogenic strains of histomonads that had been grown *in-vitro*. Subsequent challenges with virulent strains of the organism caused little or no disease but Tyzzer felt that protective immunity would lessen over time unless birds were continually exposed to the organism. Also, the author recognized that there was no way to stabilize the cultures at the desired level of attenuation.

Brackett (3) reported the development of resistance in infected turkeys after drug therapy with antihistomonal compounds. Kendall (27) found that infected birds which were treated with sodium acetarsol became resistant to reinfection with *Histomonas* via cecal worm ova. Resistance was not always protective and long lasting. Clarkson (6) reported that protective immunity was produced in turkeys after treatment with antihistomonal drugs; however, it was not possible to transfer protective immunity by administration of serum from immune birds to susceptible birds.

Culturing *Histomonas in vitro.*

Early workers used a variety of diphasic media for *in vitro* cultivation of *Histomonas* (2, 11, 55). Tyzzer et al reported that the type of bacteria species present in the cecal material during initial isolation had an important influence on successful propagation of *H. meleagridis* (52).

Dwyer formulated the most successful liquid medium for isolating histomonads, which grew large numbers of these organisms very rapidly (14). It was later modified by McDougald and Galloway, to contain 85% Hank's Medium 199, 5% chick embryo extract, 10% horse serum. To 10 ml of culture medium, 1 ml of *Histomonas* culture or fresh isolate in Hank's Balanced Salt Solution is added, using 12-15 mg of rice powder as a source of starch (40). The medium can be used as a diagnostic tool to isolate *Histomonas in vitro.* After 24-48 hours in an incubator at 41^{o} C, histomonads can be readily viewed by microscopy.

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CHAPTER 2

Immune Protection Against *Histomonas meleagridis* in Young Turkeys

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Abstract

The development of immune protection against *Histomonas meleagridis* in turkeys was studied by parenteral injection of antigens consisting of whole cultured *H. meleagridis,* and by a repeated infection/drug treatment regime. A preliminary experiment with birds 3-14 weeks old showed that birds of all ages were equally susceptible to infection. When birds were infected with cultured *H. meleagridis* and treated with dimetridazole 6-7 days later, there was considerable evidence of resistance to reinfection.

Whole *H. meleagridis* were used as antigens for single or multiple inoculations of turkey poults by the subcutaneous (SQ) or intramuscular (IM) routes. Immunized birds were exposed to histomonads by direct inoculation or by exposure to directly infected birds. Turkeys did not appear to develop significant resistance to infection after two inoculations with ~100,000 *H. meleagridis* at 7 and 14 days of age, when challenged at 28 days by the cloacal route. However, vaccinated birds placed in floor pens to commingle with directly infected birds did not develop significant liver or cecal lesions. These results suggest that while the immunity arising from inoculation with whole *H. meleagridis* cells was not adequate to protect against direct, severe inoculation, considerable benefit was observed in the resistance to infection by contact with infected birds.

Keywords: *Histomonas meleagridis*, immunization, turkey.

Introduction

Attempts to produce active immunity against histomoniasis have been largely unsuccessful or considered impractical for commercial use. Tyzzer attempted to immunize birds by cloacal inoculation with attenuated and nonpathogenic strains of *Histomonas* with limited success (22). Lund tried cloacal immunization of turkeys with nonpathogenic *H. wenrichi* but found that birds did not develop immunity to *H.* meleagridis (13). Lund also gave turkeys attenuated strains of *Histomonas* grown *in vitro* by cloacal inoculation (15). Birds could withstand a subsequent cloacal inoculation with pathogenic strains 3-6 weeks later, but became ill when challenged with *Heterakis* eggs given *per os.*

Clarkson detected precipitating antibodies to *Histomonas* in serum from drug-treated or spontaneously recovered birds and injected unexposed birds with serum from those birds (2). The author reported that it was not possible to transfer protective immunity to susceptible birds with serum from birds immune to *Histomonas*. Other workers reported that turkeys became resistant to reinfection after their infections were treated with antihistomonal drugs (12).

It would appear that avenues for immunization of birds against histomoniasis have not been adequately investigated, particularly in the use of cultured histomonas preparations for parenteral inoculation. Several experiments were planned to evaluate the protective response arising from parenteral administration of histomonads. Various routes of administration, antigen preparations, and ages of birds were studied, as well as a repeat of prior studies showing immune protection arising after infection and recovery.

Materials and Methods

Experimental Animals. Day-old, straight run turkey poults obtained from a commercial hatchery (Sleepy Creek Hatchery, Goldsboro, NC) were reared in a community brooder and fed unmedicated turkey starter crumbles *ad libitum* until two weeks of age, then they were moved into steam-sterilized finishing batteries for experimental work.

Parasites. *Histomonas meleagridis* organisms were grown *in-vitro* from an isolate obtained from infected turkeys in Buford, GA. The isolate was stored in liquid nitrogen for future use and was cultured in modified Dwyer's medium (4) as needed. These cultures were heavily contaminated with bacteria, which is normal in culture methods for *H. meleagridis.* The histomonads were estimated with a hemocytometer prior to use for infection of poults by cloacal inoculation or for use as antigens. The number of histomonads administered to groups of birds varied and was dependent on the success of *in vitro* cultures at the time of inoculation.

Antigen preparation. Histomonads cultured *in vitro* were washed 3 times in Hank's Balanced Salt Solution (HBSS) to remove soluble contaminants and some bacteria, and resuspended in HBSS prior to injection. Histomonads were given as "fresh" (presumably live, although the viability was not tested), formalin-killed (10% buffered formalin, followed by repeated washing then refrigerated at 4º C), or freeze-killed (washed as described above, then frozen). Live preparations were treated with gentamicin, amphotericin, and penicillin G (20ug/ml, 200ug/ml and 4,000units/ml) before injection into birds.

Disease exposure model. Birds designated direct exposure were given the prescribed dose of cultured *H. meleagridis* by cloacal injection with a plastic tipped syringe. Previous work has shown that infection rates of 80-100% can be expected when using this method. Birds designated "indirectly exposed" were not inoculated, but were allowed to commingle with directly exposed birds (11, 16). Previous work in our laboratory has shown that an infection rate of 50-100% can be expected by this method.

Lesion scores of histomoniasis. The lesions of the ceca and liver associated with infection of *H. meleagridis* were scored subjectively on a scale of 0-4, where normal tissues were given a score of 0 and the most severe lesions were scored 4 (17). Cecal lesions consist of thickened and disrupted mucosa, opacity of the cecal wall and formation of cheesy cores from sloughed tissues and exudates. The liver lesions consist of small to large foci of necrosis throughout the organ. In later stages of the disease, the lesions may take on a cratered appearance with well-defined edges.

Experimental design and procedures.

Experiment 1: *Susceptibility to histomoniasis of turkeys of various ages.* Turkey poults were reared in floor pens and given unmedicated turkey starter or grower feed and water *ad libitum* to ages 3 weeks (10 birds), 7 weeks (10 birds), 10 weeks (11 birds), and 13 weeks (14 birds). All birds were inoculated via the cloaca at the same time with 700,000 live histomonads as described above. Birds were observed daily for signs of infection. Birds that died during the experiment were examined for lesions in the ceca and liver. Lesions were recorded as previously described. The experiment was terminated 14 days post-inoculation (PI). Results were expressed on the basis of weight gain after challenge, mortality from histomoniasis, and lesion scores of the liver and ceca. Birds in the 3 week

age group were inadvertently given dimetridazole in water for 2 days prior to inoculation and this appeared to negatively impact the number of infected birds in this group.

Experiment 2: *Resistance to reinfection with H. meleagridis in turkeys after drug treatment to limit infection: First infection:* Fifty birds were inoculated *per cloaca* with 480,000 pathogenic histomonads at 2 weeks of age. Dimetridazole (200 ppm) was given in the drinking water for 3 days, beginning 6 days PI. Ten control birds were left unmedicated and were killed at 12 days PI to determine the overall infection rate and severity of lesions in the liver and ceca.

Second infection: Previously infected and naive control birds were challenged again 18 days after the first challenge by cloacal inoculation with 500,000 cultured histomonads. Treatment with 200 ppm dimetridazole began 5 days PI and continued for 5 days. One group of 10 naive birds and one group of 10 recovered birds were not treated, but were killed for necropsy at 10 days PI to determine the infection rate or impact of the disease on the liver and ceca.

Third infection: One group of 8 twice-recovered birds and one group of 8 same-age naive birds were challenged by cloacal inoculation with 750,000 histomonads 33 days after the second exposure. Birds were killed 14 days PI, and liver and cecal lesions were scored.

Later third infection: Another group of twice-recovered birds or naive birds was challenged with 500,000 histomonads *per cloaca* 58 days after the second exposure. All remaining birds were killed 16 days PI. Liver and cecal lesions were scored. Protective immune response was evaluated on the basis of mortality and lesion scores caused by *Histomonas.*

Experiment 3: *Vaccination of turkeys against Histomonas meleagridis in floor pens: Comparison of live vs killed preparations of Histomonas and route of inoculation.* Birds were inoculated by intramuscular (IM) or subcutaneous (SQ) routes with 125,000 cultured histomonads at 7 days of age. Forty birds were given live or killed *H. meleagridis* antigen preparations. Half of the birds received second doses of 400,000 killed cells given at 44 days of age. Birds were placed at 2 weeks of age in 2 floor pens (8 ft x 12 ft) with litter bedding as follows: Pen 1: 5 birds vaccinated once, IM or SQ with the killed preparation, 5 birds vaccinated once, IM or SQ with the live preparation. Exposure model: 12 naive birds were inoculated with 500,000 cultured histomonads *per cloaca* as seeder birds; using the model described above for simulated natural exposure to histomoniasis on litter. The remaining 15 birds were uninoculated. Pen 2: 5 birds were given a first vaccination, IM or SQ with the killed preparation, then vaccinated a second time SQ with the killed preparation. An additional 5 birds were given a first vaccination, IM or SQ with the live preparation, and a second vaccination SQ with the killed preparation. Exposure model: a group of 12 naive birds were inoculated at 49 days of age with 500,000 cultured histomonads *per cloaca* as seeder birds and the remaining 15 birds were left uninoculated. Birds were observed daily for signs of infection or mortality, and necropsies were performed to determine whether birds died of histomoniasis. Surviving birds were killed 21 days PI and examined for liver and cecal lesions. As in experiment 2, resistance to reinfection was based on mortality, and lesions scores at necropsy.

Experiment 4: *Immune protection against direct severe challenge in a battery model.* Two week old poults were randomly assigned to groups of 4 birds per cage. One

treatment group was vaccinated subcutaneously (SQ) with 0.1ml of formalin-killed histomonads at a level of $1.6x10^6$/ml. Another treatment group received SQ injections of formalin killed bacteria prepared from *Histomonas* cultures. The histomonads had been allowed to die and were passed in subculture once every 3 days for 9 days, no histomonads were visable. Cultures were washed and resuspended in HBSS. Birds were revaccinated at 40 days of age then challenged at 47 days of age by cloacal inoculation of 250,000 live, cultured histomonads. Birds were observed daily for signs of infection or mortality, and necropsy performed to determine whether birds died of histomoniasis. Surviving birds were killed 11 days PI and examined for liver and cecal lesions. Resistance to infection was based on mortality and lesion were scores found during the necropsy.

Experiment 5: *Immune protection against indirect challenge in a battery model.* Poults were assigned to treatment groups of 5 birds each: 1) unvaccinated control, 2) vaccinated at one day of age, 3) vaccinated at 7 days of age, 4) vaccinated at 14 days of age and 5) vaccinated at 1 and 14 days of age. Each vaccination contained 0.1 ml formalin-killed histomonads ($1.5x10^6$ cells/ml) given subcutaneously. At 28 days of age, birds were placed on paper-covered wire floors in battery cages. Two birds in each cage were cloacally inoculated with 200,000 live, cultured histomonads. Birds were observed daily for signs of infection or mortality, and necropsies were performed to determine whether the birds died of histomoniasis. Surviving birds were killed 11 days PI and examined for liver and cecal lesions. Resistance to infection was based on mortality, and lesion scored at necropsy.

ELISA for detection of antibodies against *H. meleagridis:* An enzyme-linked immunosorbent assay was developed to detect antibody response to *Histomonas* grown in culture. The antigen used was filtered through cheesecloth and then washed 3 times in Hank's Balanced Salt Solution (HBSS). A Lowry assay was used to determine the amount of protein present and the antigen was diluted to 20ug/ml with 1% PBS (pH adjusted to 7.4).

Each well of a flat-bottom, 96-well microtiter plate was coated with 50ul of antigen then incubated at 37ºC for 2 hours. Plates were washed 3 times in ddH_20 then blocked with 50 ul of blocking solution (100ml 1X PBS, 50ul Tween and 0.25g BSA) overnight at 4ºC. Plates were washed 3 times with ddH_20 before serum dilutions were added.

Fifty microliters of test serum diluted at 1:100 with PBS was added to each well and incubated for 2 hours at room temperature. After 3 washings with ddH_20, 50ul of goat anti-turkey IgG antibodies conjugated with alkaline phosphatase (Southern Biotech) diluted at 1:2000 was added to each well and incubated at room temperature for 2 hours. Plates were washed 3 times with ddH_20, and 50ul of chromogenic substrate, 1mg 4-nitrophenyl phosphate disodium salt hexahydrate (BioChemika) to 1 ml ELISA color reagent buffer solution (100ml ddH_20, 0.53g Na_2CO_3, 5ul 1M $MgCl_2$), was added to each well and allowed to develop for 30 minutes. The reaction was stopped by adding 50ul of 0.5 M NaOH to each well and the optical density at 405nm was measured by an automated microplate reader.

Test serum samples were compared to a positive control sample from an immune bird that was infected with *Histomonas* and treated with dimetridazole three times and a

negative control bird which had never been exposed to *Histomonas.* Test serum included samples from individual birds at 14 days of age prior to the first subcutaneous (SQ) inoculation, at 54 days of age prior to the second SQ inoculation, at 61 days of age seven days after the second SQ inoculation and at 72 days of age, 11 days post challenge with 250,000 live histomonads given cloacally.

ELISA titer ratio was calculated with the optical density values by subtracting the negative control value from the test sample value and dividing the difference by the positive control value after subtracting the negative control value (test sample-negative control/positive control-negative control).

Results

Experiment 1: *Comparison of age susceptibility:* Results of this study are presented in Table 2.1. The 3 week old poults, which had been inadvertently medicated with dimetridazole for two days prior to inoculation, had a reduced infection rate (50%) It is assumed that this was due to the drug rather than a lower susceptibility for birds of this age. Otherwise, there were no consistent differences in infection rates or lesion scores among groups of birds of different ages. Two 13-week old birds were euthanized 9 days post-inoculation (PI) due to broken wings. These birds had no liver lesions but had cecal lesions that scored 3 or 4.

Mortality began 8 days PI in the 7-week old group, 10 days PI for 10-week olds, and 11 days for 13 week-olds and 3 week olds. Mortality was 60, 40, 63, or 58%, respectively, of infected birds aged 3, 7, 10, or 13 weeks at the time of inoculation. All surviving birds from the 3 oldest groups were found to have lesions.

Experiment 2: *Resistance to reinfection after drug treatment.* Results of this study are shown in Table 2.2. Two birds in the treated group died of histomoniasis while on medication. Four of 10 control birds died of histomoniasis prior to 12 days PI during the first exposure and the 6 survivors were euthanized to determine the infection rate. Only 1 of the 10 control birds did not develop any lesions (90% infection rate). Four treated birds died 10-14 days after removal of dimetridazole, suggesting that 3 days of medication was not sufficient for complete recovery.

Birds challenged the second time developed cecal lesions (average 3.4) but not liver lesions (Table 2.2). Naive birds had average liver lesion scores of 3.7 and cecal lesion scores of 4.0 and showed a 100% infection rate. No previously infected birds succumbed to the disease after the withdrawl of dimetridazole.

Two of three birds killed prior to the third inoculation had slightly thickened ceca but no confirmed lesions, while the third bird had a ceca that appeared normal. Birds challenged a third time had no liver lesions and an average cecal lesion score of 2.6 while naive birds had cecal scores of 4.0 and liver scores of 3.8 (Table 2).

Three twice-infected birds were killed prior to the third challenge to determine the extent of ceca damage remaining from previous infections. All ceca were normal except for some slightly thickened areas. Birds challenged a third time had no liver lesions and an average cecal lesion score of 1.0. Naive birds were scored 4.0 for both liver and cecal lesions (Table 2.2).

Experiment 3: *Vaccination of turkeys in floor pens.* In pen 1, 9 of 12 seeder birds died of histomoniasis while the 3 remaining had no or low liver lesion scores, suggesting a 75% infection rate from cloacal inoculation (Table 2.3). Cecal and liver lesion scores of

indirectly exposed naive birds averaged 2.9 or 1.8, respectively, and 2 of 15 died of histomoniasis. Birds vaccinated once with the killed preparation of histomonads, SQ or IM, had no lesions in the ceca or liver. Two birds given the live preparation intramusularly died of causes unrelated to histomoniasis. One of 8 birds given the live preparation had minor cecal lesions, and none had liver or cecal lesions.

In pen 2, all 12 of the seeder birds died of histomoniasis showing a 100% infection rate (Table 2.3). Eight of the 15 naive birds (indirectly exposed) died from histomoniasis. Liver or cecal scores for indirectly exposed naive birds averaged 3.2 or 2.9 respectively, showing that the disease spread readily from directly inoculated birds. Two of the birds that were vaccinated twice with the killed preparation of *Histomonas* developed minor cecal lesions (group average score 0.5) but no liver lesions were found in that group. Only 1 of 10 birds in the group receiving the live preparation SQ during the first vaccination had cecal lesions (group average 0.2) for the group of 10 birds. No liver lesions were observed in this group.

Experiment 4: Results for this study are shown in Table 2.4. Infected control birds had liver lesion scores averaging 3.4 and cecal lesion scores of 3.9, while the average weight gain was 0.08 kg/bird. Birds inoculated with killed bacteria had liver and cecal lesions of 3.1 and 3.8, respectively, with an average weight gain of 0.11 kg/bird. Poults inoculated with histomonads and bacteria had an average liver score of 2.8, cecal score of 3.7, and weight gain of 0.17 kg/bird. No birds died of histomoniasis during the study.

Experiment 5: Group 1, unvaccinated control, had cecal and liver lesions of 0.56 and 0.97, respectively, and had an average body weight gain of 1.087 kg/bird. Group 2, vaccinated at 1 day of age, had an average cecal score of 0.78, liver score of 1.0 and

gained an average of 1.128 kg/bird. Birds vaccinated at 7 days of age had an average cecal score of 1.39, liver score of 1.67 and weight gain of 0.979 kg/bird. Group 4, vaccinated at 14 days of age, had average cecal lesion score of 0.78, liver lesion score of 1.20 and a body weight gain of 0.957 kg/bird. Birds vaccinated twice, at 1 and 14 days old had average ceca score of 0.22 and liver lesion score of 0.72, and an average body weight gain of 1.220 kg/bird.

ELISA for detection of antibodies against *H. meleagridis*. Results are presented in Table 2.6. The titer ratios comparing the antibodies produced by immune birds to vaccinated birds ranged from 0.08 to 0.13 (average 0.10) forty days after the primary vaccination and 0.66 to 1.64, with an average of 1.10, seven days after the secondary vaccination. Sample/positive control ratios 11 days after challenge ranged from 0.36 to 1.05 with an average of 0.68. Unvaccinated birds had titer ratios of 0.32 and 0.53, averaging 0.43, while unvaccinated birds 11 days post challenge had titer ratios of 0.21 and 0.05, average 0.13.

Discussion

The results of experiment 1 have shown no evidence of age-related resistance to histomoniasis to the age of 14-weeks. These results were in agreement with previous research where birds of various ages were tested, suggesting that birds do not develop resistance to histomoniasis as they mature.

In experiment 2, birds became progressively more resistant to histomoniasis with a regime of repeated infection and treatment (recovery). This is in agreement with results of Joyner (12) who found that when the disease was permitted to develop to an advanced

stage before treatment, birds become resistant to reinfection. Clarkson (2) described the development of antibodies to *Histomonas* after similar infection/treatment regimes.

It was interesting that birds developed resistance to liver infection, while remaining at least partially susceptible to cecal infection. This suggests that different mechanisms of immunity could be involved for the 2 organs. Other protozoans infecting turkeys (Coccidia) stimulate protective immunity which depends entirely on the cellular response (23). The present results suggest an involvement of humoral immune factors. Clarkson (2) and Dwyer (5) reported development of precipitating antibodies in turkeys infected with *H. meleagridis.* Recent work in our lab demonstrated the presence of antibodies in immune serum that were capable of lysing histomonads *in-vitro* in immune serum (Hu, et. al, unpublished results).

Experiment 3 demonstrated that vaccination with live or killed histomonads may limit the indirect spread of histomoniasis within turkey flocks. Immunized birds in contact with infected birds did not develop liver lesions but did have some cecal lesions. Although the cecal lesions were less severe than in naive birds, it is possible that some birds could succumb to peritonitis due to cecal necrosis.

Experiments 4 and 5, conducted with direct and indirect battery models, respectively, suggesting that the immunity developing after parenteral inoculation was not protective against severe, direct challenge. However, the indirect battery model offered some evidence that vaccination deterred spread of histomoniasis from directly challenged birds to unchallenged birds. These results are in agreement with the observations in Experiment 3. The finding of a reduction of bird to bird transmission is important, because it is believed that this is the way in which blackhead disease is able to

spread rapidly through a confinement growing system (9). The age of birds at challenge, and the age at vaccination are additional variables which have not been addressed.

Antibodies against the *H. meleagridis* preparations were detected by ELISA at 61 days of age, 7 days after the second inoculation, but were not detected at 54 days of age after a single inoculation. Antibody titers were reduced 11 days post challenge in vaccinated birds; however, it is unclear if the reduction is due to immunosuppression or antibody binding to antigen. Antibody titers increased in unvaccinated birds over the 58 day period, but were much lower than that of vaccinated birds. Challenged unvaccinated birds had lower titer ratios than unchallenged birds. Further work is needed to define the age of immune competence against this disease, and to define the roles of cellular and humoral responses in resistance to infection.

The role of bacteria in the immune response to histomoniasis was not addressed in this study. Previous work demonstrated the importance of bacteria for *H. meleagridis* to produce virulent infections in turkeys and chickens (1, 19). However, a test of antibiotics commonly used in poultry failed to show any significant reduction in severity of lesions in chickens (8). The results of ELISA tests showed that turkeys in experiments 2 and 3 developed antibodies against bacteria present in the inoculum as well as against *Histomonas* antigens. Further work will be required to define the importance of bacteria in immunity against histomoniasis.

The primary site of infection for blackhead disease is the cecum. Previous studies have shown that the infection spreads to the liver via the blood stream (3). Infections of the liver have been produced by IV inoculation of whole blood from diseased birds (18).

Thus, the histomonads are likely to come in contact with the humoral immune factors and for these factors to have an important role in immune protection.

While bird numbers were too low for statistical analysis, it appeared that antigens from cultured *H. melagridis* were effective in stimulating immune protection. The means of production (live or formalin-killed) and route of administration (IM or SQ) had little effect on the expression of a protective response.

Considerable work will be required to determine the optimum preparation method for the antigen, route of administration, and dosage. However, these results suggest that turkeys could be protected against histomoniasis by a formalin-killed histomonad preparation. Additional work is in progress to identify the *H. meleagridis* antigens most important in the immune response.

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Table 2.1 Susceptibility to histomoniasis of turkeys of various ages (Experiment 1)

Age in weeks[A]	Average liver score	Average ceca score	Mortality[B]	% Mortality	% Infected
3[C]	3.8	2.2	3/5	60	50
7	4.0	2.1	4/10	40	100
11	4.0	3.5	7/11	60	100
14	3.8	2.8	7/12	58	100

[A]All birds inoculated *per cloaca* with 700,000 histomonads grown *in-vitro.*

[B]Does not include uninfected birds or birds eunthanized due to injury.

[C]Birds were inadvertantly given dimetridazole for 2 days prior to inoculation.

Table 2.2: Resistance to reinfection with *Histomonas meleagridis* infected 1 or 2 times and treated with dimetridazole. (Exp. 2)

Treatment schedule						Average lesion scores			
	Days of age					Immune birds		Control (naïve) birds	
Group	Infected[A]	Treated[B]	Infected[C]	Treated[D]	Infected	Liver	Cecal	Liver	Cecal
1	14	+	32	-	-	0	3.4	3.7	4.0
2	14	+	32	+	65[E]	0	2.6	3.8	4.0
3	14	+	32	+	90[F]	0	1.0	4.0	4.0

[A]All birds inoculated cloacally with 480,000 *H. meleagridis*.

[B]Treatment with dimetridazole (200 ppm in water) 3 days.

[C]Inoculated cloacally with 500,000 *H. meleagridis*.

[D]Treatment with dimetridazole (200 ppm in water) 5 days.

[E]Inoculated cloacally with 750,000 *H. meleagridis***.**

[F]Inoculated cloacally with 500,000 *H. meleagridis*.

Table 2.3: Vaccination of turkeys against *H. meleagridis*: Comparison of cecal and liver lesions for live vs killed histomonad preparations and route of inoculation.[A] (Experiment 3)

	Naïve		Killed (IM)		Killed (SQ)		Live (IM)		Live (SQ)	
# dose	Liver	Ceca	Liver	Ceca	Liver	Ceca	Liver	Ceca	Liver	Ceca
1[A]	1.8	2.9	0	0	0	0	0	0.7	0	0
2[B]	2.9	3.2	0	0.2	0	0.8	0	0	0	0.2

[A]First doses were 125,000 live or killed *H. meleagridis* cells given IM or SQ as indicated

Live cells were washed 3 times in Hank's Balanced Salt Solution (HBSS) and resuspended in HBSS.

Killed cells were washed 2 times, killed with buffered 10% formalin, washed twice more and resuspended in HBSS.

Gentamicin, Penicillin G and Amphitercin B were added to all preparations.

[B]All second doses were 400,000 killed *H. meleagridis* cells given SQ.

Table 2.4: The effect of vaccination on direct challenge of *Histomonas meleagridis* in turkeys.[A B] (Experiment 4)

		Lesion scores[C]		
Immunization Regime	Pen #	Liver	Ceca	Weight gain (loss) kg
Control (unchallenged)	10	0	0	1.04
	17	0	0	1.11
	18	0	0	1.16
	mean	0	0	1.10
Control (challenged)	4	3.8	4.0	(-0.01)
	11	4.0	4.0	(-0.23)
	12	3.0	3.8	0.31
	16	3.5	4.0	0.05
	20	2.5	3.8	0.28
	mean	3.4	3.9	0.08

Bacteria (Immunized with 2 injections of killed bacteria present in *H. meleagridis* grown *in vitro)*	1	3.5	4.0	0.06
	3	3.8	4.0	0.11
	5	2.8	3.8	0.19
	6	1.8	3.0	0.30
	7	3.7	4.0	(-0.03)
	15	3.3	4.0	0.03
	mean	3.1	3.8	0.11
Histomonas + bacteria (Immunized with 2 injections of killed *Histomonas* and bacteria grown *in vitro*)	2	3.0	4.0	(-0.05)
	8	2.0	2.8	0.50
	9	2.5	3.3	0.33
	13	3.0	4.0	0.18
	14	3.5	4.0	0.22
	19	3.0	4.0	(-0.14)
	mean	2.8	3.7	0.17

[A]Two week old poults were vaccinated with preparations of *Histomonas* grown *in vitro.* Each poult received 0.1 cc subcutaneously at 14 days and 39 days of age. Cultured *H. meleagridis* and bacteria from *Histomonas* cultures grown *in vitro* were washed in Hank's Balanced Salt Solution (HBSS), killed in 10% buffered formalin and washed twice more. 200 ml of washed cultures were reduced to 5 ml after washing. Cultures containing *Histomonas* had approximately 150,000 histomonads in 0.1 cc. along with bacteria associated with normal *Histomonas* cultures.

[B]Poults were challenged by cloacal inoculation with 250,000 histomonads at 47 days.

[C]Lesions were scored 11 days post inoculation.

Table 2.5: The effect of vaccination on indirect challenge of *Histomonas meleagridis* in turkeys.[A B] (Experiment 5)

		Lesion Scores[C]		
Treatment	Replication	Liver	Ceca	Weigh gain (loss) kg
Unvaccinated control	1	0	1.00	1.165
	2	0	0.33	1.062
	3	0	0.5	1.603
	4	0	0	1.348
	5	2.00	2.67	0.565
	6	1.33	1.33	0.781
	mean	0.56	0.97	1.087
Day old vaccination	1	3.33	4.00	0.355
	2	1.33	1.33	1.047
	3	0	0.67	1.346
	4	0	0	1.431
	5	0	0	1.263
	6	0	0	1.326
	mean	0.78	1.00	1.128

7 day old vaccination	1	1.00	1.33	0.988
	2	1.33	1.33	1.062
	3	3.67	4.00	0.570
	4	0	0	1.259
	5	1.33	1.33	0.919
	6	1.00	2.00	1.078
	mean	1.39	1.67	0.979
14 day old vaccination	1	2.00	3.67	0.491
	2	0	0.50	1.206
	3	2.67	3.00	0.452
	4	0	0	1.171
	5	0	0	1.134
	6	0	0	1.289
	mean	0.78	1.20	0.957
1 & 14 day old vaccination	1	0	0.33	1.298
	2	0	0	1.230
	3	0	0	1.308
	4	0	0	1.336
	5	0.67	1.33	1.184
	6	0.67	2.67	0.962
	mean	0.22	0.72	1.220

[A]Two week old poults were vaccinated with preparations grown *in vitro.* Each poult received 0.1 cc subcutaneously at 14 days and 39 days of age. *H. meleagridis* cultures were washed in Hank's Balanced Salt Solution (HBSS) then killed in 10% buffered formalin and washed twice more. The preparation contained approximately 150,000 histomonads in 0.1 cc.

[B]Poults were challenged by cloacal inoculation of 200,000 cultured histomonads at

[C]Lesions were scored 11 days post inoculation.

Table 2.6: Analysis by indirect ELISA of IgG antibodies produced by turkeys against *Histomonas meleagridis* cultured *in vitro* [A]. (Experiment 6)

	ELISA Titer ratio (test sample/positive control)			
Bird number	Preinoculation (14 days of age)	40 dpi (primary) (54 days of age)	7 dpi (secondary) (61 days of age)	11 dpi (challenge) (72 days of age)
Naïve control birds[B]				
1	0.05	-	-	0.32
2	0.03	-	-	0.53
mean	0.04	-	-	0.43
Infected control birds[C]				
1	0.02	-	-	0.21
2	0.04	-	-	0.05
mean	0.03	-	-	0.13

Vaccinated birds[D]				
1	0.04	0.13	1.32	0.85
2	-0.03	0.09	1.26	0.73
3	0.05	0.12	1.65	1.05
4	0.04	0.08	0.66	0.36
5	0.03	0.11	1.00	0.62
6	0.02	0.09	0.75	0.48
mean	0.02	0.10	1.10	0.68

[A]The indirect ELISA used alkaline phosphatase, goat anti-turkey conjugate at a dilution of 1:2000. Test samples were diluted at 1:200. Values given are adjusted for background.

[B]Same age as inoculated poults.

[C]Inoculated *per cloca* with 250.000 live histomonads cultured *in vitro.*

[D]Two week old poults were vaccinated with *Histomonas* preparations grown *in vitro.* Each poult received 0.1 cc subcutaneously at 14 days and 39 days of age. *H. meleagridis* cultures were washed with Hank's Balanced Salt Solution (HBSS), killed in 10% buffered formalin and washed twice more. Each 0.1 ml contained approximately 150,000 histomonads.

CHAPTER 3

Influence of Bedding Material on the Transmission of *H. meleagridis* in Turkeys

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Armstrong, P.L. and McDougald, L.R.. To be submtted to *Avian Diseases.*

Abstract

Previous studies have shown that blackhead disease (histomoniasis) can spread from inoculated turkey poults to uninoculated poults in litter-floored pens and cages without the aid of invertebrate vectors. Other studies have demonstrated that birds are able to acquire histomoniasis by cloacal drinking after contact with liquid cultures. The exact mechanism by which birds acquire the infection under practical conditions is not known.

Naive poults were comingled with directly infected birds on litter, exposed to fresh contaminated litter once or multiple times, or orally provided fresh droppings from inoculated birds. Birds comingling with inoculated poults had a higher rate of infection than those exposed only to contaminated litter. Uninoculated poults developed lesions after exposure to contaminated litter, although the number of exposures did not influence the speed of infection. Poults did not develop lesions after orally ingesting fresh droppings from inoculated birds.

Poults were housed on bare wire, pine shavings, paper or fiber mats and allowed to comingle with directly inoculated birds. Some birds in each group developed lesions although the birds on bare wire had a lower infection rate than those housed on bedding. There was no significant difference in the infection rate based on the types of bedding used.

These studies were conducted to determine whether birds become infected from direct contact with infected birds or from contact with contaminated bedding and the influence of the type of bedding used.

Keywords: *Histomonas meleagridis*, transmission, turkey.

Introduction

Blackhead disease of gallinaceous birds is caused by the protozoan parasite *H. meleagridis.* The cecal worm, *Heterakis gallinarum,* was found by early workers to be an important vector in the transmission of *H. meleagridis* (2, 7). Histomonads are incorporated into the ova of the worms and are infective to the next host after the ova become embryonated (5). *Histomonas* organisms found in cecal worm eggs can survive for extended periods of time, thus providing a reservoir for the organism from one year to the next (1). Later work identified the earthworm as another vector of *Heterakis* larvae and thereby histomoniasis (6). Recently, it was demonstrated that histomoniasis could spread through flocks of turkeys on litter in the absence of worm vectors (4). The spread of infection was thought to involve the phenomenon of cloacal drinking, whereby environmental liquids are taken into the cloaca after direct contact with contaminated litter (3). This concept was developed as an experimental model for battery cage tests, wherein uninfected birds were commingled with directly infected birds. The floors of cages were lined with heavy paper or other material to encourage build-up of infective materials.

However, it was not determined whether birds became infected from contact with contaminated litter or from direct contact with infected birds. Further, there was no comparison of bedding materials for successful transmission of the infection. The present experiments were designed to determine whether direct bird contact is necessary,

and to compare bedding materials and liners for cages for success in promoting transmission of *Histomonas* infections from infected to uninfected turkey poults.

Materials and methods

Experimental Animals. Day-old, straight run turkey poults were obtained from a commercial hatchery (Sleepy Creek Hatchery, Goldsboro, NC). The poults were reared in a community brooder and fed unmedicated turkey starter crumbles *ad libitum* until two weeks of age, then moved into steam-sterilized finishing batteries for experimental work.

Parasites. *Histomonas meleagridis* organisms were grown *in-vitro* from an isolate obtained from a field outbreak in leghorn chickens near Athens, GA. The isolate was stored in liquid nitrogen for future use and then cultured in modified Dwyer's medium as needed. The histomonads were estimated on a haemocytometer prior to clocal inoculation.

Lesion scores of histomoniasis. The lesions of the ceca consist of a thickening of the mucosa, sloughing of mucosal tissues and formation of firm cheesy cores from sloughed material and exudates. Liver lesions begin as small scattered foci which enlarge and become necrotic as the infection progresses. In advanced stages, the liver lesions coalesce and involve most of the organ. Liver and cecal lesions typical of *H. meleagridis* were scored subjectively on a scale of 0-4. Normal tissues were scored 0, small or mild lesions were scored 1, moderately severe lesions were scored 2 or 3, and the most severe lesions were scored 4.

Experimental design and procedures. ***Experiment 1:*** *Lateral transmission of H. meleagridis in battery cages through contact with contaminated litter.* Two-week old turkey poults were randomly divided into 12 groups of 8 birds each to allow for 6

treatments and 2 replications. Directly exposed birds were inoculated *per cloaca* with ***H. meleagridis*** by means of a plastic pipette tip attached to a 10 ml syringe, or were orally gavaged with fresh cecal droppings from donor turkeys 4 days PI. Uninoculated birds received exposure only by contact with litter contaminated by inoculated birds 4 days PI. Treatments included 1) unexposed controls; 2) 2 of 8 directly inoculated; 3) 8 of 8 directly inoculated; 4) uninoculated birds exposed a single time to contaminated litter (one hour in cages immediately after removal of infected birds); 5) uninoculated birds exposed multiple times to contaminated litter (one hour in cages immediately after removal of infected birds, repeated hourly for 5 exposures; 6) uninoculated birds orally given 1 ml of fresh cecal droppings. Birds were observed daily for morbidity and mortality. Dead birds were examined for lesions associated with histomoniasis. Liver and cecal lesions were scored. Remaining birds were killed on day 14 post-inoculation and examined for lesions and scored. Suspicious lesions were examined by microscopy for the presence of histomonads. The experiment was repeated three times; however, in the third trial, treatment groups #5 and #6 were eliminated and the number of birds varied in the remaining 4 treatment groups due to a shortage of birds.

Experiment 2: *Influence of the choice of bedding and litter materials on lateral transmission of H. meleagridis.* Two-week old poults were randomly assigned to 12 groups of 8 birds, providing 3 replications of 4 treatments. In each cage, 4 birds were inoculated with ***H. meleagridis*** *per cloaca* as described above (directly exposed birds). Others were uninoculated, but were allowed to commingle with the directly inoculated birds throughout the study. Treatments included 1) 4 of 8 inoculated, on wire; 2) 4 of 8 inoculated, on paper, 4) 4 of 8 inoculated, on shavings; and 5) 4 of 8 inoculated and

placed on fiber (shredded wood chick box liner). Droppings were allowed to build up on bedding material. Mortality was recorded as well as lesion scores of poults that died throughout the study. The experiment was terminated 14 days post-inoculation, remaining birds were killed and examined for lesions. Any observed lesions were scored and recorded as described above.

Results

Experiment 1: *Lateral transmission of H. meleagridis through contact with contaminated litter.* The unexposed and oral treatment groups performed as expected; no birds became infected with histomoniasis. The overall infection rate in the directly inoculated poults over the course of trials 1, 2 or 3 was 87.5%, 50% or 75%, respectively. The infection spread from inoculated poults to commingled uninoculated poults in trials 1 and 3, but not trial 2. The rate of lateral transmission was 2/6 (33%), 0, or 3.5/6 (58%) in the three trials. Of the poults that contacted only contaminated droppings became infected. In trial 1, 3/16 (19%) became infected after a single exposure, and 1/16 (6%) became infected after multiple exposures. In trial 2, 1 of 16 birds (6%) given one exposure and 1 given multiple exposures developed infections. In trial 3, 1 of 6 birds (17%) given multiple exposures became infected. None of the birds given contaminated cecal droppings by the oral route developed infection with *H. meleagridis.*

Experiment 2: *Influence of bedding and litter on lateral transmission of H. meleagridis.* All directly inoculated birds became infected and had severe lesions at necropsy (Table 1). Uninoculated birds caged with paper, shavings or fiber mats as the bedding also became infected and developed severe lesions, with the exception of 1 bird on shavings.

Of birds caged with bare wire floors, only 4 of 12 birds (33%) became infected, and the lesion scores of these birds were much lower than recorded in other treatments.

Discussion

These experiments confirmed earlier results suggesting that birds became infected with histomoniasis by direct means rather than by the use of vectors (3, 4). In experiment 1 it was demonstrated that birds could become infected with *H. meleagridis* through contact with contaminated bedding, even in battery cages, although at a lower rate than that recorded with bird-to-bird contact. It was interesting that multiple exposures of the birds to contaminated litter did not appear to increase the chance of them becoming infected.

In the choice of bedding material, it made no difference whether birds were placed on paper, shavings or wood fiber mats, in the rate of infection arising from commingling, or in the severity of lesions. However, the rate of infection was much lower in cages without bedding, where birds were placed directly on wire floors which allowed most of the droppings to fall through. Thus, it would appear that the contagiousness of blackhead was best with direct bird contact, but the presence of bedding also encouraged transmission. Even though there were some infections in birds on wire floors, the birds also had physical contact, so it was not possible to decide with certainty which factor was responsible. The higher rate of infection in cages with bedding could possibly be explained if the physical conformation of the bedding encouraged contact between the birds and the contaminated droppings. Additional experiments would be needed to determine how long *H. meleagridis* can survive and remain infective in the litter.

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Table 3.1: Infections of turkey poults with H*istomonas meleagridis* by direct and indirect contact in battery cages. (Experiment 1)

		Control (Infected/Total)		Commingle (Infected/Total)		Contact with Infected droppings (Inf./ Total)	
Trial	Rep	Uninoculated	Inoculated	Inoculated	Uninoculated	Single	Multiple
1	1	0	7/8	2	4/6	2/8	0/8
	2	0	7/8	2	0/6	1/8	1/8
	Mean	0	7/8	2	2/6	1.5/8	0.5/8
2	1	0	3/8	2	0/6	1/8	0/8
	2	0	5/8	2	0/6	0/8	1/8
	Mean	0	4/8	2	0/6	0.5/8	0.5/8
3	1	-	3/6	2	5/6	-	1/6
	2	-	6/6	2	2/6	-	1/6
	Mean	-	4.5/6	2	3.5/6	-	1/6

Two week old poults were inoculated *per cloaca* with 500,000 histomonads grown *in vitro*. Necropsy at 14 days PI

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Table 3. 2: Influence of bedding on transmission of *Histomonas meleagridis* from directly inoculated to uninoculated turkeys by comingling.[A] (Experiment 2)

		Average lesion scores[B]					
Floor		Inoculated			Uninoculated		
Medium	Rep	Liver	Ceca	Infected	Liver	Ceca	Infected
Bare wire	1	3.50	4.00	4/4	0	0.25	1/4
	2	4.00	4.00	3/3	0	0.25	1/4
	3	4.00	4.00	4/4	1.75	1.50	2/4
	mean	3.83	4.00		0.58	0.67	
Paper	1	3.00	4.00	4/4	2.50	2.75	4/4
	2	3.25	3.75	4/4	3.25	4.00	4/4
	3	4.00	3.75	4/4	4.00	3.50	4/4
	mean	3.42	3.83		3.25	3.42	

Shavings	1	3.00	4.00	4/4	3.25	4.00	4/4
	2	4.00	4.00	4/4	3.00	2.75	3/4
	3	2.75	4.00	4/4	3.50	4.00	4/4
	mean	3.25	4.00		3.25	3.58	

Two week old poults were inoculated *per cloaca* with 500,000 live histomonads grown *in vitro.*

Liver and cecal lesions were scored 14 days PI.

CHAPTER 4

Conclusion

1. Turkeys showed no difference in susceptibility to *Histomonas meleagridis* between 2 and 16 weeks of age.
2. Repeated infection with *H. meleagridis* and treatment with antihistomonal medication (dimetridazole) resulted in turkeys that were highly resistant to reinfection, based on liver and cecal lesions, weight gain after inoculation, and mortality from histomoniasis.
3. Turkeys developed antibodies against *H. meleagridis* after infection, as shown by an ELISA test. Antibody titers did not necessarily correlate with protective immunity.
4. Turkeys became infected with *H. meleagridis* after commingling with infected birds (indirect infection) in battery cages. Infections were not transmitted as readily when birds were on bare wire mesh floors as when some type of bedding was used. The type of bedding made no difference in the overall transmission rate.