JINGHUI HU

STUDIES ON HISTOMONAS MELEAGRIDIS AND HISTOMONIASIS IN CHICKENS AND TURKEYS
(Under the direction of LARRY R. MCDOUGALD)

A series of studies has been conducted to expand knowledge of the pathogenicity, epidemiology, and treatment of histomoniasis in chickens and turkeys.

The interaction of cecal coccidiosis with histomoniasis in chickens was investigated, using concurrent infections. Cecal lesions from *H. meleagridis* were severe in all inoculated control groups and did not appear to be affected by the introduction of *Eimeria tenella* infections. However, the severity of liver lesions and number of birds positive for liver lesions of *H. meleagridis* increased significantly with the presence of *E. tenella.* The positive relationship between infections of cecal coccidiosis and *H. meleagridis* in chickens suggests that, under field conditions, such dual exposure may contribute to increased clinical outbreaks of histomoniasis in chickens.

The lateral transmission of *H. meleagridis* in turkeys was studied in floor pens in the absence of the carrier cecal worm *Heterakis gallinarum*. One group received no exposure. In other groups, either 10% (LE) or 25% (HE) of the birds were inoculated *per cloaca* with cultured *H. meleagridis* (200,000 cells/bird). Inoculated birds died at 10-18 days post-infection (DPI). Uninoculated-birds in the high exposure group (HE) died of histomoniasis beginning 16 DPI, and continued to 100% mortality by day 23 DPI. Uninoculated birds in low exposure group (LE) died beginning on day 19 DPI and continuing through day 31 DPI. All but one LE birds alive on day 31 DPI had severe liver and cecal lesions of histomoniasis at necropsy. There was no evidence of histomoniasis in unexposed birds at the end of experiment. These results suggested that lateral transmission of histomoniasis through a

flock can occur readily through normal contact between infected birds and uninfected birds and their droppings in the total absence of cecal worms.

Since no products are available for treatment of blackhead outbreaks, studies were conducted on other antiprotozoal or antibiotic compounds. Five anticoccidials, six antibiotics, and nine antiprotozoals drugs known from literature were tested for their efficacy against *Histomonas meleagridis* in chickens. None of 5 anticoccidials (salinomycin, diclazuril, nicarbazin, roxarsone, and lasalocid) and 6 antibiotics (bacitracin, apramycin, penicillin, chlortetracycline, tylosin, sarafloxacin) had significant efficacy in control of histomoniasis. Out of 9 antiprotozoals, the nitroimidazoles dimetridazole (control), metronidazole, ornidazole, and tinidazole suppressed growth of *H. meleagridis in vitro* at 10 ug/ml or higher. There were also highly effective in chickens at 200 ppm in the feed, preventing liver lesions and reducing cecal lesions to near zero. Paromomycin sulfate and carbadox were weakly effective with the range tested but were ineffective *in vivo*. Quinolinol, mebendazole, diloxanide furoate, and albendazole had no demonstrable efficacy *in vitro* and were not tested *in vivo*.

A new phenomenon discovered during the culture of *H. meleagridis* was that cultured *H. meleagridis* were readily agglutinated by horse serum at concentrations of 15% or higher. The agglutinating factor (AF) was active when serum regardless of whether serum was heat-inactivated, and was present in several batches of sera tested. The AF had a molecular weight similar to that of serum globulins as shown by precipitation with 25-40% ammonium sulfate. The AF was used as a means of obtaining *Histomonas* cells free of bacteria in the following way: *Histomonas* cultures were pooled, washed twice with Hank's balanced salt solution (centrifuged at 1000g for 15 min and the supernatant decanted), and applied to a

nylon wool column. Horse serum was added (15%), and filtrates were agitated gently in Petri plates, whereupon clusters of *H. meleagridis* formed. The aggregated suspensions were collected by centrifugation, loaded onto a 2-layer sucrose gradient and centrifuged at 2500 g for 10 min. A band of cells forming between the two layers was collected and washed 2 to 3 times with centrifugation. The resulting clumps of *H. meleagridis* were free of visible bacteria.

INDEX WORDS: *Histomonas meleagridis*, Histomoniasis, Blackhead Disease, Epidemiology, Pathology, Control, Chemotherapy, Purification, *Eimeria tenella*, Culture *in vitro*, Chickens, Turkeys, Agglutinins.

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by

JINGHUI HU

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STUDIES ON HISTOMONAS MELEAGRIDIS AND HISTOMONIASIS

IN CHICKENS AND TURKEYS

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JINGHUI HU

Approve

Major Professor: Larry R. McDougald

Committee: John R. Glisson
Nicholas M. Dale
Pedro N. Villegas
Roger D. Wyatt

Electrical Version Approved:

Maureen Grasso
Dean of the graduate School
The University of Georgia
December, 2002

DEDICATION

To my wife, Meihua Qiao, for her understanding, encouragement, full support and love to me. To my parents, Guanghuo Hu and Liangrong Chan, for encouraging me to aim high and pursue graduate study. To my son, Michael Zhiyuan Hu, who was born while studying for PhD in the University of Georgia.

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TABLE OF CONTENTS

Page

Acknowledgements v

Introduction 1

Literature review 3

Purification of *Histomonas meleagridis* cultured in vitro
via agglutinins in horse serum.....36

Direct lateral transmission of *Histomonas meleagridis*
in turkeys 47

The efficacy of some drugs with known antiprotozoal activity
against *Histomonas meleagridis* in chickens..... 57

Effect of anticoccidials and antibiotics bn the control of black
Head disease in broiler breeder pullets 68

Blackhead disease (H*istomonas meleagridis*) aggravated in broiler chickens
by concurrent infection with cecal coccidiosis (*Eimeria tenella*) 87

Conclusion 101

INTRODUCTION

Histomoniasis, also frequently called blackhead (Cushman, 1893) or enterohepatitis (Smith, 1895) is an infectious disease of gallinaceous birds that is caused by the protozoan, *Histomonas meleagridis*. Histomoniasis can cause 90-100% mortality in turkeys, but is relatively mild in chickens. Most studies were conducted before 1980, with little additional research done during the past 20 years.

In recent years, there was a significant increase in field cases of clinically severe histomoniasis in chickens, in both leghorn and broiler breeder pullets (Homer and Butcher, 1991; Luma *et al.*, 1999). The possible causes for this increase would include (a) increased virulence of organism, (b) increased susceptibility, (c) interaction of *Histomonas* with other infections, (d) built-up cecal worm eggs in chicken houses, (e) change in management such as feed restriction, and (f) lack of effective drugs to control the disease. But, the actual reasons for the dramatic increase in clinical outbreaks are still unclear.

H. meleagridis is known to survive for a long period in cecal worm eggs, providing a reservoir of infection from one year or place to another. Previous researcher concluded that direct bird-to-bird transmission was unimportant in birds that were well fed and cared for (Lund, 1956). But it is hard to explain that the rapid spread of histomoniasis through turkey flocks resulting in 50-100% mortality in a few weeks. While there is no doubt that cecal worm eggs may play important role in survival of histomonads in environmental conditions, but there is no research to show how lateral transmission is important in histomoniasis outbreaks in turkey or chicken flocks.

The increase in importance of blackhead disease comes at a time when use lack effective drugs effective in treatment of *H. meleagridis*. The most effective drugs including dimetridazole and ipronidazole are no longer registered for use in the USA due to action of the Food and Drug Administration (McDougald, 1997).

The objectives of this project were to:

1. Study the conditions under which H. meleagridis spreads from bird to bird after introduction of infection into a flock.
2. Demonstrate what, if any, influence parasites such as coccidia contributes to pathogenicity of *H. meleagridis* in chickens.
3. Establish a system to screen drugs *in vitro* and *in vivo* and to evaluate the activity of drugs against *H. meleagridis*, which are shown to be effective in control of bacteria and other related protozoa
4. Characterize an agglutination factor in H. meleagridis which might be useful in seperation of cells from other organisms for use in other studies.

LITERATURE REVIEW

1. Histomoniasis and Its Causative Agent

1.1. Histomoniasis in chickens and turkeys

Histomoniasis, also frequently called blackhead or enterohepatitis, is an infectious disease of gallinaceous birds caused by the protozoan, *Histomonas meleagridis*. In 1893, Cushman first brief described histomoniasis and (for no apparent reason) called it blackhead. Smith (1895) described histomoniasis as infectious entero-hepatitis and gave a thorough description of the disease after he necropsied about 50 turkeys. He also named the protozoan parasite *Amoeba meleagridis*. In fresh preparations from ceca, he found the protozoan to be circular or oval, 8-14 μm in diameter, and except for a granular nucleus, almost structureless. Fixed and stained organisms were small oval bodies of 6 to 10 μm.

Tyzzer (1919) studied the morphology of the blackhead organism and described various developing phases. The form observed in the periphery of early lesions was called the invasive phase. These long forms with a single pseudopodium were frequently 30 μm in length. In fixed tissues, some round forms 8-17 μm in diameter were observed. The endoplasm of the invasive phase contained deeply stained particles within vacuoles and an extranuclear body. The organisms in the vegetative phase had basophilic cytoplasm without the inclusions, and a small amount of reticular or granular material was distributed around the nucleus. Vegetative phase parasites were larger, varying from 12 - 21 μm. A so-called resistant phase described by Tyzzer, was smaller, measuring 5 - 22 μm in diameter.

In 1920, Tyzzer noted its flagellate characteristics and changed the name of the parasite to *Histomonas meleagridis* (Smith, 1895). Later, Tyzzer and Fabyan (1922) reported free forms with one or two flagella in material from ceca of experimentally infected turkeys. Tyzzer (1934) studied the movement of *H. meleagridis* in cecal discharges at 42 C and described rhythmically beating flagella causing it to rotate counter-clock-wise one-fourth or one-third of a turn at each stroke.

Histomoniasis was also recognized in chickens (Chester and Robin, 1900), but some differences of the disease in chickens and turkeys were noted (Curtice, 1907a). Chickens had less extensive liver lesions, and very few died. Tyzzer and Fabyan (1922) demonstrated that the protozoan and nematode parasites responsible for histomoniasis in turkeys and chickens were the same by feeding turkeys with (a) *H. gallinarum* ova of chicken origin, (b) liver tissues from an infected chicken, and (c) soil from hen yards.

Histomoniasis develops in gallinaceous birds other than turkeys and chickens, including the ruffed grouse, bobwhite quail, Japanese quail, the guinea fowl, pheasant, chukar partridge, and peafowl (Graybill, 1925; Lund and Chute, 1972, 1973, 1974, 1974a; Tyzzer and Fabyan, 1920;). *Heterakis gallinarum*, the carrier of *H. meleagridis*, can transfer the disease from birds of one species to those of the same or another species (Lund and Chute, 1972, 1973, 1974, 1974a).

The enlarged ceca and extensive liver lesions characteristic of histomoniasis are well described in the literature. The microscopic pathology of lesions of moribund and dead birds was described by Tyzzer and Fabyan (1920) and Malewitz *et al.* (1958). Farmer *et al*. (1951) gave a report on the progressive pathology of histomoniasis in turkeys following rectal inoculation with cultured *H. meleagridis*.

The signs of histomoniasis in turkeys are ruffled feathers drooping wings, drowsiness, birds huddling together, reduced food intake, reduced weight gain, and sulfur-yellow droppings. Cheesy cecal core material may appear in the droppings. These clinical signs develop from 12-15 days after infection and mortality may follow at 15 to 21 days after infection. The course of the infection is usually less severe in chickens where unthriftiness, diarrhea and cheesy cecal core material in feces are the most common signs.

The ceca are the primary sites of infection. This was demonstrated by Durant (1930) and Delaplane and Stuart (1933) when they ligated the ceca and found that histomoniasis would not develop when birds were exposed *per os* to infective worm ova. Farmer *et al*. (1951) observed hyperemia, edema, and polymorphonuclear infiltration of the mucosa and submuscosa of the ceca in early stages of infection. Later inflammatory reaction spread into the muscularis mucosa and protozoa were seen singly and in "nests' in the muscle layers. Multinucleated giant cells were numerous and often contained histomonads (Malewitz *et al.*, 1958). The ceca were often filled with adherent, hard, dry, cheesy cores of serum proteins and cellular debris. The serosal surface of the ceca frequently showed an inflammatory reaction and peritonitis and was often quite severe.

The livers of diseased birds had dense areas of cellular infiltration, cloudy swelling, degeneration, and necrosis of cells. There was congestion of the blood vessel and sinusoids. In the necrotic areas, lymphocytes, macrophages, and giant cells were found, and the latter often contained protozoa. The histomonads seen in lesions of the ceca and liver were intercellular (Smith, 1915; Tyzzer, 1920).

On the liver surface, areas of necrotic and degenerated tissues were saucer-shaped, yellowish to yellowish-green in appearance, and depressed below the surface especially in advanced cases. Such lesions extended throughout the liver parenchyma.

Venkataratnam and Clarkson (1963) studied the effect of histomoniasis on the blood cells of six-week old cockerels. A rise in total leukocytes was detected one day after infection and reached a maximum count of 70,000 /mm^3 10 days after infection. The increase in leukocytes consisted mainly of heterophils, although monocytes and eosinophils increased significantly during the recovery phase. Lymphocyte, basophils, and erythrocyte counts were not changed. Total cell counts returned to normal levels 21 days after infection. Blood changes in three week old chickens were similar (Wilson and Perie, 1967). The maximum leukocyte count was 92,000 /mm^3 on the tenth day. Basophils, eosinophils and monocytes were significantly increased but there was no significant change in lymphocyte number.

McDougald and Hansen (1970) determined the effect of histomoniasis on several plasma enzymes in chickens and turkeys. Substantial and sustained increases in glutamic-oxalacetic transaminase (GOT) and lactic dehydrogenase (LDH) were observed in infected turkeys after 9 or 12 days postinfection. GOT activity increased substantially and transiently in chickens after 9 days if liver damage was present, and was below normal when infected chickens had cecal but not liver damage. Cholinesterase was progressively depressed in turkeys but not in chickens. Glutamic-pyruvic transaminase (GPT) remained same in all instances. Glutamic dehydrogenase (GLDH) and malic dehydrogenase (MDH) were evaluated in turkeys with liver damage; amylase was elevated and MDH and LDH were depressed during cecal histomoniasis in chickens. In the absence of liver lesions, the fall in these enzymes was associated with cecal lesions, probably due to loss of plasma into cecal

lumen (Beg and Clarkson, 1970). Al-Khateeb and Hansen (1973) reported that the number of liver lesions was correlated significantly with GOT level and suggested that plasma GOT could be used as clinical evidence for histomoniasis. In turkeys, the albumin concentration fell and γ-globulin rose significantly during infection. The drop in albumin coincided with the acute inflammation of cecal mucosa and with the appearance of large quantities of serum protein in the cecal contents, the rise in γ-globulin appeared related to immunological response.

1.2. Factors affecting pathogenicity of *H. meleagridis*

1.2.1. Age

Previous reports have suggested that age of birds was a factor in histomoniasis infection. Curtice (1907b) found that 90% of the poults confined in an area became infected while only 20 percent of old turkeys confined in the same area became infected. However, Kendall (1957) found that there was no significant difference in susceptibility to the disease in turkeys 7 weeks to 20 months old when experimentally infected *per os* with *H. gallinarum* or per rectum with *H. meleagridis*.

Histomoniasis in chickens, is usually more severe in younger birds (Milks, 1908; Desowitz, 1951; and Ohara and Reid, 1961). Milks (1908) never diagnosed blackhead in chicken flocks more than six weeks old. Desowitz (1951) studied young chicks by rectal inoculation of infective material. Mortality was highest in the group inoculated at 21 days of age and lowest in those inoculated at 34 days of age. Ohara and Reid (1961) found chickens to be more susceptible to infection when they were fed *H. gallinarum* ova at 32 days of age than at 1, 46, or 64 days of age, respectively. Results with rectal inoculation of *H.*

meleagridis also indicated that birds of 3-4 weeks old were more susceptible than old or younger birds.

1.2.2. The interaction of histomonads with other pathogens or conditions

1.2.2.1. Coccidia:

Chappel (1973) reported that the histomonads appeared to affect the development of *E. tenella* in 2 ways: (1) introduction of *H. meleagridis* at the same time or 1 day after *E. tenella* apparently resulted in migration of the histomonads in mid-lamina propria simultaneous to maturation of second-generation schizonts. The degree of pathology due to rupture of schizonts was much greater than with coccidia alone, possibly due to weakening of the lamina propria from histomonal migration. Oocyst production was reduced due to lack of tissue suitable for gametogony. (2) Inoculation of *H. meleagridis* 3 day before *E. tenella* apparently resulted in the destruction of second-generation schizonts situated adjacent to multiplying histomonad colonies. Although gametocytes appeared little affected, oocyst production was drastically reduced presumably due to a reduction in the number of parasites producing sexual stages.

1.2.2.2. Bacteria:

H. meleagridis has a distinct requirement for certain species of bacteria for pathogenicity in susceptible hosts, or even in for growth *in vitro* (McDougald and Reid, 1978). Tyzzer (1921) discovered that embryonated eggs of *Heterakis* might fail to develop in young turkeys given access to only sterile feed and water, whereas the larvae developed readily in poults having access to soil. In 1934, Tyzzer first recognized that the flora accompanying the protozoa at the time of its isolation exercised an important influence on its later propagation. A similar suggestion was made by Bishop (1938), who found that some

cultures failed to support histomonad growth due to the absence of 'compatible' flora. Doll and Franker (1963) were the first to attempt to infect germ-free turkeys with *Heterakis* and *H. meleagridis*. Only one of the 12 poults given about 1000 bacteria-free *Heterakis* eggs had liver lesions when necropsied 17 days later, and 11 other poults had no macroscopic evidences of survival of either *Heterakis* or *Histomonas*. No parasites were found in stained sections of the livers of the germfree poults without visible lesions. Eleven of 12 conventional poults given similar infective doses of *Heterakis* eggs developed typical histomoniasis and died 14 to 21 days after feeding. They suggested that the bacteria present in conventional birds might contribute some heat-labile factor necessary for the growth of the parasites.

Later, Franker and Doll (1964) found that both *Heterakis* and *Histomonas* sometimes grew in poults that have only one or two species of bacteria. Bradley and Reid (1966) grew *H. meleagridis* in poults with no intestinal flora except *Escherichia coli.* When poults were supplied only heat-killed *E. coli* or an *E. coli* filtrate, *H. meleagridis* did not grow. They viewed their findings as demonstrating the existence of a synergistic relationship between the two organisms and suggested that the proper combination of organisms (*H. meleagridis* and bacteria) must be present in order to produce histomoniasis. Springer *et al*. (1970) concluded that the essential contributing factor of bacteria in the pathogenesis of *H. meleagridis* involved neither pH nor oxidation-reduction potential in the intestine. However, Kemp (1974) found evidence for a direct effect of bacteria on the environment of *H. meleagridis* through the use of turkey poults with one surgically ligated cecum. The ceca remained bacteriologically sterile when ligation was done prior to normal hatching. Such altered birds became infected in the normal cecum but will remain refractory to infection in

the ligated cecum. The difference in the bacteria requirements in chickens and turkeys could be significant with respect to the difference in pathogenicity of *H. meleagridis* in chickens and turkeys. Further study on bacterial flora of other host will be valuable in elucidation of the relationship of host flora and pathogenicity in the various host birds.

2. Transmission

2.1. Transmission aided by helminths

While studying the epidemiology of blackhead, Smith and Graybill (1920) observed the disease in turkeys penned with chickens. Examination of cecal contents from dead birds revealed the presence of *Heterakis gallinarum,* the chicken cecal worm, along with *H. meleagridis*. They suggested that the causative organism may be ingested simultaneously with *Heterakis* ova. Later, Graybill and Smith (1920) concluded that the cecal worm was an important part in the transmission of histomoniasis, although the exact role of the worm was not clearly understood. Blackhead was consistently produced by feeding embryonated ova of the cecal worm. They regarded *Heterakis papillosa* as an accessory agent needed to break down the resistance of the bird and prepare the cecal wall for invasion by histomonads.

It was Tyzzer (1926) who demonstrated that the protozoan was actually transmitted within the *Heterakis* ova. Ova embryonated in 1.5% nitric acid to render them superficially sterile, produced blackhead in turkeys. Infected, embryonated *Heterakis* ova are now regarded as the most important source of histomonads for both chickens and turkeys, and have been used experimentally as a means of infection.

Histomoniasis was transmitted to turkeys by intra-cecal injection with *Heterakis* larva surface sterilized with a 50% solution of hydrogen peroxide (Swale, 1948). He concluded that viable larvae were necessary to transmit and initiate disease. Previous attempts to transmit *Histomonas* with unembryonated *Heterakis* ova were unsuccessful (Tyzzer, 1934).

Gibbs (1962) provided convincing morphological evidence of *Histomonas* in the reproductive tract and eggs of *Heterakis* by light microscopy. He found histomonads in both sexes. In male heterakids, histomonads were in the lumen of the gut, invading gut cells, and among the sperm of the testis, vas deferens and seminal vesicle, and were identified in all parts of reproductive tracts of female *Heterakis*. Histomonads were acquired and transmitted by *Heterakis* for more than 10 days (Lund, 1971). Lund and Chute (1973) confirmed that the female heterakid was capable of transmitting *H. meleagridis* from the bird to the cecal worm egg without the intervention of the male except for fertilization.

Although Tyzzer (1934) was unable to infect turkeys by inoculation with male heterakids, quite different results were obtained by Springer, Johnson and Reid (1969). They fed turkey poults whole male or female worms, triturated worms and embryonated ova. The female worms, containing unembryonated ova, did not infect the birds with blackhead, but male worms transmitted infection in 9 of 11 poults. Triturated infections in birds with female worms and 3 of 5 with male worms. These results suggested that some type of resting phase is present in male worms and may be passed on to female copulation.

Lund and Chute (1972) found a positive correlation between the number of heterakids lost per bird and the incidence of *Histomonas* infections and suggested that liberation of histomonads occurred as the heterakids died. Several studies have shown that ova of *H. gallinarum* remained positive for *Histomonas* as long as 2 to 3 years (Niimi, 1937; Farr,

1959, 1961; Lund, 1960). Lund and Chute (1970) reported that young chickens were 16 times as important as mature chickens in contaminating soil with *Histomonas*-bearing *Heterakis* eggs. Compared with old turkeys, poults produced so few *Heterakis* eggs as to be of no importance.

2.2. Direct transmission without helminths.

In his original report (1895), Smith postulated the direct infection of turkeys by ingestion of *Amoeba* (=*Histomonas*) *meleagridis*. Pursuing Smith's hypothesis, Moore (1896) reported that histomoniasis was produced in turkeys by feeding them the droppings or diseased tissues of severely affected birds. Later, Chester and Robin (1900) thought he had produced histomoniasis in a chicken by a similar way. However, other investigators were less successful in transmitting histomoniasis in chickens and turkeys via the oral route (Curtice, 1907a; Tyzzer, 1919, 1920a, 1921; Tyzzer and Fabyan, 1920).

In 1922, Tyzzer and Fabyan produced "typical blackhead" in a poult that had received fresh liver lesion material orally when it was 4, 6, and 16 days old. Subsequently, Tyzzer and Collier (1925) produced blackhead in several 5 day old poults fed infected liver. Tyzzer (1926) demonstrated that *H. meleagridis* could be transmitted "to some extent in nature by directed ingestion of material contaminated with freshly passed discharges containing the protozoan."

Since that time, some investigators have produced blackhead by giving histomonads orally, but the method was unreliable. Farmer and Stephenson (1949) found a low infection rate using emulsified cecal lesions but no infection with emulsified liver lesions.

Horton-Smith and Long (1955, 1956), who found that *H. meleagridis* seldom survived the low pH of the upper digestive tract demonstrated the irregularity of infection by the oral

route. Only after starving chickens for 18 hours was infection produced with one ml of cecal filtrate given orally. A study of the starved digestive tract revealed an increase in pH. Further elevation of the pH with one gram of a mixture of 40% calcium carbonate, 17% magnesium trisilicate, and 43% colloidal kaolin given orally to starved birds greatly increased the incidence of histomoniasis. Lund (1956) produced infections of the ceca in 43 of 109 poults, 6 - 9 weeks old, by giving orally 10,000 - 50,000 histomonads in 1 ml of saline. However, only two birds developed liver lesions and only one died.

2.3. Earthworm transmission

The earthworm has been shown to serve as carrier of *H. gallinarum* larvae and a reservoir for *H. meleagridis* (Lund, 1969). Curtice (1907b) first demonstrated the transmission of blackhead with earthworms, but concluded, however, that the earthworm was probably a carrier of infected soil and not necessarily a second host of the parasite.

Although not producing blackhead, *H. prepicullum* was transmitted by the dung-earthworm (Scott 1913), *Helodrilus parvus*. Another cecal worm, *H. papillosa* was transmitted by earthworm, *Helodrilus gieseleri hempeli* (Ackert, 1917). It was not determined whether the relationship of nematodes was a casual association or true parasitism.

Lund, Wehr, and Ellis (1963) reported that earthworms were actually biologic vectors of *H. gallinarum*, and that, *Histomonas* could also be transmitted after a true parasitic relationship. They observed numerous larvae emerging from pores of the body when worms were warmed in the laboratory. When infected earthworms were fed to poults or young pheasants, both cecal worm and blackhead infections occurred. Lund *et al.* (1966) also demonstrated the infection of chickens with both parasites by feeding earthworms. Also,

cockerels and poults became infected with both *Histomonas* and *Heterakis* when they were fed earthworms from soil where ring-necked pheasants were raised (Kemp *et al.*, 1975).

3. Culture *In Vitro*

The first cultivation of *H. meleagridis* was by Drbohlav (1924), who reported that coagulated white of egg covered with blood bouillon containing 1% peptone was better than either blood agar overlaid with Locke's solution or coagulated egg medium with Locke's solution. Growth of *H. meleagridis* was best at a pH of 7.2-7.8. Tyzzer (1934) described a diphasic medium with rice powder and 5% horse serum in the fluid overlying agar-egg albumen slants. He reported that the nature of the bacterial flora accompanying *H. meleagridis* at the time of its initial isolation from cecal material had an important influence on its propagation success. Bishop (1937) cultured the blackhead parasite from liver lesions in inspissated horse serum slants. He later grew the same strain of histomonads with several media including one consisting only of "horse serum diluted 1:8 with solid rice starch but without a slope" (Bishop, 1938).

DeVolt (1943) developed a simpler, easily prepared medium (pH=9) consisting of Locke's solution with 2% turkey serum and 2% N/20 NaOH, autoclaved at 120 C for 20 minutes. Before use, each tube received a bit of sterile rice starch. He reported that certain bacteria existed with cultures of the blackhead parasite in some degree of symbiosis. Subsequently, other investigators modified DeVolt's media in various ways. Lesser (1960a) tested a number of substitutes for blood serum. Of these, the only one which gave results approximating that of serum was fresh cream, either pasteurized or non-pasteurized, sterilized by passage through a Selas filter (0.6 micron). The substitution of fresh cow milk for cream was not as satisfactory. Lesser (1960b) later grew *H. meleagridis* in medium 199

diluted 10-fold with distilled water and supplemented with 10% filtered cream or serum and 0.05% NaHCO3. Lesser (1961b) found that cholesterol and its esters could replace cream in the above medium. In a test of cholesterol esters, the sterate and palmitate supported histomonad growth, but not as well as cream. Growth was poor with the acetate and benzoate esters.

Lund *et al.* (1966) found that the pathogenicity of *H. meleagridis* had waned after some 500 passages *in vitro* and that its immunizing ability declined rapidly between 730 and 835 passages. The strain's observable structure and activity remained unchanged after 1000 transfers, but it had almost lost its ability to grow in either chickens or turkeys (Lund, 1967). Augustine *et al*. (1970) reported that no qualitative difference could be found by the indirect fluorescent antibody method between fresh isolates of *H. meleagridis* and those maintained in culture.

The most successful medium for cultivation of *H. meleagridis* is that of Dwyers (1970) as modified by McDougald and Galloway (1973). The medium consisted of 85% medium 199, 5% chicken embryo extract, and 10% sheep or horse serum adjusted to pH 7.8. Histomonads grow rapidly in this medium, and when used for diagnosis, the tests can be read 28-48 hrs after inoculation.

4. Diagnosis

4.1. Clinical signs

Affected birds become listless, walk slowly, stand with ruffled feathers and dropping wings, and often have their head drawn to their bodies and their eyes closed. These signs are not specific for histomoniasis. However, turkeys with histomoniasis usually void sulfur-colored droppings and have the characteristic sulfur-colored stain on feathers near the vent.

Chickens less frequently void such droppings, which seem to be passed only by birds with rather pronounced liver lesions. However, chicken often pass cores or core fragments with blood in various amounts. Turkeys usually do not void cecal cores until recovery is underway. Then, in most instances, the cores must be fragmented to be expelled.

4.2. Confirmation of organisms

A tentative confirmation of histomoniasis can be made at farm by inspection of the ceca and liver of recently dead birds or sick ones killed for such examination. A conclusive identification requires laboratory examination and, whenever possible, the demonstration of *H. meleagridis*. There are four different types of confirmative tests which can be made (McDougald, 1978).

1) Histomonads may be demonstrated in cecal fluid or mucosal scrapings by phase contrast microscopy. The use of a warm stage (40 – 45 C) promotes observation of ameboid movement.
2) Histomonads may be cultured in Dwyer's medium. Samples of cecal contents or scrapings must be obtained before much cooling has occurred. These may be inoculated into culture medium or physiological saline (40 C) for holding (McDougald and Galloway, 1973).
3) Positive cultures can be confirmed by cloacal inoculation of culture material into young turkeys or chickens (1-3 weeks old). Characteristic lesions should appear upon postmortem examination in about a week.
4) Histopathology has been of value in distinguishing histomoniasis from other diseases, particularly mycoses. *H. meleagridis* stained weakly with H & E method. However, the periodic acid-Schiff's stain technique and Grocott's stain give good

differentiation of *H. meleagridis* and *albicans* or other fungi in liver sections (Kemp and Reid, 1966).

5. Prevention and Control

5.1. Chemotherapy and chemoprophylaxis

The early work on the chemotherapy of histomoniasis was focused on arsenical compounds. After proof that the blackhead organism was a flagellate, Tyzzer (1923) tried various arsenicals for the treatment of blackhead because of prior success of these compounds against flagellates. Neoarsphenamine (sodium-3,3-diamino-4,4- dihydroxy-arseno-benzene-n-methylene sulfate) injected intravenously or subcutaneously in turkeys showed some favorable effect on the course of natural infections and lowered mortality. However, this compound proved to be instable, was toxic for young turkeys, and was not available in quantities suitable for treatment of commercial flocks. Tryparsamide (N-phenylglycenamide-p-arsonic acid) could be given intravenously or subcutaneously in doses as high as 1 g/kg of body weight without serious toxic effects. Prompt clinical improvement usually followed injection, and mortality was greatly lowered. Tryparsamide was more effective than neoarsphenamine (Tyzzer, 1923). McGuire and Morehouse (1952) screened a large number of organic arsenicals. The best of these was 4-nitrobenzenearsonic acid (Histostat®, Dr. Salsbury's Laboratories, Charles City, JA) administered in water or in feed was highly effective at 0.0075-0.075% in feed, or 0.006-0.04% in water. There was some evidence of growth stimulation with 0.01 to 0.03 % in feed. Welter and Clark (1961) reported that p-ureidobenzenearsonic acid (Carbarsone®, Whitmoyer, Philedelphia PA), administered continuously starting one week before infection, reduced mortality and lesions

due to histomoniasis in turkeys. Similar results were obtained in challenge experiments by Sullivan *et al.* (1964, 1965) when dietary treatments were initiated five days prior to exposure. Worden and Wood (1973) tested carbarsone (0.075%) for adverse effects in turkeys, and found no evidence of any effect upon food consumption or body weight gain from day old to 24 weeks, and no macroscopic evidence of any organ damage. Peardon and Eoff (1967) reported that carbarsone was effective against *H. meleagridis* in chickens when given continuously at 0.025% or 0.05% in the feed. McDougald (1979) reported that carbarsone and amprolium could be used in combination without interference in efficacy against the target diseases (coccidiosis and blackhead).

DeVolt and Holst (1948) reported that iodochlorhydroxyqiun ('Vioform') showed prophylactic activity against blackhead when used at 1 or 0.5 % in feed for 21 days. Later, they compared vioform with chlorhydroxyqinoline, and found that chlorhydroxyqinoline was more effective (DeVolt and Holst, 1949). However, in later trials against infections induced by dosing *Heterakis* ova, efficacy of chlorhydroxyqinoline was marginal (DeVolt, 1950).

Lindquist (1962) reported that paromomycin sulfate at high levels (0.1-0.2% in feed) provided 80% protection against heavy histomoniasis mortality of turkey poults, while metronidazole [1-(2-hydroxyethyl)-2 methyl-5-nitroimidazole] in feed (0.038%) provided 100% protection.

The greatest activity is to be found among heterocyclic compounds, particular those based upon a 5-membered ring. As a group, the nitrofuran have proved highly successful. In a survey by McGregor (1953) furazolidone had prophylactic activity against *Histomonas* infections when fed continuously at 0.0125% in feed. The compound had some therapeutic activity if the dose level was raised to at least 0.016% but relapses occurred when medication

was withdrawn (Horton-Smith and Long, 1956). Jerstad (1957) reported that no adverse effects on growth of poults when furazolidone at 0.02% in feed was given after poults were 7 weeks old; however, sexual maturity was delayed when the drug was fed to breeder stock. Costello and Devolt (1956) studied on the effect of Furoxone [N-(5-nitro-2-furfuryl-idene)-3-amino-2-oxazolidone] against blackhead and found that it had a protective action against infectious enterohepatitis without any detrimental effect on growth during a 4-week period of administration. However, feeding the drug at 0.033% over a longer period of time significantly retarded the growth of the birds.

Nifursol (3, 5-dinitrosalicylic acid, 5-nitrofurfurylidene hydrazide), another nitrofuran derivative, afforded excellent protection against blackhead disease at low feed concentrations, with a high margin of safety in chickens and turkeys (Vatne *et al.*, 1969a, 1969b, Sullivan *et al*., 1973.

Histomonacidal properties are especially well developed in 5-nitrothiazole derivatives and it was among this group that drugs with real practical value for the control of blackhead were first found (Waletzky *et al*., 1950). Joyner and Kendall (1955) reported that 2-amino-5-nitrothiazole was effective for prevention at a concentration of 0.05% in feed and 0.1% controlled established infections, provided that the disease had not progressed too far. However, withdrawal of the drug allowed relapse.

Nithiazide, a substituted thiazolyl urea related to 2-amini-5-nitrothiazole was effective at 0.025-0.05% in feed (Cuckler and Malanga, 1956). Regardless of whether, poults were infected by cecal inoculation of *Histomonas meleagridis* or oral dosing with *Heterakis* ova, nithiazide was more effective than similar levels of 2-amini-5-nitrothiazole, and was better tolerated. Nithiazide did not interfere with maturation or reproduction when fed

continuously for 62 weeks and was well tolerated at a level of 0.1% of the diet (Cuckler *et al.* 1957).

Lucas (1961) reported that dimetridazole (1,2-dimethyl-5-nitroimidazole) was very active against blackhead in turkeys. Compared to nithiazide and acinitrile (2-acetamino-5-nitrothiazole), dimetridazole was more effective, providing 100% protection at levels of 0.012 and 0.025%. Subsequent reports by Lucas (1962, 1963a, 1963b) and McGuire *et al*. (1964) further demonstrated a high activity of dimetridazole against histomoniasis. Treatment with 0.05% (w/v) dimetridazole permanently suppressed the histomoniasis infection in turkey poults infected via *Heterakis gallinarum ova* and treated after a delay of 2 days. After 5 days delay 1/62 died. Treatment for longer period did not increase the efficacy (Lucas, 1963a). McGuire *et al.* (1964) reported that dimetridazole feed concentrations of 0.01-0.1% provided maximum blackhead preventive efficacy in chickens and turkeys. Dimetridazole fed to laying hens for a period of seven months had no effect on egg production. Levels of the drug in the albumen were increased as the level of dimetridazole increased (Colvin *et al.*, 1963). Dimetridazole was marketed as Emtryl® (Salsbury Laboratories, Charles City IA) and was the product of choice for blackhead treatment for many years.

Mitrovic *et al*. (1968) tested a large number of 5-nitroimidazoles against *H. meleagridis* infections in turkeys. The most effective was 1-methyl-2isopropyl-5-nitroimidazole (ipronidazole), which was developed as a commercial product (Ipropran® Holfman-LaRoche, Inc. NJ) was twice as effective as dimetridazole and 4-8 times more effective than other 5-nitroimidazoles. Ipronidazole was highly effective therapeutically, especially against

advanced infection when given in either feed or water at 0.025 or 0.0125%, respectively. However, drug level, route and timing of therapy were important for best results (Sullivan *et al*., 1973). For prevention of histomoniasis, the continuous administration of ipronidazole at 0.00625% in the feed was highly effective in preventing histomoniasis mortality (Mitrovic *et al*., 1970, Sullivan, 1973). Dimetridazole and ronidazole fed at recommended levels had the same prophylactic efficacy as ipronidazole, but carbarsone, nitarsone and 2-acetylamino-5-nitrothiazole, were significantly less effective (Sullivan *et al*., 1973). Ipronidazole was also an effective growth promoter for turkeys (Marusich *et al*., 1970).

Whitemore *et al*. (1968) and Peterson (1968) reported that ronidazole (1-methyl-2-carbamoyloxymethyl-5-nitroimidazole) effectively prevented histomoniasis in turkeys. Ronidazole at 30-60 ppm in feed prevented mortality and morbidity in poults. Treatment with 30-40 ppm of ronidazole in water, initiated 10 days post-exposure and continued for at least 7-10 days was effective as a treatment (Sullivan *et al.*, 1977).

Hegngi *et al.* (1997) tested albendazole and febendazole for effectiveness in the treatment and prevention of histomoniasis. Both drugs were found to be effective as a preventive, but not as a treatment. When turkey poults were placed on contaminated litter, treatment with albendazole and febendazole was associated with a significant increase in body weight gain and a reduction in cacal and liver lesion scores. They concluded that the prophylactic effect could be attributed to the destruction of the transport vector (*Heterakis* larvae) rather than direct killing of *Histomonas* in the cacal lumen.

Callait *et al*. (2002) tested the efficacy of 10 drugs against *H. meleagridis in vitro*. Febendazole, albendazole, and sulfadiazine were ineffective against *H. meleagridis*. Nifursol, the only compound still authorized as a feed additive in Europe, is an inhibiting

agent but is not lethal *in vitro*. Roxarsone is effective at high concentration (200 μg/mL) after a long exposure (48 hrs). Dimetridazole, metronidazole, ronidazole, tinidazole, and furazolidone had lethal activity against *H. meleagridis in vitro*. Dimetridazole, which is very effective in treatment and prevention of *H. meleagridis,* is not available in USA and Europe.

5.2. Prevention by management

Since primary means of transmission of histomoniasis occurs through the vehicle of heterakid eggs, successful control measures are directed in large part toward reduction or exclusion of the eggs (McDougald, 1997).

For turkeys, exclusion of domestic chickens from turkey-raising operations is essential, since chickens may often harbor large numbers of egg-laying cecal worms. Young turkeys should be kept isolated from mature turkeys (Harwood, 1954).

Young chickens, especially pullets, become infected in problem houses where worm eggs have built up in number for several years. Anthelminthic therapy may have value in preventing histomoniasis by reducing the contamination of the premises. However, most poultry growing houses have earthen flows and contain considerable contamination, challenging the effectiveness of this approach.

5.3. Immunization

Little is known concerning the mechanism of protective immunity against this disease. Turkeys do not readily become resistant to reinfection with *H. meleagridis*. Tyzzer (1933) reported that *H. meleagridis* maintained in culture for 2 years was no longer pathogenic to chickens, but still stimulated some degree of protection immunity against reinfection. In later attempts to immunize young turkeys by the same procedure, some degree of success was attained (Tyzzer, 1934, 1936).

Lund (1959) tested immunogenicity of a nonpathogenic strain discovered in 1954. Rectal inoculation of several thousand nonpathogenic histomonads on 2 or 3 consecutive days afforded considerable protection against modest rectal challenges with pathogenic histomonads 3 to 6 weeks later, but much less effective against pathogenic histomonads introduced by feeding eggs of *H. gallinarum.* He speculated that an immunity barrier limited to the surface of the cecal mucosa was established, and that the larvae of the cecal worms often penetrated this barrier before liberating their histomonads, thus permitting blackhead to develop. Immunization by the introduction of nonpathogenic histomonads via *Heterakis* eggs was not successful.

Dwyer (1971) compared the antigens of 4 strains (one virulent, and three strains derived after 12, 24, 52 weeks of cultivation *in vitro*, respectively) using gel diffusion method. Analysis of bands revealed a common antigenic composition of the 4 strains. The concentration and the number of precipitin lines increased with the length of cultivation. These observations suggested a relationship between antigenicity and pathogenicity of histomonads. Later, Dwyer and Honigberg (1972) confirmed the above results with the more sensitive quantitative direct fluorescent antibody methods, which brought out significant differences in antigenic composition among the parental strain and three substrains.

Clarkson (1963) reported that a protective immunity was produced in drug-treated turkeys and in fowls recovering spontaneously. These birds developed precipitating antibodies in their sera to an antigen derived from *H. meleagridis*. Antigen was first detected in cecal contents 4 days after infection and serum precipitins 7 days later. It was not possible to transfer proctective immunity by injections of serum from immune to susceptible birds

(Clarkson, 1963). Kendall (1957) found no age immunity against histomoniasis in turkeys. He found those five birds that recovered from a previous infection under sodium acetarsol therapy was protected against reinfection via ova of *H. gallinarum.* Kendall found pathogenic histomonads in the ceca of resistant birds. Other research workers have reported the development of resistance in turkeys after recovery from natural infections (Sautter, Pomeroy and Roepke, 1950), or under drug therapy (Brackett and Bliznick, 1949) but this resistance was not always solid or long lasting. Thus, Lund (1959) recommended that prophylactic drugs against histomoniasis must be given continuously during periods of risk.

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PURIFICATION OF *HISTOMONAS MELEAGRIDIS* CULTURED *IN VITRO* VIA AGGLUTININS IN HORSE SERUM

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[1]**Jinghui Hu and L. R. McDougald. 2002. To be ubmitted to Parasitology Research.**

1. Abstract

Cultured *Histomonas meleagridis* were readily agglutinated by horse serum at concentrations of 15% or higher. The agglutinating factor (AF) was active in several batches of serum, regardless whether the complement was heat-inactivatedor not. The AF had a molecular weight similar to that of serum globulins as shown by tests of proteins precipitated with 25-70% ammonium sulfate. Our method of culturing *Histomonas in vitro* requires contamination by bacteria, which flourish in the rich medium, making it difficult to obtain cells free of bacteria for biochemical or immunological studies. The AF was used as a means of obtaining *Histomonas* cells free of bacteria as follows: Histomonad cultures were pooled, washed twice with Hanks balanced salt solution (centrifuged at 1000 g for 15 min and the supernate decanted), and applied to a nylon wool column. Horse serum was added (15%), and filtrates were agitated gently in Petri plates, where *H. meleagridis* cells aggregated into large clumps. The aggregates were collected by centrifugation, loaded onto a 2-layer sucrose gradient and centrifuged at 2500 g for 10 min. A band of cells forming between the two layers was collected and washed 2 to 3 times with centrifugation. The resulting clumps of *H. meleagridis* were free of visible bacteria.

2. Introduction

Histomonas meleagridis, a causative agent of blackhead disease in turkeys and chickens, has been successfully cultured *in vitro* (Lesser, 1960a, 1960b, 1963, Delappe, 1953, Devolt, 1943, McDougald and Galloway, 1973). However, the most successful methods require extensive contamination with bacteria for good growth. Thus, it is difficult to obtain pure *H. meleagridis* cells free of bacterial contamination for biochemical or immunological research.

Elimination of bacteria by gradient centrifugation is difficult because histomonads share a density with some bacteria. Further, *H. meleagridis* cells are fragile, lysing easily with most working conditions. The cells are amoeboid and pleomorphic, lending further complications in their purification. During culture and subculture of *H. meleagridis* in a medium consisting of Medium 199, chick embryo extract, and horse serum, we found that histomonad cells aggregated into clusters in the presence of 15% or higher horse serum. The clusters did not appear to include bacteria. We recognized this phenomenon as potentially useful in separating *H. meleagridis* cells from contaminating bacteria, for use in biochemical or immunological work.

3. Materials and Methods

Parasites. The parasites were cultured in modified Dwyer's Medium (Medium 199 in Hank's balanced sait solution, 85%; Chicken embryo extract, 5%; Horse serum, 10%; rice powder, 15 mg/50ml; pH 7.2) (McDougald, 1973). The cultures were harvested when the concentration of *H. meleagridis* reached more than 200,000 per ml. Pooled cultures were washed twice in HBSS + gentamycin (20 *u*g/ml) by centrifugation at 1000 g for 15 min. The pellets were resuspended in washing solution and loaded onto a nylon wool column (packed with nylon wool to depth of 2-3 cm, pre-washed with washing solution). The elute from this column (containing *H. meleagridis* cells) was used for further purification steps.

Treatment of *H. meleagridis* with horse serum and gradient centrifugation: The elute from the previous step was mixed with horse serum to a concentration of at least 15%. The suspension was mixed well and distributed to clean Petri dishes, then agitated gently. Stock sucrose solution (100 g sucrose, 120 ml of water, and 1 ml of phenol) was diluted with

water and used for layering (upper solution, 1 part sucrose + 3 parts water; lower solution 1 part sucrose + 2 parts water) (Hu, Jiang, and Zhu, 1995). The histomonad suspensions were loaded onto sucrose in centrifuge tubes, then centrifuged at 2500 g for 10 min. A band forming between the two layers was collected and washed 2-3 times in washing solution by centrifugation at 1000 g for 10 min.

Agglutinin factor in horse serum. Proteins were precipitated sequentially from horse serum by treatment with saturated ammonium sulfate solution (SASS) (25-70%). Batches of 30 ml horse serum were mixed with equal volumes of 0.01 M PBS (pH 7.2). Then 20 ml of SASS were added dropwise as diluted serum was agitated gently by magnetic stirrer in an ice bath. After an additional 10 min in ice, the mixture was centrifuged for 20 min at 3000 g. The supernate was drawn off and used in the next step by addition of more SASS. The precipitate was washed with the corresponding conc. of SASS. The precipitate was mixed with 1/4 the initial vol of PBS and dialysed against PBS (1 L of PBS for 1 hr and overnight against fresh PBS) to remove SASS. The conc. of SASS used were 25, 33, 40, 50, and 70%, respectively in successive steps. Dialysed proteins were mixed with PBS to initial sample volume. Reconstituted protein solutions were mixed with the *H. meleagridis* cells eluted from the column at a conc of 15 or 30%. Aggregation assay was done as described above.

Aggregation properties of horse sera of different brands. Batches of horse serum (4) were purchased from different suppliers and used test the agglutination properties as described above, using horse serum at 15 and 30%.

4. Results and Discussion

Histomonads were readily clumped by natural agglutinins (AF) found in horse serum, when the concentration of serum was 15% or higher. Similar results were obtained with 4 batches of horse serum from different suppliers, but not from bovine, chicken, or turkey serum (Table 3.1). The AF was not observed in serum from chickens or turkeys, even after infection with *H. melelagridis*. Heating of horse serum to inactivate complement had no effect on the AF reaction. The nature of the AF is unknown. However, the AF could be precipitated with 25-40% ammonium sulfate giving it characteristics similar to serum globulins. The presence of AF in precipitated proteins was demonstrated after precipitates were dialyzed to remove ammonium sulfate (Table 3.2).

Clumped *H. meleagridis* prepared in this way were essentially free of bacteria, and were suitable for our study of biochemical, immunologic, and structural properties (Figure 3.1, 3.2, 3.3). Figure 3.1 show the histomonads cultured *in vitro*. Figure 3.2 showed the clumping of histomonads. Figure 3.3 showed the purified histomonads.

The clumping of *H. meleagridis* cells by horse serum was surprising, as this serum has been used at 5-10% in culture media for many years (McDougald and Galloway, 1973). This phenomenon has provided us with a quick and easy procedure to clean cells from *in vitro* culture for electron microscopy and other work. We did not test to prove sterility, as it was not needed for our work, but we would not expect that the cultures be sterile. Tests are underway to further characterize the AF, to demonstrate its presence in other sera, and to expand the technique for production of cleaned *H. melelagridis* cells for other studies.

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Table 3.1. The aggregation of *Histomonas meleagridis* by sera of various sources and by 4 batches of horse serum.

Serum	Source of serum	Serum Conc.(%)	Aggregation
Chicken	Chickens infected with *H. meleagridis*	15 30	No No
	Uninfected chickens	15 30	No No
Turkey	Turkeys infected with *H. meleagridis*	15 30	No No
	Uninfected Turkeys	15 30	No No
Bovine	Life Technologies, Grand island, NY.	15 30	No No
Horse 1	Life Technologies, Grand Island, NY.	15 30	Yes Yes
Horse 2	Atlanta Biologicals, Norcross, GA.	15 30	Yes Yes
Horse 3	Pel-Freez Biologicals, Rogers, AR	15 30	Yes Yes
Horse 4	Sigma Chemical Co., St. Louis, MO.	15 30	Yes Yes

Table 3.2. The agglutinin factors in horse serum by successive precipitation method.

Protein [A] Conc (%)	Protein precipitated by indicated % of ammonium sulfate [B]					
	25%	33%	40%	50%	70%	Whole Serum
15	+ [C]	+	+	+	+	15
30	+	+	+	+	+	30

[A] Vol/vol of protein solution or whole serum mixed with eluted parasites.

[B] Proteins precipitated by 25-70% saturated ammonium sulfate were dialized overnight against PBS.

[C] *H. meleagridis* cells were aggregated (+) or not aggregated (-) by the proteins or serum.

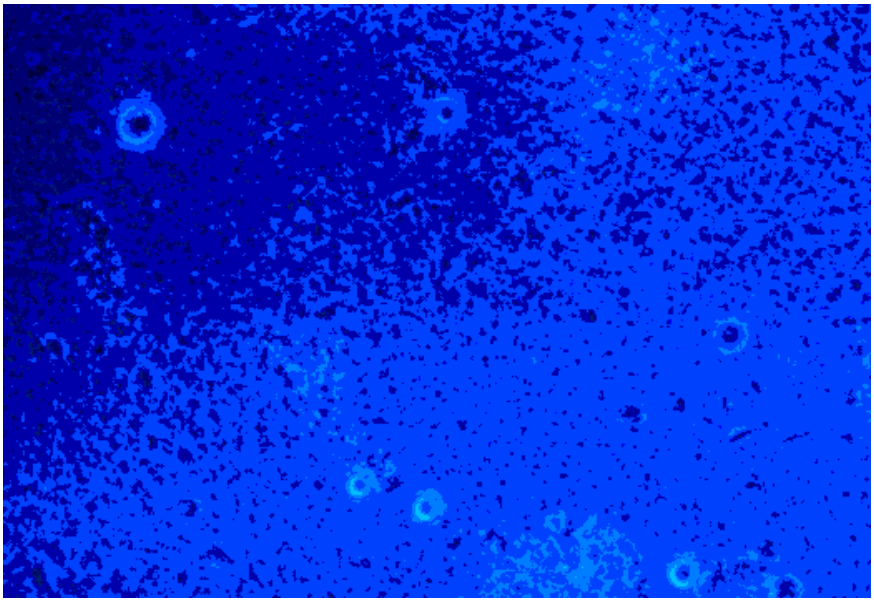

Figure 3.1. Culture of *Histomonas meleagridis in vitro*(250X).

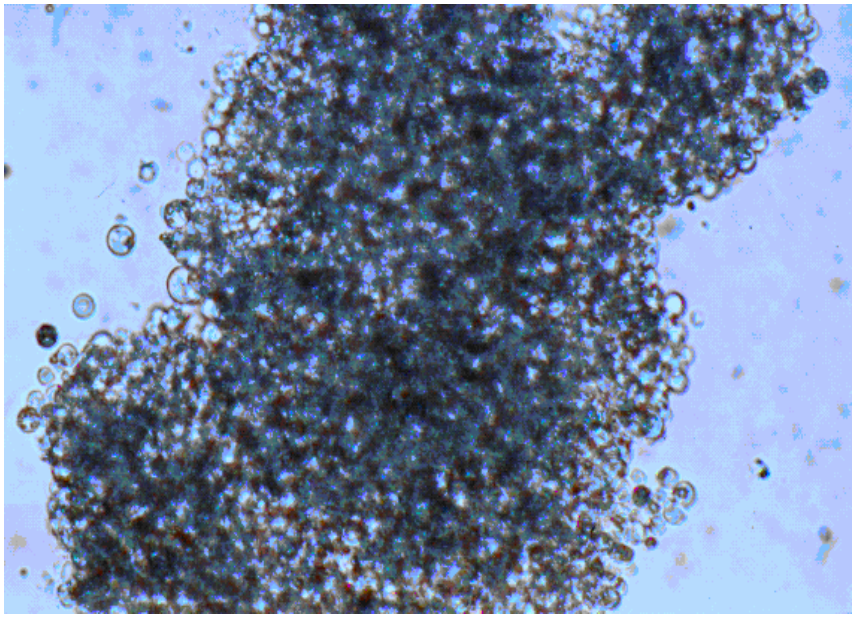

Figure 3.2. Clumping of histomonads when concentrations of horse serum are more than 15% (250X).

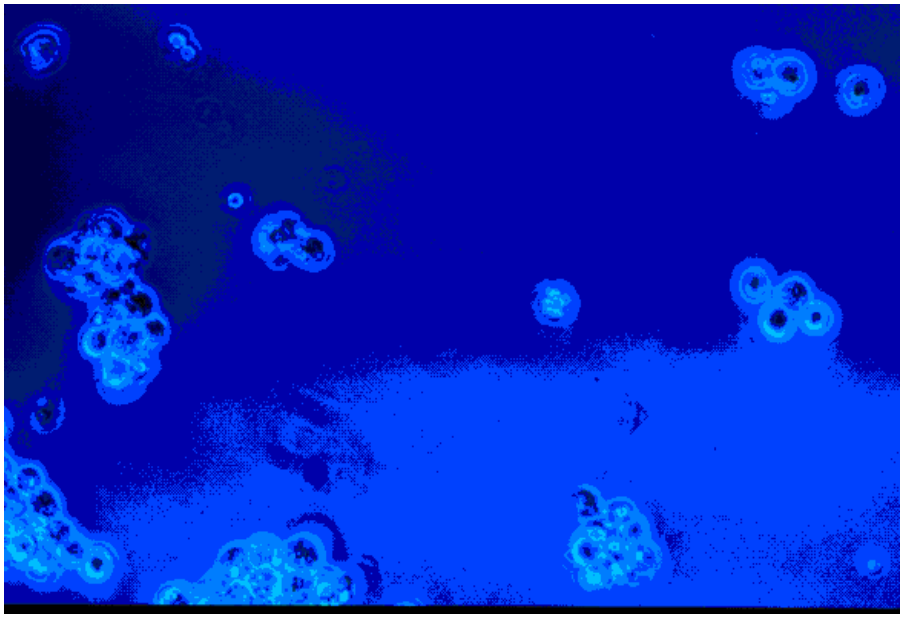

Figure 3.3. Purified histomonads (250X).

DIRECT LATERAL TRANSMISSION OF *HISTOMONAS MELEAGRIDIS* IN TURKEYS

[1]**Jinghui Hu and L. R. McDougald. 2002. Accepted by Avian Disease.**

1. Abstract

The lateral transmission of *H. meleagridis* in turkeys was studied in floor pens without the presence of *Heterakis gallinarum*. Battery-reared poults (120) were transferred at 2 wks of age to concrete-floored floor pens with fresh pine shavings litter (40/group). One group received no exposure. In other groups, either 10% or 25% of the birds were inoculated *per cloaca* with cultured *H. meleagridis* (200,000/bird), and placed in the pens as seeder birds. Inoculated birds died at 10-18 days postinfection (DPI) showing typical liver and cecal lesions of histomoniasis. Birds in the high exposure group (HE) died of histomoniasis beginning 16 DPI, and continuing to 100% mortality by day 23 DPI. Birds in the low-exposure group (LE) died beginning on day 19 DPI and continuing through day 31 DPI. All but one LE bird alive on day 31 DPI had severe liver and cecal lesions of histomoniasis at necropsy. There was no evidence of histomoniasis in unexposed birds. No cecal worms (*H. gallinarum*) were found at necropsy of dead birds or in unexposed birds at the end of the experiment. Even though *H. gallinarum* is the only known reservoir for *H. meleagridis*, these results suggest that lateral transmission of histomoniasis through a flock can occur readily through normal contact between uninfected birds and infected birds and their droppings in the total absence of cecal worms.

Key words: *Histomonas meleagridis*, turkeys, lateral transmission, histomoniasis, blackhead disease

2. Introduction

Early work with blackhead disease of turkeys identified the cecal worm (*Heterakis gallinarum*) as the main vector and reservoir of the causative protozoan *Histomonas meleagridis* [1, 2, 3, 10]. Survival of *H. meleagridis* in droppings is brief [11]. However, the infective protozoans are known to survive for long periods in cecal worm eggs, providing a reservoir of infection from one year to another [1]. Blackhead infections result from ingestion of embryonated cecal worm eggs or adult cecal worms, but not unembryonated worm eggs [9]. In some areas, earthworms may consume cecal worm eggs in soil and thus harbor the contained histomonads, transmitting the infection when the earthworm is consumed by a turkey [6].

The infectivity of the lumen form of *H. meleagridis* in droppings of infected birds was reportedly low [5] presumably because of the acidity of the crop and gizzard [4]. Lund [5] concluded that direct bird-to-bird transmission was unimportant *in birds that were well fed and cared for*. There is a general perception among scientists and poultry veterinarians that infections arise *only* from ingestion of cecal worm eggs, leading some to question whether other vectors may be involved where cecal worms cannot be demonstrated [8]. However, the exposure to cecal worm eggs cannot explain the rapid spread of histomoniasis in turkey flocks resulting in 50-90% mortality in a few weeks (H. L. Shivaprasaud, pers. comm. 2002; Karen Burns, pers. com., 2002). We conducted the present study to determine whether *H. meleagridis* could spread directly from infected bird to uninfected bird under practical conditions, in the absence of *Heterakis* worms or other potential vectors.

3. Materials and methods

Experimental Animals: Day-old turkey poults (Nicholas) were obtained from a commercial turkey hatchery and maintained until 2 weeks old in a steam-sterilized battery. Unmedicated feed and water were provided *ad libitum.*

Parasites: *Histomonas meleagridis* were isolated from ceca of infected turkeys and subcultured in modified Dwyer's medium [7]. Seeder birds were exposed by cloacal inoculation of 200,000 cells in 1 ml of culture medium via a blunt-tipped pipette. Exposed birds were returned to the pen to commingle with the others.

Floor Pen Facility and Management: Three pens of a concrete-floored building were used. No other animals were housed in the building during the experiment. The pens were approximately 2.4 x 3 m, and separated by floor to ceiling chain link fencing. Black plastic sheeting to a height of 1.2 m was used to further separate the pens. Fresh pine shaving litter was added to a depth of 10 cm. Doors were kept closed. The study was conducted in the winter, and the only arthropods observed were a few houseflies. The water supply (from chlorinated municipal water) had a pH of 6.5-7. Feed was unmedicated turkey starter ration formulated to meet National Research council (NRC) requirements. Pens were equipped with nipple waterers and tube-type manual feeders. After transfer to pens, the birds were given unmedicated feeder and water *ad libitum*.

Experimental design and procedures: The two-week old birds were divided into 3 groups of 40 birds. Pen 1 was unexposed. In Pen 2, 4 birds (10%) were inoculated with *H. meleagridis* (LE), and in Pen 3, 10 birds (25%) were inoculated with *H. meleagridis* (HE). The birds were observed twice daily for morbidity and mortality. During daily care, the UE pen was entered first, the LE pen second, and the HE pen last. Dead birds were examined for

presence of cecal or liver lesions of histomoniasis, and for any evidence of cecal worms. At termination of the study at 31 DPI, all surviving birds, including UE controls, were killed and examined for lesions of histomoniasis. Intestines were opened longitudinally and examined grossly for infection with cecal or intestinal worms. Five handfuls of litter from each pen were examined by scattering in a pan for any sign of arthropods.

4. Results and Discussion

The mortality of turkeys after introduction of *H. meleagridis* is shown in Table 4.1. No birds died or became sick in the UE controls. Death losses from histomoniasis began first with the inoculated birds in HE or LE pens. The inoculated seeder birds died 10-16 DPI. Uninoculated turkeys in the (HE) group died 16-23 DPI, and all were dead by day 23 DPI. Uninoculated turkeys in the LE group died from day 19 DPI to day 31 DPI. Yellow diarrhea was noted in the LE and HE pens. Morbidity became common in LE and HE pens as the infections progressed. Necropsy of the dead birds revealed lesions typical of histomoniasis in the liver and ceca. No worms were found in ceca or intestines of dead birds. The experiment was terminated on day 31 DPI because the surviving 11 LE birds suffered morbidity. At necropsy, 10 of the 11 survivors had typical liver lesions and cecal lesions of histomoniasis. Only one bird was normal. Examinations of the litter were negative for adults or larvae of beetles or other arthropods.

These results indicated that the rapid spread of *H. meleagridis* within a turkey flock was possible without the presence of cecal worms or other vectors. The birds in this study were

reared free from any exposure to worm eggs of any species, and without the arthropods or invertebrates frequently found in poultry houses (hister beetles, darkling beetles, earthworms, ants, and most flies). The only opportunity for the birds to become infected was from usual litter pecking and coprophagy, and possibly from contamination of the feed by infected pen-mates. The inoculum of *H. meleagridis* cultured *in vitro* contained only histomonads and bacteria. The absence of cecal worms or other potential vectors was confirmed by necropsy of death losses from histomoniasis, and necropsy of birds in the UE pen at the end of experiment. Birds in the UE pen were negative for lesions of histomoniasis, demonstrating the absence of vectors or reservoirs of infection in the experimental unit.

The feed and water were at no time interrupted during this study. Thus, previous observations that feed deprivation was necessary to allow infection with *H. meleagridis* [4,5] do not seem apropos. Even though histomonads do not survive more than a few minutes outside the bird (in droppings), and most would be killed by acid conditions in the upper digestive tract, it appears that sufficient numbers of organisms survive to precipitate infection after direct ingestion. Infection of birds by this means helps to explain the rapid dissemination of histomoniasis through commercial flocks.

When Norton *et al.* [8] were unable to find cecal worms associated with an outbreak of blackhead in turkeys, they suggested that some other vector must be responsible for spreading the infection through the flock. However, the present work suggests that the infection could easily and rapidly disseminate after focal introduction of *H. melelagridis* into the flock, not requiring all of the birds to ingest cecal worm eggs.

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Table 4.1. Mortality of turkeys after exposure to *Histomonas meleagridis* by infected seeder birds in the absence of cecal worms or other vectors. Birds were unexposed (UE), exposed to 10% seeders (LE), or exposed to 25% seeders (HE).

	UE	LE		HE	
DPI [A]	Uninoc [C]	Seeder [B]	Uninoc.[C].	Seeder [B]	Uninoc.[C]
10	0	1	0	2	0
11	0	0	0	2	0
12	0	0	0	1	0
13	0	0	0	1	0
14	0	1	0	3	0
16	0	0	0	1 [D]	3
17	0	0	0	-	9
18	0	2 [D]	0	-	3
19	0	-	2	-	8
20	0	-	7	-	3
21	0	-	2	-	1
22	0	-	2	-	0
23	0	-	7	-	3 [D]
25	0	-	2	-	-

27	0	-	1	-	-
28	0	-	2	-	-
31	0	-	10 [E]	-	-

[A] DPI: Days postinoculation.

[B] Mortality of seeder birds (inoculated *per* cloaca with 2.5×10^5 cells of *H. meleagridis*).

[C] Mortality of uninoculated birds.

[D] Denotes 100% mortality for group.

[E] Birds euthanized on DPI 31 because of morbidity; 9 of 10 had severe cecal and liver lesions of histomoniasis, and were included with mortality.

THE EFFICACY OF SOME DRUGS WITH KNOWN ANTIPROTOZOAL ACTIVITY AGAINST *HISTOMONAS MELEAGRIDIS* IN CHICKENS

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[1]**Jinghui Hu and L. R. McDougald. 2002. Submitted to Poultry Science.**

1. Abstract

Nine known antiprotozoal drugs were tested *in vitro* and *in vivo* for activity against *Histomonas meleagridis*. The nitroimidazoles dimetridazole, metronidazole, ornidazole, and tinidazole suppressed the growth of *H. meleagridis in vitro* at 10 ug/ml or higher. Paromomycin sulfate and carbadox were weakly effective within the range tested. Quinolinol, mebendazole, diloxanide furoate, and albendazole had no demonstrable efficacy *in vitro*. Drugs showing some activity were tested in young chickens inoculated with $2x10^5$ *H. meleagridis/*bird. Dimetridazole, metronidazole, ornidazole, and tinidazole were highly effective at 200 ppm in feed, preventing liver lesions and reducing cecal lesions to near zero. Paromomycin sulfate and carbadox were ineffective *in vivo*, with no improvement in liver or cecal lesion scores compared to that of infected controls.

Keywords: Histomonas meleagridis, chemotherapy, antihistomonal, antiprotozoal, dimetridazole, metronidazole, ornidazole, tinidazole, paromomycin, albendazole, mebendazole, quinolinol, carbadox.

2. Introduction

Several products were at one time registered for prevention or treatment of blackhead disease (histomoniasis) in the USA, including two arsenicals, two nitroimidazoles, and one nitrofuran (McDougald, 1997b). However, recent regulatory action has removed all but histostat (4-nitrophenyl-arsonic acid) from the market in the USA, leaving poultry producers with no products to treat outbreaks of this disease (McDougald, 1997b). Outbreaks of histomoniasis in chickens and turkeys continue to cause serious losses in young turkeys, and in broiler breeder or layer pullets (Homer and Butcher, 1991; McDougald, 1997a). Previous

attempts at protection against blackhead by immunization were largely unsuccessful, thus it is unlikely that vaccines will be developed (Lund, 1969). Management practices (segregation of turkeys from chickens, disinfection of litter and soil, prevention of worm infections, etc.) are important in preventing outbreaks, but overall have limited effectiveness. Thus, we considered it important to test new drugs identified as having antiprotozoal activity to identify possible candidates with antihistomonal activity.

Histomonas meleagridis, the causative agent of blackhead, is closely akin to other amebas and flagellates (Smith, 1895, Tyzzer, 1919, 1920). Thus, compounds active against *Entamoeba* or *Trichomonas* could be expected to have activity against *H. meleagridis*. From the literature, we selected tinidazole, metronidazole (Bhopale *et al.*, 1995), ornidazole (Leimer *et al*., 1980), and diloxanide furoate (McAuley *et al*., 1992) as candidates. Some of the benzimidazole anthelminthics also have antiprotozoal activity, including albendazole and mebendazole (Katiyar *et al*., 1994). Paromomycin sulfate 5,7-diiodo-8-hydroxy-Quinoline and carbadox are antibiotics or antibacterials with reported antiprotozoal activity (Barr *et al*. ,1994; Fournet *et al*., 1994). Dimetridazole, which was long used as the drug of choice for treating blackhead outbreaks (McDougald, 1997b), was used as a postitive control in all studies.

3. Materials and Methods

Drugs used: Dimetridazole, metronidazole, ornidazole, tinidazole, paromomycin sulfate, mebendazole, albendazole, Diloxanide furoate, and 5,7-diiodo-8-hydroxy-Quinoline were purchased commercially (Sigma-Aldrich, St. Louis, MO). Carbadox was supplied by Phibro (New York, NY).

3.1 Experiments i*n vitro*

Histomonas meleagridis were cultured in modified Dwyer's Medium (Medium 199 in HBSS, 85%; Chicken embryo extract, 5%; Horse serum, 10%; rice powder, 15 mg; pH 7.2) (McDougald, 1973). The drugs were dissolved/suspended in 0.5 ml DMF or water, then M199 was added for a concentration of 1000 μg/ml. Working dilutions of 100, 10, 1, 0.1, and 0.01 μg/ml were prepared in culture flasks, each replicated in two flasks, with 9.5 ml vol. Flasks were inoculated with 0.5 ml of seed culture containing approximately 2.5x 10^5 of *H. meleagridis*, and incubated at 40 C for 2 days. Parasites were counted by inverted phase microscopy.

3.2. Experiments i*n vivo*

Candidate compounds with significant activity *in vitro* were tested in chickens using a previously established infection model (McDougald and Hu, 2001). Infective histomonads were cultured *in vitro* as described above. Two-week-old broiler chickens were exposed by intracloacal inoculation of 2 x 10^5 *H. meleagridis*. Each bird also received 1x10^4 oocysts of cecal coccidia (*Eimeria tenella)*. The compounds tested *in vivo* were dimetridazole, metronidazole, ornidazole, tinidazole and paromomycin sulfate given in the feed at 200 or 400 ppm beginning one day before inoculation. Each treatment was replicated in 3 replicates of 10 birds each. The test was terminated 13 days postinoculation (DPI). Body wts of birds before infection and at 13 DPI were recorded by pen unit. The liver and cecal lesions of histomoniasis were scored at necropsy as previously described (McDougald and Hu, 2001).

Statistic analysis. The data were processed with General Linear Model Program of SAS. The weight gains, liver lesion scores and cecal lesion scores were analyzed by ANOVA and Duncan's multiple range test for difference between means.

4. Results and Discussion

4.1. Experiments i*n vitro*

All of the tested nitroimidazoles (dimetridazole, ornidazole, tinidazole, and metronidazole) were highly active *in vitro* (Table 5.1) as shown by near complete suppression of growth of *H. meleagridis* at 10 ug/ml or higher. Concentrations of 1 μg/ml or less were ineffective. Paromomycin sulfate reduced the growth of parasites at 10 μg/ml, and was completely effective at 100 μg/ml. Mebendazole, albendazole, Diloxanide furoate, and 5,7-diiodo-8-hydroxy-Quinoline reduced growth of *H. meleagridis* at 100 μg/ml. Carbadox reduced growth of histomonads at 100 but not 10 μg/ml. DMF had no effect on the growth of *H. meleagridis*, but the use of DMF as a dissolving/suspending agent appeared to affect observed endpoints with some compounds.

4.2. Experiments *in vivo*

Average wt gain of infected controls was depressed in comparison with uninfected controls (Table 5.2). Treatment with Dimetridazole, metronidazole, ornidazole, tinidazole at 200 or 400 ppm improved wt gain relative to the infected control. Paromomycin sulfate treatment provided no improvement in gain. The higher level (400 ppm) of tinidazole and metronidazole, but not ornidazole, appeared to depress weights, possibly a sign of toxicity. Liver scores were nil with dimetridazole, metronidazole, ornidazole, or tinidazole. The cecal

lesion scores were also nil or nearly nil with these compounds. Paromomycin sulfate was inactive, with no reduction in liver or cecal lesion scores. Carbadox did not reduce liver or cecal lesions of histomoniasis at 50 or 100 ppm.

These results indicated that dimetridazole, metronidazole, ornidazole, tinidazole were highly effective in control of *H. meleagridis* in chickens, but paromomycin sulfate and carbadox were ineffective at the tested levels.

Tests of compounds *in vitro* are probably reliable indicators of activity for selection of candidates to test in animals. However, some compounds are active only after metabolism to another form in the animal and would be missed with this system.

These results suggest that nitroimidazoles active against *Entamoeba* are also active against *Histomonas*. However, compounds of other classes were inactive.

Benzamidazole anthelminthic compounds are sometimes used for prevention of blackhead disease in chickens or turkeys (Hegngi *et al*., 1999). However, these compounds are not effective against the causative organism *H. meleagridis* and are believed to work by killing the cecal worm (*Heterakis gallinarum*) which carries the infection into cecal tissues. Paromomycin reportedly has activity against a variety of protozoans, including *H. meleagridis* (Lindquist, 1962) but had little activity against *H. meleagridis* in this study. The earlier study used high levels of paromomycin in feed (1000-2000 ppm), much higher than that used here (200-400 ppm) and higher than would be practical under commercial conditions. The authors speculated that paromomycin might be working by reduce the presence of bacteria necessary for pathogenicity of *H. meleagridis,* since they found no improvement in efficacy with increased dosage. However, recent work suggests that histomoniasis could not be controlled by antibiotic activity alone (Hu *et al*., 2002).

We found no useful activity of diloxanide furoate, carbadox or the quinoline compound. Clearly, the results of testing with *Entamoeba* and other organisms can in some cases be used as leads for activity against *H. meleagridis,* but in other cases is not a good indicator.

The poultry industry is in dire need of products for treatment or prevention of histomoniasis in chickens and turkeys to replace dimetridazole and ipronidazole, recently de-listed by the FDA. Considerable activity can be found among other antiprotozoal compounds, but unfortunately the best ones are also nitroimidazoles. Other tested antiprotozoal compounds did not possess useful activity against *H. meleagridis*.

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Table 5.1. The effect of drugs on growth of *Histomonas meleagridis in vitro* with or without Dimethyl-formamide (DMF).

Drugs	DMF	100 ppm	10 ppm	1 ppm	0.1 ppm	0.01 ppm
Dimetridazole	Yes	0	0	1.20	12.83	8.46
	No	0	0	1.98	39.11	83.17
Ornidazole	Yes	0	0	6.17	73.46	86.96
	No	0	0	1.29	73.39	89.48
Tinidazole	Yes	0	0	6.59	25.75	65.27
	No	0	0	69.74	85.41	94.64
Metronidazole	Yes	0	0	24.94	37.99	68.88
	No	0	0	35.19	69.10	93.57
Paromomycin	Yes	17.39	17.39	42.56	33.18	38.67
	No	0	1.49	71.78	91.58	75.74
Mebendazole	Yes	40.72	36.93	40.92	44.91	43.71
	No	73.13	44.55	55.45	56.93	50.50
Diloxanide	Yes	21.36	36.13	33.33	31.34	34.33
furoate	No	28.98	79.62	78.66	83.44	75.80
Albendazole	Yes	18.99	21.05	47.60	46.00	85.35
Quinolinol	Yes	10.53	19.91	27.92	40.50	72.54
Carbadox	No	5.0	95.0	80.0	85.0	n.d

Table 5.2. The result of some drugs on control of *Histomonas meleagridis* in chickens

Trt	Drugs/ppm	*E. tenella* 1 x10^4/bird	*Histomonas* 2x10^5 /bird	Wt Gain (Gram)	Liver Scores [A]	Cecal Scores [B]
1	None	-	-	808.4 bc	0 c	0 c
2	None	+	+	749.1 d	1.31 ab	3.03 b
3	Dimet/200	+	+	829.9 ab	0 c	0 c
4	Orni/200	+	+	817.2 b	0 c	0.2 c
5	Orni/400	+	+	801.5 bcd	0 c	0 c
6	Tini/200	+	+	876.7 a	0 c	0 c
7	Tini/400	+	+	817.1 b	0 c	0 c
8	Metro/200	+	+	881.7 a	0 c	0.43 c
9	Metro/400	+	+	812.8 b	0 c	0.13 c
10	Parom/200	+	+	749.7 cd	1.55 a	3.77 a
11	Parom/400	+	+	774.5 bcd	0.97 b	3.17 b
12	Carbadox 100	+	+	602 d	0.50 b	3.67 a

EFFECT OF ANTICOCCIDIALS AND ANTIBIOTICS ON THE CONTROL OF BLACKHEAD DISEASE IN BROILER BREEDER PULLETS

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1. Abstract

Broiler chicks inoculated with both *Histomonas* and cecal coccidia developed moderately severe blackhead disease. Antibiotics tested at normal feed or water additive levels had little effect on *Histomonas* lesions or weight gains. Bacitracin at 100, 200, or 300 g/ton reduced liver lesion scores somewhat ($p<0.05$), but had no other positive effects. Apramycin at 300 ppm in water reduced liver lesion scores somewhat ($p<0.05$), but did not improve cecal lesions or weight gains. Penicillin (100 ppm), chlortetracycline (100 ppm), tylosin (110 ppm), and sarafloxacin (40 ppm), in water gave no improvement in liver or cecal blackhead lesions. Weight gains were improved relative to infected controls with penicillin, tylosin, or sarafloxacin treatment ($p<0.05$).

Five anticoccidials (salinomycin, diclazuril, nicarbazin, roxarsone, and lasalocid) were tested at common use levels in two trials. Results were similar in both trials: Liver lesion scores in the nicarbazin treatment were reduced ($p<0.05$) in comparison with controls and other medicated groups, and the number of birds positive for liver lesions was lower ($p<0.05$). Otherwise, anticoccidials had no effect on liver or cecal lesion scores or weight gains. Control of coccidiosis by the anticoccidials (as shown by oocyst counts) varied from product to product, but was not correlated with severity of blackhead lesions. These results suggest that the effect of cecal coccidia on susceptibility of chickens to *H. meleagridis* is not a simple function of mechanical damage to the cecal mucosa.

2. Introduction

Histomoniasis (blackhead disease) can cause 90-100% mortality in turkeys, but is relatively mild in chickens [1,2]. However, recent outbreaks in broiler breeder pullets and layer pullets in the Southeastern USA were severe, creating a significant deterrent to the production of broiler hatching eggs and table eggs [3,4]. Outbreaks of blackhead in breeder and layer pullets are characterized by extensive mortality, morbidity, poor uniformity, and reduced potential for egg production. Morbidity and mortality from blackhead depends on *H. meleagridis* spreading from the ceca to the liver, eventually causing destruction of that organ. More typically, chickens suffer little liver involvement and the infection often passes without notice. Chickens or turkeys become infected with *H. meleagridis* by swallowing eggs of the cecal worm (*Heterakis gallinarum).* Cecal worms are widespread in poultry houses, providing almost universal exposure of chickens. However, the reasons for the present increase in clinical cases of histomoniasis in chickens are not clear. This increase in clinical blackhead outbreaks comes only a few years after FDA's action barring the use of nitroimidazole products (ipronidazole and dimetridazole) in the USA, leaving poultry producers with no effective treatment.

We recently demonstrated that concurrent exposure to cecal coccidia (*Eimeria tenella)* and *H. meleagridis* increased the severity of liver lesions caused by *H. meleagridis* in chickens [5]. The mechanism for such interaction was not clear. Possible interactions might include physical damage to the mucosa, a change in cecal microflora, and other physiological or immunological factors. Blackhead disease is dependent upon the cecal microflora for full pathogenicity [6,7].

Previously established interactions between *H. meleagridis*, bacteria, and coccidia [5], suggest that the strategic use of anticoccidials and antibiotics could offer some relief in outbreaks of blackhead. Because of misinformation and misplaced expectations, we felt that it was important to test commonly used feed and water medications available to the poultry industry, to determine whether any of these products had any significant effect on blackhead disease. These products are not known to have significant antihistomonal activity, but might have some indirect influence because of the complex relationship between *H. meleagridis* and its host.

3. Materials and Methods

Parasites and Infections: Blackhead Parasites**:** A culture of *H. meleagridis* was isolated from a field outbreak in commercial layer pullets in North Georgia in 1998. This isolate was frozen in liquid nitrogen and retrieved for use by culturing in modified Dwyer's medium [8]. This medium consists of Medium 199, horse serum, chick embryo extract, and rice powder. Thereafter, fresh cultures were isolated from young turkeys before each series of experiments, to ensure maintenance of pathogenicity. Fresh cultures were pooled and counted using a hemacytometer, and diluted to final working concentration in Medium 199. The calculated dosage of *H. meleagridis* was inoculated into chickens *per* cloaca, using a hypodermic syringe fitted with a blunt-tipped plastic pipette tip. Past experience suggested that 10-15% of the birds do not become infected after inoculation in this way, probably because the inoculum is voided by some birds. Liver and cecal lesions caused by *H. meleagridis* were scored and recorded at necropsy, as previously described [5]. On this scale, a score of 0 = normal, 1= minimal or barely detectable lesions, 2 = scattered but easily

detectable lesions, 3 = moderately severe lesions involving much of the organ, and 4 = severe lesions involving the entire organ.

Cecal Coccidia: A field isolate of *E. tenella* (Athens, GA, 1997) was prepared for use as previously described [2]. The oocysts were inoculated *per os* in 1 ml of water using a 10 ml syringe equipped with a plastic pipette tip. Our model, developed in previous experiments [5], consisted of oral gavage with 10,000 oocysts of *E. tenella* on the same day as intracloacloacal inoculation with 2.5 x 10^5 cells of *H. melelagridis*. In some experiments, a separate infected control was maintained, with only *H. meleagridis* inoculation.

3.1 Experimental Animals, Test Medicaments, and Experimental Design

Experimental Animals: Broiler chicks (Peterson x Arbor Acres) were hatched from breeder flocks maintained at the UGA poultry research farm and used straight run. No vaccinations were given. Unmedicated feed and water were given *ad libitum*. Chicks were housed in steamed-cleaned, stainless steel cages until assignment to treatments at 2 weeks old. Thereafter, birds were housed in cages with stainless steel droppings trays, feeders and waterers (Petersime).

Feed Additives and water medication: Commercial premixes of anticoccidials and antibiotics were used to prepare feeds with diclazuril, 1 ppm; lasalocid, 90 ppm; nicarbazin, 125 ppm; roxarsone, 50 ppm; salinomycin, 60 ppm; bacitracin, 50, 100, 200, or 300 ppm. Apramycin wetable powder was mixed into tap water to produce 75, 150, or 300 ppm. Penicillin, chlortetracycline, tylosin or sarafloxacin were given in the drinking water at 100, 100, 110, or 40 ppm, respectively.

Experiment I. Effect of bacitracin on blackhead disease in chickens: Four levels of bacitracin (50, 100, 200, and 300 g/ton respectively) were tested in 3 replicates of 10 birds

each. Controls were unmedicated, uninfected (UUC), and unmedicated, infected with both parasites (IIUC). Medicated feeds were given where indicated, from 14 days of age through termination when birds were 28 days old. The birds were inoculated with *H. meleagridis* and *E. tenella* at 15 days of age. Pen weights were recorded at inoculation and at necropsy at 28 days of age. Liver and cecal lesions were scored and recorded at necropsy [5].

Experiment II. The Effect of apramycin on blackhead disease in chickens. The experiment included 5 treatments with 3 replicates of 10 birds each. The concentrations of apramycin in water were 75, 150, and 300 g/ton, respectively. Controls were UUC and IIUC. Medicated water was given from 14 to 28 days of age. The birds were inoculated with *H. meleagridis* and/or *E. tenella* at 16 days of age. Pen weights were recorded on days 16 and 28. Liver and cecal lesions were scored and recorded at necropsy.

Experiment III. The effect of antibiotics on blackhead disease in chickens. This test included 7 treatments with 3 replicates of 10 birds each. Antibiotics administered in water were penicillin, 100 ppm; chlortetracycline, 100 ppm; tylosin, 100 ppm; and sarafloxacin, 40 ppm. Controls included UUC, unmedicated, inoculated with *H. meleagridis* only (IUC), and inoculated with *Histomonas* + *E. tenella* (IIUC). Medicated water was given from 14 to 28 days of age. Birds were inoculated with at 15 days of age. Pen weights were recorded at inoculation and at termination. Liver and cecal lesions were scored and recorded at necropsy on day 28.

Experiment IV. The effect of anticoccidials on blackhead disease in chickens. Two identical trials were conducted to evaluate effects of anticoccidials on histomoniasis. Eight treatments included diclazuril, 1.0 ppm, salinomycin, 60 g/ton, lasalocid, 90 g/ton, nicarbazin, 0.0125%, and roxarsone, 45.4 g/ton. Controls included UUC, IUC, and IIUC.

Each treatment was replicated in 3 groups of 10 birds each in two identical trials. Medicated feeds were given from 14 days of age through termination when birds were 28 days old. The birds were inoculated with *H. meleagridis* and *E. tenella* at 15 days of age. Pen weights were recorded at inoculation and at necropsy at 28 days of age. Liver and cecal lesions were scored and recorded at necropsy. In the second trial only, fecal samples were collected from each pen on DPI 6-8, and oocysts of *E. tenella* were counted in aliquots using a McMaster chamber.

3.2. Statistical analysis

Weight gains and lesion scores were analyzed by ANOVA for differences at $P<0.05$, followed by Duncan's multiple range test to separate means, using the general Linear Model program on SAS.

4. Results and Discussion

4.1. The effects of bacitracin on H. *meleagridis* in broiler chicks

Severe cecal lesions of blackhead developed in all birds exposed to *H. meleagridis* (Table 6.1). Weight gains were significantly depressed in all the infected birds ($p<0.05$) compared to UUC, and were not improved by bacitracin treatment. Liver lesion scores were significantly reduced in groups treated with100, 200, 300 g/ton of bacitracin, but not in birds treated with 50 gram/ton of bacitracin. However, cecal lesion scores and the number of birds with liver lesion were unchanged in treated birds, in comparison with the infected control.

4.2. The effects of apramycin on H. *meleagridis* in broiler chicks

Weight gains were depressed in comparison with the UUC ($P<0.05$) in all *H. meleagridis*-infected groups, regardless of treatment (Table 6.2). Cecal lesion scores were

numerically reduced at high concentrations of apramycin. Liver lesion scores were significantly reduced by treatment with 300 ppm of apramycin, but not with other treatment levels.

4.3. The effects of other antibiotics on H. *meleagridis* in broiler chicks

None of the tested antibiotics (penicillin, chlortetracycline, tylosin, or sarafloxacin) was effective in reduction of liver or cecal lesions caused by blackhead (Table 6.3). However, three products (penicillin, tylosin, or sarafloxacin) improved weight gains ($p<0.05$), in comparison with IIUC (Treatment 3).

4.4. The effect of anticoccidials on *H. meleagridis* in broiler chicks

In Trial 1, weight gains were significantly ($p<0.05$) depressed in all *Histomonas*-infected groups relative to the UUC, regardless of treatment (Table 6.4). Liver lesions in the nicarbazin treatment were significantly reduced ($p<0.05$), compared with the IIUC, and the number of birds positive for liver lesions was significantly lower. There was no change in average liver lesion scores in other medicated groups compared to the IIUC. Cecal lesion scores caused by blackhead were not reduced by any drug treatment. Oocysts produced were not counted in Trial 1.

In Trial 2, weight gains were significantly ($P<0.05$) depressed in all blackhead-infected birds in comparison with the UUC (Table 6.5). As in the first trial, liver lesions were reduced by nicarbazin treatment ($p<0.05$), in comparison with the IIUC, but not in comparison with other drug treatments. Cecal lesions caused by blackhead were not reduced by anticoccidial treatment. Even though coccidial oocyst counts were greatly reduced by diclazuril, salinomycin, roxarsone, and nicarbazin, only nicarbazin appeared to have an effect on the number and severity of blackhead lesions in the liver.

Synergism of blackhead by Coccidia: Liver lesions caused by *H. meleagridis* were significantly more severe in control birds inoculated with both *H. meleagridis* and *E. tenella* (IIUC), as compared with those receiving only *H. meleagridis* (IUC)(Tables 6.3, 6.4, 6.5). This confirms earlier observations on the synergism of these infections [5].

Although blackhead disease is not new in chicken flocks, and once accounted for up to 5.5% of annual diagnostic cases in some laboratories [9], clinical outbreaks in chickens in the USA and other countries have increased significantly in the past several years [3,4,10]. The reasons for this increase are not understood, but could include depressed immune function, a change in the adaptation of *H. meleagridis* to chickens, or a change in the virulence of *H. meleagridis* [5]. These infectious protozoans are carried inside the eggs of the cecal worm (*Heterakis gallinarum)*, and may lie dormant for many years [1,11]. We believe that most commercial chicken facilities are contaminated with these worms, and thus, with *H. meleagridis* [12]. Therefore, the reason for increases in clinical outbreaks in chickens, with classical liver and cecal involvement, high mortality, and morbidity, are unlikely to be a result of more widespread exposure.

Antibiotics might be expected to help control blackhead disease because of indirect effects. Bacteria are required for the full pathogenicity of *H. meleagridis* in chickens and turkeys, as shown by studies in bacteria-free and contaminated birds [6,7]. A combination of *Escherichia coli*, *Pseudomonas aeruginosa, Streptococcus,* and *Clostridium perfringens* was most successful and consistent in providing the necessary conditions for development of blackhead disease in gnotobiotic chickens [7]. The interaction of bacteria and *H. meleagridis* is less complicated in turkeys, where contamination of gnotobiotic birds with *E. coli* alone is sufficient to support development of blackhead disease [6]. The results of our studies suggest

that while some of the tested products partly reduced the liver lesion scores or improved gain when used at high levels, none was curative for blackhead disease. These studies suggest that the tested antibiotics did not adequately control the bacterial species required for pathogenicity of *H. meleagridis*. In future work, antibiotics could be administered from day one, as a means of further altering the bacterial flora.

Anticoccidials highly effective against *E. tenella* could be expected to aid in prevention of blackhead disease because of the demonstrated interaction between *E. tenella* and *H. meleagridis* [5] and between *E.adenoeides* and *H. meleagridis* in turkeys [7]. Most anticoccidials are known to allow some development of coccidia, even with sensitive laboratory strains. An exception to this is diclazuril, which under laboratory conditions can completely prevent development of *E. tenella* [13]. Thus, it was surprising that diclazuril had no effect on development of blackhead in two trials, even though it was shown in Trial 2 that no oocysts were produced in infected birds. Nicarbazin, the only product showing an effect on the liver lesions in both trials, was also highly effective against coccidia, but allowed some oocysts to be produced. It is possible that nicarbazin has some innate antihistomonal activity because of its chemical similarities to known antihistomonal compounds (nitro-phenolic ring structure), but this has not been recognized in previous tests. Thus, the mechanism for nicarbazin's interference with the synergistic effects of coccidia on blackhead remains unclear.

Roxarsone is used in broilers in combination with other anticoccidials as an aid in controlling *E. tenella* [14], or in combination with an anticoccidial vaccine [15]. Because of its chemical similarity to other organic arsenic products with known antihistomonal activity, roxarsone is sometimes given in the water during blackhead outbreaks in chickens. In this

study, we saw no evidence that roxarsone was of benefit in prevention of blackhead at the normal feed additive level of 45 g/ton.

At present, no products are approved for treatment of blackhead infections in chickens or turkeys. Only nitarsone (4-nitrophenylarsonic acid, 0.0175%) is used in feed for prevention of blackhead. Exposure of chickens to the cecal worm vector (*Heterakis gallinarum*) can be reduced by frequent worming with effective products [12].

5. Conclusions and Applications:

1) Some of the tested antibiotics had beneficial effects on weight gains in infected chickens. However, none was effective in reducing the lesions or other pathology of *H. meleagridis* at FDA-approved levels.
2) High levels of some products (300 g/ton of bacitracin or 300 ppm of apramycin) reduced liver lesion scores, but had no other positive benefits.
3) Lesions and other signs of blackhead were not reduced by most anticoccidials, regardless of the effectiveness of control of cecal coccidia.
4) Nicarbazin (0.0125% in feed) gave significant reduction in liver lesion scores in two trials. The reason for this improvement was not clear.

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Table 6.1. Effect of bacitracin on blackhead disease in broiler-type chickens inoculated with *Histomonas meleagridis* and *Eimeria tenella.*

Treatment	Bacitracin (g/ton) [A]	Infect.[B]	Av Weight gain (g)	Average Liver lesion[C]	Ave Cecal Lesion[C]
1	0	--	764.8 [a]	0 [c]	0 [c]
2	0	HC	615.9 [b]	0.93 [a]	3.66 [ab]
3	50	HC	604.2 [b]	0.51 [ab]	3.44 [b]
4	100	HC	621.7 [b]	0.34 [bc]	3.76 [a]
5	200	HC	647.1 [b]	0.31 [bc]	3.71 [a]
6	300	HC	635.9 [b]	0.44 [bc]	3.69 [a]

[A] Bacitracin methylene disalicylate mixed into the feed and given continuously from one day prior to infection until termination.

[B] The infection model consisted of 10^4 oocysts of *E. tenella* (given by oral gavage) and 2.5 x 10^5 cultured cells of *H. meleagridis* (by intracloacal inoculation) given on the same day. H= *H. meleagridis*, C= Coccidia (*E. tenella*)

[C] Lesions of histomoniasis, scored on a scale of 0-4 at necropsy on DPI 13-14 as previously published [5]. Means with similar superscript letters are not significantly different ($p<0.05$).

Table 6.2. The effect of apramycin on blackhead disease in broiler-type chicks inoculated with *Histomonas meleagridis* and *Eimeria tenella.*

Treatment	Infection[A]	Apramycin[B] ppm	Weight gain (g)	Average Liver Lesions [C]	Average Cecal Lesions [C]
1	----	0	857.5 [a]	0.00 [c]	0.00 [b]
2	HC	0	769.6 [b]	1.37 [a]	3.74 [a]
3	HC	75	702.8 [c]	0.77 [ab]	3.63 [a]
4	HC	150	786.8 [b]	1.18 [ab]	3.56 [a]
5	HC	300	701.0 [c]	0.47 [bc]	3.40 [a]

[A] The infection model consisted of 10^4 oocysts of *E. tenella* given by oral gavage (C) and 2.5 x 10^5 cultured cells of *H. meleagridis* by intracloacal inoculation (H) given on the same day.

[B] Apramycin mixed into the water and given continuously from one day pre-infection.

[C] Lesions of histomoniasis, scored on a scale of 0-4 at necropsy on DPI 13-14 as previously published [5]. Means with similar superscript letters are not significantly different ($p<0.05$).

Table 6.3. Effects of antibiotics on blackhead disease in broiler-type chicks inoculated with *Histomoniasis meleagridis* and *Eimeria tenella*.

Treatment	Infection[A]	Antibiotics in water [B]	Wt gain (g)	Avg. Liver Lesions [C]	Avg. Cecal Lesions [C]
1	--	None	715.8 a	0.00 b	0.00 c
2	H--	None	609.3 bc	0.58 a	3.67 ab
3	HC	None	586.8 c	0.82 a	3.63 ab
4	HC	Penicillin 100 ppm	648.5 b	0.78 a	3.44 ab
5	HC	Chlortetracycline 100 ppm	617.9 bc	1.14 a	3.68 ab
6	HC	Tylosin 110 ppm	658.2 b	0.91 a	3.90 a
7	HC	Sarafloxacin 40 ppm	655.8 b	1.06 a	3.21 b

[A] The infection model consisted of 2.5 x 10^5 cultured cells of *H. meleagridis* (H) by intracloacal inoculation and/or 10^4 oocysts of *E. tenella* given by oral gavage (C) given on the same day.

[B] Antibiotics mixed into the water and given continuously from one day preinfection.

[C] Lesions of histomoniasis, scored on a scale of 0-4 at necropsy on DPI 13-14 as previously published [5]. Means with similar superscript letters are not significantly different ($p<0.05$).

Table 6.4. The effect of anticoccidials on blackhead disease after inoculation of chickens with *H. meleagridis* and *E. tenella* (trial 1).

Treatment	Infection [A]	Anticoccidial [B]	Wt gain (g)	Liver Lesion [C]	Cecal Lesion [C]
1	--	None	701.5 [a]	0.00 [d]	0.00 [c]
2	--H	None	640.8 [b]	0.18 [cd]	3.59 [ab]
3	CH	None	616.7 [bc]	0.84 [a]	3.63 [ab]
4	CH	Diclazuril 1.0 ppm	557.1 [de]	0.78 [ab]	3.74 [ab]
5	CH	Salinomycin 60 g/ton	513.8 [e]	0.54 [ab]	3.78 [a]
6	CH	Roxarsone 45.4 g/ton	577.4 [cd]	0.61 [ab]	3.43 [b]
7	CH	Lasalocid 90 g/ton	593.8 [bcd]	0.68 [ab]	3.86 [a]
8	CH	Nicarbazin 125 ppm	523.7 [e]	0.48 [bc]	3.52 [ab]

[A] The infection model consisted of 2.5 x 10^5 cultured cells of *H. meleagridis* given by intracloacal inoculation (H) and/or 10^4 oocysts of *E. tenella* given by oral gavage (C) given on the same day.

[B] Anticoccidials used as commercial premixes mixed into the feed and given continuously.

[C] Lesions of histomoniasis, scored on a scale of 0-4 at necropsy on DPI 13-14, as previously published [5]. Means with similar superscripts are not significantly different ($p<0.05$).

Table 6.5. The effect of anticoccidials on blackhead disease after inoculation of chickens with *H. meleagridis* and *E. tenella* (Trial 2).

Treatment	Infection [1]	Anticocci-dial [2]	Wt gain (g)	Oocyst ($x10^6$)	Liver Lesion [3]	Cecal Lesion [3]
1	--	None	701.5 [a]	ND	0.00 [d]	0.00 [c]
2	--H	None	640.8 [b]	ND	0.18 [cd]	3.59 [ab]
3	CH	None	616.7 [bc]	ND	0.84 [a]	3.63 [ab]
4	CH	Diclazuril 1.0 ppm	557.1 [de]	0	0.78 [ab]	3.74 [ab]
5	CH	Salinomycin 60 g/ton	513.8 [e]	21	0.54 [ab]	3.78 [a]
6	CH	Roxarsone 45.4 g/ton	577.4 [cd]	285	0.61 [ab]	3.43 [b]
7	CH	Lasalocid 90 g/ton	593.8 [bcd]	44	0.68 [ab]	3.86 [a]
8	CH	Nicarbazin 0.0125%	523.7 [e]	4	0.48 [bc]	3.52 [ab]

[1] The infection model consisted of "C" (10^4 oocysts of *E. tenella* given by oral gavage) and/or "H" (2.5×10^5 cultured cells of *H. meleagridis* by intracloacal inoculation) given on the same day.

[2] Anticoccidials as commercial premixes mixed into the feed and given continuously.

[3] Lesions of histomoniasis, scored on scale of 0-4 at necropsy on DPI 13-14 as previously published [5]. Means with similar superscript letters are not significantly different ($p<0.05$).

BLACKHEAD DISEASE (*HISTOMONAS MELEAGRIDIS*) AGGRAVATED IN BROILER CHICKENS BY CONCURRENT INFECTION WITH CECAL COCCIDIOSIS (*EIMERIA TENELLA*)

L. R. McDougald and Jinghui Hu. 2001. Avian Dis. 45:307-312.

1. Abstract

The effect of concurrent cecal coccidiosis infections on severity of *Histomonas meleagridis* (blackhead disease) in chickens was investigated in a series of experiments. Cecal lesions from *H. meleagridis* were severe in all inoculated control groups and did not appear to be affected by the introduction of *Eimeria tenella* infections. However, the severity of liver lesions and number of birds positive for liver lesions of *H. meleagridis* increased significantly with the presence of *E. tenella*. The increase was similar when 10^3 or 10^4 oocysts of *E. tenella* were given, and was the same when oocysts were given at the same time as *H. meleagridis* or 4 days prior. The liver lesions increased directly as doses of *H. meleagridis* increased from 7.5x 10^3 cells to 30, 100 or 300 x 10^3, respectively, when *E. tenella* were given along with *H. melelagridis,* but not when *H. meleagridis* were given alone. Administration of a 3x dose of live coccidiosis vaccine, containing very low levels of *E. tenella,* gave a small but significant boost to liver lesions, but at a much lower level than that observed with larger doses of *E. tenella*.

The positive relationship between infections of cecal coccidiosis and *H. meleagridis* in chickens suggests that such dual exposure may contribute to increased clinical outbreaks of blackhead disease in chickens under field conditions.

2. Introduction

The flagellate *Histomonas meleagridis* is well known among parasitologists and poultry veterinarians for its spectacular pathogenicity and virulence in turkeys, causing a disease known as blackhead (6). Infectious cells carried inside eggs of the cecal worm *Heterakis gallinarum* start in the ceca and spread to the liver, then birds may die from hepatic failure.

Other gallinacious birds, particularly chickens, serve as carriers because they harbor the cecal worm, but rarely suffer the potentially fatal liver involvement with *H. meleagridis*. In recent years an increase in field cases of clinically severe blackhead disease in chickens, particularly leghorn and broiler breeder pullets (4, 5), has forced a reexamination of factors affecting pathogenicity of *H. meleagridis*. Some possible causes of this increase are (a) increased virulence of the organism, (b) increased susceptibility of chickens (either through genetic changes or immunodeficiency), and (c) interaction of *Histomonas* with other infections.

There is no literature on interaction of blackhead disease with other infectious disease agents, except for a 1973 study on the effect of blackhead disease on the severity of coccidiosis, wherein *H. meleagridis* given on or before inoculation with *E. tenella* appeared to interfere with oocyst production (1). Thus, a series of experiments was designed to determine whether concurrent cecal coccidiosis infections could increase the number or severity of liver lesions in chickens.

3. Materials and Methods

***Histomonas meleagridis* cultures for experimental infections**: A medium consisting of chick embryo extract, horse serum, medium 199, and rice powder (8) was used to culture *H. meleagridis* from a field outbreak of blackhead in Leghorn pullets. The culture was re-isolated from turkeys prior to use in each experiment to maintain pathogenicity. Fresh cultures were pooled and counted using a hemacytometer, and diluted to final working concentration made in Medium 199 (Gibco), pH 7.4, 1 ml/dose. Inoculations of *H. meleagridis* were made via the cloaca with a hypodermic syringe, using a blunt-tipped

plastic pipette tip in place of the needle. Past experience suggested that 10-15% of birds do not become infected after inoculation in this way, probably because most of the inoculum is voided.

Cecal Coccidia: A field isolate of *E. tenella* (Athens, GA, 1997) was prepared for use as previously described (7). The oocysts were inoculated *per os* in 1 ml volume using a 10 ml syringe and a plastic pipette tip in place of the needle.

Birds and facilities: Broiler chicks (Peterson x Arbor Acres) were hatched from breeder flocks maintained at the UGA poultry research center and used straight run. No vaccinations were given. Unmedicated feed and water were given *ad libitum*. Chicks were housed in steam-cleaned, stainless steel cages until used at 10 or14 days of age.

Experimental Design: Treatments were replicated in 3 cages of 10 birds each, except for 6 replicates in the test of the live coccidiosis vaccine.

Concurrent infection with *H. meleagridis* and *E. tenella*: This experiment was repeated three times. As the results were similar, the results of only one experiment are given. *Histomonas* inoculations (10^5/bird) were given when birds were 14 days old. Oocysts of *Eimeria tenella* were inoculated on day 10 (4 days prior to *Histomonas*), or on day 14. Also, *E. tenella* were given at 10^3/bird, or at 10^4/bird. Controls were a) uninoculated, b) inoculated with *E. tenella* only (10^3 or 10^4 oocysts), or c) inoculated with *H. meleagridis* only. The test was terminated when birds were 24 days old, with record of weight gains (per cage) and mortality. Birds were killed by cervical dislocation for necropsy. Liver and cecal lesions were scored on a scale of 0-4 (Table 7.1). This is a subjective system, but is considered reasonably accurate when used repeatedly by the same workers.

Titration of *H. meleagridis* inoculum in presence of *E. tenella*: *Histomonas* inoculations were given on day 14 in graded doses of $7.5x10^3$, $30x10^{3,}$, 10^5,or $3x10^5$ cells per dose, with and without concurrent inoculation with 10^4 oocysts of *E. tenella.* Controls included a) uninoculated or b) inoculated with *E. tenella* only. The test was terminated when birds were 28 days old, with record of weight gains (per cage), mortality, and necropsy of all birds. Liver and cecal lesions were scored as above.

Interaction of *H. meleagridis* with a coccidiosis vaccine: A commercial live coccidiosis vaccine (Immucox, Wingo, Inc., Watkinsville, GA), containing small numbers of live *Eimeria* oocysts, was given to day-old chicks at the recommended level or at 3x. The treatments included *Histomonas* inoculation with the vaccine at the two levels, *Histomonas* alone, and uninoculated controls, each replicated in 6 cages of 10 birds each. The test was terminated when birds were 28 days old, with data collected as in the other experiments, above.

Statistical Evaluation: Data were analyzed, where appropriate, using the GLM procedures on SAS (SAS Institute, Cary, NC), for significance at $P<0.05$.

4. Results

Interaction of *H. meleagridis* with *E. tenella*: Cecal lesions indicated that 79% of the birds inoculated with *H. meleagridis* (Treatments 2, 4, 5, 7, and 8) became infected, causing a reduction in weight gain of approximately 19% (Table 7.2). Inoculation with *E. tenella* reduced final weights by 6 or 11%, but coccidian lesions were not apparent at the time of necropsy. Cecal lesions from blackhead averaged 4.00 in positive birds of infected groups, regardless of whether birds received one or both inocula. Liver lesions averaged 0.25 in

birds inoculated only with *H. meleagridis*, while those receiving concurrent *E. tenella* inoculations averaged 0.97-1.14. The number of birds with liver lesions was higher in groups inoculated with both parasites (69-91%) compared with those receiving only *H. meleagridis* (19%). Severe liver lesions (scores of 3 or 4) were found only in birds inoculated with both parasites. The results did not appear to be correlated with the time or intensity of inoculation with *E. tenella.* Increased severity of liver lesions in the presence of coccidia was highly significant in all combination exposure groups ($P<0.05$). A similar pattern of infection and severity of liver lesions was seen in all three replicate studies.

Titration of *H. meleagridis* inoculum in the presence of *E. tenella:* Inoculation with *H. meleagridis* produced cecal lesion scores averaging 3.47-3.83, with 73-93% positive at necropsy (Table 7.3). Weight gain was reduced about 15% by *H. meleagridis* infections. Neither the number of birds infected nor the severity of cecal lesions appeared to correlate with the number of *H. meleagridis* in the inoculum in mono-infections. Liver lesions averaged 0, 0.20, 0.19, or 0.23 for birds receiving $7.5x10^3$, $3x10^4$, 10^5 or $3x10^5$ histomonads, respectively, with 0, 17, 13, or 17% positive for liver lesions. However, when *E. tenella* was given along with *H. meleagridis*, the liver lesions increased significantly ($P<0.05$) in direct proportion to inoculum dose (Table 7.3). The liver lesions averaged 0.14, 0.41, 0.47, or 0.86 for birds receiving $7.5x10^3$, $3x10^4$, 10^5 or $3x10^5$ histomonads, respectively, with 10, 30, 37 or 53% of birds positive for liver lesions.

Interaction of *H. meleagridis* with coccidiosis vaccine: Infection with *H. meleagridis* caused cecal lesions averaging 3.4-3.7, with 90B92 percent of birds positive (Table 7.4). Weight gain was reduced about 18% by blackhead infection. Liver lesions averaged 0.23 in birds receiving only *H. meleagridis*, compared with 0.35 for those with *H. meleagridis* plus a

coccidiosis vaccine and 0.45 (Significant at $P<0.05$) for those receiving *H. meleagridis* plus a 3x dose of vaccine . The number of birds positive for liver lesions was 13% with only *H. meleagridis,* 25% with *H. meleagridis* plus the vaccine, and 32% in those receiving the 3x dose of vaccine.

5. Discussion

Concurrent infections with *E. tenella* and *H. meleagridis* in chickens caused increased severity of liver lesions and an increase in the percentage of birds that suffered liver lesions. These effects were noted when coccidia were given at the same time or 4 days prior to inoculation with *H. meleagridis*. Blackhead lesions were not more severe when 10^4 oocysts of *E. tenella* were given, as opposed to 10^3 oocyts. Liver infections were more severe as the dose of *H. meleagridis* was increased from $7.5x10^3$, to 3x 10^4, 10^5 or 3x 10^5 cells. The severity of lesions was directly correlated with inoculation dose in birds receiving both parasites.

Cecal lesions were severe in all groups inoculated with *H. meleagridis*, regardless of the level of inoculation or the presence of *E. tenella*. Oral inoculation with a 3x dose of a commercial live coccidiosis vaccine (containing fewer than 100 *E. tenella*/dose) also enhanced number and severity of blackhead lesions in the liver when given at elevated doses, although at a much lower level than higher doses of coccidia. This effect was considered minor, in comparison with the results of the other experiments.

The interaction of cecal coccidiosis with blackhead disease could be expected because of the disruption of cecal mucosa by *E. tenella*, perhaps making it easier for *H. meleagridis* in the cecal tissues to enter the bloodstream and spread to the liver. Blackhead

can be transferred from bird to bird via whole blood collected from the veins draining the ceca of infected turkeys (9). Thus, we could expect that other diseases or toxins that disrupt the mucosa might also affect the spread of blackhead in the blood supply of the intestinal tract, accounting for more field reports of severe infections in chickens. However, if this was the only mechanism of interaction we might expect more severe liver lesions where higher doses of coccidia were given, but this was not observed.

Because of the well known complexity of the interaction of *H. meleagridis* with intestinal bacteria, the present observations could have other explanations. Previous work with germfree birds has shown that *H. meleagridis* requires the presence of certain species of bacteria for expression of pathogenicity in turkeys and chickens (10, 11). Coccidiosis is known to cause major increases in the intestinal population of *Clostridium perfringens* and a decrease in *Bacterioides sp.* (2).

A recent report suggests that cecal worms (*Heterakis gallinarum*), the carrier of *H. meleagridis*, is widespread and common on broiler breeder pullet farms (3). Coccidia are also prevalent in poultry(6,7), providing a high potential for the interaction of these two diseases. However, the significant effects of coccidiosis on the course of *H. meleagridis* in chickens do not completely explain the spectacular outbreaks of blackhead disease often reported from commercial layer and breeder pullet flocks. Extensive liver lesions, high morbidity, and mortality of 10-15% reported in young pullets suggest a rapidly developing disease of high virulence. *H. meleagridis* isolated from such outbreaks and tested in our laboratory show ordinary affinity for chickens (Unpublished observations). Immuno-depression from viral infections such as Marek=s disease virus, chicken anemia virus, and infectious bursal disease, which are commonly diagnosed in poultry, may contribute to the

field outbreaks. These viruses are responsible for an increased incidence and severity of other diseases where T-cell function is important for primary and secondary protection. Thus, for future research, to explain the increase in clinical blackhead disease reported from commercial poultry it may be important to focus on the interaction mucosal damage, changes in cecal microflora, and immunodeficiency produced by viral infections.

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Table 7.1. Subjective visual lesion scoring system for blackhead disease (*Histomonas meleagridis)* in chicken ceca and livers.

Organ	Score	Observations
Cecum	0	Normal cecal mucosa and lumen contents
	1	A few scattered small lesions visible from serosal or mucosal surfaces, no blood or thickening of mucosal wall
	2	Lesions prominent but discreet, some bleeding and/or thickening of mucosa, lumen contents fairly normal
	3	Cecum enlarged, opaque yellow appearance, walls thickened, lesions confluent, blood or caseous abnormal lumen contents; the entire cecum is involved
	4	Cecum distended, yellow, dense caseous cores, cecal walls thickened and fragile
Liver	0	Normal
	1	A few small foci on surface of liver. Lesions are off-white and variable in appearance
	2	5-10 small foci, or 3-5 larger lesions/lobe
	3	Lesions numerous, but much normal tissue still remaining
	4	Lesions numerous throughout, coalescing, no normal tissue

Table 7.2. Effect of concurrent cecal coccidiosis inoculations on *Histomonas* infections in chickens (Experiment 1). Means with common superscript letters are not significantly different at P<0.05.[A]

	Inoculations/Day		Average gain		Lesion Scores		% Positive	
Trt	*E. tenella* [B]	*Histomonas* [C]	g [D]	% gain	Cecal [E]	Liver	Cecal	Liver
1	-	-	884^{a}	100%	0 ^{b}	0 ^{b}	0	0
2	-	10^5	713de	81	4.00^{a}	0.25^{b}	68	19
3	10^3/ 14	-	790^{bc}	89	0 ^{b}	0 ^{b}	0	0
4	10^3/ 10	10^5	746^{cd}	84	4.00^{a}	1.10^{a}	73	73
5	10^3/ 14	10^5	752^{cd}	85	4.00^{a}	1.02^{a}	87	69
6	10^4/ 14	-	835^{ab}	95	4.00^{a}	0 ^{b}	0	0
7	10^4/ 10	10^5	662^{e}	75	4.00^{a}	0.97^{a}	90	74
8	10^4/ 14	10^5	691de	78	4.00^{a}	1.14^{a}	77	91

A This experiment was repeated three times, with similar results. Thus, the results of only one experiment are given.

B Sporulated oocysts given *per os* when birds were 10 or 14 days old.

C *Histomonas meleagridis* from culture (10^5/bird) were given *per rectum* when birds were 14 days old.

D Average gain/bird from age 14-24 days.

E Average scores *H. meleagridis* lesions in positive birds. Lesions from coccidiosis had healed, as the peak time for such lesions was more than a week prior to necropsy.

Table 7.3. Titration of infective dose of *Histomonas meleagridis* in the presence and absence of concurrent cecal coccidiosis infections (Experiment 3). Data entries with similar superscript letters are not significantly different at P<0.05.

	Inoculations		Average gain		Lesions		% Positive birds	
Group	*E. tenella* [A]	*Histomonas* [B]	g [C]	%	Cecal [D]	Liver	Cecal	Liver
1	B	B	691[a]	100	0 [c]	0 [d]	0	0
2	+	B	669[ab]	97	0.2 [c]	0 [d]	0	0
3	B	7.5	594 [c]	86	3.47 [b]	0 [d]	77	0
4	B	30	571 [c]	83	3.77 [ab]	0.20 [c]	83	17
5	B	100	618 [bc]	89	3.62 [ab]	0.19 [c]	73	13
6	B	300	550 [c]	80	3.73 [ab]	0.23 [c]	87	17
7	+	7.5	623	90	3.73 [ab]	0.14 [c]	73	10
8	+	30	597 [bc]	86	3.83 [a]	0.41 [b]	80	30
9	+	100	573 [c]	83	3.70 [ab]	0.47 [b]	87	37
10	+	300	595 [c]	86	3.6 [ab]	0.86 [a]	93	53

[A] Sporulated oocysts (10^4 /bird) given *per os* when birds were 10 or 14 days old.

[B] *H. meleagridis* cells (x 10^3/bird) from culture given *per rectum* to 14-day-old birds.

[C] Lesions primarily from *H. meleagridis*. Occasional light coccidiosis lesions.

[D] Average gain/bird from age 14-24 days.

Table 7.4. Effect of concurrent administration of live coccidiosis vaccine on severity of *Histomonas meleagridis* infections in chickens. Data entries within columns with similar superscript letters are not significantly different at P<0.05.

	Inoculations		Average gain		Lesion Score[D]		% Positive	
Trt	Vaccine[A]	*Histomonas*[B]	g [C]	%	Ceca	Liver	Ceca	Liver
1	B	B	681[a]	100	0 [b]	0 [c]	0	0
2	B	+	556[b]	82	3.5[a]	.23[b]	92	13
3	1x	+	579[b]	85	3.4[a]	.35[ab]	90	25
4	3x	+	583[b]	86	3.7[a]	.45[a]	92	32

[A] A commercial live vaccine containing low levels of *Eimeria tenella* (cecal coccidia) and other species, given to day-old chicks at the recommended level (1x) or at three times the recommended level (3x).

[B] *H. meleagridis* from cultures. Given *per rectum* at 10^5/bird at 14 days.

[C] Lesion scores graded subjectively on a scale of 0-4, for blackhead lesions.

[D] Average gain per bird calculated from day of inoculation to termination (days 14-28).

CONCLUSION

1. Coccidiosis aggregated pathogenicity of histomoniasis in chickens. Cecal lesions did not appear to be affected by the introduction of *Eimeria tenella* infections. However, the severity of liver lesions and number of birds positive for liver lesions of *H. meleagridis* increased significantly with the presence of *E. tenella.* The positive relationship between infections of cecal coccidiosis and *H. meleagridis* in chickens suggests that, under field conditions, such dual exposure may contribute to increased clinical outbreaks of histomoniasis in chickens.
2. The lateral transmission of *H. meleagridis* in turkeys was studied in floor pens in the absence of the carrier cecal worm *Heterakis gallinarum*. These results suggested that lateral transmission of histomoniasis through a turkey flock can occur readily through normal contact between infected birds and uninfected birds and their droppings in the total absence of cecal worms.
3. Five anticoccidials, six antibiotics, and nine antiprotozoals drugs known from literature were tested for their efficacy against *Histomonas meleagridis* in chickens. None of 5 anticoccidials (salinomycin, diclazuril, nicarbazin, roxarsone, and lasalocid) and 6 antibiotics (bacitracin, apramycin, penicillin, chlortetracycline, tylosin, sarafloxacin) had significant efficacy in control of histomoniasis. Out of 9 antiprotozoals, only nitroimidazoles (dimetridazole, metronidazole, ornidazole, and tinidazole) suppressed growth of *H. meleagridis in vitro* and *in vivo*.

4. A new phenomenon discovered during the culture of *H. meleagridis* was that cultured *H. meleagridis* were readily agglutinated by horse serum at concentrations of 15% or higher. The agglutinating factor (AF) was active when serum regardless of whether serum was heat-inactivated, and was present in several batches of sera tested. Histomonads were purified via AFfrom *in vitro* culture.