MOLECULAR CHARACTERIZATION OF *HISTOMONAS MELEAGRIDIS* AND OTHER PARABASALIDS IN THE UNITED STATES USING THE 5.8S, ITS-1, AND ITS-2 rRNA REGIONS

by

LORI ANN LOLLIS

(Under the Direction of Larry R. McDougald)

ABSTRACT

Extracted DNA from twenty-eight *Histomonas meleagridis*-infected avian tissue samples from multiple hosts and geographic locations was analyzed for variation in 5.8S rRNA and flanking internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S. Samples were amplified by polymerase chain reaction (PCR), sequenced, and compared with known sequences from GenBank accessions of *H. meleagridis* and other related protozoa. The analyses revealed significant genetic variation within *H. meleagridis* sequences suggesting the possibility of multiple genotypes or possible misdiagnosis. Related protozoa found in some samples were identified as *Tetratrichomonas* spp. A phylogenetic tree analyzing the 5.8S and flanking ITS regions was inconclusive. In contrast, a tree constructed only on the 5.8S rRNA, grouped all but one *H. meleagridis* sample into one clade, including GenBank accessions from Europe. This suggests that the 5.8S region is more reliable in identifying genera. No correlation between genotypes and host species or geographic location was observed.

INDEX WORDS: *Histomonas meleagridis*, *Simplicimonas similis, Tetratrichomonas*, molecular characterization, internal transcribed spacer region

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DEDICATION

I would like to dedicate this thesis to my parents, Jerry L. Lollis and Martha G. Lollis whose support, love, and wisdom has given me the courage and strength to go after any goal. They instilled in me the drive and patience that helped me complete this. I love you both and thank you for always believing in me.

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CHAPTER 1

INTRODUCTION

Histomonas meleagridis is the causative agent of histomoniasis, more commonly known as Blackhead disease. *Histomonas meleagridis* is a parabasalid protozoan parasite of the family Dientamoebidae, order Tritrichomonadida, and class Tritrichomonadea (Cepicka et al., 2010). The family Dientamoebidae includes the 4 genera *Dientamoeba, Protrichomonas, Histomonas,* and *Parahistomonas*. The family is characterized as uninucleate to binucleate, lacking an infrakinetosomal body in the mastigont, a costa, and undulating membrane. *Histomonas meleagridis* has been known to cause significant morbidity and mortality events in gallinaceous birds, especially turkeys and chicken (McDougald, 2005). It is believed the ring-neck pheasant (*Phasianus colchicus*) is the natural host of *H. meleagridis* and the introduction of pheasants into the United States coincided with the emergence of blackhead and destruction of the emerging turkey industry in the early 1890's (Lund and Chute, 1972).

Little research has been conducted on genetic variability of *H. meleagridis* and how it relates to infectivity, transmission or virulence. Most studies have focused on the internal transcribed spacer (ITS) regions of the ribosomal gene. The ITS regions are non-coding sequences, resulting in less conservational pressure, and are suitable for molecular characterization of phylogenetically related organisms (Hillis and Dixon, 1991). In other studies, the 5.8S rRNA and flanking internal transcribed spacer regions (ITS1 and ITS2) were successfully used as molecular markers for comparative sequence analysis (Bart et al., 2008; Gerhold et al., 2008; Grabensteiner et al., 2010; Sansano-Maestre et al., 2009).

In a previous study, genetic variation of the ITS region 1 of *H. meleagridis* was scrutinized using C-profiling (van der Heijden et al., 2006). In C-profiling, the cysteine nucleotide pattern is compared

between samples for evidence of genetic variation. Results yielded three significant genetic variants (Types I, II, and III) closely related to *Trichomonas vaginalis* and *Dientamoeba fragilis* (van der Heijden et al., 2006). Another resent study found four types of *H. meleagridis* using C-profiling of the 5.8S and flanking ITS regions. Analysis determined two types (A and B) were similar in both chickens and turkeys, type C was found almost exclusively in turkeys and the fourth type (D) was very rare (Hauck et al., 2010). Specific objectives of this study are:

1. Compare sequences of 5.8S rRNA and flanking internal transcribed spacer (ITS) regions and the 5.8S region exclusively of *H. meleagridis* from multiple avian species and various geographic locations in the United States.

2. To determine correlations between genetic variation and host species, and genetic variation and geographic locale.

3. Investigate occurrence of outbreaks relative to transmission determining if the same genotype is present in multiple outbreaks or if each outbreak consists of a new genotype.

4. Determine if other undescribed species are present and cause possible misdiagnosis of histomoniasis.

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CHAPTER 2

LITERATURE REVIEW

Histomonas meleagridis

History and Morphology

Histomonas meleagridis, the causative agent of histomoniasis more commonly known as blackhead, is a flagellated protozoal parasite affecting gallinaceous species. The flagellated form of *H. meleagridis* is found in the cecal lumen containing one flagellum. Interestingly, as the protozoan infects liver tissue it takes on an amoeboid form losing its flagellum. Histomoniasis was first discovered in 1893 in Rhode Island (Cushman, 1893) where it caused significant losses in the turkey industry. Early workers considered *H. meleagridis* a stage in the coccidian life cycle (Cole and Hadley, 1908; Cole and Hadley, 1910). It was also suggested that histomoniasis was a stage of *Trichomonas* (Hadley, 1920; Hadley and Amison, 1911) or *Pentatrichomonas* (Allen, 1936; Allen 1940). A commonly held European theory was that blackhead disease was caused by a yeast or mycotic organism (Enigk, 1935; Enigk 1936). It was Tyzzer who correctly identifies the organism and created the genus *Histomonas* to further distinguish it from other protozoa (Tyzzer, 1920).

Histomonas meleagridis is of the family Dientamoebidae, order Tritrichomonadida, and class Tritrichomonadea (Cepicka et al., 2010). The family Dientamoebidae includes the 4 genera *Dientamoeba, Protrichomonas, Histomonas,* and *Parahistomonas*. The family is characterized as uninucleate to binucleate, lacking an infrakinetosomal body in the mastigont, a costa, and undulating membrane. Organisms are round or amoeboid ranging in diameter from 8-15µm (Smith, 1895). Histomonads are known to infect the cecal lumen and liver tissue. *Histomonas meleagridis* is anaerobic. It lacks

mitochondria and instead contains hydrogenosomes that further converts pyruvate and malate to hydrogen and acetate for its energy requirements (Muller, 1993). Similar pathways are seen in *Giardia* (Townson et al., 1996; Brown et al., 1998) and *Entamoeba* (Reeves, 1984). The origin of the hydrogenosome is unclear but it is believed to share a common ancestor with the mitochondrion (Tachezy et al., 2001)

Until recently, it was thought that histomonads did not take on a cyst form and could only survive in droppings without the protection of an intermediate host (Graybil, 1920; Lund and Chute, 1974). Recent studies have identified a possible cyst or cyst-like form. A set of Berlin isolates, were reported to form a cyst-like stage in cultures (Munsch et al., 2008). In a follow-up study, these cyst-producing isolates were cloned and the ITS 1 and 18S rRNA regions sequenced to confirm that these cyst stages were *H. meleagridis* (Munsch et al., 2008). It was suggested that these cyst stages may have been overlooked in the past because they are found in low numbers. It was speculated that specific conditions may also be required to produce the cyst-like stage.

In another study, Zaragatzki et al., (2009) cultured trophozoite stages of multiple clonal genotypes *in vitro* and attempted to induce the cyst stage by manipulation of temperature and nutrient contents. When the temperature was lowered and medium deprived of calf serum and/or rice starch, or the pH or $MgCl_2$ concentrations changed, many histomonads were killed and some assumed a spherical stage. As conditions worsened a smaller condensed spherical stage appeared (4-7µm). It was suggested that the larger spherical stage is a pre-cyst-like form and the smaller spherical stage is representative of a true cyst form. The study also determined that acidic pH conditions resulted in fewer formed spherical stages than were seen with basic pH.

Clinical signs of histomoniasis include lethargy, decrease feed intake, necrotic foci of the liver that resemble targets (Cushman, 1893;Shivaprasaud et al., 2002), sulfur-colored droppings, and cecal cores containing a caseous exudate. The birds ultimately die from liver failure. Cushman was first to describe liver lesions associated with *H. meleagridis*, but the cecal lumen is the primary infection site.

The blood supply to the liver is connected with the blood supply of the ceca and intestines (Clarkson, 1966). Thus, histomonads can be carried from the primary site of infection, ceca, to the liver and other tissues. Lesions have also been reported in the spleen, kidney, and lungs and the bursa of fabricius (Clarkson, 1966; Levine, 1947; Malewitz and Calhoun, 1957; Malewitz 1958; Tyzzer and Fabyan, 1920); although, it has been theorized that histomonads can reach the bursa of fabricius without use of the bloodstream (Cortes et. al, 2004; Marx, 1973).

Treatments

Treatment of histomoniasis has been challenging. Many different methods have been implemented in an attempt to control this disease, and several chemical compounds were successful. However, most of these treatments for histomoniasis are no longer available and those that are still in use are only available in the United States. Several different types of antihistomonals were available in the 1960's such as arsenical compounds, nitroheterocyclic compounds, and several disinfectants. Vitamins and other nutritional compounds were thought to have an effect on histomoniasis as well (Whitmore et al., 1968).

Trivalent arsenicals were found overall to have disappointing results. Tyzzer examined several different trivalent arsenicals and concluded tryparsamide to have curative properties when injected intravenously or subcutaneously, but the other drugs produced poor results (Tyzzer, 1923). Good results using trivalent arsenicals were reported by Blount (Blout, 1938); however, a later study found no real benefits using these drugs (Jaquette and Marsden, 1947).Pentavalent arsenicals were less toxic than the trivalent arsenicals, but a narrow chemotherapeutic index was observed (Bowen, 1971). Nitrofurans were arsenical compounds and were quite effective in reducing mortality, but allowed for relapse (Bowen et. al, 1971; .Grumbles et. al, 1951, 1952, 1952). Nitarsone, a pentavalent compound, is still in use today as a feed additive and is highly effective for preventative use. However, relapse can occur after medication withdrawal (McGuire and Morehouse, 1952).

Nitroheterocyclic compounds such as nitromidazoles had exceptional curative properties against histomoniasis in all bird species (Flowers et al., 1965; McGuire et al., 1964). These drugs could be administered as feed additives or in water-dispersal and could be used as preventative treatments or as treatment against infection. Within the nitroheterocyclic compounds, the nitroimidazoles produced good results. Prevention as well as treatment was observed with little or no side effects (Flowers et. al, 1965). However, in the 1990's these drugs were banned in the United States by the Food and Drug Administration and in the European Union in 2003, because they were suspected carcinogens. This ban left the poultry industry with no available treatments for histomoniasis (Hafez et al., 2005; McDougald, 2005).

Antihelminthics have also been used to prevent histomoniasis. However, these drugs have to be given prior to exposure with the *Heterakis* eggs and have no effect on birds infected via cloacal drinking (Hegngi, 1999). Birds were also less likely to relapse with these treatments. Disinfectants such as ipecac and paromomycin were able to reduce mortality but overall were not very useful (Lindquist, 1962; Wedeforth and Wedgeforth, 1921). In the early 1990's all curative treatments for histomoniasis were banned by the FDA for being suspect carcinogens (McDougald, 2005; Hafez et al., 2005).

Recently, the effects of nifurtimox and tiamulin have been studied as possible histomoniasis treatments. Tiamulin, a semisynthetic pleuromutilin, targets the bacterial ribosome 50S subunit and strongly inhibits pepitdyl transferase (Hauck and Hafez, 2010). Studies showed that tiamulin was affective against protozoan parasites *in vitro*, but results with flock outbreaks have varied with reduced mortality observed in some flocks and failed protection in others.

Nifurtimox (NFX), also known by Lampit and Bayer A-2502, has been used in humans as an anti-trypanosomal treatment of Chagas disease (Hauck et al., 2010). NFX is a nitrohetero-cyclic compound like the Nitromidazoles that were used successfully for histomoniasis treatment. Studies showed that NFX, when given at 200 or 400ppm in the feed, reduced mortality from Histomoniasis

(Hauck et al., 2010). NFX also reduced liver lesions associated with histomoniasis, but had less effect on cecal lesions. High concentrations of NFX *in vitro* inhibited *H. meleagridis* development, but concentrations of 12.5-100ppm seemed to only delay *H. meleagridis* growth up to 48 hours.

Host Species and Transmission of *Histomonas meleagridis*

Historically, Ring-neck pheasants (*Phasianus colchicus*) are probably the natural host for *H. meleagridis*, and consequently the introduction of the Ring-neck pheasant's into the United States coincided with the emergence of Histomoniasis, causing devastating losses in the fledging American turkey industry (Lund and Chute, 1972). Turkeys (*Meleagris gallopavo*) are most susceptible to disease and often suffer high morbidity and mortality. Sometimes entire flocks are lost. Chickens are a known carrier for the parasite, but an increasing number of disease reports in broiler breeders have been observed over the last several years.

The best means of transmission of *H. meleagridis* is through ingestion of an enteric host, cecal worms (*Heterakis gallinarum*) by the host. The histomonads reside in the ova of the *H. gallinarum* and it is through the ingestion of these embryonated eggs that birds were able to become infected with this disease (Graybill and Smith, 1920; Tyzzer, 1924). Chickens, guinea fowl, chukar partridges, and pheasants are the best reservoir of infection and host for cecal worms (Lund and Chute, 1970; Lund and Chute, 1974). Cross-contamination studies demonstrated that worm eggs can survive in the soil for up to three years and still test positive for *H. meleagridis* after 150 weeks (Farr, 1961). Thus, the requirement for separate rearing of chickens and turkeys became an integral control method. Other non-gallinaceous species such as ducks and geese are poor hosts for the cecal worms, but can still act as asymptomatic carriers of *H. meleagridis* (Chaudhury et. al, 1984; Lund and Chute, 1974). Mechanical transmission (i.e. workers or other animals) and some invertebrates such as darkling beetles and house flies may be able to aid in transmission, but studies are inconclusive (McDougald, 2005).

Histomonad-infected droppings is the means of the second, direct route of transmission involving retrograde peristalsis, also referred to as cloacal drinking (Hu and McDougald, 2003). This direct route allows for birds, specifically turkeys, in high density to rapidly spread the disease resulting in mortality rates as high as 100%. Similar results could not be produced with chickens. No bird to bird transmission of histomoniasis on floor pens where uninfected birds co-mingled with infected birds occurred (Sorvari et al., 1977). Reasons for this difference in epidemiology between turkeys and chickens are still unclear.

Age was once thought to be an influencing factor of resistance. It was suggested that younger birds contracting histomoniasis are more likely to die than older birds (Higgens, 1915); and, older birds are less likely to transmit disease compared to their younger counterparts (Lund and Chute; 1970). However, other studies have found that age does not have an effect on resistance or susceptibility in turkeys (Kendall, 1957; Armstrong and McDougald, personal communication).

Pathogenicity

A distinct relationship between virulence of *H. meleagridis* and certain bacteria has been demonstrated in previous studies, but the reason for this is not well understood. Liver lesions in turkeys caused by *H. meleagridis* are often sterile, but various bacterial species have been isolated including *Escheria coli* ,the most prevalent, followed by *Streptococcus, Diplococcus, Lactobacillus, Micrococcus, Salmonella, Clostridium, and Pseudomonas* (Harrison et. al, 1954). Studies with bacteria-free turkeys demonstrated that *H. meleagridis* infections initiated by worm eggs were non-virulent, and contamination of the birds with *Clostridium perfringens*, *Bacillus subtilis*, or *E. coli* resulted in pathogenic infections (Bradley and Reid, 1966; Franker and Doll, 1964). In chickens, this relationship was more complex with *H. meleagridis* requiring multiple bacteria species to attain full virulence (Springer et. al, 1970).

Pathogenicity of *H. meleagridis* varies greatly between host species. Lund infected multiple gallinaceous species with *H. meleagridis* and noted host-specific variation in severity of infections. Turkeys were most severely affected, followed by peafowl, chukar partridges, chickens, and ring-neck

pheasants (Lund, 1967; Lund and Chute, 1972; Lund and Chute, 1972). Difference in susceptibility of three different turkey lines, wild Canadian turkeys (WTC), British United turkey (BUT-Big6), and Kelly-Bronze turkey (KBT) was observed (AbdulRahman and Hafez, 2009). The three lines were inoculated intracloacally and observed for four weeks. All lines were susceptible but mortality among the WCT line was significantly higher. Both KBT and BUT-Big6 had higher liver lesion scores and lower mortality rates than the WTC line. Multiple chicken breeds were also compared for susceptibility to histomoniasis and some strain-specific variation in severity was observed (Lund, 1967).

In vitro Culture of Histomonads

Several requirements are necessary for the successful cultivation of *H. meleagridis*. Presence of bacteria is important for growth though the reason is still unclear. It was suggested that bacteria aid in the development of an anaerobic environment (McDougald, 2005). It is also not known whether histomonads cultures without bacteria would be virulent. Some studies showed that the inclusion of antibiotics would reduce bacterial growth while still allowing histomonad growth, but supplementation with antibiotic-killed bacteria was required (Lesser, 1960; Lesser, 1960; Lesser, 1963). A starch source is also necessary for culturing histomonads. Historically, rice powder has been used but other substitutes, such as rye or oats have been successful as well (Hauck et al., 2010). Histomonads are known to be anaerobic because of their lack of mitochondria and dependence on the anaerobic hydrogenosome for energy production (Muller, 1993). In culture, the extensive bacterial growth depletes the available oxygen and histomonads were also readily propagated in anaerobic media (Delappe, 1953; Stepkowski, 1979). Maintenance of cultured histomonads for many years can be accomplished by freezing the histomonads in liquid nitrogen using a suitable cryoprotectant like DMSO (8%) with chicken serum (8%) in medium 199 (Chute and Chute, 1969; Honigberg and Dwyer, 1969).

Histomonads have been cultured using different media; however, Dwyer's medium has been used most often with consistently good results (Hu et al., 2006; Hu et al., 2005; Hu et al., 2002). Dwyer's medium consists of Medium 199, chick embryo extract, serum, and rice powder. Recent variation on

Dwyer's medium (high rice powder and no chick embryo extract) was reported to propagate histomonads almost 10-fold (van der Heijden and Landman, 2007). Aliquots of both Dwyer's medium and the modified recipe can be frozen until ready for use and function as a cryoprotectant for freeze preservation of histomonads.

Histomonads can grow rapidly in Dwyer's medium for two to five days and then numbers decline. Prolonged culturing and repeated passage can result in permanent loss of virulence and an inability to colonize in birds (Lund et. al, 1966; Lund et. al, 1967; Dwyer and Honigberg, 1970). Another issue in culturing histomonads is contamination by other organisms. An organism called *Blastocystis*, often found in the intestinal tract of turkeys, grows exceedingly well in Dwyer's medium and has been shown to compete with the histomonads *in vitro* (Tyzzer, 1936; Delappe, 1933). *Blastocystis* is phylogenetically similar to red algae and is not inhibited by antibiotics or antifungals, making it difficult to prevent or inhibit *Blastocystis* growth in histomonad cultures (Arisue et. al, 2002).

Recently, conditions required for successfully culturing *H. meleagridis* from couriered carcasses was demonstrated (Gerhold et al., 2010). Two birds were inoculated with 90,000 histomonads/ml and euthanized ten days post infection. Carcasses were incubated at room temperature for two and twenty-four hours. Ceca samples were placed in flasks containing Dwyer's media and 10% HIHS. Flasks were designated multiple time intervals from six to one hundred twenty hours and incubated at 4°C, 25°C, or 30°C. Histomonads that were stored at 30°C could survive for up to seventy-two hours, but the other time and temperatures produced negative results. This study provides a new a way to transport cultivable histomonads and allow for the possibility of earlier detection methods.

Immunity and Immunization

Immunization in turkeys has proven to be difficult. It is possible for a turkey to contract histomoniasis, recover, and if re-infection occurred the turkey would still die (Curtis, 1907). Although multiple immune studies have been conducted, little information regarding immunization or immune

response to histomoniasis has been determined. Clarkson examined the protective ability of antibodies against *H. meleagridis* by passively immunizing naïve birds with antisera from infected and treated birds. The results suggested antibodies offer no protection for birds against histomoniasis (Clarkson, 1963). However, a recent study concluded that systemic immunity by serum antibodies is not the primary means of protection against Histomoniasis. It was suggested that the mucosal immune response may be more important in disease prevention, but deserves further investigation (Windisch and Hess, 2010; Bleyen et al., 2009).

The early immune response to *H. meleagridis* in the gut was examined and compared the response of chickens to turkeys (Powell et al., 2009). A greater number of parasites migrated to turkey liver compared to the chickens. The chicken was able to mount an effective cecal innate immune response controlling parasite numbers. The turkey failed to produce this effective response. Higher antibody levels were observed in the chicken suggesting an adaptive immune response that does not occur in the turkey. However, more research is needed to determine how *H. meleagridis* interacts with the immune system.

An indirect sandwich ELISA was recently developed detecting IgG antibodies against *H. meleagridis* in the sera of chickens and turkeys (Windisch and Hess, 2009). The IgG antibody is not a major player in protective immunity against histomoniasis, but asymptomatic infected chickens could be identified as soon as fourteen days post-infection. Another study designed a specific blocking-ELISA based on monoclonal antibodies (van der Heijden et al., 2010), and was able to successfully detect antibodies without cross-reaction with a closely related parasite *T. gallinarum*. Both ELISA's are promising tools for detection and diagnostics.

Several attempts at vaccinating birds to create immune response proved to be disappointing. Tyzzer and Lund used attenuated cultures of *H. meleagridis* to vaccinate birds and obtained minor protection when challenged via cloacal inoculation. However, it was determined that immunogenicity of vaccine strains was lost from long term culture. It was theorized that the use of booster inoculations

could maintain long-term immunity, but the theory was concluded to be impractical (Lund, 1959;Tyzzer, 1933; Tyzzer, 1933; Tyzzer, 1934; Tyzzer, 1936).

Parahistomonas wenrichi, an avirulent protozoan, was used in another vaccination attempt. Cloacal infection with this organism conferred protection against *H. meleagridis* given cloacally, but did not confer protection against infections initiated with *H. meleagridis*-infected *Heterakis* ova (Lund, 1956). It is plausible that different infection routes could elicit different immune responses (Campbell and Chadee, 1997). A study from Garcia et al. (2009), found that a similar parasite's, *E. histolytica*, amebic virulence is primarily determined by its ability to adapt and survive the aerobic conditions of animal tissues. Results concluded that with amebic survival secured, inflammation is stimulated and tissue destruction occurs primarily caused by the host, taking on an autoimmune-like direction.

Recently, Liebhart investigated the effectiveness of oral vaccination using *H. meleagridis* attenuated by repeated passage *in vitro* (Liebhart et al., 2010). Day-old turkey poults were vaccinated with a dose of 10^4 attenuated histomonads, then challenged with 10^4 histomonads two or four weeks later. There were no adverse effects on body weight gain or clinical signs in vaccinated birds. Birds that were challenged two week after vaccination had a higher antibody titer than those that were challenged four weeks after, suggesting that humoral antibodies are not efficient at providing immune protection in turkeys. Whether or not a vaccine could offer efficient protection for turkeys against Histomoniasis is still unknown, but the use of attenuated histomonads seems to hold promise. More knowledge of the turkey's immune response to *H. meleagridis* would be valuable in advancing this work.

Molecular Characterization

Historically, diagnosis of histomoniasis was based on necropsy and microscopic examination. Within the last few years, use of polymerase chain reaction (PCR) has proven successful in detection of *H. meleagridis* (Hafez et. al, 2005; Huber et al., 2005). Other molecular studies on related parasites used PCR detection to detect parasite presence (Bart et al., 2008; Gerhold et al., 2008).

According to Dwyer, antigenic analysis was used to establish the taxonomic position of *H. meleagridis* near *D. fragilis*, *Trichomonas*, and *Entamoeba* based on antigenic analysis (Dwyer, 1971; Dwyer, 1972; Dwyer, 1972; Dwyer, 1974). This relationship was also confirmed by the rRNA genes from both parasites having a reduced G + C content and increased chain length (Gerbod et al., 2001).

Little research has been conducted on genetic variability of *H. meleagridis* and how it relates to infectivity, transmission or virulence. Most studies have focused on the internal transcribed spacer (ITS1 and ITS 2) regions of the ribosomal gene. The ITS regions are non-coding sequences, resulting in less conservational pressure, and are suitable for molecular characterization of phylogenetically related organisms (Hillis and Dixon, 1991). In other studies, the 5.8S rRNA and flanking internal transcribed spacer regions (ITS1 and ITS2) were used as molecular markers for comparative sequence analysis (Bart et al., 2008; Gerhold et al., 2008; Grabensteiner et al., 2010; Sansano-Maestre et al., 2009). Therefore genetic variation is easier to detect and evolves at a faster rate than protein-coding regions.

In a previous study, genetic variation of the internal transcribed spacer (ITS) region 1 of *H. meleagridis* was scrutinized using C-profiling (van der Heijden et al., 2006). In C-profiling, the cysteine nucleotide pattern is compared between samples for evidence of genetic variation. Results yielded three significant genetic variants. Variants were closely related to *Trichomonas vaginalis* and *D. fragilis* specifically Types I and II were closely related to each other and Type III was similar to *D. fragilis* (van der Heijden et al., 2006).

Another study examined *H. meleagridis* in German poultry flocks. C-profiling of the ITS 1 region disclosed four types that were similar but not identical. These four types were significantly different from the three van der Heijden types. Two types (A and B) were similar in both chickens and turkeys, Type C was found almost exclusively in turkeys, and Type D was rare. No clear correlation between host and subtype could be determined (Hauck et al., 2010).

Sequencing analysis is often used to study phylogenetic relationships to infer diversity or similarity between a set of organisms. Recently phylogenetic analysis has shed some light on the taxonomic placement of *H. meleagridis*. The Cavalier-Smith system placed *H. meleagridis* in the phylum Parabasalea, Class Trichomonadea, and Family Monocercomonadidae (Cavalier-Smith, 1998). Recently, the taxonomic placement of *H. meleagridis* was revised placing it in Class Tritrichomonadea and Family Dientamoebidae (Cepicka et al., 2010).

Phylogenetic analyses of four protein genes were examined to determine/confirm the placement of *H. meleagridis* in close proximity to *D. fragilis* and *T. foetus*. Phylogenetic trees derived from the GADPH, enolase, and the α- and β-tubulin were compared to sequences from other related parasites. The GADPH-based tree determined the histomonal sequences were closely related to both *T. foetus* and *Monocercomonas* sp. Similar results were seen in the enolase-based tree; however, *T. foetus* and *T. gallinarum* had a closer relationship to the histomonal sequences and *Monocercomonas* sp. was set apart. The α- and β-tubulin trees disclosed no significant conclusions (Hauck and Hafez, 2010). Therefore, further research is needed regarding the range of variation and possible speciation of *H. meleagridis* and any correlation to host species or geographic locale, occurrence of breakouts relative to transmission, and determining if other unknown species are present resulting in potential misdiagnosis of histomoniasis.

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CHAPTER 3

MOLECULAR CHARACTERIZATION OF *HISTOMONAS MELEAGRIDIS* AND OTHER PARABASALIDS IN THE UNITED STATES USING THE 5.8S, ITS-1, AND ITS-2 rRNA REGIONS

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ABSTRACT: Extracted DNA from twenty-eight *Histomonas meleagridis*-infected avian tissue samples from multiple hosts and geographic locations was analyzed for variation in 5.8S rRNA and flanking internal transcribed spacer regions (ITS 1 and ITS 2). Samples were amplified by polymerase chain reaction (PCR), sequenced, and compared with known sequences from GenBank accessions of *H. meleagridis* and other related protozoa. The analyses revealed significant genetic variation within *H. meleagridis* sequences and suggested the possibility of multiple genotypes within the samples or possible misdiagnosis. Related protozoa found in some samples were mostly identified as *Tetratrichomonas* spp. However, one sample had a 93% identity to *Simplicimonas similis*, a newly described organism, suggesting the possibility of a new pathogen in poultry. A phylogenetic tree analyzing the 5.8S and flanking ITS regions was inconclusive and unable to resolve all *H. meleagridis* into a single grouping. In contrast, a tree constructed only on the 5.8S rRNA, grouped all but one *H. meleagridis* sample into one clade, including GenBank accessions submitted from Europe. This suggests that the 5.8S region is more reliable in identifying *H. meleagridis* than the combined 5.8S and flanking ITS regions. There was no correlation between genotypes and host species or geographic location, suggesting that *H. meleagridis* moves freely between multiple avian species in the sampled regions.

Introduction

Histomonas meleagridis is the causative agent of histomoniasis, more commonly known as Blackhead disease. *Histomonas meleagridis* is a parabasalid protozoal parasite of the family Dientamoebidae, order Tritrichomonadida, and class Tritrichomonadea (Cepicka et al., 2010). The family Dientamoebidae includes the 4 genera *Dientamoeba, Protrichomonas, Histomonas,* and *Parahistomonas*. The family is characterized as uninucleate to binucleate, lacking an infrakinetosomal body in the mastigont, a costa, and undulating membrane. *Histomonas meleagridis* has been known to cause significant morbidity and mortality events in gallinaceous birds, especially turkeys and chicken (McDougald, 2005). It is believed the ring-neck pheasant (*Phasianus colchicus*) is the natural host of *H. meleagridis* and the introduction of pheasants into the United States coincided with the emergence of blackhead and destruction of the emerging turkey industry in the early 1890's (Lund and Chute, 1972).

Little research has been conducted on genetic variability of *H. meleagridis* and how it relates to infectivity, transmission or virulence. Most studies have focused on the internal transcribed spacer (ITS) regions of the ribosomal gene. The ITS regions are non-coding sequences, resulting in less conservational pressure, and are suitable for molecular characterization of phylogenetically related organisms (Hillis and Dixon, 1991). In other studies, the 5.8S rRNA and flanking internal transcribed spacer regions (ITS1 and ITS2) were used as molecular markers for comparative sequence analysis (Bart et al., 2008; Gerhold et al., 2008; Grabensteiner et al., 2010; Sansano-Maestre et al., 2009). In a previous study, genetic variation of the ITS region 1 of *H. meleagridis* was scrutinized using C-profiling (van der Heijden et al., 2006). In C-profiling, the cysteine nucleotide pattern is compared between samples for evidence of genetic variation. Results yielded three significant genetic variants closely related to *Trichomonas vaginalis* and *Dientamoeba fragilis* (van der Heijden et al., 2006). Another resent study found four types of *H. meleagridis* using C-profiling of the 5.8S and flanking ITS regions. Analysis determined two types (A and B) were similar in both chickens and turkeys, type C was found almost exclusively in turkeys and the fourth type (D) was very rare (Hauck et al., 2010). The goal of the present study was to compare sequences of 5.8S rRNA and flanking ITS regions and the 5.8S region

exclusively of *H. meleagridis* from multiple avian species and various geographic locations in the United States to examine: the range of variation and possible speciation of *H. meleagridis* and any correlation to host species or geographic locale, occurrence of breakouts relative to transmission, and determining if other unknown species are present resulting in potential misdiagnosis of histomoniasis.

Materials and Methods

Sample acquisition and DNA extraction. A total of 130 possible histomoniasis samples from wild or commercial turkeys (*Meleagris gallopavo*), commercial and backyard chickens (*Gallus domesticus*), Chukar partridges (*Alectoris chukar*), peafowl, and Northern Bobwhites (*Colinus virginianus*) were obtained from labs located in North Carolina, Georgia, Arkansas, and California. Most samples consisted of paraffin-embedded tissue blocks, although some were formalin fixed tissues, fresh tissue samples, or live cultured histomonads. All samples were from cases that had been diagnosed as histomoniasis because of characteristic gross lesions in the cecum and/or liver in conjunction with microscopic observation. DNA was extracted using QIAGEN DNA Extraction Mini kits (QIAGEN, Valencia, California) per the manufacturer's instructions. Extracted DNA was stored at -20 C until used for DNA amplification by polymerase chain reaction (PCR).

Molecular characterization. The 5.8S rRNA and flanking ITS1, and ITS2 regions were amplified using Trichomonadida-family wide primers ITSF (5'-TGCTTCACTTCAGCGGGTCTTCC-3') and ITSR (5'-CGGTAGGTGAACCTGCCGTTGG-3') (Felleisen, 1997; Cepicka et al., 2005). PCR components included 1-2 µl of extracted DNA in a 25µl reaction containing Ready-to-go PCR beads (GE Scientific, Piscataway, NJ) and 20 pM of ITSF and ITSR primers. Cycling parameters for the amplification were 94 C for 2 minutes followed by 40 cycles of 94 C for 30 seconds, 48 C for 30 seconds, and 72 C for 2 minutes, and a final extension at 72 C for 15 minutes. For all PCR reactions, water was used as a negative control to detect contamination, and DNA isolated from a laboratory-propagated sample of *H. meleagridis* was included as a positive control.

PCR amplicons were separated by gel electrophoresis using a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. An approximate 400–base pair amplicon was excised and the DNA purified using a QIAquick Gel Extraction kit (QIAGEN) and ligated into the pDrive vector using the QIAGEN PCR cloning kit per the manufacturer's instructions. DNA transformation procedure was performed using QIAGEN EZ competent cells and 2µl of ligation-reaction per the manufacturer's instructions. Competent cells containing the vector with PCR product insert were detected with blue/white screening by plating 50 µl of the transformation mixture on Luria-Bertani (LB) broth agar plates supplemented with 100 mg/ml carbenicillin, 100 mM of Isopropyl β-D-1-thiogalactopyranoside, and 40 mg/ml of β-Gal reagent. Two to three colonies, if available, were isolated and propagated in LB broth supplemented with 100 mg/ml carbenicillin. Plasmid DNA was isolated using Mini-prep kit (QIAGEN) per the manufacturer's instructions. Sequencing of the inserts was performed using M13 F plasmid specific primers at the Integrated Biotechnology Laboratories (The University of Georgia, Athens, GA 30602). Sequences obtained from this study and other related sequences were aligned using the multisequence alignment ClustalX program (Thompson et al., 1994). Phylogenetic relationships were analyzed using Molecular Evolutionary Genetics Analysis (Center for Evolutionary Functional Genomics, Tempe, Arizona), version 4 program (Tamura et al., 2007). Phylogenetic trees of both 5.8S and flanking ITS regions and the 5.8S region were constructed with neighbor-joining, minimum evolution, and maximum parsimony algorithms using the Kimura 2-parameter model and close neighbor interchange. Bootstrap values were constructed using Felsentein's bootstrap test (Felsentein, 1985).

Results

Of 130 samples tested, twenty-eight were successfully cloned and sequenced (Table 1). Recovery from the formalin fixed samples was low. Sequence analysis of the 5.8S and flanking ITS regions (Fig. 1) revealed significant genetic variation within *H. meleagridis*. Phylogenetic alignment of the 5.8S, and flanking ITS regions with related organisms and *Tritrichomonas nonconforma* (as out-group, AY886845) resulted in a 341-bp alignment, of which 69 were invariant, 81 variable characters

were parsimony uninformative and 191 were parsimony informative. Strong to moderate neighbor-joining (bootstrap= 98-78%), minimum evolution (bootstrap= 98-66%), and weak maximum parsimony (bootstrap=92- 52%) values support this significant variation (Tree A, Fig. 1).

Clade 1 contained sequences sharing a 78.3% conserved identity and came from multiple species and geographic locations. The unresolved sequences share a 71.6% conserved identity and consists of multiple species and geographic locations. GAQ 1-B, obtained from the same Georgia quail as GAQ 1-A, was separated from clade 1 and the unresolved with weak to moderate bootstrap values, neighbor-joining (66%), minimum evolution (58%) and maximum parsimony (72%). GABC 1 shares a 93% maximum identity with a newly described organism *Simplicimonas similis*, and phylogenetic analysis also determined GABC 1 to be closely related to *S. similis* (Cepicka et al., 2010). Three sequences (NCT 3, NCT 4, and NCT 5) share a 90.9% conserved identity and are closely related to *Tetratrichomonas gallinarum* (83.6%) and *Trichomonas* sp. (82.7%). Sequences GABB 5-A and GABB 5-B and NCT 2-A and NCT 2-B were from the same broiler breeder and turkey, respectively (Table I), and were present in clade 1 and the unresolved sequences, suggesting birds can be infected with multiple genotypes. Genotypic variation did not correlate with host species or geographic location.

Sequence analysis of the 5.8S region demonstrated high conservation among the *H. meleagridis* sequences (Tree B, Fig. 2). Phylogenetic alignment of the 5.8S region with related organisms using *Tritrichomonas nonconforma* (as out-group, AY886845) resulted in a 116-bp alignment, of which 39 were invariant, 47 variable characters were parsimony uninformative and 30 were parsimony informative. Moderate to weak bootstrap values support this conservation: neighbor-joining (58%), minimum evolution (56%), and maximum parsimony (87%).

Histomonas clade (Fig. 2) consisted of 26 sequences; including all the GenBank *H. meleagridis* sequences sharing an 89.7% conserved identity. GACL 1 was separated completely from the other *H. meleagridis* sequences by moderately strong bootstrap values: neighbor-joining (87%) and minimum evolution (87%). Sequence GABC 1 was determined to be more closely related to the simplicimonads

and trichomonads with moderate bootstrap values: neighbor-joining (71%), minimum evolution (66%), and maximum parsimony (63%). Three sequences from North Carolina (NCT 3, NCT 4, and NCT 5) share a 94.8% conserved identity and are more closely related to *Tetratrichomonas gallinarum* (93.1%) and *Trichomonas* sp. (93.1%) (AY245156; AF236105.1). GAQ 1-B was determined to be related to *T. gallinarum* and *Trichomonas* sp. sharing a 94.8% conserved identity.

Discussion

In this study, a total of 130 samples of possible histomoniasis cases were collected, and from these, twenty-eight sequences were produced. Formalin fixed tissues contain fragmented or otherwise unsuitable products leading to our low number of positive clones. Other studies with *H. meleagridis* and *T. gallinae* using similar materials also reported low recovery rates (Hauck et al., 2010; Gerhold, unpublished data).

Significant genetic variation among isolates of *H. meleagridis* was observed by both sequence and phylogenetic analyses of the 5.8S rRNA and the flanking ITS regions (Tree A). Our data are consistent with the findings of genetic variation in Dutch and German isolates where C-profiling of the ITS1 region was used to compare isolates (van der Heijden et al., 2006; Hauck et al., 2010). Our study differed from their work by comparing full sequences of 5.8S rRNA and flanking ITS regions (Tree A), or the 5.8S region alone (Tree B), of samples collected from several geographic regions in the USA. These results not only detected significant variation, but also indicated the presence of other species in the samples. It was not possible to make direct comparisons of our work with C-profiling studies because base sequences are not determined in the latter method.

Construction of two phylogenetic trees with different regions (A=5.8S rRNA + flanking ITS regions vs. B=5.8S rRNA exclusively) resulted in different associations. With tree A, not all of the *Histomonas*-like sequences were resolved into the same clade. Only one *Histomonas* clade was observed and a set of unresolved *H. meleagridis* sequences including one of European origin (DQ167587). All of the sequences from Germany (HM229778-HM229787) were placed into clade 1 sharing a maximum

identity of 89-99% with the sequences from our data. Several of the GenBank *H. meleagridis* sequences (DQ167587, Reis et al., 2009; HM229778-HM229787, Hauck et al., 2010) were from turkeys in the Netherlands and Germany.

In contrast, construction of a tree using only the 5.8S region (Tree B) disclosed the strong conservation of this region among *Histomonas*-like organisms, while offering a clear separation of other known genera and species. This suggests that the 5.8S region used alone is best for separation of genera and species in the parabasalids. In this tree, the *Histomonas* clade contains all the GenBank sequences and all but one of the *Histomonas*-like samples. The bootstrap values are only moderate to weak (58, 56, and 87) but sequence comparison disclosed an 89.7% conserved identity within this clade.

GACL 1, from a Georgia commercial layer, was separated completely from the other *H. meleagridis* sequences in Tree B by moderately strong bootstrap values (87, 87). In Tree A, this sequence was included with the other unresolved *H. meleagridis* sequences. GACL 1 shares an 88.8% conserved identity with *H. meleagridis* and an 87.9% conserved identity with *D. fragilis*. One possibility is that GACL 1 could be *Parahistomonas wenrichi,* a described species for which there are no reference specimens (Lund, 1963).

One sample obtained from a backyard chicken (GABC 1) was closely related to the newly erected genus *Simplicimonas* (Cepicka et al., 2010). *S. similis,* a parabasalid from the order Tritrichomonadida, class Dientamoebidae was described from a gecko (*Uroplatus lineatus*). This appears to be the first report of a *Simplicimonas*-like organism in birds. It was suggested by Cepicka that GABC 1 could be *Monocercomonas gallinarum* (Personal communication). However, sequence analysis showed a 56% identity of the 5.8S and flanking ITS regions and 85.3% identity of the 5.8S region shared between *M. gallinarum*, compared with 75.9% and 94% identity, respectively, for *S. similis*. Thus, it is likely that GABC 1 is an un-described *Simplicimonas*-like species. Further characterization of this organism will require collection of more specimens, so that morphology, infectivity, and pathogenicity can be studied.

NCT 3, NCT 4, and NCT 5, obtained from commercial turkeys in North Carolina shared a 94.8% and 90.9% conserved identity within the 5.8S region and the 5.8S and flanking ITS regions, respectively.

Analyses suggest this group is closely related to *T. gallinarum* (AY245156) and *Trichomonas* sp. (AF236105.1) sharing an 83.6% and 93.1% conserved identity with *T. gallinarum* and an 82.7% and 93.1% conserved identity with *Trichomonas* sp. for the Tree B and Tree A, respectively. Further work will be required to identify the species represented by these samples.

Sequences GAQ 1-A and GAQ 1-B were isolated from the same tissue sample and apparently represent different species. GAQ 1-B was completely separated from the *H. meleagridis* sequences in both phylogenetic trees. However, phylogenetically in Tree A, it was more similar to *H. meleagridis* and *D. fragilis*, and in Tree B this sequence was closer to *T. gallinarum* and *Trichomonas* sp.

Significant correlations with host species or geographic location could not be determined from this study. Samples NCT 1 and NCT 2-A and B were from different farms in the same turkey production complex, but were identified as different based on sequence analysis. This suggests that cross contamination of the complex by workers was not the source of contamination. The poultry industries in the USA are vast and complex, and may contribute to this lack of correlations with frequent movements of birds and equipment. Entire flocks of turkeys or chickens used for breeding purposes may be reared in one state, and then moved several states away for production. Young turkeys may be brooded to a certain age in one locale, and then moved some distance away for grow out and marketing. The effect of such movements could be responsible for sequence group 1 being found in both Arkansas and Georgia turkeys. It seems unlikely that shipment of birds from a common hatchery to multiple locations could account for the spread of genotypic variants unless the birds were held in brooding facilities for some time before movement.

The study has suggested that undescribed species may cause clinical signs similar to histomoniasis. It is well known that other protozoans occasionally cause such lesions (Allen, 1941; Olson et al., 1940). It is not unusual for poultry to harbor more than one species of protozoan in the gut (Hauck et al 2010)**.** Some of these even cause lesions similar to those of histomoniasis. For many years, a strongly held view was that some species of *Trichomonas* or *Pentatrichomonas* was responsible for blackhead disease (Allen, 1941; Hadley, 1916). Unfortunately, the etiology of trichomoniasis-like

diseases in poultry has not been pursued in recent years. It would be valuable to obtain live samples of variants of *H. meleagridis* and other detected protozoans for infection experiments, and for further comparison.

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Table 3.1. List of *Histomonas meleagridis* samples obtained from diagnostic labs.

Isolate/Host	GenBank Accession Number	Chart ID
Arkansas Samples		
Turkey 1	HQ334189	Sequence group 1*
Turkey 2	HQ334173	ART 2
California Samples		
Turkey 3	HQ334174	CAT 3
Turkey 4	HQ334175	CAT 4
Georgia Samples		
Backyard chicken 1	HQ334182	GABC 1
Broiler Breeder 1	HQ334193	Sequence group 3*
Broiler Breeder 2	HQ334178	GABB 2
Broiler Breeder 3	HQ334179	GABB 3
Broiler Breeder 4	HQ334180	GABB 4
Commercial Layer 1	HQ334183	GACL 1
Quail 1	HQ334184	GAQ 1-A†
Quail 2	HQ334185	GAQ 1-B†
Broiler Breeder 5	HQ334176	GABB 5-A†
Broiler Breeder 6	HQ334177	GABB 5-B†
Broiler Breeder 7	HQ334181	GABB 7
Quail 3	HQ334194	Sequence group 3*
Peafowl 1	HQ334191	Sequence group 2*
Turkey 5	HQ334190	Sequence group 1*
Turkey 6	HQ334192	Sequence group 2*
Turkey 7	HQ334186	GAT7

Turkey 8	HQ334187	GAT8
North Carolina Samples		
Turkey 10	HQ540394	NCT 1
Turkey 11	HQ540395	NCT 2-A†
Turkey 12	HQ540396	NCT 2-B†
Turkey 13	HQ540397	NCT 3
Turkey 14	HQ540398	NCT 4
Turkey 15	HQ540399	NCT 5
Turkey 16	HQ334188	NCT 1O
GenBank *Histomonas meleagridis* Sequences		
Host	**Accession Numbers**	**Location**
Turkey (Type A+C)	HM229778	Germany
Turkey (Type A)	HM229779	Germany
Turkey (Type A)	HM229780	Germany
Turkey (Type D)	HM229784	Germany
Turkey (Type C)	HM229786	Germany
Turkey	DQ167587	Dutch
Chukar Partridge	GQ872347	Georgia, United States

* Indicates sequences obtained from different samples that are identical. These sequences were allocated to sequence group 1, 2, or 3.

† Sequences with the same number and different letter indicate those sequences that were not similar but were obtained from the same tissue sample.

FIGURE 3.1: Phylogenetic analysis of *Histomonas meleagridis* sequences and other closely related parabasalids based on the 5.8S rRNA and flanking ITS regions. The tree was constructed using a neighbor-joining algorithm with 500 replications in a Kimura 2-parameter model based on the 341-bp aligned nucleotide positions. *Tritrichomonas nonconforma* was used as an out-group. Unique sequences from *H. meleagridis* sequences obtained in this study are designated by letters; see Table I for avian species and geographic location of each sequence group. Bootstrap values for neighbor-joining, minimum evolution, and maximum parsimony algorithms are located at nodes. Asterisks indicate bootstrap values below 50%.

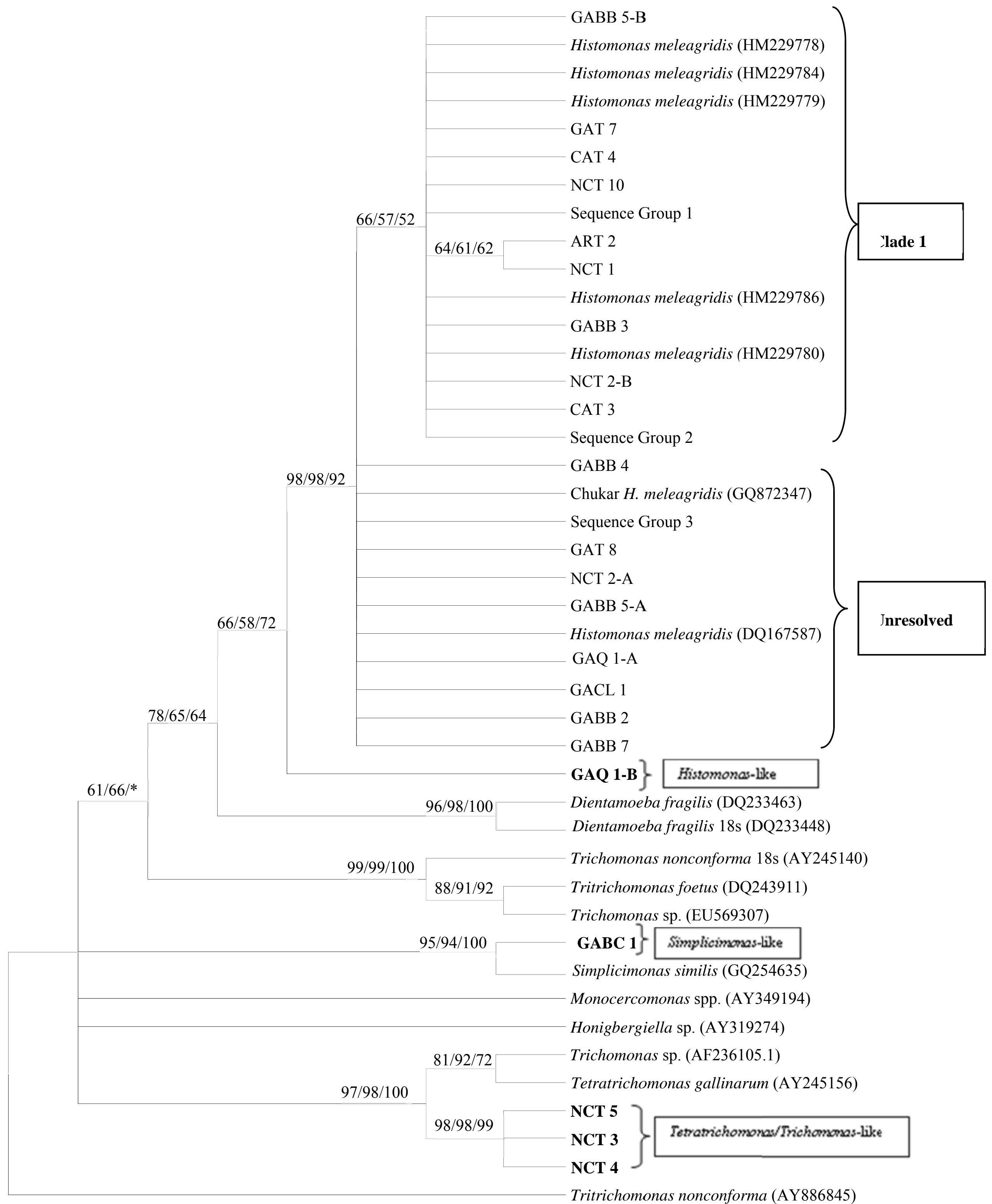
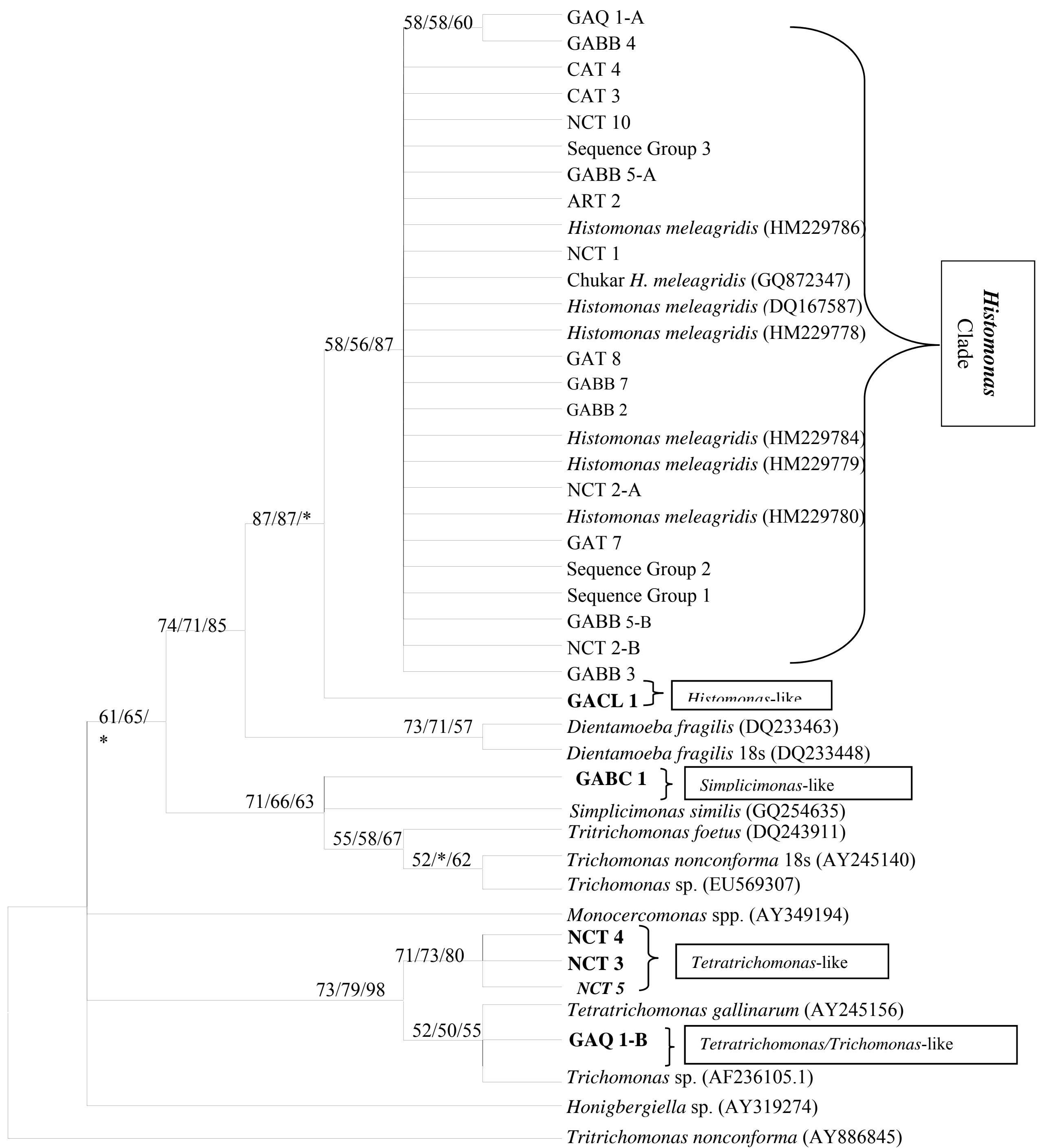

GABB 5-B
Histomonas meleagridis (HM229778)
Histomonas meleagridis (HM229784)
Histomonas meleagridis (HM229779)
GAT 7
CAT 4
NCT 10
Sequence Group 1
ART 2
NCT 1
Histomonas meleagridis (HM229786)
GABB 3
Histomonas meleagridis (HM229780)
NCT 2-B
CAT 3
Sequence Group 2
GABB 4
Chukar H. meleagridis (GQ872347)
Sequence Group 3
GAT 8
NCT 2-A
GABB 5-A
Histomonas meleagridis (DQ167587)
GAQ 1-A
GACL 1
GABB 2
GABB 7
GAQ 1-B
Dientamoeba fragilis (DQ233463)
Dientamoeba fragilis 18s (DQ233448)
Trichomonas nonconforma 18s (AY245140)
Tritrichomonas foetus (DQ243911)
Trichomonas sp. (EU569307)
GABC 1
Simplicimonas similis (GQ254635)
Monocercomonas spp. (AY349194)
Honigbergiella sp. (AY319274)
Trichomonas sp. (AF236105.1)
Tetratrichomonas gallinarum (AY245156)
NCT 5
NCT 3
NCT 4
Tritrichomonas nonconforma (AY886845)
66/57/52
64/61/62
98/98/92
66/58/72
78/65/64
61/66/*
96/98/100
99/99/100
88/91/92
95/94/100
81/92/72
97/98/100
98/98/99
Clade 1
Unresolved
Histomonas-like
Simplicimonas-like
Tetratrichomonas/Trichomonas-like

FIGURE 3.2: Phylogenetic analysis of *Histomonas meleagridis* sequences and other closely related parabasalids based on the 5.8S rRNA region. The tree was constructed using a neighbor-joining algorithm with 500 replications in a Kimura 2-parameter model based on the 116-bp aligned nucleotide positions. *Tritrichomonas nonconforma* was used as an out-group. Unique sequences from *H. meleagridis* sequences obtained in this study are designated by letters; see Table I for avian species and geographic location of each sequence group. Bootstrap values for neighbor-joining, minimum evolution, and maximum parsimony algorithms are located at nodes. Asterisks indicate bootstrap values below 50%.


58/58/60
GAQ 1-A
GABB 4
CAT 4
CAT 3
NCT 10
Sequence Group 3
GABB 5-A
ART 2
Histomonas meleagridis (HM229786)
NCT 1
Chukar H. meleagridis (GQ872347)
Histomonas meleagridis (DQ167587)
Histomonas meleagridis (HM229778)
58/56/87
GAT 8
GABB 7
GABB 2
Histomonas meleagridis (HM229784)
Histomonas meleagridis (HM229779)
NCT 2-A
87/87/*
Histomonas meleagridis (HM229780)
GAT 7
Sequence Group 2
Sequence Group 1
74/71/85
GABB 5-B
NCT 2-B
GABB 3
GACL 1
Histomonas-like
Histomonas Clade
61/65/*
73/71/57
Dientamoeba fragilis (DQ233463)
Dientamoeba fragilis 18s (DQ233448)
GABC 1
Simplicimonas-like
71/66/63
Simplicimonas similis (GQ254635)
55/58/67
Tritrichomonas foetus (DQ243911)
52/*/62
Trichomonas nonconforma 18s (AY245140)
Trichomonas sp. (EU569307)
Monocercomonas spp. (AY349194)
NCT 4
71/73/80
NCT 3
Tetratrichomonas-like
NCT 5
73/79/98
Tetratrichomonas gallinarum (AY245156)
52/50/55
GAQ 1-B
Tetratrichomonas/Trichomonas-like
Trichomonas sp. (AF236105.1)
Honigbergiella sp. (AY319274)
Tritrichomonas nonconforma (AY886845)

CHAPTER 4

CONCLUSIONS

The goal of the present study was to compare sequences of 5.8S rRNA and flanking ITS regions and the 5.8S region exclusively of *H. meleagridis* from multiple avian species and various geographic locations in the United States to examine: the range of variation and possible speciation of *H. meleagridis* and any correlation to host species or geographic locale, occurrence of breakouts relative to transmission, and determining if other unknown species are present resulting in potential misdiagnosis of histomoniasis.

As seen in other studies (van der Heijden et al., 2006; Hauck et al., 2010), significant genotypic variation is present in *H. meleagridis*. Our data demonstrated vast amounts of variation that has not been previously described. The data suggests that possible speciation of *H. meleagridis* occurs, and that several of the specific genotypes are present throughout the United States and in the European Union.

Our study was also interested in observing any correlations between genotypes and host species or geographic locale. Both van der Heijden and Hauck's studies were not able to make any clear inferences about correlations with host species or geographic location; in part because both of the European studies had samples collected from a small geographic region and only from turkeys or chickens. Our data was comprised of samples from all over the United States and from multiple avian species including peafowl, chukars, Northern Bobwhites, wild and commercial turkeys, and wild and commercial chickens. Results disclosed no apparent correlations between both host species and geographic location. Instead, genotypes from the same clade were found in all of the states and in multiple species.

Interestingly, several new species and possible pathogens were revealed in this study. Some of the samples contained both *H. meleagridis* and an un-described species, and other samples were identified as an un-described species with no *H. meleagridis* detected. This finding is important for two main reasons. Firstly, these species represent a possible new pathogen in poultry and secondly these new pathogens could be causing misdiagnoses of histomoniasis.

Sequence analysis of our data led to a very interesting discovery. Several of the samples were from different farms in the same complex. It was assumed that the outbreaks of histomoniasis were all caused from one original source. However, the sequences obtained for these samples were all significantly different. This suggests that these outbreaks are caused from a different genotype and not transmitted from one farm to the other. It is important to realize that this sheds new light on transmission and the occurrence of outbreaks.

From this study, the importance of further research on *H. meleagridis* is demonstrated. Not only are there a significant amount of possible genotypes, but un-described pathogens may be causing histomoniasis-like outbreaks. Most importantly, it was deemed that outbreaks thought to be transmitted from one farm to another are in fact completely separate outbreaks. Histomoniasis is now a re-emerging disease that causes significant loss to the commercial poultry industry and with lack of treatments and control options research and understanding of this protozoan parasite is exceedingly important.

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APPENDICES

APPENDIX A

HISTOMONAS MELEAGRIDIS SURVIVAL IN TRANSIT: ESTABLISHMENT OF CULTURE CONDITIONS FOR SURVIVAL OF *HISTOMONAS MELEAGRIDIS* IN TRANSIT

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Summary: Fresh cecal samples from turkeys in North Carolina infected with *Histomonas meleagridis* were collected at necropsy, inoculated into warmed Dwyer's medium, and sent by overnight courier to our laboratory at the University of Georgia. Further incubation, at 40 °C, yielded positive cultures from all four samples. PCR and DNA sequencing confirmed the presence of *H. meleagridis*. To further establish conditions for survival in transit, we infected turkeys with *H. meleagridis*, euthanized the birds 10 days post-infection and allowed carcasses to incubate at room temperature for either 2 or 24 hrs. Following incubation, samples of cecal contents (0.5 g) were placed in Dwyer's media and held at 4, 25, or 30 C, for 6, 18, 24, 48, 72, 96, or 120 hrs, respectively, simulating holding conditions during transit. Samples were placed in a 40 C incubator at the specified times and examined daily for histomonad growth by light microscopy. Positive histomonad growth was detected from cecal samples obtained from the 2 hr incubated carcass and from cultures held at 30 C for 6, 18, 24, 48 and 72 hrs. No growth was seen from cultures held at 25 C or 4 C or at any temperature from the carcass allowed to incubate for 24 hrs at room temperature. These results suggest that positive isolation can be made from field samples, provided that material is collected at warm temperatures and transported rapidly to the laboratory.

Index words: Culture, Dwyer's media, *Histomonas meleagridis*, histomonosis

Histomonosis, caused by the protozoal parasite *Histomonas meleagridis*, has been reported from turkeys, chickens, and other poultry (11), numerous wild birds (3, 12), and several zoo birds (4) and thorough reviews of research on *H. meleagridis* have been published (3, 11). Histomonosis outbreaks have become more frequent and severe in United States and Europe due to the ban of nitroimidazole products (13). Although *H. meleagridis* is readily cultured in several media preparations (9), parasite culture is often not used for diagnosis. Acquisition of live cultures from field outbreaks is difficult because it is considered necessary to bring infected live birds to the laboratory. The organism is considered too fragile for even short term cooling. Culture of live organisms could greatly assist diagnosticians and allow for banking of live cultures for future work.

An opportunity to study the survival of field isolates occurred when we successfully isolated *H. meleagridis* from samples collected on farms in North Carolina and shipped to our laboratory by overnight courier. This manuscript details the collection of suspected histomonosis samples in the field for shipment to the lab, and further experiments on survival of *H. meleagridis* subject to cooling in transit.

MATERIALS AND METHODS

Diagnosis of histomonosis and acquisition of field isolates: In June 2009, a veterinarian contacted our laboratory to discuss a probable histomonosis outbreak in four separate turkey facilities. To confirm the diagnosis and attempt collection of live histomonads for future work we shipped culture flasks containing complete Dwyer's medium (5) by overnight courier to the North Carolina facility. The cooperating veterinarian collected samples from 4 production farms. Sections of ceca from euthanized clinically morbid birds were placed into a flask of medium. The medium was at ambient temperature, probably about 30 C. The culture flasks were sealed tightly, placed in an insulated container, and returned to our laboratory by overnight courier. The samples were immediately placed in an incubator at 40 C for 24 hrs. Approximately 1 ml of the liquid in each sample was sub-cultured into fresh, warmed (40 C) Dwyer's medium, incubated at 40 C, and examined daily for *H. meleagridis* growth by light microscopy

at 100X and 200X magnification. From subcultures, 1ml aliquots of late logarithmic cultures were cryopreserved in liquid nitrogen using Dwyer's medium supplemented with 8% DMSO (Sigma-Aldrich) (2).

To confirm the presence *H*. *meleagridis* by PCR and DNA sequence, a total of $5x10^6$ late logarithmic-growth phase histomonads were harvested by centrifugation (750*g* for 10 min) and DNA extracted using Qiagen Mini kits (Qiagen Inc., Valencia, California) per the manufacturer's instructions. DNA amplification of the internal transcribed spacer (ITS)-1, 5.8S rRNA, and ITS2 regions was performed using primers TFR1 (5'-TGCTTCAGTTCAGCGGGTCTTCC-3') and TFR2 (5'-CGGTAGGTGAACCTGCCGTTGG-3') (6). These primers amplify an approximately 400bp product covering the ITS1, 5.8S, and ITS-2 regions of the ribosomal RNA (rRNA) gene in trichomonadid protozoa (6). PCR reaction recipe, cycling parameters, PCR product visualization, and amplicon extraction and sequencing was performed as previously described (12). For PCR extraction, a negative water control was included to detect contamination and a water control was included in the PCR reaction.

Experimental study of survival:

Experimental animals. Domestic turkey poults were obtained from Sleepy Creek Hatchery, Goldsboro NC, in June, 2009. The birds were housed in colony cages in a room used only for brooding and given nonmedicated turkey starter feed and water *ad libitum*. At three weeks of age, birds were moved into infection rooms and placed in Petersime broiler finishing batteries consisting of 15 cages each, arranged in 5 tiers of 3 cages each, with stainless steel feeding and watering troughs.

Reference parasite and culture. Cryopreserved *H*. *meleagridis* cultures obtained from a histomonosis outbreak in Georgia (USA) were rapidly thawed and placed in Dwyer's medium and incubated at 40 C. After culture for approximately 72 hrs, late logarithmic-growth phase histomonads were harvested by centrifugation (750*g* for 10 min), counted using a hemocytometer, and adjusted to 90,000 histomonads/ ml for inoculation.

Experimental infection and test of survival conditions. Two birds were inoculated with 90,000 histomonads each via cloacal inoculation and given nonmedicated turkey starter feed and water *ad libitum*. Ten days post-infection, the birds were euthanized by cervical dislocation and carcasses were incubated at room temperature (about 25 C) for either 2 or 24 hrs. Next, the carcasses were opened and ceca were incised with sterile scissors. Samples of cecal contents (0.5 g) from each bird were placed in 22 separate culture flasks with Dwyer's medium. The flasks were held at 4, 25, or 30 C, for 6, 18, 24, 48, 72, 96, or 120 hrs, respectively, simulating transportation delays. Additionally one flask was inoculated at the time of cecal content collection and incubated immediately at 40C. At the specified times, samples from each of the three temperatures were moved into 40C incubator and examined for five consecutive days by light microscopy for histomonad growth.

RESULTS

Diagnosis of *H. meleagridis* and acquisition of live field isolates. All original samples and subcultures submitted from the North Carolina turkey facilities were positive for *H. meleagridis* within 48 hrs of arrival (original samples) and within 24 hrs of subculture by light microscopy. DNA extraction from all four samples was positive for *H. meleagridis* by PCR. Nucleotide sequence analysis of the 337bp PCR product from one of the four samples revealed a 98% identity to *H. meleagridis* as compared to the Genbank® database accession number sequences GQ872347. The sequence of this isolate will be published in a separate manuscript. Cultures frozen in liquid nitrogen from these isolates have been successfully resuscitated and used for experimental study.

Isolation and culture of *H. meleagridis* from experimentally infected birds. Both inoculated birds had clinical signs consisting of listlessness, drooping wings, and depression at the time of euthanasia. Additionally, both birds had sulfur yellow diarrhea, indicating liver failure. On gross examination, the ceca were markedly thickened and the lumen was distended by a large amount of caseous necrotic and

hemorrhagic material consistent with cecal cores. The liver of both birds contained scattered target-shaped foci of necrosis ranging from 0.5-2 cm in diameter. Other gross lesions were not apparent.

Positive histomonad growth was detected in cultures originating from the 2 hr pre-incubation at 30C for 6, 18, 24, 48 and 72 hrs (Table 1). No growth was seen from cultures incubated at 25C or 4C and no growth was seen at any temperature from the carcass held for 24 hrs at room temperature (Table 1).

DISCUSSION

Acquisition of field isolates of *H. meleagridis* has generally depended upon the transport of live birds to the laboratory where fresh cecal material can be directly inoculated into culture media. Otherwise, the ova of cecal worms (*Heterakis gallinarum)* can be inoculated into turkey poults in the laboratory and histomonads cultured when the birds become sick. Consequently, most research with *H. meleagridis* has been with organisms obtained from chickens (the main source of *Heterakis* ova). In the present study we have demonstrated that fresh material could be inoculated into culture medium on the farm, transported by overnight courier to the lab, and successfully cultured for diagnostic or experimental study.

In a follow-up study, we were successful in culturing *H. meleagridis* from infected birds that were killed for necropsy, incubated at room temperature for 2 hrs, and followed by a holding period of up to 72 hrs at 30 C. From these results we could suggest several conditions for successful isolation from the field: The initial inoculation medium should be warmed to 35-40 C; samples should be sealed immediately and placed in an insulated container; the package is sent by overnight courier to the lab. The failure to isolate *H. meleagridis* from a bird dead for 24 hrs or from samples held at 4 or 25 C emphasizes the importance of starting with warm medium and maintenance of some warmth until the sample reaches the laboratory.

Currently, the diagnosis of histomonosis in birds relies on identification of characteristic gross lesions in the liver and ceca along with histopathological identification of histomonads. Increasingly,

PCR analysis is employed for confirmation (1,6,7,8,10,). ELISA is also used (15). The ability to submit live cultures of *H. meleagridis* to diagnostic laboratories for culture identification will potentially allow for earlier detection methods compared to the postmortem examinations.

Dwyer's medium (5) is often used for cultivation of *H. meleagridis* and is easily made in a diagnostic laboratory. It consists of a cell culture medium (M199), chick embryo extract (CEE_{50}), and horse serum, with 10-20 mg of rice flour added at time of inoculation. This and related media can be frozen at -20 C until ready for use. Recent studies showed that several other cell culture media can be substituted for M199, and that chick embryo extract is not essential for histomonad growth (9, 14). Other types of serum and grain flours are also acceptable (9).

Submission of inoculated culture flasks during the summer when the ambient temperature is often >30 C would allow for survival of histomonads. However, during other parts of the year when temperatures are often <30 C, the sample packages may need sources of heat to allow for histomonad survival. A potential source of heat could include re-freezeable gel packs that are heated for approximately 1-2 minutes in a microwave and placed in the container used for overnight transport. This technique has been used for maintaining survival of *Trichomonas gallinae* cultures during overnight transport (Gerhold, unpublished data).

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Table A.1. *Histomonas meleagridis* growth depending on carcass and culture incubation durations and culture incubation temperature. Detectable growth indicated by + and no detectable growth indicated by -.

	Carcass cooling time[a]					
	2hr postmortem			24hr postmortem		
Holding period (hrs)[b,c]	Holding temperature (C)			Holding temperature (C)		
	4	25	30	4	25	30
6	-	-	+	-	-	-
18	-	-	+	-	-	-
24	-	-	+	-	-	-
48	-	-	+	-	-	-
72	-	-	+	-	-	-
96	-	-	-	-	-	-
120	-	-	-	-	-	-

[a] Duration of postmortem carcass cooling at room temperature (25 C)

[b] Duration of sample holding at respective temperatures, prior to incubation at 40 C.

[c] Sample from 2 hr cooling taken for immediate incubation was positive. Sample taken from 24 hr cooling and incubated immediately was not positive.

APPENDIX B

PARTIAL SEQUENCE OF THE ALPHA-TUBULIN GENE FROM *HISTOMONAS MELEAGRIDIS* ISOLATES FROM THE UNITED STATES

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ABSTRACT: *Histomonas meleagridis*, the causative agent of histomoniasis, is a protozoan parasite classified in the Dientamoebidae (order Tritrichomonadida). The α-tubulin gene of 7 *H. meleagridis* isolates originating from either domestic chickens or turkeys from the United States was amplified by nested PCR and sequenced. A 91.4-99.8 % nucleotide identity was shared among the 7 different sequences and phylogenetic analysis disclosed that the 7 isolates were divided into at least 3 clades. These sequences had a 91-99% nucleotide identity and a 96-100% amino acid identity compared to 3 *H. meleagridis* α-tubulin sequences obtained from isolates originating from turkeys in Germany. Further α-tubulin gene analysis from protozoa in the Dientamoebidae will be useful in elucidating the evolutionary relationship of these protozoans.

Histomonas meleagridis, the causative agent of histomoniasis (blackhead disease), is a parabasalid protozoan parasite of the Dientamoebidae (order Tritrichomonadida: class Tritrichomonadea) (Cepicka et al., 2010). Dientamoebids include species of *Dientamoeba*, *Protrichomonas*, *Histomonas*, and *Parahistomonas* and are characterized as uninucleate to binucleate, lacking an infrakinetosomal body in the mastigont, and lacking a costa and undulating membrane (Cepicka et al., 2010). The most important human pathogen in this family is *Dientamoeba fragilis*, which causes chronic diarrhea and has been implicated in irritable bowel syndrome (Stark et al., 2006). Other pathogenic protozoans causing either human or animal diseases belong to Tritrichomonadea, and include *Trichomonas vaginalis*, *Trichomonas gallinae*, and *Tritrichomonas foetus* (Honigberg et al., 1984; BonDurant and Honigberg, 1994).

Histomoniasis is commonly reported from turkeys, chickens, and other poultry (McDougald, 2005), numerous wild birds (Davidson, 2008; Reiss et al., 2009), and several zoo birds (Douglas, 1981); a thorough review of research on *H. meleagridis* has been published (McDougald, 2005). The parasite has a wide spectrum of virulence and variable tissue tropism (Senties-Cue et al., 2009). Outbreaks in poultry have become more frequent and severe in United States and Europe after nitroimidazole treatment products were banned (Hauck and Hafez, 2009; Sentis-Cue et al., 2009).

Recent α-tubulin sequence analysis from 3 clones originating from 2 *H. meleagridis* isolates in domestic turkeys from Germany demonstrated that *H. meleagridis* was most closely related to *Tetratrichomonas gallinarum* and *T. foetus* (Hauck and Hafez, 2010). Constructed consensus phylogenetic trees based on glyceraldehydes 3-phosphate dehydrogenase (GAPDH), endolase, α-tubulin, β-tubulin, and 18S rRNA sequences showed a close relationship to *T. foetus* and to a lesser extent *Monocercomonas* sp. (Hauck and Hafez, 2010). However, *H. meleagridis* β-tubulin sequence analysis disclosed a close relationship to *D. fragilis,* but separate from *Monocercomonas* sp. and *T. foetus* sequences (Hauck and Hafez, 2009). Similarly, the 18S rRNA phylogenetic analysis demonstrated a close relationship between *H. meleagridis* and *D. fragilis* and separate from *T. foetus* (Gerbod et al., 2001). Further phylogenetic analysis of *H. meleagridis* protein-coding regions from other geographical

areas and avian hosts would be useful in understanding the evolutionary relationship of these protozoans and the epidemiology of histomoniasis. This report describes the α-tubulin sequences of 7 *H. meleagridis* isolates originating from either domestic chickens or turkeys from the United States.

DNA was extracted from 7 *H. meleagridis* isolates listed in Table I, using Qiagen Mini kits (Qiagen Inc., Valencia, California) per the manufacturer's instructions. DNA amplification of the α-tubulin gene was performed using a nested PCR protocol with the primary reaction consisting of 5 µl of DNA in a 50-µl reaction using primers α-tubA (5'-RGTNGGNAAYGCNTGYTGGGA-3') and α-tubB (5'-CCATNCCYTCNCCNACRTACCA-3') and the secondary reaction consisted of 1 µl of DNA from the primary reaction placed into a 50-µl reaction using primers α-tubF1 (5'-TAYTGYYWNGARCAYGGNAT-3') and α-tubR1 (5'-ACRAANGCNCGYTTNGMRWACAT-3') (Edgcomb et al., 2001). PCR components and cycling parameters were the same as previously described (Gerhold et al, 2008). For all PCR extractions, a negative water control was included to detect contamination and water controls were included in both primary and secondary reactions. One PCR amplicon was generated per unique *H. meleagridis* isolate. PCR amplicon separation and extraction and bi-directional nucleotide sequencing, using amplification and internal primers, were the same as previously described (Gerhold et al, 2008). Sequences obtained from this study and from other similar protozoa stored in GenBank were aligned using the multisequence alignment ClustalX program (Thompson et al., 1994). Phylogenetic analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 program (Tamura et al., 2007). The neighbor-joining and minimum evolution algorithms use the Kimura 2-parameter model and maximum parsimony uses a heuristic search.

The 7 sequences in this study shared a 91.4-99.8% nucleotide identity and a 96-100% amino acid identity among each other. No consistent nucleotide polymorphisms were found in isolates originating from the different avian hosts or from different areas within the United States (US). The α-tubulin sequences of the 7 *H. meleagridis* isolates had an 91-99% nucleotide identity to 3 *H. meleagridis* sequences from isolates originating from Germany (accessions GQ409855, GQ409854, FJ710160), and a

85-87% identity to *Tritrichomonas foetus* (accession AY277784). A protein BLAST revealed the sequences had a 96-100% amino acid identity to the 3 German *H. meleagridis* sequences (accessions GQ409855, GQ409854, FJ710160), a 93-96% amino acid identity to *Tritrichomonas foetus* (accession AY277784), and a 93-97% identity to *T. gallinae* (accession ABZ81810) and *T. vaginalis* (accession XP 1299097). Alignment of the partial α-tubulin gene sequence with related organisms and *Tetratrichomonas* sp. (as outgroup, Genbank accession AY886885) resulted in an alignment 909 bp in length, of which 600 were invariant, 54 variable characters were parsimony uninformative, and 253 were parsimony informative. Robust to weak neighbor joining (100-75%) and minimum evolution (100-59%) bootstrap values supported the separation of the 7 sequences in this study into 4 clades (Fig. 1). In contrast, maximum parsimony analysis failed to resolve the NC turkey 1 and GA chicken 4 sequences into separate clades; however**,** it gave robust support (97-100% bootstrap values) for the separation of the 7 sequences into 3 clades. Four of the US isolates sequences were grouped into a single clade that included 2 of the German isolates and the remaining German isolate grouped with GA turkey 1, which originated from a histomoniasis outbreak in domestic turkeys. Although, moderate neighbor joining (86%) and minimum evolution (77%) bootstrap values supported the separation of all *H. meleagridis* isolates from *T. foetus* and *Monocercomonas* sp., maximum parsimony analysis failed to resolve this separation.

Our data revealed similar phylogenetic relationships as the previous α-tubulin and β-tubulin analysis of *H. meleagridis* from Germany; however, our study revealed at least 1 separate clade formed by a single sequence (Georgia Chicken 4) that had robust separational support from all other sequences (Fig. 1). Additionally, no consistent sequence or phylogenetic differences were found from isolates originating from different avian hosts or geographical areas. Interestingly, when we performed our analyses using the previously published German sequences, it was found that two German sequences (Genbank accessions GQ409854 and GQ409855), originating from the same turkey, were placed into separate clades. Although nucleotide identities of the 7 isolates in this study ranged from 91-99%

compared to the German isolates, the amino acid identities ranged from 96-100% compared to the German isolates, suggesting conservational pressure exists at the amino acid level. This would be expected given the necessary function of the α-tubulin protein. Unfortunately, no sequences of the *D. fragilis* α-tubulin gene were available in Genbank to add to the phylogenetic analysis to determine if the α-tubulin genes of *D. fragilis* and *H. meleagridis* a have similar relationship to that of the β-tubulin.

Further work genetic analysis of *H. meleagridis* protein coding genes should be conducted from isolates from various hosts and locations to determine if any host-parasite relationships exist at either the nucleotide or amino acid level. The α-tubulins are potential targets for chemotherapy and research should be conducted to determine if histomoniasis control can be achieved with compounds that target these proteins. Inhibitors of α-tubulins, including dinitroanilines, are known to have broad antiprotozoal efficacy against related parasites (Morrissette et al., 2004) and**,** as such**,** would be potential candidates for chemotherapeutic trials.

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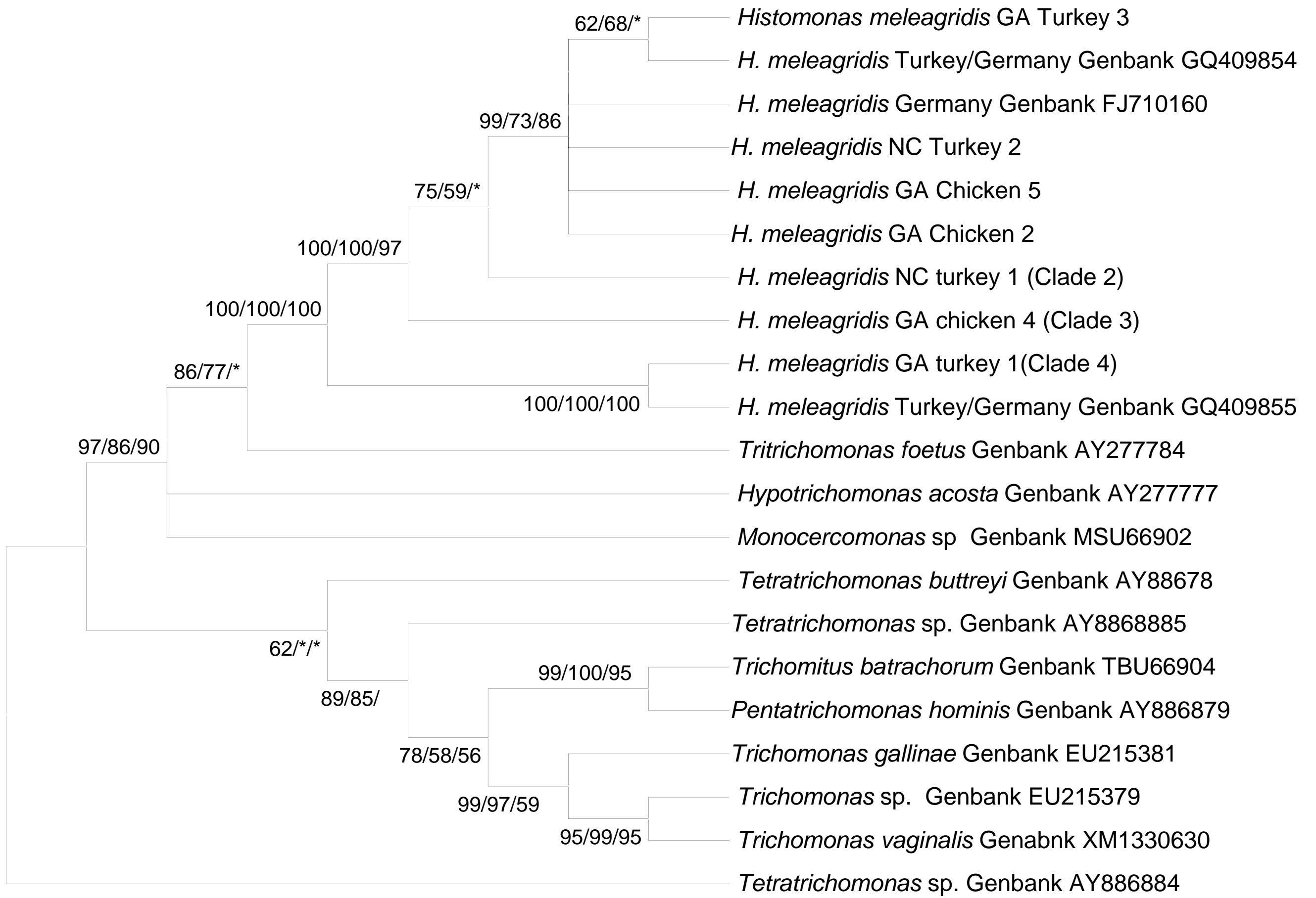
Table B.1. *Histomonas meleagridis* isolates used in this investigation.

Isolate	Host	Location of isolate origin	Genbank accession
GA Turkey 1	Turkey	Georgia	HQ416408
GA Turkey 3	Turkey	Georgia	HQ416409
GA Chicken 2	Chicken	Georgia	HQ416410
GA Chicken 4	Chicken	Georgia	HQ416411
GA Chicken 5	Chicken	Georgia	HQ416412
NC Turkey 1	Turkey	North Carolina	HQ416413
NC Turkey 2	Turkey	North Carolina	HQ416414

FIGURE B.1. Phylogenetic analysis of *Histomonas meleagridis* isolate from this study and other trichomonads based on sequence alignment of overlapping 909 bp α-tubulin gene. The tree was constructed using a minimum evolution algorithm with 500 replications in a Kimura 2-parameter model using a heuristic search with *Tetratrichomonas* sp. as an outgroup. Bootstrap values for neighbor-joining/minimum evolution/ maximum parsimony values are shown at the nodes. Asterisks indicate nodes with bootstrap values below 50%.


62/68/*
Histomonas meleagridis GA Turkey 3
H. meleagridis Turkey/Germany Genbank GQ409854
H. meleagridis Germany Genbank FJ710160
99/73/86
H. meleagridis NC Turkey 2
75/59/*
H. meleagridis GA Chicken 5
100/100/97
H. meleagridis GA Chicken 2
H. meleagridis NC turkey 1 (Clade 2)
100/100/100
H. meleagridis GA chicken 4 (Clade 3)
86/77/*
H. meleagridis GA turkey 1(Clade 4)
100/100/100
H. meleagridis Turkey/Germany Genbank GQ409855
97/86/90
Tritrichomonas foetus Genbank AY277784
Hypotrichomonas acosta Genbank AY277777
Monocercomonas sp Genbank MSU66902
Tetratrichomonas buttreyi Genbank AY88678
Tetratrichomonas sp. Genbank AY8868885
62/*/*
99/100/95
Trichomitus batrachorum Genbank TBU66904
89/85/
Pentatrichomonas hominis Genbank AY886879
78/58/56
Trichomonas gallinae Genbank EU215381
99/97/59
Trichomonas sp. Genbank EU215379
95/99/95
Trichomonas vaginalis Genabnk XM1330630
Tetratrichomonas sp. Genbank AY886884