MOLECULAR CHARACTERIZATION, HEMOLYTIC ACTIVITY, AND INVESTIGATION OF THE PRESENCE OF INTRACELLULAR RNA VIRUSES IN *TRICHOMONAS GALLINAE*

by

RICHARD WILLIAM GERHOLD JR

(Under the Direction of John R. Fischer)

ABSTRACT

DNA amplification by PCR and sequence analysis of the 5.8S rRNA and flanking internal transcribed spacer regions was performed on forty-two *Trichomonas gallinae* isolates. The analysis suggests three different species exists within the *T. gallinae* morphologic complex. One group demonstrated high homology to *T. gallinae* GenBank sequences; whereas the second group was more closely related to *T. vaginalis* (98%) than to *T. gallinae* (92%). The third group shared a 92% identity with *T. vaginalis, T. gallinae,* and *T. tenax.* Sequence analysis of the 18S rRNA and alpha-tubulin genes supports the results.

Virus purification was performed on twelve *T. gallinae* isolates to determine if intracellular RNA viruses exist. Viruses were not detected by RNA extraction, reverse transcriptase-PCR, or by electron microscopy following purification attempts.

The hemolytic activity of twenty-two *T. gallinae* isolates was investigated. Mean hemolytic activity ranged from 3.5% to 53.4% and hemolytic activity was not associated with virulence.

INDEX WORDS: Hemolytic activity, intracellular viruses, molecular characterization, *Trichomonas gallinae, Trichomonas vaginalis*, virulence

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DEDICATION

I would like to dedicate this thesis to my grandfather, Mr. Joseph A. Puelo whose dedication, devotion, diligence, and self-reliance were contagious to those who surrounded him. Many of the opportunities that I have been given in life were due to the many contributions that he bestowed upon me and our family.

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CHAPTER 1

INTRODUCTION

Trichomonas gallinae, the etiologic agent of avian trichomonosis, is a flagellated protozoal parasite in the class Zoomastigophorea and order Trichomonadida (Stabler, 1954). The protozoa are found in the upper digestive tract of clinically and subclinically infected birds within numerous avian orders. Trichomonosis generally manifests as a caseous, ulcerative mass within the oral cavity and the disease has been reported in several avian species, particularly those in the orders Columbiformes and Falconiformes (Stabler, 1954). Avian trichomonosis has been reported from most continents and is considered the most important disease for several species including mourning doves (*Zenaida macroura*), Mauritian pink pigeons (*Columba mayeri*), and focal populations of Cooper's hawks (*Accipiter cooperii*) (Stabler, 1954; Boal et al., 1998; Swinnerton et al., 2005).

Direct contact during nestling feeding and adult breeding behavior of columbids facilitates the transmission of *T. gallinae* (Kocan and Herman, 1971; Kietzmann, 1990). Adults in the order Columbiformes feed their nestlings with crop milk, a mixture containing sloughed crop epithelial cells, water, minerals, and nutrients (Beams and Meyer, 1931). During courtship, adult columbids perform a behavior termed "billing," in which birds place their bills within the bills of their mate (Sayer et al., 1993). *Trichomonas gallinae* also may be acquired via contaminated feed and water and contaminated bird feeders and birdbaths can serve as a source of exposure (Kocan, 1969).

Rock pigeons (*Columba livia*) are the natural host for *T. gallinae* and most pigeons harbor these protozoa, but rarely have clinical disease (Stabler, 1954; Tudor, 1991). *Trichomonas gallinae* has a wide spectrum of virulence, but the factors that control virulence are incompletely known (Honigberg et al., 1971; Honigberg, 1979). Previous investigations with known virulent and avirulent *T. gallinae* isolates suggest virulence and pathogenicity are controlled genetically within the organism (Honigberg et al., 1971); however, genetic investigations of *T. gallinae* have not been conducted. Infection with an avirulent isolate or survival of infection with a virulent isolate of *T. gallinae* provides columbids with protective immunity, resulting in individuals that are refractory to clinical disease (Stabler, 1948; Kocan and Amend, 1972). Therefore, previously infected pigeons and doves may serve as inapparent carriers of virulent isolates of *T. gallinae* and are potential sources of infection for naive birds (Stabler, 1954). Pigeons and doves also may serve as a source of infection for raptors, in which the disease can have focal population impacts (Boal et al., 1998).

A similar spectrum of virulence has been documented in *T. vaginalis*, a sexually transmitted protozoan that is the cause of human trichomonosis. Approximately fifty percent of *T. vaginalis* isolates are infected with double-stranded RNA (dsRNA) viruses or virus-like particles (VLP) (Wang and Wang, 1985; Benchimol et al., 2002). The presence of dsRNA viruses or VLP within *T. vaginalis* is associated with expression of immunogenic proteins on the trichomonad surface, variations in protozoal phenotypes, and up-regulation of certain proteins, including known virulence factors (Wang et al., 1987; Alderete, 1999). Similar dsRNA viruses or VLPs have been found in several other protozoal parasites including *Tritrichomonas foetus* (Vancini and Benchimol, 2005), *Cryptosporidium parvum* (Kniel et al., 2004), *Giardia lambia* (Wang et al., 1993), and *Leishmania braziliensis* (Widmer et al., 1989).

Erythrocyte hemolysis has been proposed as a valid *in vitro* virulence assay for *T. gallinae* (DeCarli et al., 1996; DeCarli and Tasca, 2002). The investigators examined the hemolytic activity of five *T. gallinae* isolates and disclosed that hemolysis of chicken erythrocytes ranged from 72% to 91%. Scanning electron microscopy demonstrated trichomonads attaching to the erythrocytes and suggested that hemolysis was due to a contact-dependent mechanism (DeCarli and Tasca, 2002). The hemolytic activity of *T. vaginalis* previously has been correlated with clinical virulence (DeCarli et al., 1989; Dailey et al., 1990).

Trichomonas gallinae is an important wildlife disease agent and can have significant population impacts for several avian species. However, little is known regarding virulence factors and genetic differences among these protozoa. To this end, we investigated several aspects of *T. gallinae* to determine if genetic differences are apparent between virulent and avirulent isolates and if intracellular viruses are associated with virulence. Additionally, we examined the hemolytic activity of *T. gallinae* isolates to determine if hemolytic activity is a valid *in-vitro* virulence assay.

Specific objectives of this study are:

1. To characterize the 5.8S ribosomal RNA and flanking internal transcribed spacer (ITS) genes of *T. gallinae* and determine if a genetic difference is apparent between clinically virulent and avirulent isolates and if a host-parasite genotype associations exist. Virulence of the isolates was determined based on the presence or absence of trichomonad-associated lesions within the avian host from which the isolate was obtained

2. To determine if intracellular viruses exist and are associated with virulence in *T. gallinae*, we investigated the presence of intracellular viruses in both clinically virulent and avirulent *T. gallinae* isolates.

3. Investigate if hemolytic activity of *T. gallinae* is a valid *in-vitro* virulence assay by determining the hemolytic activity of clinically virulent and avirulent isolates.

4. Compile diagnostic findings from mourning doves submitted to the Southeastern Cooperative Wildlife Disease Study from southeastern states.

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CHAPTER 2

LITERATURE REVIEW

Trichomonas gallinae

History and structure

Trichomonas gallinae, the etiologic agent of avian trichomonosis, is a flagellated protozoal parasite found in the upper digestive tract of both clinically affected and clinically normal birds, particularly those in the order Columbiformes (Stabler, 1954). In 1878, Rivolta originally described *T. gallinae* as *Cercomans gallinae* when he examined an oral swab from a pigeon; however, the disease was recognized by falconers hundreds of years earlier in birds of prey that fed on infected pigeons (Stabler, 1954). Trichomonosis generally manifests as a caseous, ulcerative mass in the oral cavity and esophagus and the disease has been reported in birds from several orders. Numerous significant trichmonosis epornitics have been documented in several avian species, particularly those in the orders Columbiformes and Falconiformes (Haugen and Keeler, 1952; Boal et al., 1998). Avian trichomonosis has been reported from several continents and is considered the most important disease for mourning doves (*Zenaida macroura*), Mauritian pink pigeons (*Columba mayeri*), and focal populations of Cooper's hawks (*Accipiter cooperii*) (Stabler, 1954; Boal et al., 1998; Swinnerton et al., 2005).

Trichomonas gallinae is in the class Zoomastigophorea and order Trichomonadida. The protozoa have an elongated ellipsoid shape with a mean length: width ratio of 1:8, but many

different shapes have been observed, depending on culture media or phase of growth (BonDurant and Honiberg, 1994). Organisms range in length from 12.5 to 20.0 μm with an average of 16.7 μm. Four anterior flagella originate in the kinetosome complex and a fifth recurrent flagellum forms a well developed undulating membrane. *Trichomonas gallinae* lacks a free posterior flagellum that is an important diagnostic feature separating it from *Tetratrichomonas gallinarum* which often is found in ceca of many avian species, particularly those in the order Galliformes (BonDurant and Honiberg, 1994).

Protozoa in the order Trichomonadida lack mitochondria, but instead contain energy producing organelles termed hydrogenosomes (BonDurant and Honiberg, 1994). Hydrogenosomes participate in substrate-level phosphorylation, converting pyruvate to acetate, CO_2, and molecular hydrogen as ATP is produced. Due to the lack of mitochondria and peroxisomes and the presence of hydrogenosomes, trichomonads are believed to have diverged early from the main branch of eukaryotic evolution (Muller, 1993). However, recent findings suggest that hydrogenosomes and mitochondria share a common origin which is supported by the detection of genes encoding the soluble enzymes cysteine desulphurase from *T. vaginalis* (Tachezy et al., 2001). Phylogenetic analysis of these genes indicates a common mechanism of FeS cluster formation in mitochondriate as well as in amitochondriate eukaryotes.

Trichomonas gallinae replicate by binary fission of the trophozoites and are transferred from one avian host to another by direct contact or ingestion of contaminated food and water (Stabler, 1954). Scanning electron microscopy investigations disclosed a previously unrecognized pseudocyst form (Tasca and De Carli, 2003), which may aid in the short-term survival of the protozoa outside of the avian host. Additionally, the pseudocysts may allow the protozoa to survive in carcasses of birds that have succumbed to trichomonosis. Erwin et al.,

(2000) demonstrated that live cultures of *T. gallinae* could be obtained up to 24 hrs postmortem from white-winged dove carcasses.

Epidemiology

Trichomonosis, also known as crop canker in columbids and frounce in raptors, is considered a major disease for numerous avian species in the orders Columbiformes and Falconiformes (Stabler, 1954; Forrester and Spalding, 2003; Villanua et al., 2006). Direct contact during nestling feeding and adult breeding behavior of columbids facilitates transmission of *T. gallinae* (Kocan and Herman, 1971; Kietzmann, 1990). Adults in the order Columbiformes feed their nestlings with crop milk, a mixture containing sloughed crop epithelial cells, water, minerals, and nutrients (Beams and Meyer, 1931). During courtship, adult columbids perform a behavior termed "billing," in which each bird of the adult pair places its bill within the bill of its mate (Sayer et al., 1993). *Trichomonas gallinae* also may be acquired via contaminated feed and water, and contaminated bird feeders and birdbaths can serve as a source of infection (Kocan, 1969a).

Trichomonas gallinae has been reported worldwide in rock pigeons and was first recorded in the United States from this avian species (Cauthen, 1934; Waller, 1934). The first documentation of *T. gallinae* in mourning doves was from a captive colony in New York (USA) that contained infected doves and rock pigeons (Cauthen, 1934). Rock pigeons are considered the natural host and "ultimate source" for *T. gallinae* and most pigeons harbor this protozoan, but rarely exhibit clinical disease (Stabler, 1954; Tudor, 1991; BonDurant and Honiberg, 1994). Infection with an avirulent isolate or survival of infection with a virulent isolate of *T. gallinae* provides columbids with protective immunity, resulting in individuals that are refractory to

clinical disease (Stabler, 1948; Kocan and Amend, 1972). Kocan and Knisley (1970) demonstrated that birds retained protective immunity even when they were culture-negative at the time they were challenged with known virulent isolates. Therefore, previously infected pigeons and doves may serve as inapparent carriers of virulent isolates of *T. gallinae* and are potential sources of infection for naive birds (Stabler, 1954).

Trichomonosis is considered the most important disease in free-ranging mourning doves (Conti, 1993; Forrester and Spalding, 2003). During a two-year outbreak in multiple southeastern states, an estimated 50,000 to 100,000 mourning doves died of trichomonosis in Alabama alone (Haugen and Keeler, 1952). The disease occurs in mourning doves and other columbids throughout North America annually and large mortality events frequently are reported (Conti, 1993; Forrester and Spalding, 2003; Rosenstock et al., 2004). Recent population census information indicates that mourning doves have declined over much of the United States and particularly in the eastern United States during the last few decades (Dolton and Rau, 2003); however, it is unknown if trichomonosis is associated with the population decline.

Previous surveys of *T. gallinae* in mourning doves have disclosed prevalences ranging from 1% to 38%, with most surveys reporting 5-15% (Locke and Herman, 1961; Donnelly, 1962; Carpenter et al., 1972; Barrow and Hayes, 1977; Conti and Forester, 1981; Rupiper and Wallace, 1988; Schulz et al., 2005). Although Barrow and Hayes (1977) reported higher infection rates in juveniles than adults, Schulz et al. (2005) monitored the annual variation of *T. gallinae* from hunter-killed mourning doves without clinical trichomonosis and found *T. gallinae* in essentially equal portions (5.5%) of hatch year and after hatch year birds. Prevalence rates of *T. gallinae* reported from band-tailed pigeons (*Columba fasciata*) are similar to those of mourning doves (Stabler, 1951); whereas high prevalence rates (>75%) of *T. gallinae* have been

reported from rock pigeons and white-winged doves (*Zenaida asiatica*) (Stabler, 1961; Conti and Forrester, 1981; Glass et al., 2001). Both mourning doves and band-tailed pigeons are native North American columbids; whereas rock pigeons were introduced from Europe by early colonists in the 1600s (Schorger, 1952).

The native range of white-winged doves extends from northern Chile to the southwestern United States including Arizona, Texas, and New Mexico. In Texas, a significant northward range expansion of the white-winged dove population has occurred over the past seven decades (Swanson and Rappole, 1992). Surveys of *T. gallinae* in the expanding Texas white-winged dove populations disclosed prevalences of nearly 100% (Glass et al., 2001). Similarly high *T. gallinae* rates (97%) were found in a breeding population of white-winged doves accidentally introduced into South Florida (Conti and Forrester, 1981). Interestingly, mourning doves sympatric with white-winged doves in Florida had a 17% prevalence of *T. gallinae*; whereas mourning doves from areas in Florida without white-winged doves had 1%. Concerns for the potential transmission of virulent *T. gallinae* from white-winged doves to mourning doves led investigators to experimentally inoculate mourning doves with isolates cultured from white-winged doves. Results from this experiment revealed that one out of 25 infected mourning doves developed trichomonosis (Conti et al., 1985).

Trichomonosis has been reported in other avian species including the endangered Mauritian pink pigeon, (*Columba mayeri*), numerous passerine species, and several raptors including Cooper's hawks (*Accipiter cooperii*), barn owls (*Tyto alba*), Northern goshawks (*Accipiter gentilis*), and the endangered Bonelli's eagle (*Hieraaetus fasciatus*) (Work and Hale, 1996; Boal et al., 1998; Real et al., 2000; Bunbury et al., 2005; Krone et al., 2005; Pennycoat et al., 2005; Swinnerton et al., 2005). In several of these avian species, trichomonosis has caused

both focal and species-wide conservation issues (Boal et al., 1998; Bunbury et al., 2005). In one study, trichomonosis was diagnosed in 49% of all Mauritian pink pigeon squabs (Swinnerton et al., 2005) and the disease is considered the main factor inhibiting the recovery of the endangered pink pigeons. In Tucson, Arizona, 85% of Cooper's hawk nestlings were positive for *T. gallinae* and the disease was associated with a significant decline in recruitment (Boal et al., 1998; Estes and Mannan, 2003). Interestingly, Cooper's hawks from rural locations in the southern Tucson area had a significantly lower prevalence of trichomonosis (19%) than hawks in the greater Tucson metropolitan area (Estes and Mannan, 2003). The authors noted that doves and pigeons constituted a significantly higher proportion of the diet of urban hawks as compared to the rural hawks, which could explain the different prevalences.

Virulence

Trichomonas gallinae is found in clinically normal as well as diseased birds, so presence of the parasite does not indicate disease (Stabler, 1954). Studies with *T. gallinae* have demonstrated a wide spectrum of virulence. With highly virulent isolates, a bird can succumb to infection within 14 days after inoculation with a single trichomonad; whereas with avirulent isolates, a bird may fail to even seroconvert after being inoculated with $1x10^6$ organisms (Stabler and Kihara, 1954). Previous investigations with clinically virulent and avirulent *T. gallinae* isolates suggest virulence and pathogenicity are controlled genetically within the organism (Honigberg et al., 1971). The investigator exposed a clinically avirulent *T. gallinae* isolate to the cell-free homogenate of the highly virulent Jones' barn (JB) *T. gallinae* isolate and demonstrated increased pathogenicity of the former isolate by subcutaneous mouse assay. Additionally, it was determined that the increase in pathogenicity of the transformed isolate was a function of the duration the isolate was exposed to the JB cell-free homogenate (Honigberg et al., 1971). The

transformation of the avirulent *T. gallinae* isolate could be eliminated when DNAase or RNAase was added to the cell-free homogenate of the virulent isolate or when there was omission of nucleic acids from the transformation media. The pathogenicity of the transformed isolate never reached that of the original JB isolate; however, the transformed trichomonads maintained an established infection in birds; whereas the non-transformed avirulent isolates caused transient infection. Further studies suggested that low molecular weight RNA may be responsible for the entire RNA effect (Honigberg, 1979).

Previous infection with an avirulent isolate or survival after infection with a virulent isolate of *T. gallinae* provided columbids with protective immunity for a period of time against future clinical disease from known virulent isolates (Stabler, 1948; Kocan and Amend, 1972). Kocan (1970) demonstrated that naïve pigeons inoculated with serum from pigeons exposed to avirulent *T. gallinae* isolates were immune to disease when inoculated with a virulent isolate. The length of protective immunity has not been determined and it is hypothesized that resistant doves may serve as carriers of virulent strains of *T. gallinae* and potentially serve as a source of infection for susceptible doves (Stabler, 1954; Stabler and Kihara, 1954). Pigeons and doves also may serve as a source of infection for raptors (Boal et al., 1998).

The virulence of *T. gallinae* isolates can be influenced by temperature and culture methods. Stabler et al., (1964) could decrease or increase the virulence of the highly virulent JB isolate by altering the media, duration of cultivation, or the temperature in which the organism was cultured. Attenuation of the JB isolate was noted after 17 weeks of *in vitro* cultivation and by 21 weeks of culture, the virulence was so attenuated that the organism did not even induce an immune response in inoculated birds. However, serial passage of the attenuated JB trichomonad from pigeon to pigeon could restore the full virulence to the organism (Stabler et al., 1964). The

addition of streptomycin and penicillin in the culture media resulted in more rapid attenuation of virulence and the JB isolate became avirulent for naïve birds in only 7.5 weeks of cultivation with antibiotics (Stabler et al., 1964). If *in vitro* culture of the JB isolate was performed using chick liver cells, the isolate retained its full virulence after one year of culture (Honigberg et al., 1970). It was concluded that some factor or factors in the chick liver cells allowed for retention of virulence. Cryopreservation of isolates for 12 years did not affect virulence (BonDurant and Honiberg, 1994).

Avirulent isolates are richer in antigenic compounds in the avian host as compared to virulent isolates and virulence appeared to be directly correlated to the antigenic composition of the isolate (Stepkowski and Honigberg, 1972). Investigations into the effects of both clinically virulent and avirulent *T. gallinae* on trypsin-dispersed chick liver cell cultures disclosed that virulent isolates were more motile and evoked a more active phagocyte response than avirulent isolates (Honigberg et al., 1964). Phagocytes were able to eliminate infection with avirulent isolates; whereas with virulent isolates, the organisms were initially engulfed by the macrophages, but continued to multiply inside them, eventually leading to cell rupture and release of trichomonads (Honigberg et al., 1964).

Due to the possibility of preexisting immunity to *T. gallinae*, clinically avirulent isolates potentially could be virulent to naïve birds. To differentiate between clinical and actual virulence, inoculation of naïve columbids is used often. However, live animal experiments are expensive and potentially unattractive to prospective funding agencies. Hemolytic activity has been proposed as a valid *in vitro* virulence assay for *T. gallinae* (DeCarli et al., 1996; DeCarli and Tasca, 2002). The investigators examined the hemolytic activity of five *T. gallinae* isolates and disclosed that hemolysis of chicken erythrocytes ranged from 72% to 91%. Scanning

electron microscopy demonstrated trichomonads attaching to the erythrocytes and suggested that hemolysis was due to a contact-dependent mechanism (DeCarli and Tasca, 2002). Additionally soluble hemolysins were not detected in the assay supernatant. The hemolytic activity of a closely related trichomonad, *T. vaginalis*, previously has been correlated with clinical virulence of the isolate (DeCarli et al., 1989; Dailey et al., 1990).

Pathology

Trichomonas gallinae causes erosive and ulcerative caseous stomatitis and esophagitis, as well as necrotic and caseous lesions in the liver, heart, brain, orbital sinuses, pancreas, and occasionally other abdominal viscera (Kocan and Herman 1971; Narcisi et al., 1991). Interestingly, Kocan (1969b) demonstrated that a particular *T. gallinae* isolate caused lesions in the liver of pigeons, but the same isolate caused esophageal and lung lesions without hepatic lesions in inoculated mourning doves. The lesions due to *T. gallinae* infection can occur rapidly with some isolates and ulceration of the mucosa has been reported within four days of inoculation (BonDurant and Honiberg, 1994). The inflammatory response in the esophagus and oral cavity is composed primarily of heterophils whereas in the liver heterophils and macrophages are apparent within the necrotic regions (BonDurant and Honiberg, 1994).

Ultrastructural studies of *T. gallinae* infection in ring-necked doves (*Streptopelia risoria*) disclosed that between 6 to 15 hours post-infection (PI), a few trichomonads were attached by pseudopod like ruffles to squamous cells at the palatal-esophageal junction (PEJ) (Kietzmann, 1993). Between 19 and 24 hours PI, trichomonads dislodged epithelial cells with more severe lesions occurring in areas with higher numbers of organisms. Trichomonad populations increased greatly between 48 and 72 hours PI, expansive monolayers of trichomonads were

noted, and individual trichomonads formed vertical, palisading positions on the epithelium. During this period, desquamation of the mucosa increased and deep epithelial layers were invaded prior to complete loss of superficial layers (Kietzmann, 1993). Microscopic cankers were apparent by 48 hours PI, but gross cankers were not apparent until 96 hours. At 168 hour PI, epithelial cells sloughed off in large sheets and by 240 hours, the majority of the palate and anterior esophagus were necrotic.

Molecular characterization

There is a relatively close evolutionary relationship between *T. gallinae* and *T. vaginalis*. Comparative sequence analysis of the 5.8S ribosomal RNA (rRNA) genes and internal transcribed spacer (ITS) regions of multiple trichomonad species revealed the 5.8S gene sequence of *T. vaginalis* shared a 94% identity with *T. gallinae*; and the two ITS regions flanking the 5.8S gene shared a 92.6 % identity (Felleisen, 1997). The *T. gallinae* 5.8S gene had the highest identity to *T. vaginalis* compared to any other trichomonad species that were evaluated. Sequence analysis of the 5.8S-ITS regions of twenty-four *T. gallinae* isolates from pink pigeons or Madagascar turtle doves from Mauritius disclosed no sequence variation among the isolates or to two GenBank sequences from isolates cultured from rock pigeons in the United States (Silva et al., 2007). However, the Mauritian pink pigeon isolates could be distinguished by random amplified polymorphic DNA (RAPD) analysis (Silva et al., 2007).

Recent investigations characterizing isolates from the genus *Tetratrichomonas* by sequence analysis of the 5.8S rRNA and flanking ITS regions disclosed several cryptic species and unexpected diversity within the genus (Cepicka et al., 2005; Cepicka et al., 2006). To date, *Tetratrichomonas* is the only trichomonad genus to be molecularly examined in detail and the

results indicate there are at least sixteen distinct monophyletic groups within the genus (Cepicka et al., 2005). Additionally, most of these monophyletic groups are host-specific (Cepicka et al., 2006). These data suggest that each group may represent an individual trichomonad species associated with a particular host species.

Trichomonads and viruses

Approximately one half of all *T. vaginalis* isolates are infected with double-stranded RNA (dsRNA) viruses, termed *Trichomonas vaginalis* virus (TVV). Multiple TVVs have been found and described as heterogeneous populations of icosahedral, filamentous, cylindrical, or spherical virus particles ranging from 33-200 nm in diameter (Wang and Wang, 1985; Benchimol et al., 2002). A South African study disclosed that 82% of *T. vaginalis* isolates were infected with dsRNA viruses (Weber et al., 2003). It also was determined that a single *T. vaginalis* organism may contain several dsRNA viruses and/or virus-like particles (VLP) simultaneously, and the investigators suggested *T. vaginalis* organisms may be a reservoir for dsRNA viruses (Benchimol et al., 2002). The authors also observed virus particles extracted from *T. vaginalis* being adsorbed onto the plasma membrane and subsequently concentrated in vacuoles of previously virus-free isolates of *T. vaginalis*. Additionally, immunocytochemical testing detected cytoplasmic evidence of virus proteins suggesting virus replication within the protozoa (Benchimol et al., 2002).

Investigations of *T. vaginalis* and dsRNA viruses disclosed an association between the presence of virus particles and phenotypic variations, expression of immunogens on the cell membrane, and upregulation of certain proteins (Wang et al., 1987; Alderete, 1999). In a study investigating protein expression of virus-infected and virus-free *T. vaginalis* it was revealed that

virus-positive isolates had at least forty-seven unique proteins compared to the virus-free organisms of the same strain (Provenzano et al., 1997). In the virus-infected organisms there was upregulation of other proteins including the highly immunogenic p270 protein, as well as cysteine proteinases that degrade the extracellular matrix and allow the parasites access to the epithelial cells of the host (Alderete et al., 1986; Khoshnan and Alderete, 1994; Provenzano et al., 1997). Although the exact roles of the dsRNA viruses and VLPs are not known, the up-regulation of proteins and phenotypic change that occur with virus infection suggest dsRNA viruses are associated with the virulence of *T. vaginalis*. Similar dsRNA viruses or VLPs have been found in several other protozoal parasites including *Tritrichomonas foetus* (Vancini and Benchimol, 2005), *Cryptosporidium parvum* (Kniel et al., 2004), *Giardia lambia* (Wang et al., 1993), and *Leishmania braziliensis* (Widmer et al., 1989). To date, the presence of dsRNA viruses or VLP within *T. gallinae* has not been investigated.

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CHAPTER 3

IDENTIFICATION OF *TRICHOMONAS VAGINALIS*-LIKE CRYPTIC SPECIES IN THE *TRICHOMONAS GALLINAE* MORPHOLOGIC COMPLEX[1]

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[1]R. W. Gerhold, M. J. Yabsley, A. J. Smith, and J. R. Fischer. To be submitted to the *Journal of Parasitology*.

ABSTRACT

Trichomonas gallinae has a wide spectrum of virulence that is hypothesized to be controlled genetically; however, genetic sequence analysis of *T. gallinae* isolates has not been investigated. Forty-two *T. gallinae* isolates were molecularly characterized from several avian species obtained from a wide-ranging geographic distribution to determine if *T. gallinae* isolates differed in their genetic sequence depending on virulence, host species, or geographical location. Isolates were obtained from the American Type Culture Collection or cultured from ten avian species representing three families from a widespread geographical range within the United States. The 5.8S rRNA and flanking internal transcribed spacer (ITS) genes were amplified by PCR from extracted trichomonad DNA and the sequences of the overlapping 228 bp amplicons were analyzed phylogenetically. A consistent genetic difference was not apparent between virulent and avirulent isolates; however, the results of the sequence analysis strongly suggest at least three different species may exist within the *T. gallinae* morphologic complex. One group demonstrated high nucleotide homology to the three *T. gallinae* sequences available in Genbank; whereas the second group was more closely related to *T. vaginalis* (98%) than to *T. gallinae* (92%). The third group, comprised of two common ground-dove isolates, shared a 92% identity with *T. vaginalis*, *T. gallinae*, and *T. tenax* sequences from GenBank. Sequence analysis of both the 18S rRNA and alpha-tubulin genes from a subset of the isolates supports the 5.8S-ITS sequence results. All of the *T. vaginalis*-like isolates originated from Arizona, California, or Texas; whereas *T. gallinae* isolates were found in all of the sampled states. Both *T. vaginalis*-like and *T. gallinae* isolates were involved in focal trichomonosis outbreaks in both California and Arizona.

INTRODUCTION

Avian trichomonosis, caused by the protozoan *Trichomonas gallinae*, has been reported from several continents and is considered a major disease for numerous avian species in the orders Columbiformes and Falconiformes (Stabler, 1954; Forrester and Spalding, 2003; Villanua et al., 2006). In the southeastern United States, trichomonosis is considered the most important disease of free-ranging mourning doves (*Zenaida macroura*) (Forrester and Spalding, 2003; Gerhold et al., 2007). During a two-year outbreak in multiple southeastern states, an estimated 50,000 to 100,000 mourning doves died of trichomonosis in Alabama alone (Haugen and Keeler, 1952). The disease occurs annually in mourning doves and other columbids throughout North America and large mortality events frequently are reported (Forrester and Spalding, 2003; Rosenstock et al., 2004; Gerhold et al., 2007). Recent population census information indicates mourning doves have declined over much of the United States, particularly in the eastern United States during the last few decades, (Dolton and Rau, 2003), but it is unknown if trichomonosis is associated with the population decline.

Trichomonosis has significant population impacts in other columbids including the endangered Mauritian pink pigeon, (*Columba mayeri*), numerous passerine species, and a number of raptors including Cooper's hawks (*Accipiter cooperii*), barn owls (*Tyto alba*), and the endangered Bonelli's eagle (*Hieraaetus fasciatus*) (Work and Hale, 1996; Boal et al., 1998; Real et al., 2000; Bunbury et al., 2005; Pennycoat et al., 2005). In Tucson, Arizona, approximately 85% of Cooper's hawk's nestlings were positive for *T. gallinae* and trichomonosis was associated with a significant decline in recruitment (Boal et al., 1998; Estes and Mannan, 2003).

Studies with *T. gallinae* have demonstrated a wide spectrum of virulence. With highly virulent isolates, a bird can succumb to infection within 14 days after inoculation with a single trichomonad; whereas with some avirulent isolates, a bird may fail to seroconvert after inoculation with $1x10^6$ organisms (Stabler and Kihara, 1954; Honigberg, 1979). Previous investigations with clinically virulent and avirulent isolates of *T. gallinae* suggested that virulence and pathogenicity are controlled genetically within the organism (Honigberg et al., 1971). However, virulent and avirulent isolates previously have not been analyzed genetically.

Sequence analysis of the 5.8S ribosomal RNA (rRNA) and flanking internal transcribed spacer (ITS) genes of organisms within the genus *Tetratrichomonas* disclosed unexpected diversity and host-parasite associations (Cepicka et al., 2005; Cepicka et al., 2006). The results indicated that at least sixteen distinct monophyletic groups exist within the genus and most of these monophyletic groups are host-specific (Cepicka et al., 2006). Sequence analysis of the ITS1, 5.8S, and ITS2 rRNA regions of twenty-four *T. gallinae* isolates from Mauritian pink pigeons and Madagascar turtle-doves (*Streptopelia picturata*) from the island of Mauritius disclosed no nucleotide polymorphisms among any of the isolates or to two rock pigeons isolates from the United States (Silva et al., 2007). The goal of our study was to analyze sequences of *T. gallinae* isolates from several avian species obtained from a wide-ranging geographic distribution to determine if *T. gallinae* isolates differed genetically depending on virulence, host species, or geographical location.

MATERIAL AND METHODS

Parasite culture

Isolates collected during this study were acquired from hunter-killed birds, necropsy and arbovirus-testing submissions to the Southeastern Cooperative Wildlife Disease Study (SCWDS) College of Veterinary Medicine, The University of Georgia, Athens, Georgia (USA), or from birds captured in the field. Virulence of the isolates was determined based on the presence or absence of trichomonad-associated lesions within the avian host from which the isolate was obtained (Table 3.1). Oral swabs were initially cultured in In-Pouch™ TF kits (BioMed Diagnostics, White City, Oregon, USA), incubated at 37 C, and examined for five consecutive days for trichomonad growth. The In-Pouch™ TF kit has been shown to be as sensitive as Diamond's media for isolating *T. gallinae* and the kit has practical advantages for isolation of organisms, especially in field conditions (Cover et al., 1994). Subcultures were performed using Diamond's media (pH 7.0) supplemented with 10% heat-inactivated horse serum (HIHS) (Sigma-Aldrich, St. Louis, Missouri, USA) (Diamond, 1957). Initially subcultures were supplemented with antibiotics as previously described (Diamond, 1957), but once axenic cultures were established, the use of antibiotics was discontinued. Axenic cultures in late logarithmic growth were harvested by centrifugation (750 x *g* for 10 min) and cryopreserved in liquid nitrogen using HIHS supplemented with 8% dimethyl sulfoxide (Sigma-Aldrich) until further use. Three additional isolates, two cultured from rock pigeons and one from a mourning dove, were purchased from the American Type Culture Collection (Manassas, Virginia, USA). Virulence was determined based on the presence or absence of trichomonad-associated lesions within the avian host from which the isolate was obtained.

Molecular characterization

A total of $5x10^6$ late logarithmic-growth phase trichomonads were harvested by centrifugation (750 x *g* for 10 min) and DNA extracted using Qiagen Mini kits (Qiagen Inc., Valencia, California, USA) per the manufacturer's instructions. DNA amplification of the ITS1, 5.8S rRNA, and ITS2 regions was performed using trichomonad specific primers TFR1 (5'-TGCTTCAGTTCAGCGGGTCTTCC-3') and TFR2 (5'-CGGTAGGTGAACCTGCCGTTGG-3') (Felleisin, 1997). PCR components included 1-2.5µl of DNA in a 50-µl reaction containing 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 0.01% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Promega, Madison, Wisconsin, USA), 0.75 U *Taq* DNA polymerase (Promega), and 0.5 µM of primers TFR1 and TFR2. Cycling parameters for the amplification were 94 C for 2 min followed by 40 cycles of 94 C for 30 sec, 66 C for 30 sec, and 72 C for 2 min, and a final extension at 72 C for 15 min. For amplification of the 18S small subunit (SSU) rRNA, 1-2.5µl of DNA was used in a 50-µl reaction containing the same PCR components as above except primers 16S1 (5'-TACTTGGTTGATCCTGCC-3') and 16SR (5'-TGATCCTTCTGCAGGTTCACC-3') (Cepicka et al., 2006). Cycling parameters were the same as the 5.8S/ITS amplification, except an annealing temperature of 50 C was used. Amplification of the alpha-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 alpha-tubA (5'-RGTNGGNAAYGCNTGYTGGGA-3') and alpha-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). The PCR components for

the primary and secondary reactions were the same as stated above and cycling parameters were the same except an annealing temperature of 45 C was used. For all PCR extractions, a negative water control was included to detect contamination and water controls were included in both primary and secondary reactions.

PCR amplicons were separated by gel electrophoreses using a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. An approximate 350 bp (5.8S and ITS regions), 1,600 bp (18S SSU rRNA), or 1,150 bp (alpha-tubulin) amplicon was excised and the DNA purified using a QIAquick® Gel Extraction kit (Qiagen Inc.) per the manufacturer's instructions. Sequencing of the 5.8S and the flanking ITS regions were performed using amplification primers TFR1 and TFR2. Overlapping segments of the SSU rRNA were obtained using the external primers 16S1 and 16SR and the internal primers 514F (5'-GTGCCAGCMGCCGCGG-3'), 1055F (5'-CGGCCATGCACCACC-3'), 1055R (5'-CGGCCATGCACCACC-3'), and1385R (5'-GATCCTAACATTGTAGC-3') (Cepicka et al., 2006). To obtain full length alpha-tubulin gene sequence, overlapping sequences of the external primers α-tubF1 and α-tubR1 and internal primers trichtubF1 (5'-CTCMTTCGGTGGTGG-3') and trichtubR1 (5'-KGGGAAGTGGATACG-3') (Edgcomb et al., 2001). Amplicons were sequenced at the Integrated Biotechnology Laboratories, University of Georgia, Athens, Georgia (USA). Sequences obtained from this study and from other trichomonads 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 3.1 program (Kumar et al., 1993) using the neighbor-joining and minimum evolution algorithms using the Kimura 2-parameter model and maximum parsimony using a heuristic search.

RESULTS

Seventeen clinically virulent isolates were obtained from band-tailed pigeons (*Columba fasciata*), broad-winged hawk (*Buteo platypterus*), Cooper's hawk (*Accipiter cooperii*), mourning doves (*Zenaida macroura*), and a house finch (*Carpodacus mexicanus*) from six states (Table 3.1). Twenty-five clinically avirulent isolates were obtained from common ground-doves (*Columbina passerina*), Eurasian collared-doves (*Streptopelia decaocto*), mourning doves, rock pigeons (*Columba livia*), white-winged doves (*Zenaida asiatica*), and a captive ring-necked dove (*Streptopelia risoria*) from four states (Table 3.1). One virulent and two avirulent isolates were obtained from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA). The GenBank accession numbers of the sequences obtained in this study are listed in Table 3.1.

The analysis revealed two distinct clades (Fig. 3.1). One group of 27 isolates, exclusive of the two ground-dove isolates, shared $\geq$98.8 % identity to the 228 overlapping bp of the ITS1, 5.8S, and ITS2 of three *T. gallinae* sequences (GenBank accessions EF208019, AY349182, U86614), whereas the other group of 13 isolates had $\geq$98.4% homology to the 228 overlapping bp of *T. vaginalis* (GenBank accession AY871048) and $\leq$ 92.7% homology to *T. gallinae*. Alignment of the entire ITS1, 5.8S, and ITS2 regions with related organisms and *Tritrichomonas nonconforma* (as outgroup, AY886845) resulted in an alignment 228 bp in length, of which, 152 were invariant, 53 variable characters were parsimony uninformative, and 35 of the 88 variable characters were parsimony informative. Robust neighbor-joining (bootstrap=100%), minimum evolution (bootstrap=100%), and maximum parsimony (bootstrap=99%) values supported the separation of the two clades (Fig. 3.1). Fifteen of the 228 nucleotides of the ground-dove isolates were unique compared to all other sequences and the isolates shared only an approximate

92% identity with *T. vaginalis*, *T. gallinae*, and *T. tenax* (GenBank accession U86615). The ground-dove isolates had weak support for inclusion into either clade (Fig. 3.1).

Isolates belonging to the *T. gallinae* clade were obtained from all sampled states (Arizona, California, Colorado, Florida, Georgia, Kentucky, Pennsylvania, Tennessee, and Texas). Both *T. gallinae* and *T. vaginalis*-like isolates were obtained from Arizona, California, and Texas. Although a host-parasite association was noted for rock pigeons (*T. gallinae*), Eurasian collared-doves (*T. gallinae*), white-winged doves (*T. vaginalis*-like), and common ground-doves (*Trichomonas* sp.), no association was noted for isolates from Cooper's hawks, band-tailed pigeons, or mourning doves (Fig. 3.1). *Trichomonas vaginalis*-like isolates were only detected in western states (Arizona, California, and Texas).

The 18S rRNA sequence results supported the 5.8S/ ITS results. The *T. vaginalis*-like isolates showed a 98.5% identity to a *T. vaginalis* (accession AY338473) and only 95.2% (white-winged dove 1200) and 95.8% (mourning dove 9) identities to the sequence of one isolate (Cooper's hawk 4) belonging to the *T. gallinae* clade (no 18S rRNA sequence data from GenBank was available from *T. gallinae* for comparison). The 18S rRNA sequence of the common ground-dove isolates were 97.7% identical to *T. gallinae*, and 95.6% to *T. vaginalis*. Alignment of the entire 18S rRNA region with related organisms and *Pentatrichomonas hominis* (as outgroup, DQ412643) resulted in an alignment 1,005 bp in length, of which, 892 were invariant, 62 variable characters were parsimony uninformative, and 47 were parsimony informative. Phylogenetic analysis of two *T. vaginalis*-like isolates demonstrated moderate to strong neighbor-joining (bootstrap=73%), minimum evolution (bootstrap=77%), and maximum parsimony (bootstrap=99%) separation support from the sequences of the four *T. gallinae* isolates, *T. tenax* (accession D49495) and *T. canistomae* (accession AY247748) (Fig. 3.2).

Although neighbor-joining and minimum evolution gave strong (bootstrap=99%) support for the separation of the ground-dove isolate from the *T. gallinae* and *T. vaginalis*-like clades, maximum parsimony failed to resolve the ground-dove isolate as a separate clade.

The alpha-tubulin sequences of the *T. vaginalis*-like, *T. gallinae*, and common ground-dove isolates had a 98%, 95%, and 91% identity, respectively to *T. vaginalis* (accession XM1323317). Alignment of the partial α-tubulin gene with related organisms and *Tritrichomonas foetus* (as outgroup, AY277785) resulted in an alignment 705 bp in length, of which, 543 were invariant, 53 variable characters were parsimony uninformative, and 108 were parsimony informative. Phylogenetic analyses gave strong neighbor-joining (bootstrap=89%) and minimum evolution (bootstrap=91%) support for separation of four *T. vaginalis*-like isolates from six *T. gallinae* isolates (Fig. 3.3). Maximum parsimony analysis failed to resolve the *T. vaginalis*-like isolates from *T. gallinae* isolates (Fig. 3.3). The analysis also failed to resolve the common ground-dove isolate from either *Trichomonas* group or from two *Tetratrichomonas* species (Fig. 3.3).

DISCUSSION

Sequence and phylogenetic analysis of the ITS1, 5.8S, and ITS2 regions of the isolates did not support our hypothesis of observable genetic differences between clinically virulent and avirulent isolates. However, the combined results of our phylogenetic analyses gave strong support for two or three distinct clades within the *T. gallinae* morphologic complex. One clade was closely related to three *T. gallinae* sequences available in GenBank and the other clade was more similar to *T. vaginalis* sequences (Fig. 3.1). Analysis failed to resolve the relationship of common ground-dove isolates to other trichomonads.

The isolates were initially analyzed by comparing the ITS and 5.8S rRNA sequences. The ITS regions are non-coding and hence evolve more rapidly, and previous investigations have demonstrated analysis of the ITS regions useful in molecular comparison and distinguishing intraspecific variations of trichomonads (Felleisen, 1997) and other protozoa including *Perkinsus marinus* (Brown et al., 2004) and *Entamoeba* spp. (Som et al., 2000). To confirm the identification of multiple clades based on the 5.8S-ITS regions, we examined the sequences of two coding genes, the alpha-tubulin and 18S SSU rRNA genes, from a subset of isolates representing the two different clades. The analysis of both of these gene targets supported the separation of the *T. vaginalis*-like and *T. gallinae* isolates into separate clades (Fig. 3.2 and Fig. 3.3). These findings suggest that there are at least two species within the *T. gallinae* morphologic complex.

The common ground-dove isolates contained considerable genetic polymorphisms in the 5.8S-ITS regions that were not present in any of the sequences from either the *T. gallinae* or *T. vaginalis*-like groups. The maximum parsimony phylogenetic analysis of the 5.8S-ITS regions gave minimal support (bootstrap=55%) for the ground-dove isolates to be a subclade of the historic *T. gallinae* clade (Fig. 3.1). However, the neighbor-joining and minimum evolution analysis failed to resolve the ground-dove isolates into either clade. Analysis of the 18S rRNA and alpha-tubulin genes supported the separation of the ground-dove isolates from the *T. gallinae* and *T. vaginalis*-like clades. Unfortunately, we only had two isolates from common ground-doves to characterize; however, isolates from white-winged doves, Eurasian collared-doves, and mourning dove were collected in the same geographical locality as the ground-dove isolates and these isolates were genetically distinct from the ground-dove isolates. It would be interesting to

molecularly characterize trichomonad isolates from common ground-doves throughout their range to determine if there is a distinct host-parasite association.

Few nucleotide polymorphisms have been reported during three previous studies on the 5.8S-ITS regions of *T. gallinae* (Fellisen, 1997; Kleina et al., 2004; Silva et al., 2006). Our findings of two or three distinct phylogenetic groups is of particular interest given all twenty-four isolates originating from pink pigeons or turtle doves from Mauritius had nearly identical sequences to the two GenBank *T. gallinae* sequences that originated from rock pigeons in the United States (Silva et al., 2006). Because rock pigeons are considered the natural host for *T. gallinae* (Stabler, 1954; Tudor, 1991), intentional and accidental introduction of rock pigeons likely has contributed to the spread of *T. gallinae* worldwide. All of the rock pigeon isolates in our study belonged to the *T. gallinae* clade; however, a distinct host-parasite association can not be definitively concluded because, except for one Colorado isolate, all rock pigeon isolates originated from Georgia. A more comprehensive examination of rock pigeon isolates from several geographical regions is needed to support a host-parasite association. Isolates from Eurasian collared-doves (*Streptopelia decaocto*), an invasive species that is frequently reported in the southern United States and has expanded its range northward in recent years, (Romagosa and Labinsky, 2000) demonstrated a similar phylogenetic relationship as rock pigeon isolates (Fig. 3.1, Fig. 3.2, and Fig. 3.3).

Isolates in the *T. vaginalis*-like clade originated from several avian species and included all isolates from white-winged doves (Fig. 3.1). Surveys of white-winged doves in Texas disclosed *T. gallinae* prevalence rates of nearly 100% (Glass et al., 2001) and surveys in Florida demonstrated similar high (97%) prevalence rates (Conti and Forrester, 1981). However, none of the isolates were genetically characterized. It is plausible given the high prevalence of *T.*

gallinae in white-winged doves and the data from this study that white-winged doves are infected with their own trichomonad species. With exception of the band-tailed pigeon isolates from California, the *T. vaginalis*-like isolates originated from geographical regions associated with known white-winged dove populations. Molecular characterization of white-winged dove and band-tailed pigeon isolates in areas where the two species are sympatric and allopatric would be useful to clarify the epidemiology. Additionally, it would be useful to characterize isolates from white-winged doves throughout their entire range to determine if a host-parasite relationship exists.

In this investigation, mourning dove, band-tailed pigeon, and raptor isolates belonged to both *T. gallinae* and *T. vaginalis*-like clades. Previous surveys of *T. gallinae* in mourning doves and band-tailed pigeons have disclosed prevalences ranging from 1% to 38%, with most reports indicating 5-15% (Stabler, 1951; Locke and Herman, 1961; Donnelly, 1962; Carpenter et al., 1972; Barrow and Hayes, 1977; Conti and Forester, 1981; Rupiper and Wallace, 1988; Schulz et al., 2005). Conti and Forrester (1981) found mourning doves sympatric with white-winged doves in Florida had a 17% *T. gallinae* prevalence; whereas in areas of Florida without white-winged doves, mourning doves had 1%.

Interestingly, our data demonstrated both *T. vaginalis*-like and *T. gallinae* isolates were cultured during recent large trichomonosis outbreaks from trichomonosis-affected Cooper's hawks and mourning doves in Arizona and from band-tailed pigeons in California indicating at least two separate trichomonad species were involved in each of the outbreaks. If it is found that white-winged doves and rock pigeons do indeed have separate trichomonad species, it would support the prevalence data of mourning doves and band-tailed pigeons being infected by direct and indirect (sharing birdfeeders) exposure to white-winged doves and rock pigeons. Future

molecular characterization of *Trichomonas* spp. isolates from both clinically affected birds as well as non-clinically affected columbids may be useful in interpreting the epidemiology of trichomonosis outbreaks.

Although a genetic difference was not apparent between clinically virulent and avirulent isolates, clinical virulence of trichomonads must be viewed with caution. Previous investigations disclosed that domestic pigeons previously inoculated with a known avirulent isolate or surviving infection with a virulent isolate had protective immunity when challenged with a known virulent isolate (Stabler, 1948). Additionally it has been demonstrated that clinically normal pigeons can harbor both avirulent and virulent isolates; however, naïve doves and pigeons challenged with a mixture of virulent and avirulent isolates will succumb to infection (Stabler, 1954). Thus, the actual virulence of the clinically avirulent isolates examined in this study is unknown. However, the clinically virulent isolates were cultured from birds with trichomonosis and these isolates belonged to both clades. This supports our conclusion that a genetic association was not apparent among virulent isolates. Experimental infection of naïve doves would aid in clarifying the actual virulence of the clinically avirulent isolates.

To further characterize isolates from both clades and investigate intraspecific variation within the clades, additional molecular techniques including random amplified polymorphic DNA (RAPD) analysis or amplified restriction fragment polymorphism (AFLP) would be useful. In addition, cloning and molecular characterization of individual trichomonads from several isolates from both clades could demonstrate if isolates are comprised of identical trichomonad organisms or a mixture of multiple species. Analysis of additional samples collected from common ground-doves, white-winged doves, rock pigeons, and Eurasian collared-doves from a

greater number of geographic localities would be useful to determine if there are specific host-parasite relationships in the *T. gallinae* morphologic complex.

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TABLE 3.1 List of *Trichomonas gallinae* isolates obtained from free-ranging birds (unless stated otherwise) and included in the molecular analyses.

			GenBank accession numbers		
Isolate/Host	State of origin	Clinical virulence[d]	Internal transcribed regions	18S rRNA	α-tubulin
Band-tailed pigeon 1	CA	Virulent	EU215369	nd[c]	nd
Band-tailed pigeon 2	CA	Virulent	EU215367	nd	nd
Band-tailed pigeon 3	CA	Virulent	EU215367	nd	nd
Band-tailed pigeon 4	CA	Virulent	EU215367	nd	EU215380
Broad-winged hawk 1	FL	Virulent	EU215368	EU215375	nd
Cooper's hawk 1	AZ	Virulent	EU215366	nd	nd
Cooper's hawk 3	AZ	Virulent	EU215366	nd	nd
Cooper's hawk 4	AZ	Virulent	EU215369	EU215372	EU215382
Eurasian collared-dove 829	TX	Avirulent	EU215364	EU215374	EU215381
Eurasian collared-dove 858	TX	Avirulent	EU215363	nd	nd
Eurasian collared-dove 1617	TX	Avirulent	EU215363	nd	nd
Common ground-dove 1	TX	Avirulent	EU215359	EU215371	EU215376
Common ground-dove 1321	TX	Avirulent	EU215358	nd	nd
House finch 1	KY	Virulent	EU215369	nd	EU215382
Mourning dove ATCC 30095[a]	PA	Virulent	EU215369	nd	nd
Mourning dove 2	GA	Virulent	EU215369	nd	nd
Mourning dove 5	KY	Virulent	EU215369	nd	nd
Mourning dove 6	AZ	Virulent	EU215369	nd	nd
Mourning dove 9	AZ	Virulent	EU215366	EU215370	EU215377
Mourning dove 11	GA	Avirulent	EU215369	nd	EU215382
Mourning dove 18	GA	Virulent	EU215369	nd	nd
Mourning dove 20	GA	Virulent	EU215369	nd	nd
Mourning dove 22	TX	Avirulent	EU215365	nd	EU215379
Ring-necked dove 3[b]	TN	Avirulent	EU215364	nd	nd
Rock pigeon 8	GA	Avirulent	EU215369	nd	nd
Rock pigeon 11	GA	Avirulent	EU215369	nd	nd
Rock pigeon 14	GA	Avirulent	EU215369	nd	nd
Rock pigeon 15	GA	Avirulent	EU215369	nd	nd
Rock pigeon 20	GA	Avirulent	EU215364	EU215373	nd
Rock pigeon 22	GA	Avirulent	EU215369	nd	nd
Rock pigeon 27	GA	Avirulent	EU215369	nd	nd
Rock pigeon 28	GA	Avirulent	EU215364	nd	EU215382
Rock pigeon 32	GA	Avirulent	EU215369	nd	nd

Rock pigeon 52	GA	Avirulent	EU215364	nd	nd
Rock pigeon ATCC 30230[a]	Unknown	Avirulent	EU215363	nd	nd
Rock pigeon ATCC 30228[a]	CO	Avirulent	EU215362	nd	nd
White-winged dove 840	TX	Avirulent	EU215360	nd	nd
White-winged dove 947	TX	Avirulent	EU215366	nd	nd
White-winged dove 1159	TX	Avirulent	EU215361	nd	EU215378
White-winged dove 1200	TX	Avirulent	EU215366	EU215370	nd
White-winged dove 1208	TX	Avirulent	EU215366	nd	nd
White-winged dove 1323	TX	Avirulent	EU215366	nd	nd

[a] = Obtained from American Type Culture Collection

[b] = Captive dove

[c] = Sequencing not performed on isolates

d = Virulence of the isolates was determined based on the presence or absence of trichomonad-associated lesions within the avian host from which the isolate was obtained

FIGURE 3.1. Phylogenetic analysis of *Trichomonas gallinae* isolates and other trichomonads based on the 5.8S rRNA and surrounding internal transcribed spacer regions sequences. The tree was constructed using 228 aligned nucleotide positions using a minimum evolution algorithm with 500 replications in a Kimura 2-parameter model with *Tritrichomonas nonconforma* as an outgroup. Bootstrap values for neighbor-joining/minimum evolution/maximum parsimony are shown at the nodes. Asterisks indicate nodes with bootstrap value below 50%.

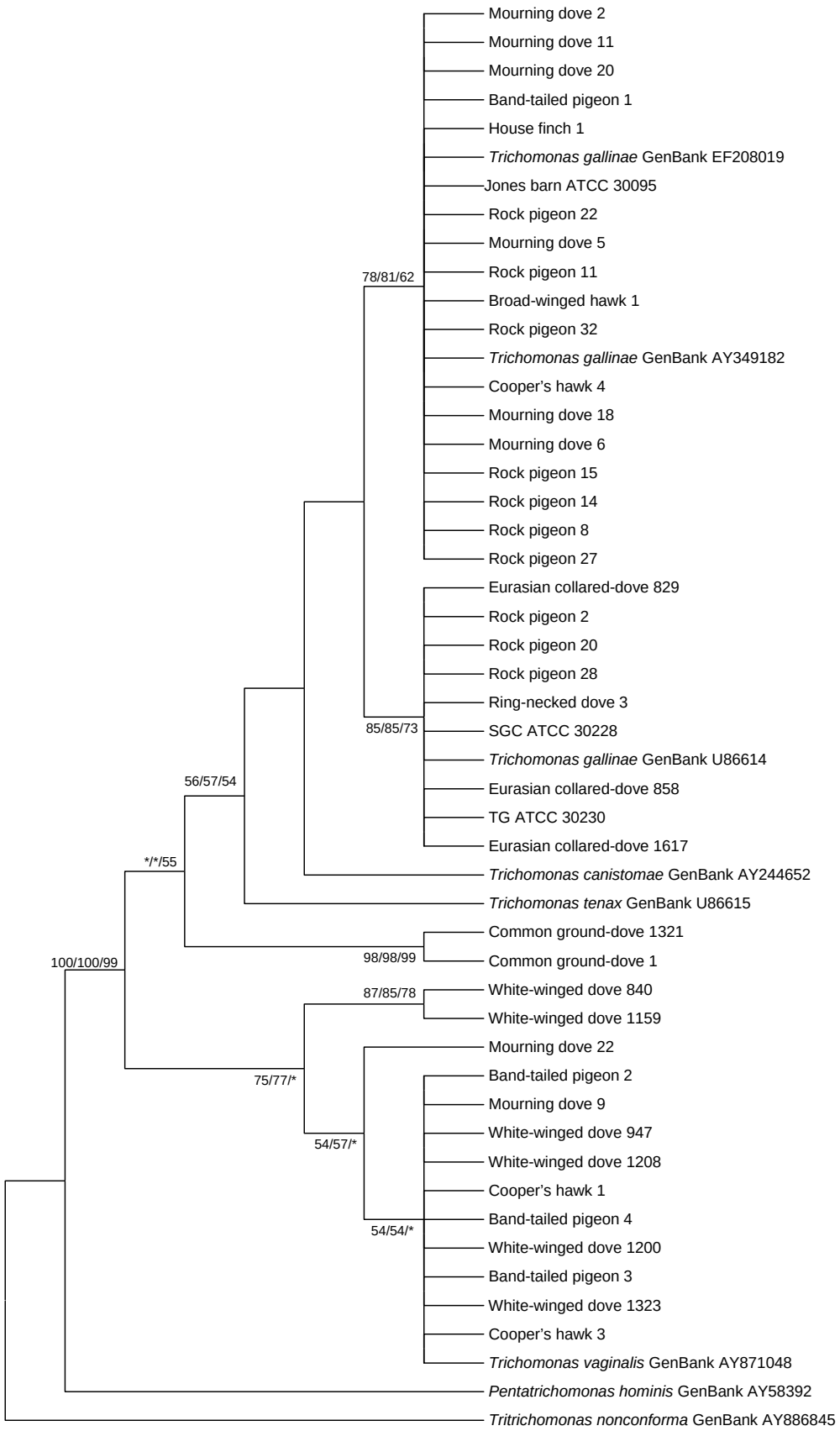

Mourning dove 2
Mourning dove 11
Mourning dove 20
Band-tailed pigeon 1
House finch 1
Trichomonas gallinae GenBank EF208019
Jones barn ATCC 30095
Rock pigeon 22
Mourning dove 5
Rock pigeon 11
Broad-winged hawk 1
Rock pigeon 32
Trichomonas gallinae GenBank AY349182
Cooper's hawk 4
Mourning dove 18
Mourning dove 6
Rock pigeon 15
Rock pigeon 14
Rock pigeon 8
Rock pigeon 27
Eurasian collared-dove 829
Rock pigeon 2
Rock pigeon 20
Rock pigeon 28
Ring-necked dove 3
SGC ATCC 30228
Trichomonas gallinae GenBank U86614
Eurasian collared-dove 858
TG ATCC 30230
Eurasian collared-dove 1617
Trichomonas canistomae GenBank AY244652
Trichomonas tenax GenBank U86615
Common ground-dove 1321
Common ground-dove 1
White-winged dove 840
White-winged dove 1159
Mourning dove 22
Band-tailed pigeon 2
Mourning dove 9
White-winged dove 947
White-winged dove 1208
Cooper's hawk 1
Band-tailed pigeon 4
White-winged dove 1200
Band-tailed pigeon 3
White-winged dove 1323
Cooper's hawk 3
Trichomonas vaginalis GenBank AY871048
Pentatrichomonas hominis GenBank AY58392
Tritrichomonas nonconforma GenBank AY886845
78/81/62
85/85/73
56/57/54
//55
100/100/99
98/98/99
87/85/78
75/77/*
54/57/*
54/54/*

FIGURE 3.2. Phylogenetic analysis of *Trichomonas gallinae* isolates and other trichomonads based on alignment of overlapping 1,005 bp 18S rRNA gene. The tree was constructed using a minimum evolution algorithm with 500 replications in a Kimura 2-parameter model with *Pentatrichomonas hominis* as an outgroup. Bootstrap values for neighbor-joining/minimum evolution/ maximum parsimony values are shown at the nodes. Asterisks indicate nodes with bootstrap value below 50%.

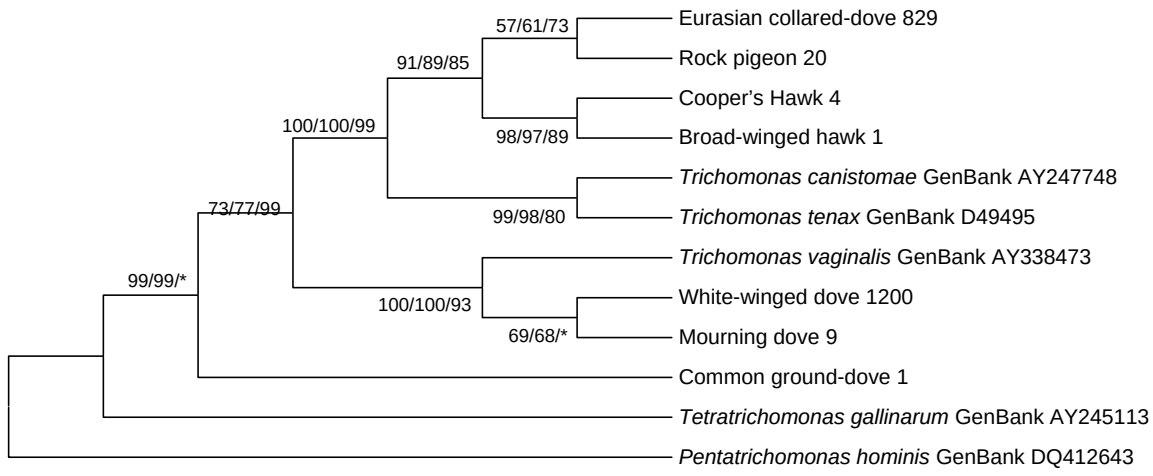
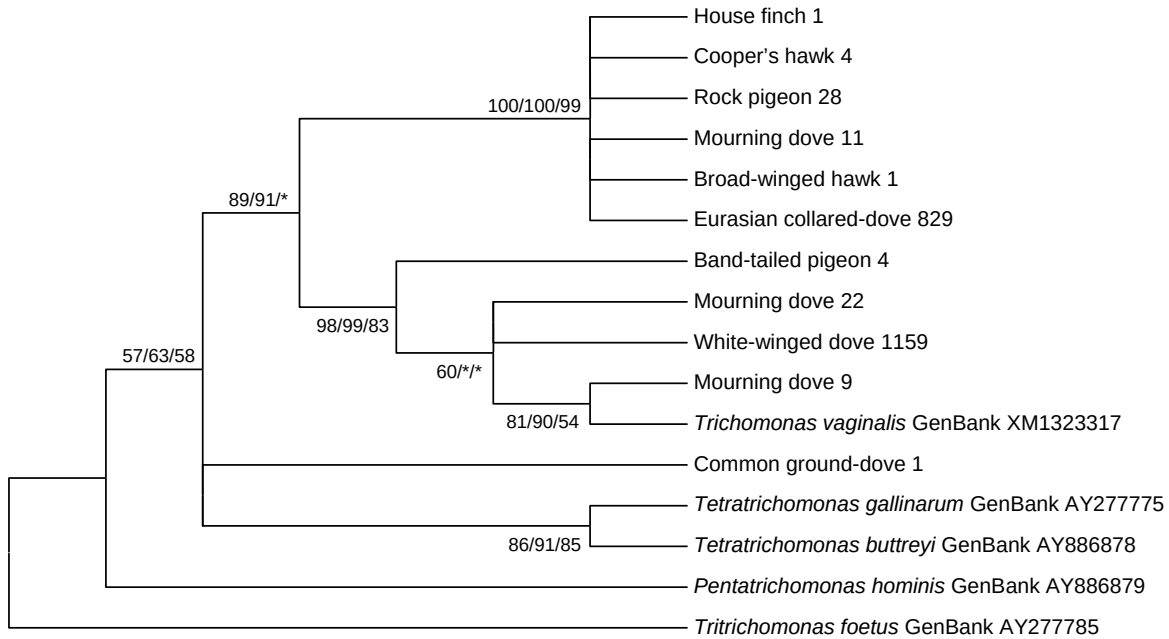

57/61/73
Eurasian collared-dove 829
91/89/85
Rock pigeon 20
Cooper's Hawk 4
100/100/99
98/97/89
Broad-winged hawk 1
Trichomonas canistomae GenBank AY247748
73/77/99
99/98/80
Trichomonas tenax GenBank D49495
Trichomonas vaginalis GenBank AY338473
99/99/*
White-winged dove 1200
100/100/93
69/68/*
Mourning dove 9
Common ground-dove 1
Tetratrichomonas gallinarum GenBank AY245113
Pentatrichomonas hominis GenBank DQ412643

FIGURE 3.3. Phylogenetic analysis of *Trichomonas gallinae* isolates and other trichomonads based on alignment of overlapping 705 bp alpha-tubulin gene. The tree was constructed using a minimum evolution algorithm with 500 replications in a Kimura 2-parameter model using an heuristic search with *Tritrichomonas foetus* as an outgroup. Bootstrap values for neighbor-joining/minimum evolution/ maximum parsimony values are shown at the nodes. Asterisks indicate nodes with bootstrap value below 50%.


House finch 1
Cooper's hawk 4
Rock pigeon 28
Mourning dove 11
Broad-winged hawk 1
Eurasian collared-dove 829
Band-tailed pigeon 4
Mourning dove 22
White-winged dove 1159
Mourning dove 9
Trichomonas vaginalis GenBank XM1323317
Common ground-dove 1
Tetratrichomonas gallinarum GenBank AY277775
Tetratrichomonas buttreyi GenBank AY886878
Pentatrichomonas hominis GenBank AY886879
Tritrichomonas foetus GenBank AY277785
100/100/99
89/91/*
98/99/83
60/*/*
81/90/54
57/63/58
86/91/85

CHAPTER 4

FAILURE TO DETECT INTRACELLULAR DOUBLE-STRANDED RNA VIRUSES IN *TRICHOMONAS GALLINAE* AND IDENTIFICATION OF A NOVEL SEQUENCE OF A *TRICHOMONAS VAGINALIS* VIRUS[1]

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[1]R. W. Gerhold, A. B. Allison, H. Sellers, J. F. Alderete, T. H. Chang, and J. R. Fischer. To be submitted to the *Journal of Virology*.

ABSTRACT

To determine if intracellular double-stranded RNA (dsRNA) viruses exist in *Trichomonas gallinae*, virus purification via ultracentrifugation was followed by gel electrophoresis of extracted RNA, reverse transcriptase-polymerase chain reaction, and transmission electron microscopy. Double-stranded RNA viruses were not detected in any of the twelve examined *T. gallinae* isolates. Sequence analysis of a dsRNA virus from a previously determined virus-infected *T. vaginalis* positive control isolate revealed a unique sequence of the RNA-dependent RNA polymerase gene of Trichomonas vaginalis virus (TVV) or related virus.

INTRODUCTION

Avian trichomonosis, caused by the protozoan parasite *Trichomonas gallinae*, has been reported from several continents and is considered a major disease for numerous avian species in the orders Columbiformes and Falconiformes (Stabler, 1954; Forrester and Spalding, 2003; Villanua et al., 2006). Previous investigations with *T. gallinae* demonstrated a wide spectrum of virulence ranging from subclinical to virulent. With highly virulent isolates, a bird can succumb to infection within 14 days of inoculation with a single trichomonad, whereas with avirulent isolates, a bird may fail to seroconvert after inoculation with $1x10^6$ organisms (Stabler and Kihara, 1954; Honigberg, 1979). Although the factors affecting the virulence of *Trichomonas* spp. are incompletely known; previous research suggests virulence may be associated with viral infection. For *T. vaginalis*, a sexually transmitted protozoan and the cause of human trichomonosis, approximately one half of all isolates are infected with double-stranded RNA (dsRNA) viruses or virus-like particles (VLP) described as heterogeneous populations of icosahedral, filamentous, cylindrical, and/or spherical virus particles ranging from 33-200 nm in

diameter (Wang and Wang, 1985; Benchimol et al., 2002). Similar dsRNA viruses or VLPs have been found in several other protozoal parasites including *Tritrichomonas foetus* (Vancini and Benchimol, 2005), *Cryptosporidium parvum* (Kniel et al., 2004), *Giardia lambia* (Wang et al., 1993), and *Leishmania braziliensis* (Widmer et al., 1989).

The presence of dsRNA viruses or VLP within *T. vaginalis* is associated with expression of immunogenic proteins on the trichomonad surface, variations in protozoal phenotypes, and up-regulation of certain proteins, including known virulence factors (Alderete et al., 1986; Wang et al., 1987; Provenzano et al., 1997; Alderete, 1999). Although the exact roles of the dsRNA viruses and VLP are not known, the up-regulation of proteins and phenotypic change associated with virus infection suggest that intracellular viruses may be associated with virulence in *T. vaginalis*. To determine if intracellular viruses exist and are associated with virulence in *T. gallinae*, we investigated the presence of intracellular viruses in both clinically virulent and avirulent *T. gallinae* isolates.

MATERIALS AND METHODS

Parasite isolation

Seven virulent and five avirulent isolates of *T. gallinae* were acquired from oral swabs from several avian species. Clinically virulent isolates were cultured from birds with trichomonad-associated lesions, whereas clinically avirulent isolates were cultured from birds lacking corresponding gross lesions. The isolates initially were cultured in In-Pouch™ TF kits (BioMed Diagnostics, White City, Oregon, USA), incubated at 37 C, and examined for five consecutive days for trichomonad growth. Further subcultures were performed using Diamond's media (pH 7.0) (Diamond, 1957) supplemented with 10% heat-inactivated horse serum (HIHS)

(Sigma-Aldrich, St. Louis, Missouri, USA). Initially cultures were supplemented with antibiotics as previously described (Diamond, 1957). Once axenic cultures were established, the use of antibiotics was discontinued. Axenic isolates in late logarithmic growth were harvested by centrifugation (750 x *g* for 10 min) and cryopreserved in liquid nitrogen using HIHS supplemented with 8% dimethyl sulfoxide (Sigma-Aldrich) until further use.

Virus purification

Approximately 2 X 10^9 trichomonads from the twelve *T. gallinae* isolates and one virus-positive *T. vaginalis* control isolate (generously donated by J. Alderete) were resuspended in approximately 30 ml TNM buffer (150 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris, pH 7.5), subjected to three freeze-thaw cycles, and sonicated for 2 min. The lysate was clarified by centrifugation at least twice at 10,000 x g for 20 min at 4 C. The supernatant was then pelleted through a 20% sucrose cushion prepared in TNM buffer at 100,000 X g for 2 hr at 4 C. The sediment was resuspended in approximately 5 ml of TNM buffer, equilibrated to a density of 1.35 gm/ml with CsCl and centrifuged at 100,000 x g for 24 hr at 4 C. Fractions were collected from the bottom of each tube and concentrated and desalted with TNM buffer using Amicon ultra-15 centrifugal filter devices (Millipore Co., Billerica, Massachusetts, USA) per the manufacturer's instructions.

Molecular Identification

Following concentration, RNA was extracted from 140µl of the Amicon filter retentate using viral RNA mini kits (Qiagen Inc., Valencia, California, USA) per the manufacturer's instructions. Extracted RNA was separated by gel electrophoreses using a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. Fragments were excised and the

RNA purified using a QIAquick® Gel Extraction kit (Qiagen Inc.). Reverse transcription-polymerase chain reaction (RT-PCR) was performed on gel extracted RNA (*T. vaginalis* positive control) as well as directly from the filter retentate extracted RNA (*T. gallinae* and *T. vaginalis* positive control) using the random decamer primers OPD-1 (5'-ACCGCGAAGG-3'), OPD-2 (5'-GGACCCAACC-3'), OPD-3 (5'-GTCGCCGTCA-3'), OPD-4 (5'-TCTGGTGAGG-3'), and OPD-5 (5'-TGAGCGGACA-3'). RT-PCR components included 2.5-µl of RNA in a 50-µl reaction containing 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 0.01% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Promega, Madison, Wisconsin, USA), 0.75 U *Taq* DNA polymerase (Promega), 0.75 U of AMV reverse transcriptase (Promega) , and 1 µM of primer. Cycling parameters for the amplification were 25 C for 5 min, 42 C for 1 hr, 94 C for 2 min followed by 40 cycles of 94 C for 45 sec, 36 C for 60 sec, and 72 C for 90 sec, and a final extension at 72 C for 10 min. Amplicons were separated by gel electrophoresis using a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. Amplicons were excised and the DNA purified using a QIAquick® Gel Extraction kit (Qiagen Inc.). Extracted DNA was then cloned using a Qiagen PCR Cloning Plus kit per the manufacturer's instructions (Qiagen Inc.). Selected recombinant colonies were cultured overnight in Luria Bertani (LB) broth supplemented with 100 µg/ml ampicillin at 37 C with shaking. Plasmid DNA was purified using a QIAprep® Spin Miniprep kit (Qiagen Inc.) according to the manufacturer's instructions. DNA inserts of plasmids were sequenced using T7 and SP6 primers. Sequences were obtained using an ABI PRISM® 3100 Genetic Analyzer at the Integrated Biotechnology Laboratories, University of Georgia, Athens, Georgia (USA).

Electron microscopy

To further investigate the presence of viruses, fomar-coated nickel grids were floated on 40 µl of the Amicon filter retentate. Negative staining was performed using 2% uranyl acetate and grids were observed in a JEOL 1210 electron microscope at 120 kV.

RESULTS

Double-stranded RNA viruses and VLP were not detected in any of the twelve *T. gallinae* isolates by gel electrophoresis of RNA extractions, RT-PCR, or negative-staining electron microscopy following the virus purification and CsCl density gradient attempts. An approximate 4.5 kb fragment was detected on gel electrophoresis of the RNA extracted Amicon filter retentate from the *T. vaginalis* virus-infected positive control. RT-PCR using OPD-5 amplified an approximately 650 bp product. Sequence analysis of the overlapping 561 bp cloned segments revealed an 81% to 84% nucleotide and an 86% to 90% amino acid identity to four *T. vaginalis* virus RNA-dependent RNA polymerase partial sequences by BLAST analysis (GenBank accessions U08999.1, DQ270032.1, DQ528812.1, and U57898.1). Electron microscopic examination of the Amicon filter retentate of the *T. vaginalis* virus purified material revealed several 33 nm diameter icosahedral virus-like particles (Fig. 4.1).

DISCUSSION

The inability to detect intracellular dsRNA viruses in the twelve examined *T. gallinae* isolates could be due to the lack of dsRNA viruses within the protozoa or inability to detect dsRNA viruses using the chosen techniques. Virus purification by ultracentrifugation was successfully performed with a positive control *T. vaginalis* isolate in our laboratory and has been shown to have a high success rate in detecting viruses in other organisms (Benchimol et al.,

2002; Poulos et al., 2006). Virus-like particles were detected in *Tritrichomonas foetus* by electron microscopy only after the trichomonads were treated with cytoskeleton-affecting chemicals including colchicine, vinblastine, taxol, nocodazole, and griseofulvin (Vancini and Benchimol, 2005). Future attempts to detect viruses in *T. gallinae* should include such protocols. Our findings suggest that intracellular viruses are not associated with the virulence of *T. gallinae*. Further research is needed to determine the role of viruses in the virulence of virus-infected trichomonads.

The partial RNA-dependent RNA polymerase sequence of the *T. vaginalis* dsRNA virus identified in this investigation is unique compared to other *T. vaginalis* virus sequences in GenBank and the homology between *T. vaginalis* virus sequences is variable, which may be expected given *T. vaginalis* isolates are infected with several different dsRNA viruses (Khoshnan and Alderete, 1993; Benchimol et al., 2002). The virus particles observed on electron microscopic examination from the *T. vaginalis* ultracentrifugation retentate are consistent with the size and shape of viruses previously detected in the isolate (Benchimol et al., 2002). These findings suggest the techniques used in this investigation are adequate to detect intracellular dsRNA viruses in trichomonads.

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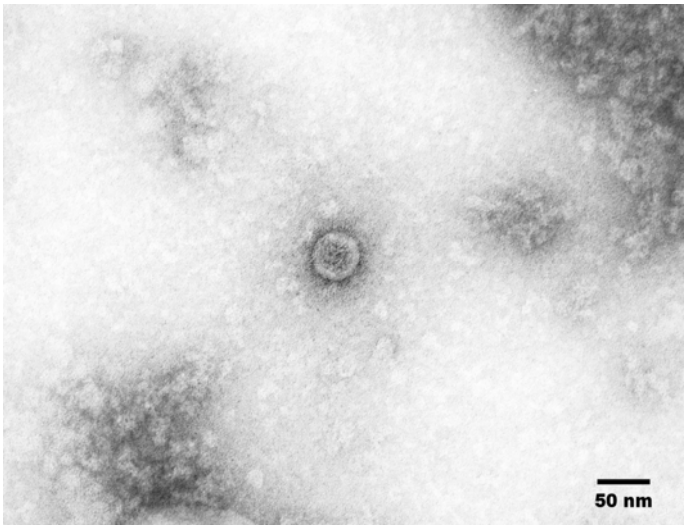
FIGURE 4.1. Negative-staining electron microscopy of virus-like particle from *Trichomonas vaginalis* virus-infected isolate. Negative stain performed with 2% uranyl acetate.


50 nm

CHAPTER 5

HEMOLYTIC ACTIVITY OF CLINICALLY VIRULENT AND AVIRULENT *TRICHOMONAS GALLINAE* ISOLATES[1]

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[1]R. W. Gerhold and J. R. Fischer. To be submitted to *Veterinary Parasitology*

ABSTRACT

The hemolytic activity of twenty-two live *Trichomonas gallinae* isolates was investigated using an 18 hr avian erythrocyte hemolysis assay. Absorbance of the assay supernatants was measured at 540 nm and expressed as percentage of complete hemolysis. Mean hemolytic activity ranged from 3.5% to 53.4% and did not correlate with clinical virulence. The results of this investigation suggest hemolytic activity is not a useful *in-vitro* virulence assay for *T. gallinae*.

INTRODUCTION

Avian trichomonosis, caused by the protozoan *Trichomonas gallinae*, has been reported from several continents and is considered a major disease for numerous avian species in the orders Columbiformes and Falconiformes (Stabler, 1954; Forrester and Spalding, 2003; Villanua et al., 2006). Studies with *T. gallinae* have demonstrated a wide spectrum of virulence. With highly virulent isolates, a bird can succumb to infection within 14 days after inoculation with a single trichomonad; whereas with avirulent isolates, a bird may fail to seroconvert after inoculation with $1x10^6$ organisms (Stabler and Kihara, 1954; Honigberg, 1979). Previous investigations disclosed that domestic pigeons previously inoculated with a known avirulent isolate or surviving infection by a virulent isolate had protective immunity when challenged with a known virulent isolate (Stabler, 1948). Additionally, it has been demonstrated that clinically normal pigeons can harbor both avirulent and virulent isolates; however, naïve doves and pigeons challenged with a mixture of virulent and avirulent isolates will succumb to infection (Stabler, 1954). Thus clinical virulence of an isolate must be viewed with caution and experimental infections of naïve birds have been used to determine actual virulence.

Erythrocyte hemolysis has been suggested as a possible *in vitro* virulence assay for *T. gallinae*. Previous investigations of five *T. gallinae* isolates demonstrated approximately 72%-91% hemolysis of chicken erythrocytes (DeCarli et al., 1994; DeCarli et al., 1996; DeCarli and Tasca, 2002). Additionally, the hemolytic activity of *T. gallinae* was observed in erythrocytes from adult humans, rabbits, rat, horse, bovine, and sheep (DeCarli and Tasca, 2002). Scanning electron microscopy demonstrated *T. gallinae* organisms attaching to erythrocytes and suggested that hemolysis was due to a contact-dependent mechanism (DeCarli and Tasca, 2002). Additionally, parasite-produced hemolysins were not detected from the assay supernatants. The hemolytic activity of *T. vaginalis* previously has been correlated with clinical virulence of isolates (DeCarli et al., 1989; Dailey et al., 1990). The goal of this investigation was to evaluate the hemolytic activity of numerous clinically virulent and avirulent *T. gallinae* isolates and determine if hemolysis is an indicator of virulence.

MATERIALS AND METHODS

Parasite culture

The origin and clinical virulence of the twenty-two *T. gallinae* isolates examined in this investigation is shown in Table 5.1. Clinically virulent isolates were obtained from birds with lesions consistent with trichomonosis; whereas clinically avirulent isolates were obtained from birds lacking corresponding lesions. Isolates were obtained from oral swabs initially cultured in In-Pouch™ TF kits (BioMed Diagnostics, White City, Oregon, USA), incubated at 37 C, and examined for five consecutive days for trichomonad growth. Further subcultures were performed using Diamond's media (pH 7.0) supplemented with 10% heat-inactivated horse serum (HIHS) (Sigma-Aldrich, St. Louis, Missouri, USA) (Diamond, 1957). Initially cultures

were supplemented with antibiotics as previously described (Diamond, 1957). Once axenic cultures were established, the use of antibiotics was discontinued. Axenic cultures in late logarithmic growth were harvested by centrifugation (750 x *g* for 10 min) and cryopreserved in liquid nitrogen using HIHS supplemented with 8% dimethyl sulfoxide (Sigma-Aldrich) until further use.

Erythrocytes

Fresh chicken erythrocytes were obtained from adult chickens housed at the Poultry Disease and Research Center, College of Veterinary Medicine, The University of Georgia, Athens, GA (USA). Blood was collected in an equal volume of Alsever's solution (Sigma-Aldrich). Following centrifugation at (250 x g for 5 min), the supernatant containing the plasma fraction was discarded. The erythrocytes were washed three times in sterile HI buffer (145 mM NaCl, 1.2 mM KH_2PO_4, and 3.8 mM Na_2HPO_4, pH 7.2). Washed erythrocytes were stored in HI Buffer at 4 C. Fresh erythrocytes were used in each experiment.

Hemolysis assay

Trichomonads were harvested from 24 hr axenic cultures at 37 C in Diamond's media (pH 7.0) supplemented with 10% HIHS. The trichomonads were washed three times in pre-warmed Diamond's media (pH 7.0) by centrifugation at 750 x g for 10 min at 25 C. A volume of 50 µl of fresh, washed, undiluted erythrocytes was mixed with a total of 1 X 10^6 trichomonads in 2.5 ml of Diamond's media (pH 7.0). Controls consisting of erythrocytes mixed with trichomonad-free Diamond's media were used to measure spontaneous hemolysis in each experiment. Following 18 hr of incubation at 37 C, cultures were centrifuged at 250 x g for 5 min and absorbance of the supernatants and controls were analyzed at 540 nm. Hemolysis of

experimental cultures was expressed as a percentage of 100% hemolysis. In each experiment, a 60 sec sonication of a post-incubational tube containing 50 µl erythrocytes mixed with trichomonad-free Diamond's media was used to determine 100% hemolysis. Calculation of the mean and standard deviation of the hemolytic activity was performed for each isolate after performing the hemolysis assay at least three times in triplicate.

RESULTS

Eleven virulent and eleven avirulent isolates originating from six avian species were examined (Table 5.1). The average hemolysis of clinically virulent and avirulent isolates ranged from 3.5% to 41.2% and from 4.3% to 53.4%, respectively (Table 5.1, Fig 5.1, and Fig. 5.2). The standard deviation of clinically virulent isolates ranged from 0.7% to 12.64% with an average of 5.5% and the standard deviation of clinically avirulent isolates ranged from 0.6% to 27.9% with an average of 8.6% (Table 5.1, Fig. 5.1, and Fig. 5.2).

DISCUSSION

The hemolytic activity of the twenty-two *T. gallinae* isolates was investigated and the results suggest that hemolytic activity does not correlate with clinical virulence. Of the eleven clinically virulent isolates cultured from birds with fulminant trichomonosis, four isolates had a mean hemolytic activity of $\leq$ l0%; whereas only two of the clinically avirulent isolates had mean hemolytic activity of $\leq$ l0% (Table 5.1, Fig. 5.1, and Fig. 5.2). Two of the clinically avirulent isolates (RODO 20 and RODO 27) had a higher mean hemolytic activity than MODO 20, which had the highest hemolytic activity of any clinically virulent isolate (Table 5.1, Fig. 5.1, and Fig. 5.2). Additionally, the results often were inconsistent between trials as evidenced by the standard deviations ranging from 0.6% to 27.9% (Table 5.1).

De Carli et al. (1996) reported 72%-91% avian erythrocyte hemolysis from five *T. gallinae* isolates; however, none of the twenty-two isolates used in our investigation approached this degree of hemolysis. Unfortunately, none of the five isolates used in the previous investigation were available for our investigation. It would be worthwhile to repeat the assay with our isolates and the five isolates used by De Carli et al. (1994), and determine if the results are consistent between investigations. Potential reasons for the differences in hemolytic activity between our and previous investigations include possible differences in methods used in determining 100% hemolysis values.

Dailey et al. (1990), demonstrated the hemolytic activity of the NYH 286 *T. vaginalis* isolate ranged from >80% to <20% when tested daily for 8 days; however, none of the other fourteen *T. vaginalis* isolates they examined showed the degree of variation in hemolytic activity as NYH 286. Hemolytic activity of *T. vaginalis* is due to direct (protozoa-erythrocyte contact) and indirect (hemolysins) mechanisms and it was proposed that low levels of hemolysis of NYH 286 were due to the isolate undergoing phenotypic variation which affected the hemolytic activity (Dailey et al., 1990). Inhibitors of cysteine proteinases greatly reduced or abolished hemolysis of *T. vaginalis* isolates indicating cysteine proteinases may be potentiators of erythrocyte lysis (Coombs and North, 1983). It would be interesting to determine what factor or factors are responsible for the *T. gallinae* -erythrocyte interaction and how the relative concentrations of these factors are associated with virulence. Additionally, it would be worthwhile to determine if the relative concentrations of these unknown factors explain the varying degrees of hemolysis observed between assays in several of the *T. gallinae* isolates.

The three band-tailed pigeon isolates (BTPN 1, BTPN 3, and BTPN 4) originated from a focally significant trichomonosis outbreak in Monterey County, California (USA). BTPN 1 and

BTPN4 had mean hemolytic activities of 3.5% and 3.9%, respectively; whereas BTPN 3 had a mean hemolytic activity of 18.8%. This is particularly interesting given the sequence analysis of the 5.8S ribosomal RNA and flanking internal transcribed spacer regions of BTPN 3 and BTPN 4 were identical; however BTPN 1 was significantly different from BTPN 3 and BTPN 4 (Gerhold, MS Thesis). These findings suggest that genetically similar isolates may have varying degrees of hemolytic activity.

Clinical virulence of trichomonads must be viewed with caution. Previous investigations disclosed that domestic pigeons previously inoculated with a known avirulent isolate or surviving infection by a virulent isolate had protective immunity when challenged with a known virulent isolate (Stabler, 1948). Although the true virulence of the clinically avirulent isolates is not known, clinically virulent isolates originated from birds that died of trichomonosis. The hemolytic activity of our virulent isolates was variable and results for individual isolates were often inconsistent between experiments.

Virulence of *T. gallinae* is influenced by temperature and culture methods (Stabler et al., 1964). Attenuation of virulent *T. gallinae* isolates was noted after 17 weeks of continuous *in vitro* cultivation and by 21 weeks of culture, the virulence of the isolates was so attenuated that the trichomonads did not even induce an immune response in inoculated birds (Stabler et al., 1964). The addition of streptomycin and penicillin in the culture media resulted in more rapid attenuation of virulence (Stabler et al., 1964). In our investigation, antibiotics were used only until axenic cultures were established and isolates were cryopreserved between experiments, which does not affect virulence (BonDurant and Honiberg, 1994).

The findings of our investigation indicate that hemolytic activity may not be a reliable indicator of virulence in *T. gallinae*. Future investigations of *in vitro* virulence assays of *T. gallinae* should include cell culture techniques. Honigberg et al. (1964) examined the behavior of virulent and avirulent *T. gallinae* isolates in trypsin-dispersed chick liver cell cultures. Virulent *T. gallinae* isolates were more motile and evoked a more active macrophage response than avirulent isolates. Phagocytes were able to eliminate infection of avirulent isolates; whereas virulent isolates were initially engulfed by the macrophages, but continued to multiply, eventually leading to macrophage rupture and subsequent release of trichomonads. Additionally, virulent isolates caused extensive degenerative changes of invaded and non-invaded cells and suppressed divisions of the fibroblast-like cell culture elements (Honigberg et al., 1964). An efficient technique capable of quantifying the cell culture variables listed above could be useful as an *in vitro* virulence assay.

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TABLE 5.1. List of *Trichomonas gallinae* isolates cultured from free-ranging birds and examined for hemolytic activity.

Isolate	Host	State of origin (USA)	Clinical virulence	Hemolysis (%)*
BTPN 1	Band-tailed pigeon (*Columba fasciata*)	California	Virulent	3.5±0.7
BTPN 3	Band-tailed pigeon	California	Virulent	18.8±8.7
BTPN 4	Band-tailed pigeon	California	Virulent	3.9±0.9
BWHA 1	Broad-winged hawk (*Buteo platypterus*)	Florida	Virulent	9.3±5.1
COHA 4	Cooper's hawk (*Accipiter cooperii*)	Arizona	Virulent	14.8±8.9
HOFN 1	House finch (*Carpodacus cassinii*)	Kentucky	Virulent	11.3±3.6
MODO 2	Mourning dove (*Zenaida macroura*)	Georgia	Virulent	10.7±12.6
MODO 5	Mourning dove	Kentucky	Virulent	14.0±4.1
MODO 11	Mourning dove	Georgia	Avirulent	31.8±10.2
MODO 18	Mourning dove	Georgia	Virulent	18.7±10.2
MODO 20	Mourning dove	Georgia	Virulent	41.1±8.7
MODO 21	Mourning dove	Georgia	Virulent	6.5±0.9
RODO 1	Rock pigeon (*Columba livia*)	Georgia	Avirulent	19.7±5.6
RODO 3	Rock pigeon	Georgia	Avirulent	11.0±5.7
RODO 6	Rock pigeon	Georgia	Avirulent	23.2±13.4
RODO 11	Rock pigeon	Georgia	Avirulent	4.3±1.5
RODO 14	Rock pigeon	Georgia	Avirulent	6.4±2.3
RODO 19	Rock pigeon	Georgia	Avirulent	11.0±7.6
RODO 20	Rock pigeon	Georgia	Avirulent	53.4±13.2
RODO 22	Rock pigeon	Georgia	Avirulent	4.4±0.6
RODO 27	Rock pigeon	Georgia	Avirulent	45.0±27.9
RODO 28	Rock pigeon	Georgia	Avirulent	16.0±6.6

*Percentage hemolysis represents mean ± the standard deviation of triplicate samples

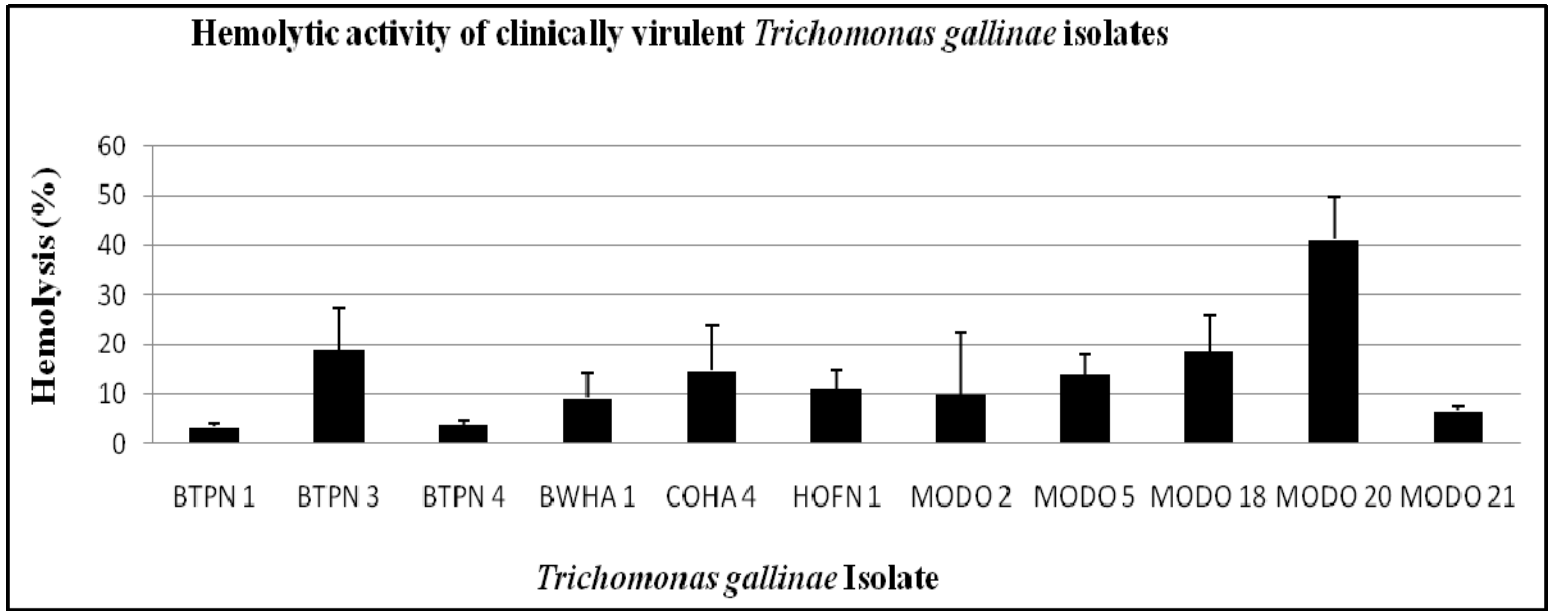

FIGURE 5.1. Hemolytic activity of clinically virulent *Trichomonas gallinae* isolates using avian erythrocytes.

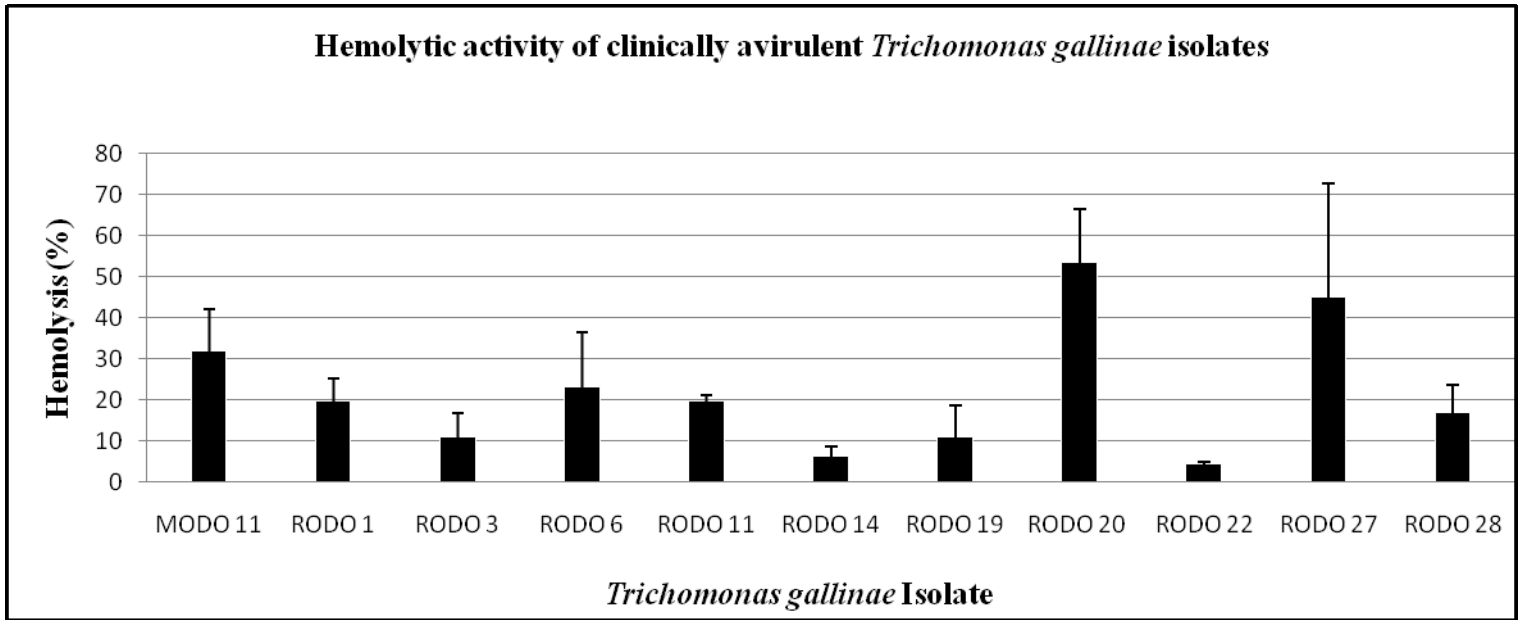

FIGURE 5.2. Hemolytic activity of clinically avirulent *Trichomonas gallinae* isolates using avian erythrocytes.

CHAPTER 6

NECROPSY FINDINGS AND ARBOVIRUS SURVEILLANCE IN MOURNING DOVES (ZENAIDA MACROURA) FROM THE SOUTHEASTERN UNITED STATES[1]

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[1]R. W. Gerhold, C.M. Tate , S. E. Gibbs, D. G. Mead, A. B. Allison, and J. R. Fischer. 2007. *Journal of Wildlife Diseases* 4:129-135. Reprinted here with permission of editor.

ABSTRACT

Mourning doves (Zenaida macroura) are the most abundant and widespread native member of the columbid family, as well as a major migratory game species, in the United States. However, there is little information on mortality factors in mourning doves. Records of necropsy accessions at the Southeastern Cooperative Wildlife Disease Study (SCWDS) from 15 southeastern states, from 1971 through 2005, were reviewed. In total, 135 mourning doves were submitted from nine states during the thirty-five year period. Trichomonosis comprised 40% (N=54) of all diagnoses and was the most frequent diagnosis. Toxicoses and avian pox comprised 18.5% (N=25) and 14.8% (N=20) of all diagnoses, respectively. The remaining diagnoses included trauma, suspected toxicosis, Ascaridia columbae infection, suspected tick paralysis, and undetermined. Adults were observed more frequently with trichomonosis (94.1%) and toxicoses (68%) as compared to juveniles, but a gender predisposition was not apparent for either disease. Age and gender predilections were not apparent for cases of avian pox. The majority of the trichomonosis and avian pox cases were observed in the spring-summer, whereas the majority of the toxicosis cases were observed in the winter-spring. Additionally, Georgia Department of Human Resources-Division of Public Health and West Virginia Department of Health and Human Resources submitted 809 mourning doves to SCWDS from 2001 through 2005 for West Nile virus (WNV) surveillance efforts. West Nile virus was isolated from 2.1% (N=17) and Eastern equine encephalitis virus (EEEV) was isolated from 0.2% (N=2) of the submitted birds.

Key words: Columbid, diseases, mourning dove, southeast United States, Zenaida macroura

Mourning doves (Zenaida macroura) are the most abundant native columbid and represent the most popular avian game species within the United States (Dolton and Rau, 2003). Populations have fluctuated throughout the United States and have declined over much of the eastern United States during the last few decades (Dolton and Rau, 2003). Previous morbidity and mortality investigations in mourning doves have disclosed several diseases that may have potential population implications (Conti, 1993; Forrester and Spalding, 2003). Trichomonosis, caused by the protozoan Trichomonas gallinae, is considered the most important disease in mourning doves and several large epidemics have been reported (Stabler, 1954; Conti, 1993; Forrester and Spalding, 2003). One of the largest outbreaks occurred in 1950 and 1951 in multiple southeastern states. During this 2-yr outbreak, in Alabama alone, an estimated 50,000 to 100,000 mourning doves died of trichomonosis (Haugen and Keeler, 1952). Avian pox, toxicoses, and trauma have also been identified as other diseases with potential population implications (Conti, 1993; Forrester and Spalding, 2003). In this review, we examined mourning dove diagnoses from the Southeastern Cooperative Wildlife Disease Study (SCWDS) necropsy records and investigated whether common causes of morbidity and mortality were associated with gender, age, or seasonal patterns. Additionally, records of mourning doves submitted from two states for West Nile virus (WNV) surveillance from 2001 through 2005 were reviewed.

Records of mourning dove necropsy accessions, received at SCWDS from 15 southeastern states from 1971 through 2005, were examined. Accessions were subjected to a complete necropsy, which included both gross and histopathologic examination of all major organs. The findings were categorized into cause of mortality, age, gender, season of mortality, and state of origin. A chi-squared test (alpha=0.05) was used to determine if significant differences existed between the categories of interest. In addition to necropsy accessions, the

Georgia Department of Human Resources-Division of Public Health and West Virginia Department of Health and Human Resources submitted dead mourning doves to SCWDS from 2001 through 2005 as part of their WNV surveillance programs. The virus isolation records were examined for mourning doves from which arboviruses were isolated. Unlike complete necropsies, WNV submission protocols consisted only of extraction of a brain sample for virus isolation without histopathological examination of tissues. Therefore, the cause of death could not be directly attributed to virus infection. During 2001, gross findings were recorded for birds submitted for WNV testing; however, these findings were not recorded for the subsequent years.

From 1971 through 2005, 135 mourning doves from nine southeastern states (Alabama, Florida, Georgia, Louisiana, North Carolina, South Carolina, Tennessee, Virginia, and West Virginia) were submitted to SCWDS for necropsy. A significantly greater number of adult (X^2=33.3, 1 df, P<0.001) and male (X^2=6.02, 1 df, P<0.02) mourning doves were submitted as compared to juvenile and female doves, respectively. Trichomonosis, avian pox, and toxicoses were the three most frequently diagnosed diseases and they constituted 73% (N=99) of all diagnoses. Of these three diseases, trichomonosis was diagnosed most frequently (X^2=13.4, 2 df, P<0.005) followed by toxicoses and avian pox (Table 6.1). The number of mortality events for each of the three diseases was analyzed and trichomonosis was the most frequent event followed by avian pox and toxicosis (Table 6.1). A significantly greater number of mortality events were due to trichomonosis (X^2=15.4, 2 df, P<0.001) and a significantly lesser number were due to toxicoses (X^2=9.3, 2 df, P<0.01). Mortality events of avian pox were not significantly different than trichomonosis or toxicoses events (X^2=0.76, 2 df, P>0.05). When suspected toxicoses were included with trichomonosis, avian pox, and confirmed toxicoses, the combined diagnoses

constituted 89.6% (N=121) of all diagnoses. Other diagnoses included trauma, Ascaridia columbae infection, suspected tick paralysis, and undetermined (Table 6.1).

Trichomonosis was diagnosed by identifying flagellated protozoa consistent with Trichomonas spp. on wet mount, culture, or histological preparations demonstrating intralesional protozoa. Trichomonads were cultured in Diamond's media supplemented with 10% horse serum (Diamond, 1957). Trichomonosis was diagnosed more frequently in adults (N=51, 94%), as were toxicoses (N=21, 84%), but a gender predisposition was not apparent for either disease. Of the identified organic chemicals causing confirmed mortality in doves, two mortality events were due to carbofuran and one event each was due to famphur and toxaphene. The remaining doves diagnosed as toxicosis had non-diagnostic findings on chemical analysis and the diagnosis was based solely on >50% decrease in brain cholinesterase (ChE) concentrations as compared to brain ChE concentrations for control doves. For doves diagnosed with avian pox, no age or gender predisposition was apparent. Ninety-eight percent of the avian pox diagnoses were made from identifying characteristic eosinophilic inclusion bodies of avian pox virus on impression smears or histological examination. The remaining avian pox diagnoses were made solely by observations of gross lesions.

More mourning doves were submitted in the spring and summer months (X^2=9.6, 1 df, P< 0.0025), than the autumn and winter months. Toxicoses were diagnosed more frequently in the spring (90%; X^2=32.6, 3 df, P<0.001) than other seasons, whereas avian pox was diagnosed more frequently in the summer (90%; X^2=28.8, 3 df, P<0.001). Trichomonosis was diagnosed more often in the spring and summer months (70%; X^2=4.4, 1 df, P<0.05) than in autumn and winter months.

West Nile virus was isolated from 2.1% (N=17) and Eastern equine encephalitis virus (EEEV) was isolated from 0.2% (N=2) of the 809 doves submitted from Georgia and West Virginia. Virus isolation and reverse-transcriptase polymerase chain reaction (R-T PCR) for EEEV and WNV were performed as previously described (Gottdenker et al., 2003; Allison et al., 2004). Gross findings recorded for doves submitted in 2001 indicated that 43.4% (N=36) had lesions consistent with trichomonosis, 19.3% (N=16) had lesions consistent with trauma, and 3.6% (N=3) had lesions consistent with avian pox. These findings must be viewed with caution, because confirmatory diagnostic testing was not conducted.

Trichomonosis was the most frequently diagnosed disease in this retrospective study. Mourning doves may transmit or acquire T. gallinae through feeding crop milk to nestlings, billing courtship during mating, or by ingestion of contaminated food or water (Kocan, 1969; Kocan and Herman, 1971). The high frequency of trichomonosis diagnoses in the present review is consistent with previous reports of diseases in mourning doves (Conti, 1993; Forrester and Spalding, 2003). Population impacts of trichomonosis outbreaks in mourning doves are variable and, generally, the disease is observed in juveniles more than adults (Conti and Forrester, 1981; Haugen and Keeler, 1952; Ostrand et al., 1995). Schulz et al. (2005) monitored the annual variation of T. gallinae from hunter-killed mourning doves without clinical trichomonosis and found that T. gallinae was isolated from essentially an equal portion (5.5%) of hatch year and after hatch year birds. We observed trichomonosis in 48% (N=51) of the adults and in 20% (N=3) of the juveniles which may be due to a significantly greater number of adults being submitted.

Rock pigeons (Columba livia) are the natural host for T. gallinae and most pigeons harbor this protozoan, but rarely have clinical disease (Stabler, 1954; Tudor, 1991).

Trichomonas gallinae is known to have a wide spectrum of virulence, but the factors that control virulence are incompletely known (Honigberg et al., 1971; Honigberg, 1979). Infection with an avirulent strain or survival of infection with a virulent strain of T. gallinae provides columbids with protective immunity, resulting in individuals that are refractory to clinical disease (Stabler, 1948; Kocan, 1972). Therefore, previously infected pigeons and doves may serve as inapparent carriers of virulent strains of T. gallinae and are potential sources of infection for naive birds (Stabler, 1954). Pigeons and doves also may serve as a source of infection for raptors, in which the disease can have focal population impacts (Boal et al., 1998).

Brains and gastrointestinal contents were collected from dead mourning doves and frozen at -20 C for brain ChE assays as described by Hill (1988). When available, brains from healthy mourning doves were used for control specimens. Following the initial brain ChE assay, the samples were incubated at 37 C for 18 hours to evaluate enzyme reactivation. Reactivation of ChE is characteristic of a carbamate poisoning, but not an organophosphorus poisoning (Smith et al., 1995).

Chemical analyses of the gastrointestinal contents were performed as previously described in White et al. (1989) and Holstege et al. (1994) at the Cooperative Extension Service (College of Agriculture, the University of Georgia, Athens, Georgia 30602, USA) or the University of Pennsylvania Veterinary Diagnostic Laboratory (College of Veterinary Medicine, Kennett Square, Pennsylvania 19348, USA). Gastrointestinal contents were screened for organic compounds including organophosphorus and carbamate pesticides. In general, the list of screened compounds included chlorpyriphos, diazinon, disulfoton, famphur, fenthion, parathion, terbufos, aldicarb, aldicarb sulfone, aldicarb sulfoxide, bendiocarb, carbaryl, carbofuran,

carbosulfan, formetanate HCl, 3-hydroxy carbofuran, toxaphene, methiocarb, methomyl, mexacarbate, oxamyl, primicarb, propoxur, thiodicarb, and trimethacarb.

Confirmed toxicosis cases were diagnosed by significantly decreased brain ChE concentration alone or in conjunction with identification of a specific compound. Diagnoses of suspected toxicosis were based on a history strongly suggestive of poisoning, ingesta in gastrointestinal tract, and unremarkable necropsy findings, but with negative toxicological findings or detection of a compound at non-diagnostic levels in addition to <50% decrease in brain ChE concentrations as compared to ChE concentrations for control doves.

Confirmed toxicoses were observed most frequently in the months of late winter and early spring, which may coincide with application of pesticides on lawns, fields, and planted crops. Additionally, several of the case histories indicated possible intentional poisoning. Carbofuran was the most frequently identified compound in our retrospective study, followed by famphur and toxaphene. A 20-year retrospective summary from the National Wildlife Health Center disclosed that 18 mortality events, involving 302 individual columbids, were due to anticholinesterase pesticides (Fleischli et al., 2004). Of the identified compounds in this study, three mortality events were due to carbofuran, two events were due to fenthion, one event was due to famphur, and twelve events were due to unspecified anticholinesterase pesticides. Heavy metal toxicosis has been a concern in doves, especially with the use of lead shot at dove fields (Schulz et al., 2006). However, there were no cases of heavy metal toxicoses in mourning doves in our records.

Avian pox was diagnosed more frequently in the summer, which would be expected because the virus is believed to be predominately transmitted by blood-feeding arthropods (Akey

et al., 1981). In Florida, cases of avian pox in wild turkeys (Meleagris gallopavo) have been documented primarily in the months of September through December, which corresponds to the peak of two mosquito populations known to transmit avian pox virus (Akey et al., 1981). Because avian pox infections often are easily recognized by visual examination, field biologists may not submit these carcasses for laboratory testing; thus, avian pox may be underrepresented in our necropsy accessions.

The two doves diagnosed with A. columbae infection had more than one hundred parasites each within the intestinal tract. Previous reports documented that A. columbae occurred in 2.0 to 25.8% of examined doves, with infections generally consisting of less than 30 parasites (Barrows and Hayes, 1977; Conti and Forrester, 1981; Lee et al., 2004). Interestingly, a study comparing the relative helminth intensities in mourning doves and the interrelationships with the introduced white-winged dove (Zenaida asiatica) in Florida disclosed a higher intensity of helminths in mourning doves in areas where white-winged doves were present (Conti and Forrester, 1981). Bean et al. (2005) reported that helminth intensity in the introduced Eurasian collared-dove (Streptopelia decaocto) was similar to that of the white-winged doves, but significantly higher than that of mourning doves. However, it is unknown if Eurasian collared-doves directly affect mourning doves helminth intensities in areas where the two species are sympatric. The two doves diagnosed with A. columbae infection originated in areas in which Eurasian collared-doves are present (Romagosa and Labisky, 2000).

Two ticks identified as Ixodes brunneus were removed from the dove diagnosed with suspected tick paralysis. Ixodes brunneus infection has been associated with tick paralysis syndrome in birds; however, definitive diagnosis of the syndrome requires removal of the ticks and regression of clinical signs (Luttrell et al., 1996), which was not possible because the bird

was dead upon submission. However, histological and gross examination did not disclose additional causes of mortality.

West Nile virus was isolated from 2.1% of submitted doves and EEEV was isolated from 0.2% of submitted doves. Tesh et al. (2004) reported that 2.2% (N=6) of mourning doves, 2.1% (N=1) of rock pigeons, and 2.6% (N=1) of Inca doves (Columbina inca), tested as part of dead bird surveillance in Texas, were positive for WNV. In a separate investigation, 12.3% (N=30) of free-ranging, healthy mourning doves, 28.9% (N=153) of rock pigeons, and 26.8% (N=15) of common ground doves (Columbina passerine) from Georgia were seropositive to WNV (Gibbs et al., 2006). Comparably, 4.3% (N=14) of blue jays (Cyanocitta cristata) and 9.7% (N=3) of American crows (Corvus brachyrhynchos) were WNV seropositive. The relatively low virus isolation rates from dead bird submissions in conjunction with high seroprevalence rates from clinically healthy birds, suggests that mourning doves may be relatively less susceptible to WNV-induced disease in comparison to corvids and several other passerine species (Gibbs et al., 2006). Forrester and Spalding (2003) found < 1% of tested doves were positive for EEEV, which is consistent with our findings.

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TABLE 6.1. Diagnostic findings in 135 mourning doves from the southeastern United States (1971-2005).

Diagnosis	Number of Doves (%)	Number of mortality events (%)	States	Frequency[a]
Trichomonosis	54 (40)	39 (47)	AL, FL, GA, NC, SC, TN, VA, WV	18
Toxicosis	25 (18.5)	7 (8.4)	GA, SC, VA, WV	7
Suspected toxicosis	22 (16.2)	6 (7.2)	GA, SC, WV	6
Avian pox	20 (14.8)	17 (20.4)	GA, SC, VA, WV	10
Undetermined	6 (4.4)	6 (7.2)	FL, GA, VA	6
Trauma	5 (3.7)	5 (6.0)	GA	5
Ascaridia columbae infection	2 (1.5)	2 (2.4)	FL, GA	2
Suspected tick paralysis	1 (<0.1)	1 (1.2)	GA	1

[a] = Number of years disease diagnosed during the thirty-five year retrospective study.

AL= Alabama SC= South Carolina

FL= Florida TN= Tennessee

GA= Georgia VA= Virginia

NC= North Carolina WV= West Virginia

CHAPTER 7

CONCLUSIONS

The major objective of this research was to gain a better understanding of *Trichomonas gallinae*, the causative agent of avian trichomonosis. The disease has been diagnosed in numerous avian species and has caused conservation issues in several species including endangered and game birds.

Study 1 (Chapter 3)

The goal of this project was to molecularly characterize *T. gallinae* isolates from several avian species obtained from a wide-ranging geographic distribution to determine if *T. gallinae* isolates differed genetically depending on virulence, host species, or geographical location. This was performed by analyzing the phylogenetic relationships of the 5.8S rRNA-ITS, 18S rRNA, and alpha-tubulin sequences. The results demonstrated the following: 1) a genetic difference was not apparent between clinically virulent and avirulent isolates in the analyzed three gene targets, 2) the sequence analysis strongly suggests at least three different species exist within the *T. gallinae* morphologic complex, 3) a potential host-parasite genotype association exists, and 4) at least two separate trichomonad species were involved in two major outbreaks in western states (USA). The findings of a potential host-parasite genotype association are consistent with the molecular investigations of *Tetratrichomonas* spp. (Cepicka et al., 2006). Additionally, the

finding of multiple trichomonad species involved in trichomonosis outbreaks is important in wildlife management and conservation.

Study 2 (Chapter 4)

The presence of intracellular viruses in clinically virulent and avirulent *T. gallinae* isolates was investigated. The presence of double-stranded RNA (dsRNA) viruses within *T. vaginalis* is associated with up-regulation of certain proteins, including known virulence factors (Alderete et al., 1986; Provenzano et al., 1997). We proposed that intracellular viruses may be associated with virulence of *T. gallinae*. To this end, we examined twelve *T. gallinae* isolates for intracellular dsRNA viruses and the data demonstrated that dsRNA viruses were not detected in the *T. gallinae* isolates by virus purification and CsCl density gradient. Additionally, a novel RNA-dependent RNA polymerase partial sequence was identified from the known dsRNA virus-infected *T. vaginalis* isolate serving as a positive control.

Study 3 (Chapter 5)

This study investigated the validity of *T. gallinae* hemolytic activity as an *in-vitro* virulence assay. Clinical virulence of *T. gallinae* must be viewed with caution due to protective immunity from previous infection with the protozoa. Experimental infections of naïve birds have been used to determine actual virulence. Erythrocyte hemolysis has been suggested as a possible *in vitro* indicator of virulence for *T. gallinae*. Previous investigations demonstrated an approximate 90% hemolysis of chicken erythrocytes by a *T. gallinae* isolate (DeCarli and Tasca, 2002). The hemolytic activity of *T. vaginalis* previously has been correlated with clinical virulence of the isolate (DeCarli et al., 1989; Dailey et al., 1990). The hemolytic activity of twenty-two live *T. gallinae* isolates was determined and the results disclosed that hemolytic

activity was not associated with clinical virulence and the results of the assay were inconsistent between experiments for many of the isolates.

Study 4 (Chapter 6)

Mourning doves represent the most popular avian game species within the United States and populations have fluctuated throughout the United States and declined over much of the eastern United States during the last few decades (Dolton and Rau, 2003). Little is known about mourning dove diseases and their potential to impact conservation efforts. To this end, we examined mourning dove diagnoses (n=135) from the Southeastern Cooperative Wildlife Disease Study (SCWDS) necropsy records over a thirty-five year period and determined if common causes of morbidity and mortality were associated with gender, age, or seasonal patterns. Additionally, records of mourning doves (n=809) submitted from two states for West Nile virus surveillance from 2001 through 2005 were reviewed. The results indicated the following: 1) trichomonosis comprised 40% (n=54) of all diagnoses and was the most frequent diagnosis, 2) toxicoses and avian pox comprised 18.5% (n=25) and 14.8% (n=20) of all diagnoses, respectively, 3) adults were observed more frequently with trichomonosis (94.1%) and toxicoses (68%) as compared to juveniles, but a gender predisposition was not apparent for either disease; age and gender predilections were not apparent for cases of avian pox, 4) the majority of the trichomonosis and avian pox cases were observed in the spring-summer, whereas the majority of the toxicosis cases were observed in the winter-spring, and 5) West Nile virus was isolated from 2.1% (n=17) and Eastern equine encephalitis virus was isolated from 0.2% (n=2) of the submitted doves. These data suggests that trichomonosis, avian pox, and toxicoses are the three major diseases of mourning doves in the southeastern United States; furthermore, it

suggests that trichomonosis is the most important disease in mourning dove conservation if submissions to SCWDS are a true indicator of diseases occurring in nature.

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