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Molecular confirmation of *Trichomonas gallinae* and other parabasalids from Brazil using the 5.8S and ITS-1 rRNA regions

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ABSTRACT

Clinical, gross, and histopathology lesions and molecular characterization of *Trichomonas* spp. infection were described in two striped owls (Asio (*Rhinoptynx*) *clamator*), one American kestrel (*Falco sparverius*), two green-winged saltators (*Saltator similis*), and in a toco toucan (*Ramphastos toco*) from Brazil. These birds presented clinical signs including emaciation, ruffled feathers, abundant salivation and open mouth breathing presumably due to abundant caseous material. Gross lesions were characterized by multifocal yellow friable plaques on the surface of the tongue, pharynx and/or caseous masses partially occluding the laryngeal entrance. In the owls, the caseous material extended into the mandibular muscles and invaded the sinuses of the skull. Histopathologically, marked necrotic and inflammatory lesions were associated with numerous round to oval, pale eosinophilic structures (6–10 μm) with basophilic nuclei, consistent with trichomonads. Organisms similar to those described above also were found in the liver of the two green-winged saltators. To the authors' knowledge, this is the first report of trichomonosis in a striped owl and a toco toucan. Sequence analysis of the *Trichomonas* spp. internal transcribed spacer 1 (ITS-1) region and partial 5.8S of the ribosomal RNA (rRNA) disclosed significant genetic diversity. Two sequences had 100% identity to *Trichomonas gallinae*, whereas two sequences had a 99% and 92% identity to a *Trichomonas vaginalis*-like sequence, respectively. One sequence (green-winged saltator 502-08) had a 100% identity to a newly recognized genus *Simplicomonas*.

1. Introduction

Avian trichomonosis, also known as pigeon trichomonosis or canker, is caused by the flagellated protozoan parasite *Trichomonas gallinae*. The parasite primarily infects the upper digestive tract of birds and lesions vary from mild ulceration of the mucosa to large caseous masses (Narcisi et al., 1991). Trichomonosis has been reported in turkeys and chickens (Stabler, 1954), raptors, columbids, passerines (Forrester and Foster, 2008; Stoute et al., 2009), budgerigars (Mckeon et al., 1997), house finches (Gerhold et al., 2008) and in wild finches (Neimanis et al., 2010). Multiple large mortality events have been associated with trichomonosis (Forrester and Foster, 2008; Robinson et al., 2010). Previous molecular characterization of *T. gallinae* isolates in the United States, disclosed

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multiple genotypes and potential host-parasite associations (Gerhold et al., 2008; Anderson et al., 2009; Lawson et al., 2011). Here we describe the lesions and molecular characterization of *Trichomonas* spp. infection in two striped owls (*Asio* (*Rhinoptynx*) *clamator*), one American kestrel (*Falco sparverius*), two green-winged Saltators (*Saltator similis*), and in a toco toucan (*Ramphastos toco*) from Brazil. All birds are listed as those of least concern species by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN, 2009).

2. Material and methods

2.1. Birds and history

A mature male free-ranging striped owl (726-06) was found dead in a local forest near the Veterinary School at the Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. An immature male free-ranged striped owl (966-08) and one mature female American kestrel (1357-08) and two green-winged Saltators (500-08 & 502-08) were recovered from illegal trade and housed in the Center for Triage of Wild Animals (Centro de Triagem de Animais Selvagens – CETAS, Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis – Brazilian Institute of Environment and Renewable Natural Resources – IBAMA), Belo Horizonte, Minas Gerais State. A free-ranging mature female toco toucan (614-06) was recovered from a forest area near Pampulha Lake and housed in Belo Horizonte Zoo (Fundação Zoo-Botânica). Sick birds were submitted for veterinary care at the CETAS and Zoo but they died a short time after admission.

2.2. Pathology

Necropsy was performed and gross lesions were recorded. Section of brain, tongue, oropharynx, mandibular muscles, esophagus, crop, proventriculus, ventriculus, intestine, lung, liver, kidney, spleen and heart were collected and fixed in 10% neutral formalin until processing for histologic examination. Fixed tissues were trimmed, embedded in paraffin, sectioned at 5 μm of thickness, stained with hematoxylin and eosin (H&E), and examined by bright field microscopy.

2.3. Molecular characterization

DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissues using QIAGEN DNA Extraction Mini kits (QIAGEN, Valencia, CA) per the manufacturer's instructions. Extracted DNA was stored at −20 °C until used for DNA amplification by polymerase chain reaction (PCR). The internal transcribed spacer 1 (ITS-1) and partial 5.8S rRNA regions were amplified using Trichomonadida-family wide primers ITS1F (5′-AGCGCAATTTGCATTCAA-3′) and ITS1R (5′-CGGTAGGTGAACCTGCCGTTGG-3′) that were modified from Felleisen (1997) and Cepicka et al. (2005). PCR components included 1–2 μl of extracted DNA in a 25 μl reaction containing Ready-to-go PCR beads (GE Scientific, Piscataway, NJ) and 20 pM of ITS1F and ITS1R primers. Cycling parameters for the amplification were 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 15 min. A water control was included in DNA extraction and water was used for all PCR reactions as a negative control to detect contamination. DNA isolated from a laboratory-propagated sample of *T. gallinae* was included as a positive control.

PCR amplicons were separated by gel electrophoresis using a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. An approximate 200-base pair product was excised and the DNA purified using a QIAquick Gel Extraction kit (QIAGEN) and ligated into the pDrive vector using the QIAGEN PCR cloning kit per the manufacturer's instructions. DNA transformation procedure was performed using QIAGEN EZ competent cells and 2 μl of ligation-reaction per the manufacturer's instructions. Competent cells containing the vector with PCR product insert were detected with blue/white screening by plating 50 μl of the transformation mixture on Luria-Bertani (LB) broth agar plates supplemented with 100 mg/ml carbenicillin, 100 mM of Isopropyl β-D-1-thiogalactopyranoside, and 40 mg/ml of β-Gal reagent. Two to three colonies, if available, were isolated and propagated in LB broth supplemented with 100 mg/ml carbenicillin. Plasmid DNA was isolated using Mini-prep kit (QIAGEN) per the manufacturer's instructions. Bi-directional sequencing of the inserts from the clones (2–3 per isolate) was performed using M13 F and M13 R plasmid specific primers at the Integrated Biotechnology Laboratories (The University of Georgia, Athens, GA 30602). The sequences obtained were compared to those in GenBank. Sequences were also aligned with other related sequences using the multisequence alignment ClustalX program and the chromatograms were manually examined to detect polymorphisms (Thompson et al., 1994). Phylogenetic analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 program (Kumar et al., 1993). The neighbor-joining and minimum evolution algorithms use the Kimura 2-parameter model and maximum parsimony uses a heuristic search. The GenBank accession numbers of sequences obtained in this study are listed in Table 1.

3. Results

3.1. Clinical signs

The two green-winged saltators were found dead while housed in the CETAS-IBAMA and clinical signs were not recorded. All others birds demonstrated difficulty eating and drinking and at physical examination there were yellow and friable plaques on the tongue and oral mucosa consistent with trichomonosis. The immature striped owl was lethargic, emaciated, had ruffled feathers, salivated excessively, had difficultly closing its mouth due to abundant caseous material, and exhibited open mouth and loud breathing. The immature American kestrel had multifocal yellow plaques on the oral mucosa when presented to the veterinarian and during hospitalization the plaques increased in size and became diffuse on the oral mucosa and tongue. Four days later the kestrel demonstrated severe

Table 1
Birds examined from Brazil in this study. Percent identity of ITS-1 and partial 5.8S rRNA to three parabasalids sequences from GenBank are listed for each isolate.

Bird ID	Species	Tissue sample	% identity to *Trichomonas-gallinae* GenBank accession AY349182	% identity to *T. vaginalis*-like GenBank accession EU215365	% identity to *Simplicomonas* sp. GenBank accession HQ334182	GenBank accession number for respective isolates in this study
500-08	Green-winged Saltator (*Saltator similis*)	Liver	89%	92%	53%	JX089389
502-08	Green-winged Saltator	Liver	74%	79%	100%	JX089388
966-08	Immature striped owl (*Asioclamtor*)	Oral mucosa	100%	94%	77%	JX089390
1357-08	American kestrel (*Falco sparverius*)	Oral mucosa	100%	94%	77%	JX089391
726-06	Mature striped owl	Oral mucosa and pharynx	91%	99%	70%	JX089392
614/06	Toucan (*Toco toucan*)	Oral mucosa and proximal esophagus	ND	ND	ND	N/A

ND, PCR unrewarding on multiple attempts; N/A, not applicable.

dyspnea and died the following day. The toco toucan was lethargic and had difficulty standing.

3.2. Pathology

Necropsy revealed that all birds were thin and dehydrated. Multifocal yellow, friable plaques were observed on the surface of the tongue in the owls, toucan and American Kestrel. Friable and yellow plaques and/or nodules also were observed on the oral mucosa, upper mouth, pharynx and larynx of the American kestrel (Fig. 1). Abundant gray to yellow caseous masses were observed covering the oral mucosa of adult (Fig. 2) and immature owl. The masses partially occluded the laryngeal entrance. On cut surface, the caseous material extended into the submucosa and into the mandibular muscles (Fig. 3). In the immature owl, caseous coalescing masses invaded the sinuses of the skull. The toco toucan also showed two caseous masses on the surface of the pharynx and esophagus. In the two green-winged saltators the liver was moderately pale and had mild hepatomegaly and lesions in the oral cavity were not found.

Histopathologically, marked diffuse necrotizing stomatitis and pharyngitis characterized by caseous necrosis

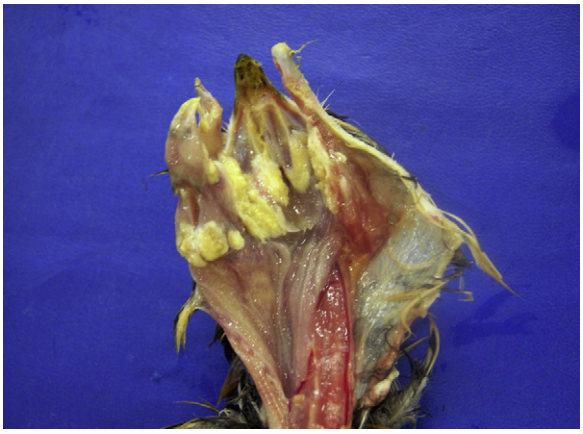

Fig. 1. Gross lesions of an American kestrel. Multifocal, friable, yellow, raised plaques on the oral mucosa.

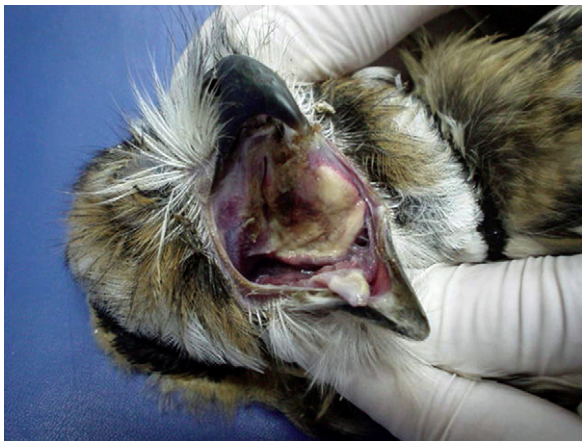

Fig. 2. Gross lesions of a mature striped owl with trichomonosis. Abundant yellow, caseous, material covering part of the oral mucosa and partially occluding the entrance of the larynx.

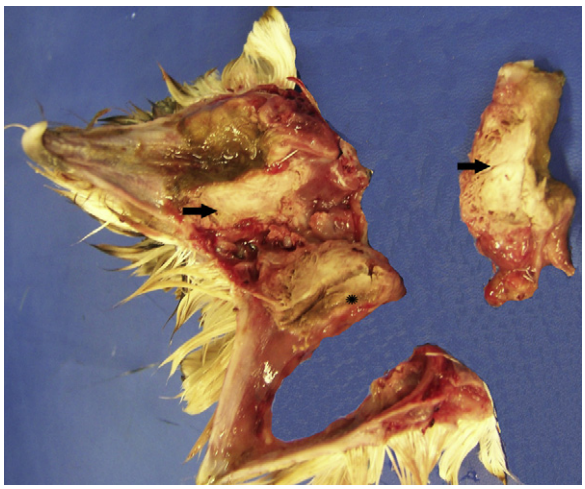

Fig. 3. Gross lesions of an immature striped owl with trichomonosis. Cut surface: caseous necrosis extends into submucosa (arrows) and adjacent muscles (*).

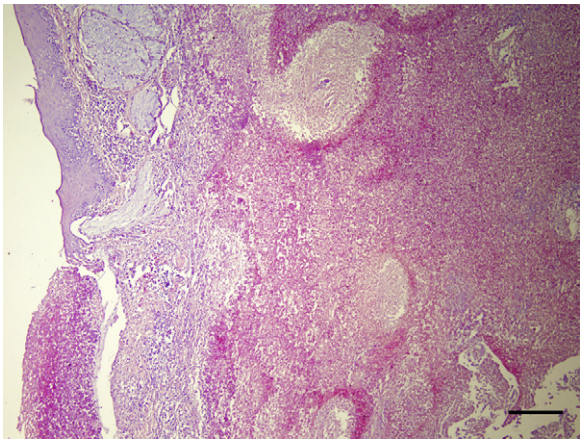

Fig. 4. Oropharynx of the American kestrel. Marked, diffuse, caseous, necrosis and partial loss of epithelial mucosa. H&E. Bar: 50 μm.

and loss of epithelial mucosa was present (Fig. 4) in the American kestrel, toucan, and owls. The necrotic areas were surrounded by many degenerate heterophils, macrophages, lymphocytes, and plasma cells that extended into the submucosa. Caseous necrosis also was observed in the muscles of mandible, mucosa of sinus (immature owl) and proximal esophagus (toco toucan). Surrounding the caseous areas there was coagulative necrosis in the muscle fibers associated with heterophils and macrophages. In the owls and toco toucan, fibroblasts were observed in moderate quantity surrounding necrotic areas. There were also foci of mineralization and fibrinoid necrosis of vessel walls. The necrotic areas contained numerous round to oval, pale eosinophilic structures (6–10 μm) with basophilic nuclei, compatible with trichomonads (Fig. 5). In the immature owl, salivary glands contained similar protozoal organisms associated with plasma cells and lymphocytes. In the two green-winged saltators, several organisms similar to those described above were diffusely observed in the liver. In addition, there were moderate multifocal to coalescing

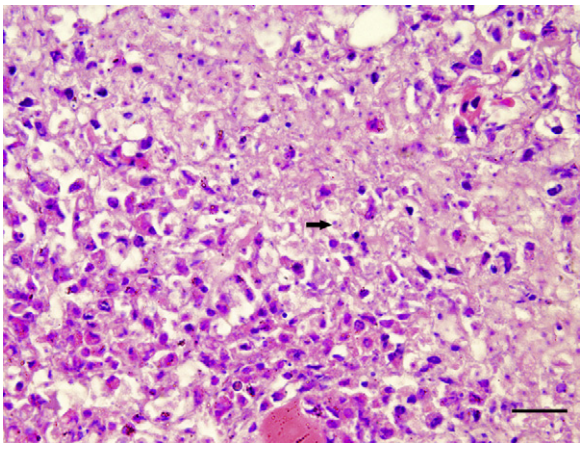

Fig. 5. Immature striped owl. Mandible muscle. Lymphocytes and plasma cells infiltration associate with numerous round to oval, pale eosinophilic structures (6–10 μm) with basophilic nuclei (arrow head), consistent with trichomonads. H&E. Bar: 50 μm.

plasma cells and lymphocytes infiltration associated with random coagulative necrosis. Mild to moderate acute urate nephropathy was also observed in the mature owl and American kestrel.

4. Molecular characterization

4.1. PCR analysis, molecular characterization, and phylogenetic analysis

Sequence analysis of the ITS1-5.8S rRNA region using FFPE tissues revealed significant genetic variation (Table 1). Two sequences (immature owl and American kestrel 966-08 & 1357-07) had a 100% identity to the *T. gallinae* (GenBank accession AY349182). A sequence from the mature owl (726-06) had a 99% identity to *Trichomonas vaginalis*-like organism (GenBank accession EU215365), whereas it had only a 91% identity to *T. gallinae*. One sequence (green-winged saltator 500-08) had a 92% identity to *T. vaginalis*-like and an 89% identity to *T. gallinae*. The last sequence from the green-winged saltator (502-08) had a 100% identity to a newly recognized genus *Simplicomonas* (GenBank accession HQ334182). PCR testing of the toucan samples were unrewarding on multiple attempts. Alignment of the ITS1 and 5.8S of these sequences along with other *Trichomonas* sequences from an outbreak in the Caribbean on St. Kitts, West Indies (Stimmelmayr et al., in press) and related organisms from GenBank and *Pentatrichomonas hominis* (as outgroup, AY758392) resulted in a 93-bp alignment. Phylogenetic analyses divided the isolates into two distinct clades (Fig. 6). One group containing 2 of the isolates from this study (966-08 & 1357-08), grouped with other prototypical *T. gallinae* isolates from GenBank and one isolate (502-08) grouped with a *Simplicomonas* sp. (GenBank accession HQ334182) isolated from a backyard chicken in Georgia (USA) (Lollis et al., 2011). Phylogenetic analysis failed to resolve isolates 500-08 and 726-08 into a particular clade. One isolate from the St. Kits outbreak (#21) grouped with the prototypical *T. gallinae* isolates from GenBank; whereas the other St. Kits isolates grouped with *Histomonas*-like organism (GenBank accession HQ334182) found within the liver of a bobwhite quail in Georgia, USA (Lollis et al., 2011).

5. Discussion

The changes reported in these birds are consistent with upper digestive tract lesions reported with avian trichomonosis (Stabler, 1954; Narcisi et al., 1991). The identification of intralesional trichomonads associated with classical lesions indicates that the birds were infected with virulent *Trichomonas* isolates. DNA amplification and nucleotide sequencing confirmed the presence of *Trichomonas* spp. in four birds in this study. Additionally, the sequence from one green-winged saltator had 100% sequence to recently described parabasalid genus, *Simplicomonas* (Cepicka et al., 2010; Lollis et al., 2011). This information suggests that *Simplicomonas* has similar histological morphological characteristics as *Trichomonas*, although lesions were found in the liver only.

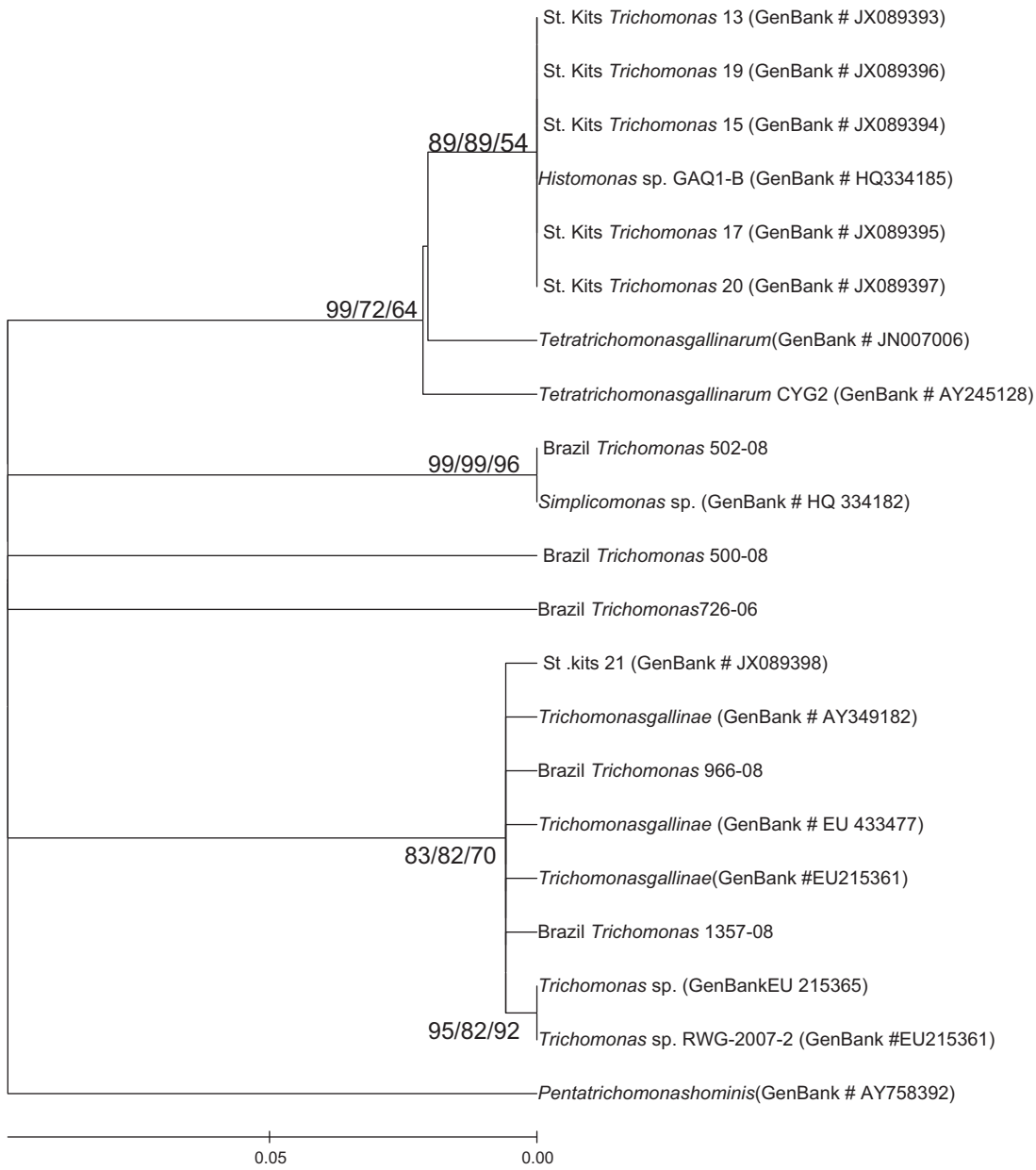

Fig. 6. Phylogenetic analysis of *Trichomonas* spp. isolates from this study and other trichomonads based on sequencing of the 5.8S rRNA and flanking ITS-1 regions. The tree was constructed using 93-aligned nucleotide positions using a minimum evolution algorithm with 500 replications in a Kimura 2-parameter model with *Pentatrichomonas hominis* as an outgroup. Bootstrap values for neighbor-joining/minimum evolution/maximum parsimony are shown at the nodes.

Domestic pigeons (*Columbia livia*) are the primary host of *T. gallinae*. In columbids, the protozoan is transferred in the "pigeon milk" from the crop of an infected parent to the newly-hatched nestling. Sources of infection to others birds can be the water, contaminated seeds (Stabler, 1954; Forrester and Foster, 2008) or when avian predators (such as owl or American kestrel) feed on infected prey (Erwin et al., 2000). The striped owl is a nocturnal medium-sized raptor found in Central and South America and it primarily preys on small mammals (rats, bats and opossums) and birds (sparrows, feral doves and others) (Sick, 1997). The American kestrel is one of the smallest raptors of the world and occurs all over the Western Hemisphere in a great variety of habitat types. In tropical areas like Brazil and, especially in the Cerrado biome, the American kestrel apparently displayed a higher consumption of insects, arthropods and occasionally avian prey (Cabral et al., 2006). Toco Toucan (*R. toco*) is one of the largest frugivorous birds and it usually consumes fruits (e.g. figs), but also will eat insects, frogs, small reptiles, eggs and avian nestlings. It is found in semi-open habitats throughout a large part of central and eastern South America (Cubas, 2007).

Trichomonas-associated mortality is often attributed to esophageal obstruction by caseous masses leading to emaciation, dehydration, or asphyxiation (Narcisi et al., 1991). The birds in this study were emaciated and alimentary contents were not found in the digestive tract. Also, two birds showed acute urate nephropathy, indicating dehydration. When trichomonads colonize the upper digestive tract, they incite progressive inflammation and necrosis. Trichomonads attach to the surface epithelium and use amoeboid motion and hydrolytic enzymes to separate epithelial cell junctions to enable invasion and progressive movement deeper into the submucosa. Once lesions become marked, swallowing is seriously impeded, leading to regurgitation and accumulation of food in the oral cavity and crop (Neimanis et al., 2010). Death occurs as a result of starvation, respiratory failure (if the lesion blocks the trachea) or hepatic dysfunction if organisms invade the liver (Forrester and Foster, 2008), which was observed in the green-winged saltators in this report. There is a close correlation between the lesion intensity and pathogenicity, larger lesions resulting from highly pathogenic isolates (Honigberg, 1979). Infection with mild pathogenic isolates results in mild inflammation in the oral mucosa and pharynx. However, infection with more virulent isolates results in marked caseous necrosis in the upper digestive tract. Some highly virulent isolates invade sinuses, skull and internal organs such as liver, lungs, air sacs, pericardium and pancreas (Forrester and Foster, 2008). In this study, an immature owl showed invasion in the sinus, mandibular muscles and salivary glands suggesting that it was infected with a highly virulent isolate.

Studies to determine hemolytic activity (DeCarli and Tasca, 2002; Gerhold et al., 2009) with different avirulent and virulent strains of *T. gallinae* demonstrated varying results. Gerhold et al. (2009) suggested that hemolytic activity does not correspond with clinical virulence. Narcisi et al. (1991) report that hemoglobin levels did not change significantly during the course of *T. gallinae* infection in pigeons. These results correspond with our pathologic data. Gross and histological lesions consistent with intravascular and/or intracellular hemolytic anemia (Valli and Gentry, 2007) were not observed.

Positive PCR resultant sequences indicated *Trichomonas* or other parabasild infections in all birds except to the toucan. Considering that histopathology analysis was strongly compatible with trichomonad organisms, it is possible that the duration of the tissues in formalin lead to negative PCR results due to the DNA crosslinking associated with formalin fixation (Lin et al., 2009). Similar difficulty with formalin fixed tissues was found in certain *Histomonas meleagridis* samples (Lollis et al., 2011).

It appears that multiple genotypes of trichomonad species were found in these birds from Brazil. Similarly various genotypes, and likely species, were associated with large trichomonosis mortality events in mourning doves and Cooper's hawks from Arizona and band-tailed pigeons (*Patagioenas fasciata*) from California during 2005–2006 (Gerhold et al., 2008). In contrast, no genetic variation was apparent in the *Trichomonas* sequences obtained from isolates causing morbidity in mortality in passerines in the UK (Robinson et al., 2010). Of particular interest is the finding of the *T. vaginalis*-like organism in the owl. Gerhold et al. (2008) found these sequences associated with white-winged doves in Arizona, Texas and California. Although similar white-winged doves are not found in Brazil, picazuro Pigeon (*Patagioenas picazuro*) are common. It would be useful to survey and sequence positive trichomonad isolates from picazuro pigeons to determine if they contain similar sequence identity to the *T. vaginalis*-like isolate from this study. One sequence, from a green-winged saltator with inflammatory and necrotic lesions in the liver, was 100% identical to a *Simplicomonas* sp. sequence that caused hepatitis-associated mortality in a backyard chicken in Georgia (USA) (Lollis et al., 2011). Given that the saltators were confiscated during attempts to smuggle birds into other states or countries, the illegal trade market may explain the appearance of the *Simplicomonas* sp. in the United States. Further surveillance and molecular genotyping of illegally and legally traded birds is needed to determine the transmission risk of novel protozoal infections in native wild birds and domestic poultry. To our knowledge, this is the first report of a *Simplicomonas* sp. causing disease in a free ranging bird.

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References

Anderson, N.L., Grahn, R.A., VanHooser, K.A., BonDurant, R.H., 2009. Studies of trichomonad protozoa in free ranging songbirds: prevalence of *Trichomonas gallinae* in house finches (*Carpodacus mexicanus*) and corvids and a novel Trichomonad in mockingbirds (*Mimus polyglottos*). Vet. Parasitol. 161, 178–186.

BirdLife International, 2009. *Asio* (*Pseudoscops*) *clamator*, *Falco sparverius*, *Saltator similis* and *Ramphastos toco*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2011.2. www.iucnredlist.org (downloaded on 24.01.12).

Cabral, J.C., Granzinolli, M.A.M., Motta-Junior, J.C., 2006. Dieta do quiriquiri, *Falco sparverius* (Aves: Falconiformes), na Estação Ecológica de Itirapina, SP. Rev. Bras. Ornitol. 14, 393–399.

Cepicka, I., Kutisová, K., Tachezy, J., Kulda, J., Flegr, J., 2005. Cryptic species within the *Tetratrichomonas gallinarum* species complex revealed by molecular polymorphism. Vet. Parasitol. 128, 11–21.

Cepicka, I., Hampl, V., Kulda, J., 2010. Critical taxonomic revision of parabasalids with description of one new genus and three new species. Protist 161, 400–433.

Cubas, Z.S., 2007. Piciformes (Tucano, Aracari, Pica-pau). In: Cubas, Z.S., Silva, J.C.R., Catão-Dias, J.L. (Eds.), Tratado de animais selvagens. Medicina Veterinária. , 1st ed. São Paulo, Roca, p. 1354.

DeCarli, G.A., Tasca, T., 2002. Trichomonas gallinae: a possible contact-dependent mechanism in the hemolytic activity. Vet. Parasitol. 106, 277–283.

Erwin, K.G., Kloss, C., Lyles, J., Feiderhoff, J., Fedynich, A.M., Henke, S.E., Roberson, J.A., 2000. Survival of *Trichomonas gallinae* in White-winged doves carcasses. J. Wildl. Dis. 36, 551–554.

Felleisen, R.S., 1997. Comparative sequence analysis of 5.8S rRNA genes and internal transcribed spacer (ITS) regions of Trichomonadid protozoa. Parasitology 115, 111–119.

Forrester, D.J., Foster, G.W., 2008. Parasitic diseases of wild birds. In: Atkinson, C.T., Thompson, N.J., Hunter, D.B. (Eds.), Trichomonosis. Wiley-Blackwell, Ames, IA.

Gerhold, R.W., Yabsley, M.J., Smith, A.J., Ostergaard, E., Manna, W., Cann, J.D., Fischer, J.R., 2008. Molecular characterization of the *Trichomonas gallinae* morphologic complex in the United States. J. Parasitol. 94, 1335–1341.

Gerhold, R.W., Yabsley, M.J., Fischer, J.R., 2009. Hemolytic activity of *Trichomonas gallinae* isolates does not correspond with clinical virulence. Vet. Parasitol. 160, 221–224.

Honigberg, B.M., 1979. Biological and physiological factors affecting pathogenicity of trichomonads. In: Levandowsky, M., Hunter, S.H. (Eds.), Biochemistry and Physiology of Protozoa. Academic Press, New York, pp. 409–427.

Kumar, S., Tamura, K., Masatoshi, N., 1993. MEGA: molecular evolutionary genetics analysis, version 1.01. The Pennsylvania State University, University Park.

Lawson, B., Cunningham, A., Chantrey, J., Hughes, L., John, S., Bunbury, N., Bell, D., Tyler, K., 2011. A clonal strain of *Trichomonas gallinae* is the aetiological agent of an emerging avian epidemic disease. Infect. Genet. Evol. 11, 1638–1645.

Lin, J., Kennedya, S.H., Svarovskyc, T., Rogers, J., Kemnitz, J.W., Xu, A., Zondervan, K.T., 2009. High-quality genomic DNA extraction from formalin-fixed and paraffin-embedded samples deparaffinized using mineral oil. Anal. Biochem. 395, 265–267.

Lollis, L.A., Gerhold, R.W., McDougald, L.R., Beckstead, R.B., 2011. Molecular characterization of *Histomonas meleagridis* and other parabasalids in the United States using the 5.8S, ITS-1, and ITS-2 rRNA regions. J. Parasitol. 97, 610–615.

Mckeon, T., Dunsmore, J., Raidal, S.R., 1997. *Trichomonas gallinae* in budgerigars and columbid birds in Perth, Western Australia. Aust. Vet. J. 75, 652–654.

Narcisi, E.M., Sevolan, M., Honigberg, B.M., 1991. Pathologic changes in pigeons infected with a virulent *Trichomonas gallinae* strain (Eiberg). Avian Dis. 35, 55–61.

Neimanis, A.S., Handeland, K., Isomursu, M., Agren, E., Mattsson, R., Hamnes, I.S., Bergsjø, B., Hirvelä-Koski, V., 2010. First report of epizootic Trichomoniasis in Wild Finches (Family Fringillidae) in Southern Fennoscandia. Avian Dis. 54, 136–141.

Robinson, R.A., Lawson, B., Toms, M., Peck, K., Kirkwood, J., Chantry, J., Clatworthy, I., Evans, A., Hughes, L., Hutchinson, O., John, S., Pennycoat, T., Perkins, M., Rowley, P., Simpson, V., Tyler, K., Cunningham, A., 2010. Emerging infectious disease leads to rapid population declines of common British birds. PLoS One 5, 1–12.

Sick, H., 1997. Ornitologia Brasileira. Nova Fronteira, Rio de Janeiro, p. 862.

Stabler, R.M., 1954. *Trichomonas gallinae*: a review. Exp. Parasitol. 3, 368–402.

Stimmelmayr, R., Stefani, L.M., Thrall, M.A., Landers, K., Revan, F., Miller, A., Beckstead, R., Gerhold, R. Trichomonosis in free-ranging Eurasian collared doves (*Streptopelia decaocto*) and African collared dove hybrids (*Streptopelia risoria*) in the Caribbean and description of ITS-1 region genotypes. Avian Dis., in press.

Stoute, S.T., Charlton, B.R., Bickford, A.A., Bland, M.C., 2009. Respiratory tract trichomoniasis in breeder squab candidates in Northern California. Avian Dis. 53, 139–142.

Thompson, J.D., Higgins, D.G., Giboson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Valli, V.E.O., Gentry, P.A., 2007. Hematopoietic system. In: Jubb, Kennedy, Palmer, Grant, M. (Eds.), Pathology of Domestic Animals. , 5th ed. Elsevier Sauders, Philadelphia, PA, pp. 107–324.