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Investigation of *Trichomonas gallinae* in passerines

Investigação de *Trichomonas gallinae* em passeriformes

Jenny Paola Hidalgo Martínez[1]*; Alexia Brauner de Mello[2]; Marjorie de Giacometi[2]; Rodrigo Casquero Cunha[3]; Camila Belmonte Oliveira[4]; Marlete Brum Cleff[5]; Raqueli Teresinha França[5]

Highlights

T. gallinae has pathogenic potential for birds, causes caseous lesions.
Direct contact during feeding is the most efficient way to establish an infection.
The migratory habit favors the dissemination of pathogens.
Risk factors for trichomoniasis are associated with the host and/or parasite.

Abstract

Trichomoniasis, caused by the protozoan *Trichomonas gallinae,* has as main hosts birds of the Columbidae family, which have a high prevalence of the protozoan without manifesting the disease. The continuous growth of the pigeon population and its cosmopolitan nature mean that today there is a worldwide distribution of this species, being responsible for the distribution and maintenance of the prevalence of trichomoniasis in almost the entire world. The transmission of the disease may be by direct contact, or indirect, through food or water. This indirect route is the reason why such a wide range of bird orders can be infected, very different from columbids such as falconiformes, strigiformes, passerines, piciformes, psittaciformes, gruiformes, galliformes or anseriformes. Thus, the objective of this study was to evaluate the presence of *T. gallinae* in passerines received at a Wild Animal Screening Center. In order to carry out this study, 300 birds of the order Passerine corresponding to 23 different species were analyzed, received at the Wild Fauna Rehabilitation Center (NURFS) of the Federal University of Pelotas (UFPel), in different seasons of the year between the months of March to October 2021. Samples of swabs from the oropharynx were collected from all individuals, the material was immediately placed in a falcon tube containing

[1] Student of the Master's Course of the Graduate Program in Veterinary, Universidade Federal de Pelotas, UFPEL, Pelotas, RS, Brazil. E-mail: jhidalgo24@unisalle.edu.co
[2] Students of the Doctoral Course of the Graduate Program in Microbiology and Parasitology, UFPEL, Pelotas, RS, Brasil. E-mail: alexiabraunermello@gmail.com; marjorie.giacometi@gmail.com
[3] Prof. Dr., Departament of Medicine Veterinary Preventive, UFPEL, Pelotas, RS, Brasil. E-mail: rodrigocunha_vet@hotmail.com
[4] Profª Drª, Departament of Microbiology and Parasitology, UFPEL, Pelotas, RS, Brasil. E-mail: camilabelmontevet@yahoo.com.br
[5] Profªs Drªs, Department of Veterinary Clinics, UFPEL, Pelotas, RS, Brasil. E-mail: marletecleff@gmail.com; raquelifranca@gmail.com
* Author for correspondence

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Trypticase-Yeast Extract-Maltose (TYM) culture medium and sent to the Laboratory of Protozoology and Entomology (LAPEn), for incubation in a bacteriological growth greenhouse and subsequent identification of the protozoan on a slide in wet mounting under an optical microscope in a 40X objective. Wet mounting on a slide was performed in triplicate and analyzed in its entirety. Of the 300 birds evaluated in in vitro culture for *T. gallinae*, 25 had inconclusive results and were submitted to PCR analysis, being negative for *T. gallinae.* Although no positive Passeriformes was found, the monitoring of the occurrence of this protozoan must continue, as it is known that it may easily cause a possible epidemic, leading to losses for the wild fauna that has endangered birds.
Key words: Parasites. Protozoa. Wild birds.

Resumo

Tricomoníase, causada pelo protozoário *Trichomonas gallinae*, tem como principais hospedeiros aves da família Columbidae, que apresentam alta prevalência do protozoário sem manifestar a doença. O contínuo crescimento da população de pombos e sua natureza cosmopolita fazem com que hoje exista uma distribuição mundial desta espécie, sendo responsável pela distribuição e manutenção da prevalência da tricomoníase em quase todo o mundo. A transmissão da doença pode ser por contato direto, ou indireto, por meio de alimentos ou água. Essa rota indireta é a razão pela qual uma gama tão ampla de ordens de aves pode ser infectada, muito diferente dos columbídeos, como falconiformes, strigiformes, passeriformes, piciformes, psittaciformes, gruiformes, galliformes ou anseriformes. Desta forma, o objetivo desse estudo foi avaliar a presença de *T. gallinae* em passeriformes recebidos em um Centro de Triagem de Animais Silvestres. Para a realização deste estudo foram analisados 300 aves por conveniência da ordem Passeriforme correpondente a 23 espécies diferentes recebidos no Núcleo de Reabilitação da Fauna Silvestres (NURFS) da Universidade Federal de Pelotas (UFPel), em estações distintas do ano comprendidas entre os meses de março a outubro de 2021. De todos os indivíduos foram colhidas amostra de suabe da orofaringe, o material foi imediatamente acondicionado em tubo falcon contendo meio de cultura Tripticase-Yeast Extract-Maltose (TYM) e encaminhado ao Laboratório de Protozoologia e Entomologia (LAPEn), para incubação em estufa de crecimento bacteriologico e posterior identificação em lamina em montagem úmida no microscópio óptico em objetivo de 40X do protozoário. A montagem úmida em lamina, foi feita em triplicata e analisada em sua totalidade. Das 300 aves avaliadas no cultivo *in vitro* para *T. gallinae*, 25 tiveram resultado inconclusivo e foram sumetidas a análise de PCR, sendo negativas para *T. gallinae.* Embora não tenha sido encontrado nenhum Passeriformes positivo, o monitoramento da ocorrência desse protozoário deve continuar, pois sabe-se que ele pode causar uma possível epidemia facilmente, levando a perdas para a fauna silvestre que possuí aves ameaçadas de extinção.
Palavras-chave: Aves silvestres. Parasitas. Protozoários.

Introduction

Trichomoniasis is a parasitic disease caused by the flagellate protozoan *Trichomonas gallinae* that mainly affects columbiformes and birds of prey, and occasionally, galliformes, passerines, piciformes and psitaciformes (Grave, 1996). Pigeons (Columba livia) and other species of the Columbidae family are the main hosts

of *Trichomonas gallinae* (Stabler, 1954). In the host, the parasites are found in the upper digestive tract: oral cavity, pharynx, esophagus and crop (Stabler, 1954). Transmission from one bird to another does not require an intermediate host or vector, occurring through direct contact through the ingestion of contaminated food and water, with the regurgitation of food between parents and their children being relevant (Greiner & Ritchie, 1994).

Given the strong seasonal trends in the prevalence of infection, the age range of the population during peak transmission and pathogenesis in adults appears to be driven by environmental factors, physiological and behavioral changes that accompany the annual cycle of birds. As observed in other avian pathogens, the energy load and stress of migration, or even pre-migration activity, is linked to impaired host immunity, with increased susceptibility to infections (Lloyd, 1995). The trichomoniasis is also an emerging infectious disease of passerine birds that are likely to become exposed to *T. gallinae* in artificial feed and water sources shared by pigeons (Forzan et al., 2010; Neimanis et al., 2010; Zadravec et al., 2012).

Trichomoniasis in chaffinches emerged in Great Britain in 2005, causing a significant decline in the breeding populations of White-tailed Woodpecker (*Carduelis chloris*) and Chaffinch (*Fringilla coelebs*) (Robinson et al., 2010; Lawson et al., 2012; Chi et al., 2013). In 2008, the *T. gallinae* epidemic in Great Britain spread to southern Fennoscandia, causing multiple mortality events over a two-year period (Lawson et al., 2012; Neimanis et al., 2010). In 2007 and 2008, multiple outbreaks of trichomoniasis were identified in Canada involving purple finches (*Carpodacus purpureus*) and goldfinches (*Carduelis tristis*) (Forzan et al., 2010).

Trichomonosis epidemics in pigeons have been recorded since 1945, with morbidity and mortality involving thousands of individuals, often in winter or during spring migration (Cole & Friend, 1999; Stabler & Braun, 1975; Stromberg et al., 2008). In this context, the objective of this study was to evaluate the presence of *T. gallinae* in passerines received at the Wild Fauna Rehabilitation Center (NURFS) of the Federal University of Pelotas (UFPel) during the months of March to October 2021.

Methodology

We analyzed 300 samples of adult birds of the Passerine order, received at the Wild Fauna Rehabilitation Center (NURFS) of Federal University of Pelotas (UFPel), municipality of Capão do Leão, Pelotas, State of Rio Grande do Sul. Samples were collected between March and October 2021, of which thirteen samples were collected in summer, eighty-seven in autumn, one hundred and sixty-eight in winter, and thirty-two in spring (Table 1). All sample collection procedures were performed in strict accordance with the recommendations of SISBIO 78754-3 and Comissão de Ética em Experimentação Animal 160/2021.

Table 1
Passerines species evaluated to the presence of *T. gallinae* during the months of March to October of the year 2021

Species	No. tested (n)	Migratory habit	Habitat	Food
Paroaria coronata	66	No	Rural	Seeds and arthropods
Cyanocompsa brissonii	51	No	Rural	Seeds, fruits and insects
Sicalis flaveola	39	No	Rural	Seeds
Saltator similis	34	No	Rural and urban	Omnivorous
Saltator aurantiirostris	25	Yes	Rural and urban	Fruits and seeds
Cacicus haemorrhous	21	No	Rural	Omnivorous
Sporophila caerulescens	20	Yes	Rural and urban	Seeds and grasses
Stephanophorus diadematus	7	No	Rural	Fruits
Coryphospingus pileatus	7	No	Rural	Seeds, insects, arthropods and fruits
Sporophila collaris	5	No	Rural	Seeds
Pitangus sulphuratus	4	No	Rural and urban	Insects, fruits, carnivore, arachnid
Spinus magellanicus	4	No	Rural and urban	Seeds
Saltator fuliginosus	3	No	Rural	Fruits and seeds
Zonotrichia capensis	3	No	Urban	Fruits, insects and seeds
Tachycineta leucorrhoa	2	Yes	Rural	Insects
Sicalis luteola	2	Yes	Rural and urban	Seeds
Sporophila palustres	1	Yes	Rural	Seeds
Amblyramphus holosericeus	1	No	Rural	Seeds, arthropods, fruits
Cyclarhis gujanensis	1	No	Urban	invertebrates, carnivore
Sturnella superciliaris	1	No	Rural and urban	Larvae, insects and seeds
Turdus rufiventris	1	Yes	Urban	Larvae, insects, invertebrates, fruit and dog food
Tangaragona sayaca	1	No	Rural	Fruits, berries and insects
Molothrus bonariensis	1	Yes	Rural and urban	Omnivorous
Total	300			

Initially, the animals underwent clinical evaluation, any clinical sign observed during the evaluation was recorded in an individualized form. To collect the swab from the oropharynx, the birds were physically restrained, the beak was opened and a swab was introduced into the oropharynx, the material was immediately placed in a falcon tube containing Trypticase-Yeast Extract-Maltose (TYM) culture medium, content 10 % inactivated bovine serum, antibiotic (streptomycin) and antifungal (amphotericin) at pH 7.2 (Diamond, 1957); which was kept at a temperature of 37 ºC in a water bath until

sent to the laboratory (Sansano-Maestre et al., 2009). The animals that presented lesions in the oral cavity and the samples considered inconclusive at the time of reading, which presented a structure similar to *T. gallinae,* were submitted to PCR, totaling 25 samples.

Laboratory evaluation

After collection, the material was sent to the Laboratory of Protozoology and Entomology (LAPEn), where the falcon tubes with the culture medium and the oropharyngeal samples were placed in a bacteriological growth oven at 37ºC. Reading was performed 24 and 48 hours after collection. The tubes were centrifuged at 1500 rpm for 10 min and then an aliquot of the sediment was analyzed under an optical microscope at 40x magnification on a wet mounting slide which was made in triplicate and the reading was performed in the entire area where contained a sample and the result was recorded by the presence or absence of trophozoite, being positive for presence and negative for absence (Seddiek et al., 2014).

Twenty-five samples were chosen where the result of the parasitological reading under the microscope was not sure, the medium was centrifuged to separate the aliquot of the parasite and later exchange of the culture medium to keep it in freezing and continue with the DNA separation to perform the PCR

To confirm the identity of *T. gallinae,* DNA was extracted from 300 µL of the culture, using Brazol® reagent (Invitrogen, UK), according to the manufacturer's instructions, and diluted in 100 µL of endonuclease-free water. The concentration of the extracted DNA was quantified by reading in a spectrophotometer (NanoDrop Lite) with an absorbance of 260 nm. The extracted DNA was electrophoresed on a 1% agarose gel stained with ethidium bromide and visualized using UV transillumination. DNA sample, ITS rDNA, (370 bp) and using oligonucleotide sequences TFR 1 (5'-TGCTTCAGCTCAGCGGGTCTTCC-3') and TFR 2 (5'-CGGTAGGTGAACCTGCCGTTGG-3') were amplified by conventional PCR using ITS1F and ITS1R primers. The PCR reaction was performed in a final volume of 25 µL containing 0.5 µM of each primer, 0.8 mM of dNTP, 2.0 mM of MgCl2, 2.5 U of Taq DNA Polymerase (Ludwig Biotecnologia Ltda, RS, Brazil) and 100-150 ng of the DNA template. DNA amplification consisted of a denaturation step carried out at 95°C for 5 minutes, followed by 40 cycles at 95°C for 60 seconds, 56°C for 30 seconds and 72°C for 30 seconds, and an extension final at 72°C for 10 min. Positive and negative control reactions were included in the assay. The amplicons were analyzed by agarose gel electrophoresis (2%) and visualized by ethidium bromide staining to confirm their specificity and size.

Results and Discussion

In the 300 samples de birds of 23 species of Passeriformes were analyzed, of these samples, 32 were collected in spring, 13 in summer, 87 in autumn and 168 in winter. In the analyzed samples, no positives were found for *T.gallinae,* both in the parasitological and molecular diagnosis. Of the animals evaluated, four samples came from animals that presented lesions in the clinical examination, but negative to the microscopic test and negative to the PCR. Of the samples

processed by PCR (n=25), four were from animals that presented lesions in the clinical examination, but negative in the microscopic test and 21 samples had an inconclusive result, since they presented mobile forms in the microscopic reading, but by the PCR technique all the samples were negative.

The literature describes that most sick birds are adults (Girard et al., 2014; Begum et al., 2008; Fadhil & Faraj, 2019). In contrast, in the study carried out by Al-Sadi and Hamodi (2011), the highest frequency of positive animals was in young pigeons. Regarding seasonality, Fadhil and Faraj (2019) reports a higher incidence of the protozoan in spring compared to winter. In spring, the highest transmission is due to mating, in spring and summer, parental transmission is greater by feeding, and by the decrease in food amounts. Stress, nutritional deficiency, efforts and strains in the search for food may also be added, as well as changes of all kinds, which make birds more susceptible to the presentation of the parasite and the disease (Villanúa et al., 2006). The animals evaluated in our study were adult birds from illegal wildlife trafficking. Most of the evaluated birds arrived in the winter period, followed by autumn, periods in which there is a shortage of food. It is also important to point out that our study was carried out between the months of March and October, it was not possible to collect birds in all of some seasons such as summer and spring, which is why our data differ a little from the reports in the bibliography such as those reported by Fadhil and Faraj (2019), where the highest incidence of the protozoan was in spring and summer.

It is important to note that some of the evaluated species are migratory birds, such as T*achycineta leucorrhoa, Sporophila palustris, Sporophila caerulescens, Saltator aurantiirostris, Turdus rufiventris, Sicalis luteola* e *Molothrus bonariensis* (Timm & Timm, 2021). These habits are presented at the beginning of winter, when they look for warmer weather and greater possibilities of food, or it coincides with the breeding season. This migratory habit favors the spread of microorganisms that may not be occurring in a given region (Timm & Timm, 2021).

As can be seen, most birds in our study have similar eating habits, such as eating seeds and fruits, and some being insectivores (Timm & Timm, 2021). The shared consumption of fruits and seeds could favor contamination in the case of an infected bird. Most of the evaluated birds inhabit rural areas. Birds living in urban areas could be more exposed to the agent, since in most cities there are many pigeons (*Columbia livia*). The importance of the prevention and surveillance of this disease is due to the presence of pigeons worldwide, only not being found in the poles. Pigeons are the main hosts of *Trichomonas* spp., the transmission of these birds to others of different species and families is easy, harming the world fauna, as already evidenced by the outbreak that occurred in Europe in 2005, which caused a mortality of 35% of the passerine population. Or the outbreak that happened in North America in 2012, with the collar and flock pigeons.

Of the animals evaluated, four samples came from animals that presented lesions in the clinical examination, but negative to the microscopic test and negative to the PCR. Cultures of oropharyngeal trichomonads in specific media is regardedas the most sensitive method for the diagnos of the infection (Wieliczko et

al., 2003). Trichomoniasis is characterized by lesions with a caseous appearance that can be located in the oral cavity, pharynx and esophagus (Krone & Cooper, 2002). Occasionally the lesions may involve the ear canal, larynx and respiratory tract (Krone & Cooper, 2002). The formation of yellow-white nodules and plaques in the oral cavity are characteristics of avian trichomonosis but are not pathognomonic lesions. Capillariasis, salmonellosis, hypovitaminosis, candidiosis, poxvirus infection, aspergillosis, might display similar gross appearance (Alkharigy et al., 2018; Martínez-Herrero et al., 2020).

The diagnosis of trichomonosis is often complicated by the fragility of the parasite. To ensure valid test results, it is essential to collect and handle samples correctly, avoiding pre-analytical errors. Culture tests, parasite-specific amplification, or a combination of both test methods is the most efficient and the most commonly used way to diagnose trichomonosis in animals (Collántes-Fernández et al., 2018). In birds the use of culture for the detection of trichomonas is clearly superior in sensitivity as compared to direct microscopy (Cooper & Petty, 1988; Bunbury et al., 2005). In our study, the lack of positive results in the microscopic examination and subsequent PCR may be due to the climate, since most of the samples were collected in winter, which makes it difficult for the protozoan to appear. It would also be important to perform PCR on all study samples in order to reduce false negative reading errors and be sure of this result.

Conclusion

In our study, no positive samples were found. however, this result does not exclude the possibility that passerines are hosts for this protozoan, including being potential transmitters for birds other than columbiforms or birds of raptor.

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