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Molecular methods for detection and genetic characterization of *Trichomonas gallinae* in birds

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ABSTRACT

The parasite Trichomonas gallinae causes trichomonosis in avian and has been reported on several birds, meaning a worldwide prevalence. Infected birds usually become malnourished, because huge necrotic and inflammatory lesions in the mouth and crop prohibit them from swallowing water and food. T. gallinae threatens bird populations, including endangered birds, so it is important to improve methods for detecting infected birds. Currently, the used method to identify T. gallinae depend on direct observation of the parasites via light microscopy, but it has failed to detect dangerous strains. Here, we describe a detailed molecular method that will enable researchers and veterinarians to detect and diagnose avian trichomonosis. Polymerase chain reaction (PCR) can be an extremely sensitive and specific tool for identifying and studying T. gallinae genetics. In this efficient method, samples from birds are cultured and amplified via PCR and then the products are sequenced to allow genetic characterization of T. gallinae and construction of its phylogenetic tree.

1. Introduction

The parasite *Trichomonas gallinae* causes avian trichomonosis, infecting the upper digestive tract the mouth, esophagus, crop and pharynx—in many species of birds worldwide, including columbiformes, raptors, gallinaceous birds and passerines [1-5]. Trichomonosis causes neck swelling that may be seen by human eyes, and the sickness can last for several days or more. Infected birds usually become malnourished, because huge necrotic and inflammatory lesions in the mouth and crop prohibit them from swallowing water and food. Although trichomonas parasitize a variety of avian species, not all of them show the same clinical indications or diseases. Previous investigations have suggested that clinically virulent and avirulent *T. gallinae* isolates indicate that pathogenicity and virulence are genetically controlled within the organism [6]. Studies of *T. gallinae* have shown a wide range of virulence by evaluating parasite isolates to verify or disprove the presence of both virulent and avirulent strains.

The parasite *Trichomonas gallinae* causes trichomonosis in avian and has been reported on several birds, meaning a worldwide prevalence. Moreover, the incidence in the worldwide distribution of *T. gallinae*, especially in Columbidae, is 5.6% [7, 8]. Indeed, the disease has been reported in the white-winged dove (*Zenaida asiatica*) in Florida and Texas with an average prevalence of up to 95% [9, 10]. The largest outbreak occurred in the southeastern United States between 1950 and 1951. In Alabama alone, an estimated 50,000 to 100,000 mourning doves (*Z. macroura*) died from trichomonosis [11]. According to Forrester and Spalding [12] and Rosenstock, Rabe [13], this disease causes high mortality in the mourning dove and other columbids throughout North America. Trichomonasis has been seen in passerine birds, mainly finches, across Europe [14, 15] and North America in recent years [16]. Trichomonosis was originally identified as a new infectious illness in the UK's wild finch population [14, 17]. Since 2005, this disease has been reported in passerine birds in the UK, especially European greenfinches (*Chloris chloris)* and common chaffinches (*Fringilla coelebs*; [18]. Between 2005 and 2006, finch species exhibited 84% of the trichomonosis cases that were examined post-mortem in the UK; collectively, greenfinches and chaffinches accounted for 80% of these birds, with another 11% being columbid species [18].

Birds are the natural hosts of *T. gallinae*. Stabler [1] speculated that the primary host of *T. gallinae* is the rock dove (*Columba livia*). The worldwide distribution of this species is embroiled in the spread of trichomonosis; high rates of infection have been recorded in this species, which can also act as carrier is asymptomatic and reservoir of *T. gallinae* [19, 20]. In the early 1930s, *T. gallinae* was reported in a captive population of mourning doves in New York (USA) that also contained infected doves and rock pigeons [1, 21]. *T. gallinae* also infects gallinaceous birds and passerine birds [2, 4, 5], as mentioned above. In addition, *Trichomonas gallinae* has also been reported in birds of prey, including Cooper's hawks (*Accipiter cooperi*), Bonelli's eagles (*Aquila fasciatus*) and goshawks (*Accipiter gentilis*; [22-24].

Given this information, it is important to use an efficient method to detect and diagnose *Trichomonas gallinae* infection at the molecular level, and to tackle avian trichomonosis and its prevalence. There are currently two methods used to diagnose and detect *T. gallinae*. The first one depends on direct observation of the parasites via swabbed mucosal samples from the crop and esophagus were examined under a light microscope., or by culture amplification in a culture medium. This method, however, is not highly effective at identifying *T. gallinae* [25]. The second method involves molecular analysis of *T. gallinae* by amplifying samples using the polymerase chain reaction (PCR) [3, 24, 26-32].

2. Results and discussion

In this section, we described and discussed the steps of a detailed molecular protocol that will enable veterinarians and researchers to detect and diagnose T. gallinae infection with high accuracy as well as complements the use of light microscopic examination. Here, we described a detailed molecular method that will enable researchers and veterinarians to detect and diagnose *T. gallinae* infection and complement the first method. It is a protocol based on PCR, which is an extremely sensitive and specific tool for identifying and studying the genetics of *T. gallinae*. In the first step of the method, samples are collected and cultured. DNA is extracted from parasite cultures using DNAzol® and its quality is measured and assessed. Molecular detection via ribotyping of PCR-amplified internal transcribed spacers or Fe-hydrogenase gene from isolated DNA is then performed. At the end stage of this protocol, DNA (PCR products) is sequenced, and genetic characterizations can be performed.

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2.1. Sample collection and culturing

Sample collection and culturing are the most essential step in diagnosis of *Trichomonas gallinae* infection. Firstly, in Sterile cotton swabs is used to swab live and hunter-killed species of birds (Figure 1). To mirror the natural anatomical curvature of the mouth cavity and crop, the swab is bent into a gentle curve at an angle of roughly 120°. After bending, the swab is moistened with sterile saline or water and gently pushed into the esophagus to the level of the crop. The swab is then gently rubbed about the oral cavity and crop mucosa four or five times in a figure-eight motion to ensure that an adequate sample is captured. Secondly, after collection, each swab is immediately used to inoculate a new individual InPouch™ TV kit (BioMed Diagnostics, USA) following to the manufacturer's steps (BioMed Diagnostics, Santa Clara, California, USA), or a trypticase-yeast extract-maltose medium (TYM). The culture packs are then transported to a laboratory within 3 hours and incubated at 36°C. Thirdly, samples are checked 24 h after incubation via a microscope at 10X magnification, and then at 24 h intervals for up to eight days to examine for the growth of *T. gallinae*. Infection status is confirmed to be positive if motile parasites are seen in the culture tests for each bird; samples are recorded as negative if no parasites are found after 10 days of growth.

2.2. DNA isolation

In this step, it is very important to extract high quality of *T. gallinae* DNA from parasite cultures. Genomic DNA is extracted from parasite cultures by the use of DNAzol® (Invitrogen, UK) with these modifications: the TYM or InPouch™ TV having *T. gallinae* is moved to a 1-ml Eppendorf® tube and centrifuged at 13,000 rpm (5 min) (Figure 2). The culture media is discarded, but the pellet is kept, and then to lyse the cells, 1000 µl of DNAzol® is added to each sample and pipetted up and down quickly. The samples are then centrifuged for 10 minutes at 4°C at 4000 rpm. The resultant supernatant is transferred to a new Eppendorf tube to precipitate DNA, and each tube receives 500 µl of absolute ethanol, which is mixed through inversion before being centrifuged for 5 minutes at 4000 rpm at 4°C. Then, the DNA pellet is cleaned with 100 µl of 75% ethanol after the liquid is removed.

The liquid is removed after centrifuging the samples for another minute. The DNA pellets are air-dried for 3 minutes before being re-suspended in 100 µl of nuclease-free water. Extracted DNA from samples are stored at -20°C for further use. DNA concentrations for each isolate are determined by the use of spectrophotometry. Finally, under an ultraviolet transilluminator, the quality of the extracted DNA is visually validated on a 1% agarose gel stained with ethidium bromide.

2.3. Molecular detection via ribotyping of PCR amplification of ITS and Fe-hydrogenase gene

Here, it is very critical to determine the dangerous strains of *T. gallinae* infection. Following the protocol reported by Robinson et al. (2010), PCR is conducted to amplify either the internal transcribed spacer (ITS) region, using the forward primer TFR1 (5'-TGCTTCAGTTCAGCGGGTCTTCC-3') and the reverse primer TFR2 (5'-CGGTAGGTGAACCTGCCGTTGG-3'; [3, 24, 26], or the Fe-hydrogenase gene (FeHyd) as a single-marker subtyping tool, using the forward primer TrichhydFOR (5'-GTTTGGGATGGCCTCAGAAT- 3′) and reverse primer TrichhydREV (5'- AGCCGAAGATGTTGTCGAAT-3′; [27, 29] (Figure 2). PCR reactions were run with 15 µl BioMix™ (Bioline, UK), 3 µl of 10 pg/µl of each F and R primer solution (Eurofins Genomics, Germany), 3 µl of nuclease-free water and 1 µl of extracted DNA, to complete a 25-µl reaction mix. Each PCR run contained a negative control of nuclease-free water and positive control containing *T. gallinae* DNA previously extracted from different birds. The PCR program to amplify the ITS1/5.8S rRNA/ITS2 region began with 94°C for 15 min, followed by 35 cycles of 94°C for 1 min, annealing at 65°C for 30 s and extension at 72°C for 1 min, and ended with a final extension at 72°C for 5 min. The same program is used to amplify the Fe-hydrogenase gene, except that the annealing temperature was lowered from 72°C to 52°C. A 1% agarose gel stained with ethidium bromide is used to visually confirm PCR amplification success under ultraviolet light and a Ready-Load™ varied 100-1000 bp DNA ladder (Promega, USA) with the expected fragment lengths (Figure 2). The size of PCR products is 400 bp for ITS and 1 kb for the Fe-hydrogenase gene and then the rest of PCR products are stored at -20°C for further use or prepared for sequencing.

2.4. DNA sequencing and genetic characterization

In this step, molecular analysis is performed in DNA sequencing for genetic characterization. The prepared PCR products to be DNA sequenced, a 25-µl DNA template, and forward and reverse primers are sent for sequencing using Life Technologies 3730XL sequencers. The phylogenetic and molecular relationships of trichomonas parasites sequences are determined using Molecular Evolutionary Genetics Analysis (MEGA) software version 7 [33] (Figure 3). All sequence data are aligned using the F and R complements of the reverse primer sequences are aligned together and compared, and then one of them is removed to make sure both sequences were identical (Figure 3). For all phylogenetic trees, all positions containing gaps and missing data are eliminated. Alignment regions containing missing or erroneous (non-specific nucleotides) sequence data in any of the strains are removed across all included sequences, ensuring that only confident consensus sequence data were included in the phylogenetic comparisons (Figure 3). The new data results are then compared to published *T. gallinae* sequences imported from the NCBI GenBank database. The datasets of phylogenetic trees are obtained from the ITS1/5.8S and rRNA/ITS2 regions and Fe-hydrogenase gene sequences are constructed separately using maximum likelihood and the Tamura-Nei model is utilize to analyze taxa relationships [33, 34]. Felsenstein's bootstrap test is used to determine associated taxa clustered in the bootstrap values (1,000 times) [35].

2.5. Figures

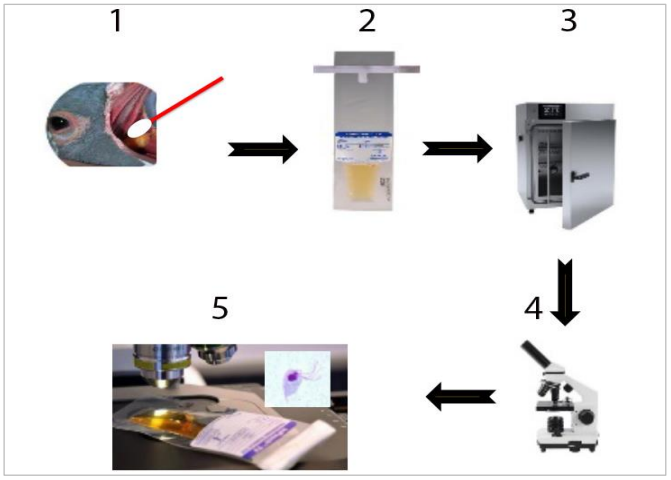

Figure 1 Schematic showing methods for detecting the presence of *Trichomonas gallinae*. (1) Samples are swabbed from birds. (2) Transfer the swabbed samples to InPouchTM TV (3) Inoculated the sample for 24hrs. (4,5) visualize the sample using light microscopy.

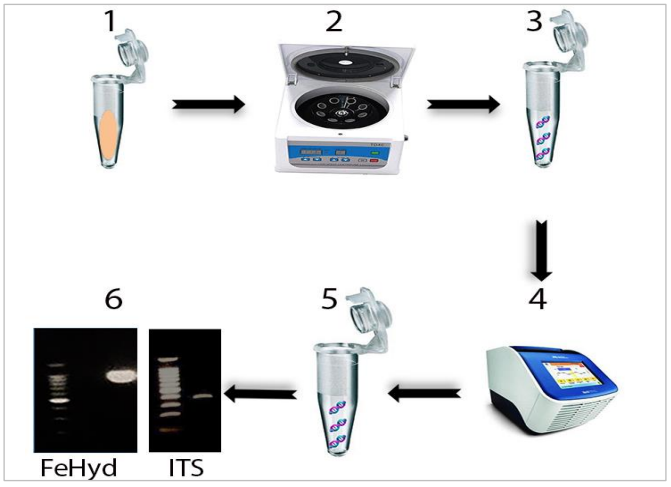

Figure 2 Schematic showing the proto**col** steps of DNA extraction (1,2,3). PCR amplification of *Trichomonas gallinae* genes from sampled birds (4,5). DNA

extraction and PCR amplification were confirmed visually on a 1% agarose gel stained with ethidium bromide, under an ultraviolet transilluminator (6).

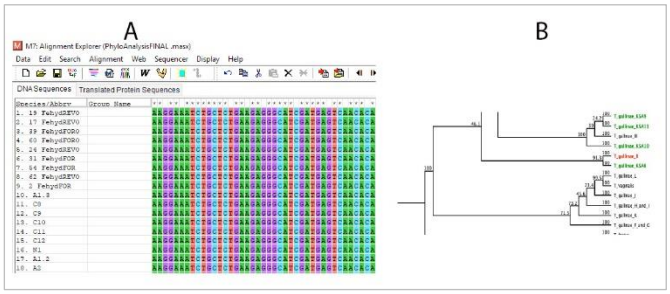

Figure 3 Schematic showing methods for DNA sequencing analysis of *Trichomonas gallinae* samples from infected birds (A) and a phylogenetic tree based on partial sequences constructed using MEGA software (B).

3. Conclusions

Trichomonas gallinae causes avian trichomonosis, which leads to the formation of large necrotic and inflammatory lesions in the mouth and crop. Thus, infected birds are not able to swallow water and food, causing them to starve. Because the prevalence of *T. gallinae* threatens bird populations, especially endangered ones**,** it is important to use an efficient and sensitive method to detect and diagnose *T. gallinae* infection at the molecular level. Here we explained a detailed molecular protocol to detect *T. gallinae* involves light microscopic examination and PCR amplification. This protocol is an extremely sensitive and specific tool for identifying trichomonads, because it combines molecular tools with the traditional method based on light microscopic examination.

The 5.8S rRNA is the commonly gene region that used to diagnose and genotype *T. gallinae*, which possesses ITS region sequences that are non-coding and evolve rapidly, making them suitable for characterizing phylogenetic trees for closely related organisms [26]. The ITS region is a highly repetitive sequence that is conserved in the genomes of organisms, that produce decent markers of greater taxa relationships [36]. Due to its potential to detect neutrally evolving fine-scale variation in a mitochondrial protists, we also recommend the use of the Fe-hydrogenase gene as a second locus for genotyping trichomonad parasites [27-29]. The single copy Fe-hydrogenase gene provides higher resolution genotyping, thus can be used as an additional single-marker subtyping tool for cultured isolates [29]. The analysis of Random amplified polymorphic DNA is also a molecular tool that researchers can use is to test the variation between T. gallinae genotypes by studying more variable sites within genomes [27]. Finally, this detailed molecular method that will help researchers and veterinarians to detect and diagnose avian trichomonosis infection with a high level of confidence and accuracy as well as help in genetic characterization of dangerous strains.

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