

# Free thesis on sampling method and lab work

[Environment](#), [Water](#)



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## Introduction

Trichomonas gallinae causes avian trichomoniasis infection, which is often a lethal disease. The parasite is considered to primarily affect columbiform birds like pigeons and doves, but can affect other wild and domestic birds as well (Stabler, 1954). Diagnosis of the infection can be reliably done by collecting and studying material from the bird's mouth and crop.

I carefully collected oropharyngeal swabs from the oral cavities of hunter-killed Woodpigeons in eight areas of Norfolk and one area of Suffolk counties in the three months' time period between April and June. Using sterile cotton swabs, I collected crop swabs from the pigeons and inoculated them into the InPouch™ TV culture kits (BioMed Diagnostics, US) as per the manufacturer's instructions. I then labeled the inoculated packs, sealed them properly, and placed them vertically in a vacuum flask with a heating pad to maintain temperatures above 28°C. These culture kits were then transported to the laboratory within three hours and incubated at 35°C. I examined the

samples immediately under a microscope at 10X magnification followed by re-examination 24 hours after incubation, and thereafter every day at 24-hour interval for up to 10 days. This was with an aim of examining the growth of *T. gallinae* parasites.

After completion of the study period of 10 days, I assessed the incubated samples for presence of an infection; the infection status was determined as positive if parasites were found in any of the culture tests, whereas the status was confirmed as negative if the parasites were not seen under the microscope even after 10 days of incubation.

## **Extraction of DNA**

I extracted the DNA from the samples in the following way. Isolation of DNA was done by the DNAzol® (Invitrogen, UK) from culture kits containing the *T. gallinae* parasites that were in an exponential growing phase. I transferred the InPouch™ TV cultures containing the parasites to 1.5 ml Eppendorf tubes. The tubes were centrifuged at 14,000 rpm for sixteen minutes at 4°C. The cultures were removed and 0.5 ml of DNAzol® was added to each sample precipitate and pipetted up and down rapidly to lyse the cells. The samples were again centrifuged at 10,300 rpm for twelve minutes at 4°C. To precipitate DNA, the resulting supernatant was transferred to a new Eppendorf tube, and 0.25 ml of 100% ethanol was added to each tube. This was followed by mixing through inversion. I then left the solution to stand and kept it on ice for thirty minutes. It was centrifuged again at 7000 rpm for three minutes at 4°C. Following this, I removed the liquid portion and left the DNA pellets to air-dry for three minutes before adding 0.5 ml of free water. This helped in re-suspending the DNA. The extracted DNA samples were

stored at -20°C and the concentrations for each isolate were measured by spectrophotometry, based on an absorbance reading of 260nm.

### **PCR amplification of the ITS1/5.8 rRNA/ITS2 region**

As previously described by Robinson et al (2010), I obtained primers to amplify the ITS region, using TFR1 (TGCTTCAGTTCAGCGGGTCTTCC) and TFR2 (CGGTAGGTGAACCTGCCGTTGG) (Germany Eurofins MWG Operon Sequencing Department Anzinger) (Felleisen, 1997; Gaspar da Silva et al, 2007). The polymerase chain reaction (PCR) components included 1µL of DNA in a 49µL reaction containing 10µL of 5X buffer (Promega, USA), 3µL of 25mM MgCl<sub>2</sub> (Promega, USA), 0.4µL of 10mM dNTP Mix (Promega, USA), 3µL each of 5µM forward and reverse primers (Eurofins, Germany), 0.25µL of 5U/µM HotStarTaq Plus DNA polymerase (Promega, USA), and 29.35µL of nuclease-free water (Promega, USA). Each PCR run contained a negative control of water. I performed the PCR amplification using the following temperature cycles: 94°C for fifteen minutes, followed by 35 cycles of 94°C for one minute and 65°C for 30 seconds, followed by 72°C for one minute, and a final extension at 72°C for five minutes. This was visually confirmed under UV by using a 1% agarose gel stained with ethidium bromide. An approximately sized band 400-base pair (bp) was referenced using a Ready-Load 100bp DNA ladder (Promega, USA). I submitted the preparation of PCR products for DNA sequencing, 25µL per DNA template, and forward and reverse primers for sequencing at the Genome Analysis Centre (Genome Enterprise Limited at Norwich Research Park) using Life Technologies 3730XL sequencers.

## **PCR for the Fe-hydrogenase gene**

I performed the DNA extraction from those culture kits that were confirmed positive for *T. gallinae* under the ITS1/5.8 rRNA/ITS2 region. The PCR amplification was further done for a fragment of the Fe-hydrogenase gene using the primers TrichhydFOR (GTTTGGGATGGCCTCAGAAT) and TrichhydREV (AGCCGAAGATGTTGTCGAAT), as described in Lawson et al. (2011). The PCR components included 1 µL of DNA in a 49 µL reaction containing 10 µL of 5X buffer (Promega, USA), 3 µL of 25mM MgCl<sub>2</sub> (Promega, USA), 0.4 µL of 10mM dNTP Mix (Promega, USA), 2.5 µL each of 5mM forward and reverse primers (Eurofins, Germany), 0.25 µL of 5U/µL HotStarTaq Plus DNA polymerase (Promega, USA), and 30.35 µL of nuclease-free water (Promega, USA). Each PCR run contained a negative control of water. The PCR amplification was performed using the following temperature cycle: 94°C for fifteen minutes, followed by 35 cycles of 94°C for one minute, and 52°C for thirty seconds, then 72°C for one minute, and a final extension at 72°C for five minutes. The PCR amplification was confirmed visually under UV by using a 1% agarose gel, stained with ethidium bromide, and the expected product size was about 1kb. I then submitted the PCR products for sequencing with both TrichhydFOR and TrichhydREV primers as described above.

## **ITS region sequence and phylogenetic analysis**

I used the Molecular Evolutionary Genetics Analysis (MEGA) software version 5.1 to conduct the molecular and phylogenetic analyses (Tamura et al., 2011). I inspected the Chromatograph files and refined them using the MEGA 5 TraceEditor extension, and aligned all sequences data using the forward

and reverse complement of the reverse primer.

In this study, I relied on the published *T. gallinae* sequences obtained from the NCBI GenBank database to compare the ITS1/5. 8S/ITS2 region and Fe-hydrogenase sequences. The repeat sequences in GenBank were deleted from the analyses, and the longest sequences were used as a consensus sequence of this type. Phylogenetic trees of the datasets obtained from the ITS1/5. 8S rRNA/ITS2 region and Fe-hydrogenase sequences were constructed using the Neighbour-Joining method and Maximum Likelihood with the Tamura-Nei model (Saitou & Nei 1987; Tamura et al. 2011). I used the Felsenstein's bootstrap test to calculate the associated taxa clustered together in the bootstrap values test (2, 000 replicates) (Felsenstein, 1985).

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