Research Article

Trichomonas Gallinae Prevalence and Genetic Characteristics from Racing Pigeons in Thi-Qar Province, Iraq

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Abstract | *Trichomonas gallinae*, a protozoan widely found in pigeons and other wild birds, causes the parasitic disease known as "avian trichomoniasis". The complications of this illness include lesions in the upper gastrointestinal tract. The present study's goal would be to study the prevalence and genetic characteristics of *T. gallinae* in racing pigeons in Thi-Qar province, Iraq, utilizing the ITS1-5.8s rRNA-ITS2 gene. 100 samples were collected from racing pigeons between September and December 2022. The swabs were taken from the throat, pharynx, crop, and esophagus with a cotton swab. Utilizing direct smear and polymerase chain reaction (PCR) techniques, 30 (30%) of the 100 collected samples were found to be positive. A DNA sequence of 372 bp length that partly encompassed the coding region of the ITS1-5.8s rRNA-ITS2 gene within ten samples (assigned 1-10) was amplified in this study. The PCR amplicons that were found in the amplified genetic region were directly sequenced. The detected variations were then used to build a phylogenetic tree, which was used to evaluate the precise phylogenetic distances in addition to other relative parastic sequences. According to the present findings, there is almost 99% homology between the sequences of *T. gallinae* and the samples that were under investigation. In this investigation, the genetic variants C64T, G236A, C243T, and A363C were found.

Keywords | *Thi-Qar* province , genetic variation, , *T.gallina*e, rtPCR, *racing pigeons*.

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INTRODUCTION

Trichomonas gallinae is a protozoan that causes an important disease in pigeons known as avian trichomoniasis. It is anaerobic, flagellates and classified in the class Zoomastigophorea and the phylum Trichomonadida (Muzeal & Khudeir, 2022). *T. gallinae* primarily affects the digestive and respiratory systems of birds.

This disease is usually referred to as "canker" when it affects pigeons. Some strains are virulent, while others are asymptomatic or have mild symptoms. Yellow-green secretions with an unpleasant odor from the mouth, diarrhea, emaciation, severe weight loss, The onset of decaying lesions, the appearance of cheddar-like sores, and oral irritations that prevent swallowing and cause catastrophic respiratory failure are all symptoms of this condition.

T. gallinae is spread between birds through parents feeding their young and ingesting contaminated food and water. It is also spread among raptors and carnivorous birds by consuming infected birds. The parasite can be diagnosed by the following methods: observing the characteristic lesions of the disease; observing the protozoa and its flagella microscopically; culturing the parasite; and using molecular methods.

In the subject of molecular epidemiology of microorgan-

isms, polymeras chain reaction and related methods are often recognized as sensitive and reliable methodologies for genetic investigations (Thomas, 2017).

Around the world, different *T. gallinae* genotypes have been identified, including genotypes A and B in China (S. Y. Feng et al., 2018) , in Spain (Quillfeldt et al., 2018), in Iran (Arabkhazaeli et al., 2020) ,whereas genotype A has been discovered in Iraq (Fadhil & Faraj, 2019).

Although trichomoniasis in Iraq has been the subject of numerous investigations, there have been few attempts to genetically characterize this parasite. The significance of trichomoniasis and the absence of thorough research on phylogenetic distribution and diversity in the past drove the need for such a study to be carried out in Thi-Qar province, Iraq. The current investigation sought to determine the frequency and genetic makeup of T.gallinae in racing pigeons in the Iraqi province of Thi-Qar.

MATERIALS AND METHODS

Samples collection

Between September 2022 and December 2022, 100 domestic pigeon mouth, pharynx, crop, and esophagus samples were taken in Thi-Qar Province of Iraq.

Microscopic Examination

Using sterile, cotton-tipped applicators that had been previously moistened, samples were randomly obtained from the mouths, pharynx, crop, and esophagus of pigeons (wet mount method). If, under a light microscope, motile and flagellated protozoa were seen, the Trichomonas were recognized (Collántes-Fernández et al., 2018).

DNA extraction

Using the gSYNCTM DNA Extraction Kit, Geneaid UK, in accordance with the manufacturer's protocol, genomic DNA was extracted from each and every isolate of *Trichomonas gallinae*. Until PCR analysis, samples were kept frozen at a temperature of -20 oC.

PCR detection of genes

The primers given in Table 1 were used to amplify the ITS1-5.8s rRNA-ITS2 gene. The 20 µL total reaction tube volume is composed of 5 µL of master mix, 1.5 µL of forward and reverse primers specifically designed for each gene, and 5 µL of DNA template. To finish off the volume, water free of nucleases was added. By combining 5 µL of DNA with loading dye (safety dye), loading the mixture into the designated wells, and subjecting the mixture to an electric field (70 volts for 45–60 min), the separated DNA samples were electrophoresed. The thermocycling program of the gene began with initial denaturation for ten minute at 95 oC, one cycle, proceeded by 35 cycles, including 30 second at 95 oC, 30 second at 60 oC,and 1 minute at 72 oC, then last extension at 72 oC for ten minute and 1 cycle.

Table 1: Primer sequences for amplifying genes

Source	Size of the item	Primer Sequences (5′- 3′)	Gene
(Felleisen, 1997)	372bp	F*:TGCTTCAGTTCAGCGGGTCTTCC R: * CGG TAG GTG AACCTG CCG TTG	ITS1-5.8s rRNA-ITS2

Real time PCR

The primer sequences of the ITS1–5.8s rRNA–ITS2 gene are listed in Table 2. The RT-PCR reaction mixture (20 µL) is composed of sample DNA (3 µL), each forward and reverse primer (1 µL), syber green master mix (12.5 µL), and sterile deionized water. The qPCR procedure was performed in triplicate. The qPCR program began with initial denaturation for 3 minute at 94 oC, one cycle, following 40 cycles, including 30 second at 94 oC, 1 minute at 60 oC and extention for 30 second at 72 oC.

Table 2: Primer sequences used for realtime PCR.

Source	Size of the item	Primer Sequences (5′- 3′)	Gene
Mohammed et al. 2023	372bp	F:CCTGCCGTTGGATCAGTTCT R:AGGAGCCAAGACATCCGTTG	ITS1-5.8s rRNA-ITS2

DNA Sequencing of PCR amplicons

The strong positive samples (10) were amplified with conventional PCR and then send for made sequence and sending to koria. According to the instructions provided by the sequencing provider (Macrogen Inc., Geumchen, Seoul, South Korea), the resolved PCR amplicons were commercially sequenced starting at the forward termini. The annotation and variances were only discernible in clean chromatographs produced from ABI sequence data, proving that they are not the result of PCR or sequencing artifacts. By contrasting the observed nucleic acid sequences of the parasite samples with the retrieved reference sequences from the parasitic database, it was possible to identify the virtual posi positions and other details of the retrieved PCR fragments.

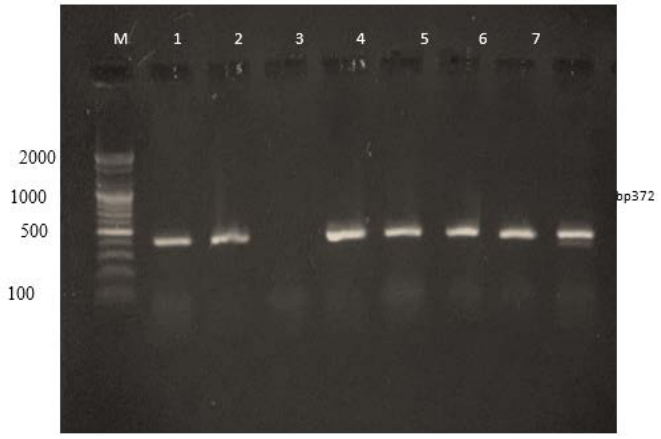

Figure 1: ITS1-5.8s rRNA-ITS2 gene amplification in an agarose gel, with M standing for the ladder, 1-2,4-8 for positive results, and 3 for negative results

Analysis of sequencing data

As long as the corresponding sequences in the reference database matched, PCR product sequencing results were edited, aligned, and evaluated using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). Both in PCR amplicons and in the proper places within the reference genome, the detected nucleic acids were given numbers. Each variant discovered in the *T. gallinae* gene was annotated by SnapGene Viewer version 4.0.4 (https://www.snapgene.com).

Creation of phylogenetic tree

Spesific phylogenetic tree was constructed in this study. Using the NCBI-BLASTn service, the identified variations were compared to their neighboring homologous reference sequences (S.-Y. Feng et al., 2018). The newly identified variant was then incorporated into an inclusive tree made by applying the neighbor-joining method and visualized with the use of the iTOL suit to produce a traditional clades formation tool. (Letunic & Bork, 2019). As a result, each phylogenetic species group's sequences were annotated in the phylogenetic tree.

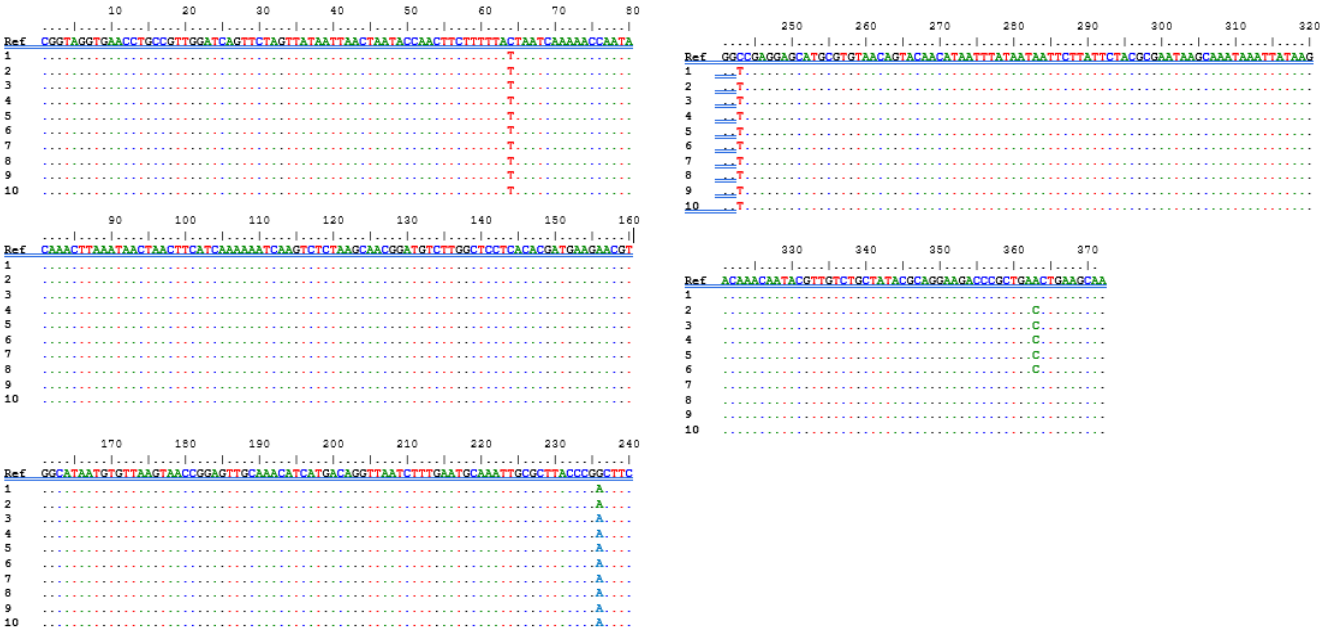

Figure 2: An alignment of the ITS1-5.8s rRNA-ITS2 sequences from the genomic DNA of 10 parasite samples from *Trichomonas gallinae* with the proper reference sequences. the NCBI reference sequences are indicated by the notation "ref".

RESULTS

Occurrence of T. gallinae in pigeons

In this investication, 30 of the 100 samples examined yielded positive results for microscopy and PCR, indicating that this protozoan is common in 30% of domestic pigeons. All 30 samples that tested positive for *T. gallinae* upon microscopic examination were confirmed to be positive by the PCR assay. Furthermore, the ten isolates' ITS1-5.8s rRNA-ITS2 sequences were successfully amplified by PCR using the designated primers, as shown by the band size of about 372 bp (Figure 1).

ITS1, 5.8S, and ITS2 sequence phylogenetic analyses

Ten samples were included inside this locus, with an amplicon length of roughly 372 bp. Before being sent for sequencing, the amplified amplicons were all examined to make sure they all showed sharp, specific, and clean bands. By using an NCBI blast, the sequencing reactions revealed the amplified products' confirmed identities. Regarding the 372 bp PCR amplicons of the currently targeted ITS1-

5.8 rRNA-ITS2 sequences, the NCBI BLASTn engine showed a high degree of sequence similarity between the sequenced samples and *T. gallinae* sequences. The NCBI BLASTn engine showed that there was approximately 99% homology with the expected target, which partially encompassed the coding section of the ITS1-5.8s rRNA-ITS2 gene sequences, by comparing the observed DNA sequences of the samples under investigation with the returned DNA sequences. Comparing the 372 bp samples' alignment findings to the matching *T. gallinae* referencing sequences, it was possible to identify four nucleic acid variations (Fig. 2). These sequences were created by comparing the samples we looked at with the most similar sequences that were stored in the NCBI database.

The exact sites of the identified alterations were documented in (Table 3) to provide a summary of all the findings from the sequenced 372bp segments.

Table 3: The pattern of the differences that have been noticed in the 372bp of the ITS1-5.8s rRNA-ITS2 amplicons when compared to the NCBI reference sequences.

Sample	Variant	Position in the PCR fragment
1,2,3,4,5,6,7,8,9,10	C64T	64
1,2,3,4,5,6,7,8,9,10	G236A	236
1,2,3,4,5,6,7,8,9,10	C243T	243
1,2,3,4,5,6	A363C	363

A phylogenetic tree was built using the analyzed ITS1-5.8s rRNA-ITS2 nucleic acid sequences in the examined parasite samples. The total number of aligned nucleic acid sequences in the currently built tree is 27. *Trichomonas gallinae*, the only species contained in this tree, is also the sole nucleic acid sequence that has been included. The ITS1-5.8s rRNA-ITS2 sequences of *T. gallinae* were found to be highly diverse in this organism, clustering into numerous neighboring phylogenetic branches based on the genetic sequences that were examined (Fig. 3).

Submission in NCBI

The local isolates submitted in gene bank under accessions number: **LC768806, LC768807, LC768808, LC768809, LC7688010, LC7688011, LC7688012, LC7688013, LC7688014, LC7688015.**

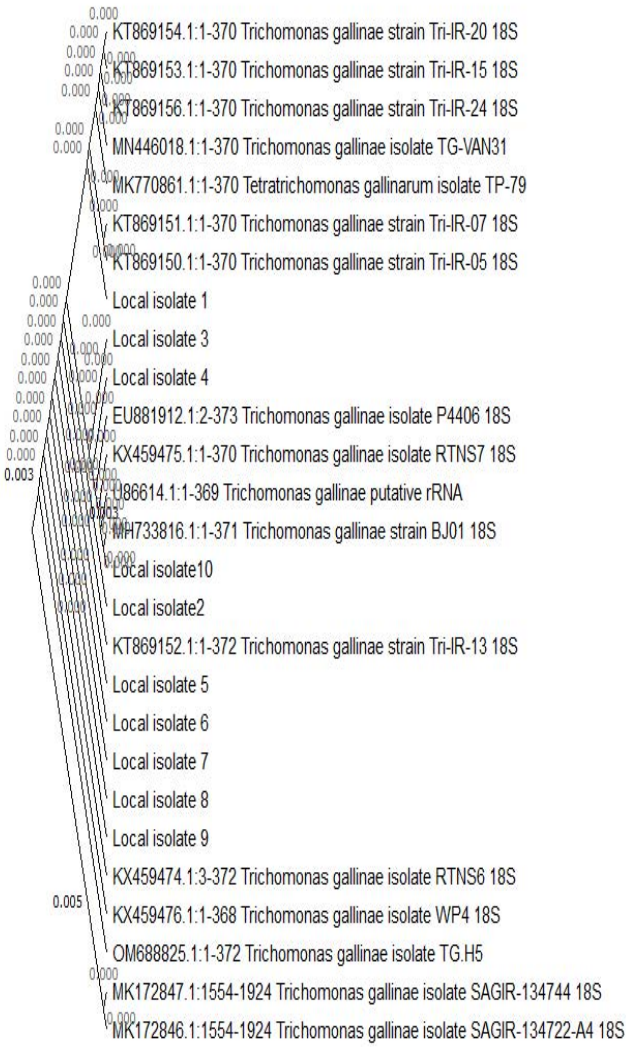

Figure 3: The Tamura-Nei model and the Maximum Likelihood approach were used to infer the evolutionary history. The tree that has the highest log probability is displayed (-528.91). By automatically applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances calculated using the Tamura-Nei model, and then choosing the topology with the highest log likelihood value, the initial tree(s) for the heuristic search were created. The rate variation model permitted some sites ([+I], 0.00% sites) to be evolutionarily invariable. There were 27 nucleotide sequences in this analysis. Codon positions 1st+2nd+3rd+Noncoding were included. The final dataset contained 374 locations altogether. In MEGA11, evolutionary analyses were carried out (Tamura et al., 2021).

DISCUSSION

Using sequence analysis of the ITS1-5.8S rRNA-ITS2 gene in 10 samples, this study was carried out on racing pigeons in Thi-Qar Governorate, Iraq, to discover the genetic variations of *Trichomonas gallinae* for the first time. Because it affects a variety of birds and shows high varia-

tion across *T. gallinae* isolates, avian trichomonosis, which is caused by *T. gallinae*, is significant. (Ayati et al., 2023). The ability to identify intraspecific genetic variations among Trichomonads utilizing sequence analysis of the ITS1- 5.8S rRNA-ITS2 was established in several studies. (Alrefaei, 2020).

The diversity observed within the majority of studied samples might be related to the four variations detected in the majority of investigated samples. The genetic diversity of *T. gallinae* was studied using sequence analysis of ITS1-5.8S-ITS2 in three types of falcons in Saudi Arabia (Alrefaei, 2020).

All study samples (1–10) were exposed to three variations (C64T, G236A, and C243T) in the position (64, 236, and 243). While samples (7, 8, 9, and 10), which did not exhibit variation in position 363 (A363C), were detected in the other studied samples (1, 2, 3, 4, and 6).

According to the present findings, there is 99% similarity between the sequences of *T. gallinae* and the isolates that were under investigation.

Some research found different proportions of similarity between the studied samples and those registered in the Gen Bank, such as OM688823 from Egypt, which matched 64% with MK771128.1 from KSA, and isolate OM679422.1 from Egypt, which was 85% identical to MT300160.1 from KSA (Mohamed et al., 2023).

Other research found 100% similarity with a number of sequences, including sequences MK932773, isolated from a European turtle dove by (Santos et al., 2019).

It was found that there is a link between the appearance of necrotic ingluvitis lesions and genetic diversity when using ITS region sequencing of *T. gallinae* in Europe, the UK, and the USA (Girard et al., 2014).

There were two genotypes of ITS1–5.8S-rRNA –ITS2 widely distributed among bird populations, a new variant, and also two sequences with mixed patterns. Genotype ITS-OBT-Tg-1 was associated with the existence of visible lesions in birds (Sansano-Maestre et al., 2016).

T. gallinae isolates from several bird species were sequenced and found to contain 29 distinct ITS 1 genotypes. Positive samples of *T. gallinae* sequence C obtained from pigeons and hens displayed obvious clinical signs or visual lesions (Albeshr & Alrefaei, 2020).

The 18S rRNA gene for 10 sequence analysis findings showed the number and locations of variations and revealed that 5 samples were 100% identical and 5 samples had variation in the gene's sequence. Following sequence analysis, 10 samples showed genotype A (Fadhil & Faraj, 2019).

The results of this study are consistent with studies that recorded genetic variations in *T. gallinae* protozoa. Including the study conducted in Iraq by (Fadhil & Faraj, 2019) and the study in Egypt by (Mohamed et al., 2023), while disagreeing with some of the findings by (Santos et al., 2019).

The most closely related sequences to the examined samples (1–10), according to the phylogenetic tree, belonged to *T. gallinae*.

Two phylogenetic locations were seen from the studied samples depending on whether they were endemic to the main *T. gallinae* branches. Nearly all of the samples' observed differences led to their varying neighboring phylogenetic locations in the tree. These neighboring positions were linked to the regularity of changes within the examined samples, which resulted in varying distributions of these samples in these referred phylogenetic positions. The isolate 1 sample, however, was also discovered to be in a different branch and close to the GenBank accession numbers KT869154.1, KT869153.1, KT869156.1, MN446018.1, MK770861.1, KT869151.1, and KT869150.1. Other study samples, however, were found in different branches.

The investigated samples (isolate 3, isolate 4, and isolate 10) were positioned nearness with GenBank accession numbers: EU881912.1, KX459475.1, U86614.1, and MH733816.1. While, other local samples (isolate2, isolate5, isolate6, isolate7, isolate8, and isolate9) were located closeness with KX459474.1, KX459476.1, OM688825.1, MK172847.1, MK172846.1, and KT869152.1 .

At least two *T. gallinae* genotypes (A and B) were discovered in birds in the United States, according to phylogenetic analysis of the ITS1-5.8s rRNA-ITS2 region from this study (Girard et al., 2014), as well as to the occurrence of gross lesions in infected animals (Martínez-Herrero et al., 2014).

CONCLUSION

Canker disease (*Trichomonas gallinae*) infection is still endemic in pigeons in Thi-Qar province, Iraq. The prevalence rate of the disease in this governorate is close to the prevalence rates recorded in local and international studies. There is genetic diversity in the *T. gallinae* parasite in the mentioned governorate.

RECOMMENDATIONS

Conducting more molecular studies on the parasite in Iraq and other countries, investigating theeffect of the parasite on the blood picture, and investigating the possibility of transmission of this species to humans.

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CONFLICTS OF INTEREST

The investigation did not involve any conflicts of interest.

NOVELTY STATEMENT

The study was conducted to investigate the spread of the *T. gallinae* parasite in racing pigeons and identify the genetic variation by analysis of the sequencing of selected samples because there are no records of avian trichomoniasis in the Thi-Qar province of Iraq, despite its widespread prevalence and the veterinary and economic importance of the disease.

AUTHORS CONTRIBUTION

Ahmed J. Mousa contributed to the conception and design of the research, the gathering, analysis, and interpretation of data, as well as the writing and revision of the manuscript. Nothaila R. Hamid contributed to data collection, analysis, and interpretation, as well as paper revision.

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