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MOLECULAR DETECTION OF *TRICHOMONAS GALLINAE* INFECTION OF LOCAL PIGEONS (COLUMBA LIVIA DOMESTICA) IN AL-QADISIYAH PROVINCE, IRAQ

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Abstract : *Trichomonas gallinae* isolates from local pigeons in Al-Diwaniya province, Iraq were amplified by polymerase chain reaction using ITS1- 5.8S and *Fe hydrogenase* gene and characterized by molecular tools to determine the geographical location and the sequences were analyzed phylogenetically. The isolates in the present study were 100% similar to *T. gallinae* isolates of Iran and Austria. Random samples found positive for trichomoniasis in culture and throat swab in live birds were confirmed by PCR. The positive samples showed clear band at 370 and 528 bp for ITS1- 5.8S and *Fe hydrogenase* gene respectively.
Keywords: *Trichomonas Gallinae,* Pigeons, PCR, Fe hydrogenase and ITS1- 5.8S

Introduction : Infectious diseases and parasites are both regulate populations, producing marked effects on host abundance and evolution (Begon et al., 1999; Alrefaei et al., 2021). Parasites are an important component of ecosystems at various levels, and understanding the influences that underlie parasite diversity is vital to identifying ecological principles that govern biodiversity. The relationship between parasitic diseases and birds differs significantly in their health consequences and ecological complexity (Friend, 2006). Disease can also be a factor in species decline to threatened, endangered or extinct status (Van Riper et al., 1986). Trichomonosis is a well-known disease caused by a flagellated protozoan parasite, *Trichomonas gallinae*. The disease is widespread among avian species, commonly affecting Columbiformes (Bunbury et al., 2008). In addition, *T. gallinae* is also predominantly found in the rock dove (*Columba livia*), making it one of the primary hosts for this parasite (Stabler, 1954). The major factor contributing to the dispersion of trichomonosis is the worldwide distribution of *C. livia*, which is a clinical carrier and reservoir of *T. gallinae* (Atkinson et al., 2009). However, trichomonosis less frequently infects *Gallus gallus* (Albeshr and Alrefaei , 2020). The disease predominantly spreads when one or both infected parents feed their squabs, and when adult birds share the same food and water sources (Bunbury et al.,2008 ;Kocan,1969). Further, transmission can occur to birds of prey via feeding on infected birds, such as pigeons or doves (Urban and Mannan , 2014). Molecular characterization has identified different strains of *T. gallinae*. The most common gene used to sequence *T. gallinae* is 5.8S ribosomal RNA (rRNA; ITS ribotype) (Sansano et al., 2009). Internal transcribed spacer (ITS) region sequences are non-coding and evolve rapidly, which makes them suitable for construction of phylogenetic trees of closely related organisms (Felleisen, 1997). Another subtype marker used to differentiate *T. gallinae* isolates is the gene encoding Fe-hydrogenase (Alrefaei, 2019). This gene can be targeted as a single marker locus for genotyping, specifically for a mitochondrial protist, and for detecting fine-scale differences in sequence variation within isolates

(Lawson, 2011). In the present study, we selected the Fe-hydrogenase gene and ITS1-5.8S as a marker subtyping tool for trichomonad parasites. This selection takes advantages of the genes ability to provide additional resolution for discrimination of *T. gallinae* strains (Kumar and Tamura , 2016).

Materials and methods

Source of samples

The current investigation comprised a total of 150 local pigeons, technically known as Columba livia domestica. The study was conducted in Al-Qadisiyah Province, Iraq, with a sample length of about one year, from September 2022 to September 2023. The process of collecting samples is crucial in diagnosing Trichomonas gallinae infection. The local markets in the Al-Diwaniya Governorate served as a primary source for Columba livia domestica pigeons. Sampling was conducted on the oral cavities of birds as conducted by (Qiu et al., 2017**)**. By using sterile cotton-tipped applicators to mix swabs with phosphate buffered saline (PBS). The swabs were then studied under a light microscope at 400x magnification to observe T gallinae in a wet mount.

DNA extraction and Ethical Approval

DNA was extracted from all isolates of *T. gallinae* in this study following the protocol as previously described by Albeshr and Alrefaei (Albeshr and Alrefaei, 2020), and stored at 20 ◦C. Approval granted based on ethical considerations The authors of this study affirm that all methods in our experiment were conducted in accordance with the authorized Ethical Norms by the scientific board of the College of Veterinary Medicine, University of Al-Qadisiyah (committee permission number 1422 on 18/10/2023).

Amplification of ITS1- 5.8S and Fe hydrogenase genes

The ITS1-5.8S-ITS2 gene was amplified from ten out of fifty samples using ITS1-5.8S rRNA-IT2 and Fe hydrogenase (Albeshr and Alrefaei, 2020; Alrefaei et al., 2021). specific primers for the Trichomonas genus. The following primer set was used in this study:

Fw-ITS1-5.8S-ITS2: 5’-TGCTTCAGTTCAGCGGGTCTTCC-3’

Rv-ITS1-5.8S-ITS2: 5’-CGGTAGGTGAACCTGCCGTTGG-3’

Fw-Fe hydrogenase- 5’-GTTTGGGATGGCCTCAGAAT-3′

Rv-Fe hydrogenase - 5’- AGCCGAAGATGTTGTCGAAT-3′

The PCR was performed in a 50 µL reaction mixture containing 25 µL of PCR Master Mix, 1.5 µM of each primer, and nuclease-free water to bring the final volume to 50 µL. The PCR conditions in the thermocycler (Biometra, Analytik Jena, Germany) were as follows: initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute, extension at 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. The expected PCR product length was 372 and 528 bp for ITS1-5.8S-ITS2 and Fe hydrogenase respectively. After the PCR, aliquots of the PCR product were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. The electrophoresis was run at an electric current of 100 volts for 25 minutes, and the results were visualized under a UV transilluminator.

Purification of PCR product, DNA sequencing, sequence analysis, and phylogeny

The PCR products were purified using the GeneJET PCR Purification Kit (ThermoFisher

Scientific™) according to the manufacturer's instructions. The purified PCR product was sent for sequencing using the automatic chain termination method (Chen et al., 2022). The nucleotide sequence was then subjected to sequence analysis search using the online program BLASTN (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information). Multiple sequence alignment was conducted using CLC Sequence Viewer 8.4. A phylogenetic tree was constructed using CLC Sequence Viewer 8.4 to determine the genetic affiliations of the ITS1-5.8S rRNA-ITS2 and Fe hydrogenase nucleotide sequences in correlation to closely related parasites.

Result

PCR technique

The DNA samples characterized with high concentration 400 to 710 ng/µl and purity ranged between 1.6 to 1.8 were subjected for PCR. By using PCR techniques, *Trichomonas gallinae* in Al-Diwaniya province was identified as the etiological agent of Trichomoniasis in pigeons utilizing ITS1-5.8S rRNA-ITS2 and Fe hydrogenase genes in 100 oropharyngeal swab samples revealed that 91% (table 1).

Table 1: Infection rate of T.gallinae in domestic pigeon by PCR.

Test	No. of swap samples	No. of positive	Percentage %
PCR	**100**	**91**	**91%**

Sequence Alignment of ITS1-5.8S-IT2 and Fe hydrogenase genes

Table 4.5 demonstrates that the ITS1-5.8S-IT2 and Fe hydrogenase regions 18S rRNA ribosomal gene (372, 528 bp) of each isolate was effectively amplified and sequenced. The results of the 18S RNA gene sequence analysis for 15 samples revealed the Sequencing analysis was done by Mega 6 software and showed the presence of changes in nucleotide sequence of 18S ribosomal RNA gene compared to other globally known. These were transition or transversion. These changes are illustrated in (Figures 1 and 2).

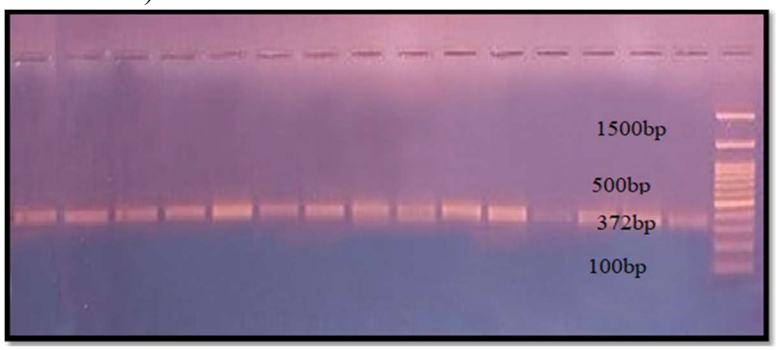

Figure (1): Agarose gel electrophoresis (1%) showing the PCR product of ITS1-5.8S-IT2 gene from 15 samples randomly selected. Lane M: DNA ladder. Lanes 1-10: PCR product of ITS1/1.5.8S/ITS2 gene of 372 bp from 15 randomly selected samples.

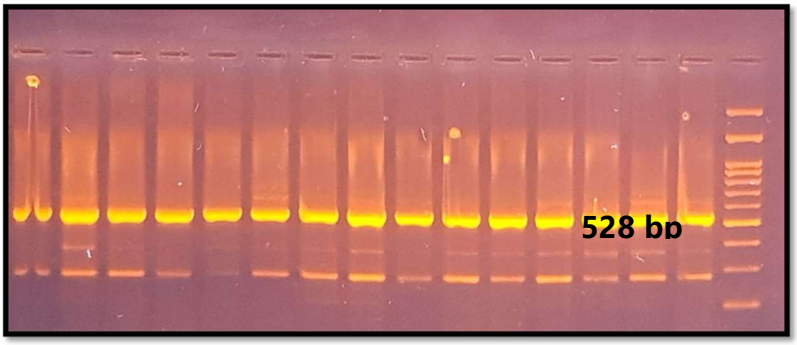

Figure (2): Agarose gel electrophoresis (1%) showing the PCR product of Fe hydrogenase gene from 15 samples randomly selected. Lane M: DNA ladder. Lanes 1-10: PCR product of Fe hydrogenase gene of 528 bp from 10 randomly selected samples.

Phylogenetic analysis of ITS1-5.8-ITS2

A phylogenetic tree based on ITS1-5.8S-ITS2 (rRNA) gene sequence of Al Diwaniyah city isolates clarify that there were relation between *T.gallinae* local isolates and other global isolates obtained from GenBank. The result showed identity 100% with Iran, Chain, Italy, Spain, Germany and Switzerland strains, 99% identity with USA, Italy, France, Malta. Slovenia. And Czech Republic strains. table (2) and Fig (3).

Table 2: Trichomonas gallinae compared with other registered global of ITS1-5.8S-IT2 gene.

Local Isolate No.	Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Identical to	Genbank Accession Number	Country	dentity (%)
No.1	OR074515	Trichomona gallinae	EU881912	Spain	97%
No.2	OR074516	Trichomona gallinae	EU881912	Spain	98%
No.3	OR074517	Trichomona gallinae	MG733819	Chian	97%
No.4	OR074518	Trichomona gallinae	KT869156.	Iran	99%
No.5	OR074519	Trichomona gallinae	KT869156.	Iran	97%
No.6	OR074520	Trichomona gallinae	KT869156.	Iran	99%
No.7	OR074521	Trichomona gallinae	JN007005	Switzerland	98%
No.8	OR074522	Trichomona gallinae	KT869156.	Iran	97%
No.9	OR074523	Trichomona gallinae	MG733819	Chian	99%
No.10	OR074524	Trichomona gallinae	MG733819	Chian	98%

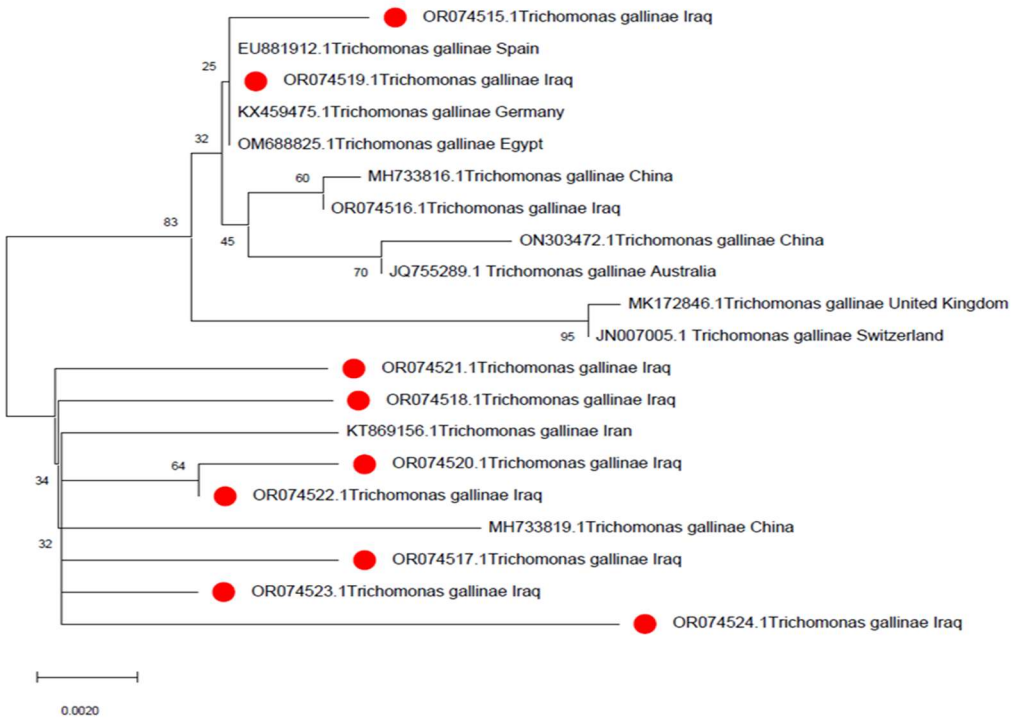

Figure (3): Phylogenetic tree analysis of Trichomonas gallinae 18S rRNA of ITS1-5.8S-IT2 gene that used for country relationship. Red circle: Trichomonas gallinae isolated from Iraq. Mega 6+ NCBI.

Phylogenetic analysis of Fe hydrogenase gene

A phylogenetic tree based on Fe hydrogenase (rRNA) gene sequence that there were relation between *T.gallinae* local isolates and other global isolates obtained from GenBank. The result showed identity 99% with Iran, Austria and USA while 98% with Spain, Austria and USA and 97% in Austria and USA. table (3) and figure (4).

Table 3: Trichomonas gallinae compared with other registered global of Fe hydrogenase gene.

No.	Accession number	NCBI-BLAST Homology Sequence	Country	identity (%)
No.1	ART89070.1	Trichomona gallinae	Iran	99%
No.2	UK159204.1	Trichomona gallinae	Austria	98%
No.3	ALB76773.1	Trichomona gallinae	Spain	99%
No.4	ALB76773.1	Trichomona gallinae	Spain	98%
No.5	CDF66387.1	Trichomona gallinae	Austria	97%
No.6	AOG61487.1	Trichomona gallinae	Austria	99%

No.7	**AOG61487.1**	**Trichomona gallinae**	**USA**	**98%**
No.8	**AGM46589.1**	**Trichomona gallinae**	**USA**	**97%**
No.9	**AGM46589.1**	**Trichomona gallinae**	**USA**	**99%**
No.10	**AGM46589.1**	**Trichomona gallinae**	**USA**	**99%**

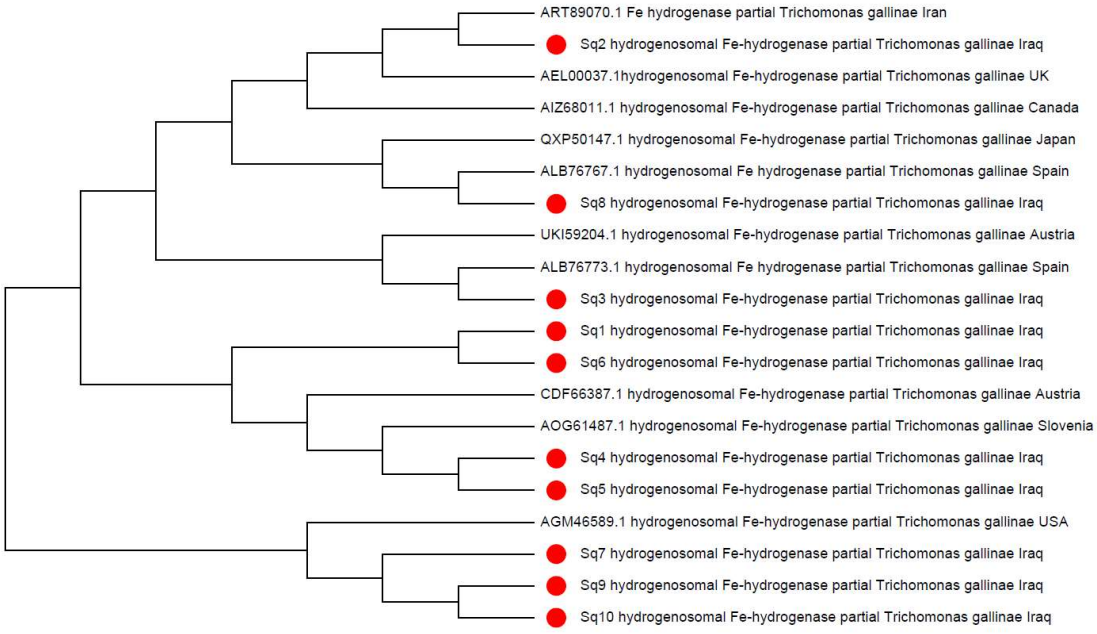

Figure (4): Phylogenetic tree analysis of Trichomonas gallinae 18S rRNA of Fe hydrogenase gene that used for country relationship. Red circle: Trichomonas gallinae isolated from Iraq. Mega 6+ NCBI.

Discussion

Microscopic inspection alone is insufficient to distinguish Trichomonas spp. Molecular techniques such as polymerase chain reaction (PCR) have been utilized to diagnose trichomoniasis disease in Columbids and to distinguish Trichomonas spp. (Quillfeldt et al., 2018).PCR is increasingly being utilized to detect and discriminate Trichomonas spp. It recognized quite small amounts of parasite DNA. Using particular primers for amplification of the ITS1/5.8/ITS2 18S rRNA ribosome gene, PCR testing results revealed a high infection rate of 85% in domestic pigeons with Trichomonas gallinae. Similar investigations, such as Chi et al (2013), recorded results that were closer to the current study by recording 100% in British. In Europe, the was 74% (Marx et al., 2017). In Egypt, EL-Khatam et al (2016) recorded 89.97%-100%. Geographical areas, sample numbers, and diverse hosts could all be factors in this infection rate difference (EL-Khatam et al.,2016). The majority of studies on the molecular detection and characterization of T. gallinae in various nations have relied on the ITS1/5.8S rRNA/ITS gene and the Fe hydrogenase gene. In line with the present

investigation, Saikia et al., (2023) confirmed T. gallinae infection in domestic pigeons of India by amplification of ITS1/5.8S rRNA/ITS and Fe hydrogenase gene by PCR and obtained positive fragment at 290 bp while Forzan et al. (2010) reported that T. gallinae was found in 37.5% of finches in Canada based on histology and PCR amplification of the ITS1/5.8S r RNA/ITS2 region, yielding a 290-bp fragment. Similarly, Sansano-Maestra et al. (2009) investigated the prevalence of T. gallinae in wild and domestic pigeons and raptors in eastern Spain and amplified a 369 bp band fragment by PCR using the 5.8S rRNA gene and the surrounding ITS1 and ITS2 regions. The findings of the molecular diagnosis of avian Trichomoniasis are consistent with the findings of Fadhil and Faraj (2020) who utilized PCR and specific primers for the 18S ribosomal gene to detect a high rate of infection in pigeons. Furthermore, the findings coincide with Chen et al., (2022). Based on what the results we have, this molecular technique is a great way to study how common trichomoniasis is among pigeons in large-scale epidemiological studies. But when a quick diagnosis needs to be made on a small, random group, clinical signs are often enough as described by Hammadi et al., (2023). Furthermore, the phylogenic tree analysis showed the 18S ribosomal RNA genes of Trichomonas gallinea detected strain was similar to the many countries, their similarities indicate that they were all from the same location of origin. Due to the fact that distinct sequences in his analysis shared a single-nucleotide polymorphism in the ITS region with other GenBank-registered sequences, this was true for all of the sequences (Alrefaei et al., 2021). In contrast, it was found many differences with European strains, these differences may be due to mutations or species variations as described by Ayati, et.al. (2023) who founded the similarity between isolates and those registered in GenBank; the genotype B isolates from LC136936 (Egypt), T869155 (Tehran, Iran), MH733822 (China), and EU881912 (Spain) had the highest similarity, whereas the genotype A isolate JQ755283 (Australia) had the most significant difference. The relationship between the strains that have been isolated in Iraq and those that have been isolated in groups far from Iraq may be the result of international trade or an open border between Iraq and its neighbors. According to Sansano-Maestre et al. (2009), genotype A is more prevalent in Columbiformes. The genotype A found in domestic pigeons is consistent with this finding.

Conclusions

This is first study for using PCR technique with phylogenetic tree of *Trichomonas gallinae* in domestic pigeons in Iraq. There is no sequences information for any ITS1,5.85, rRNA ITS2 region and Fe hydrogenase *18s* rRNA genes of *T. gallinae* obtainable from Iraq other than the sequence reported here.

Novelty

This research is new, original in detection of *Trichomonas gallinae* in domestic pigeons in AL-Diwanyia, Iraq.

Authors Contribution

All authors contributed equally to the manuscript and agree to the conditions outlined in the copyright assignment form included.

Conflict of interest

The authors have declared no conflict of interest.

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