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# Genetic Sequence and Phylogenetic Analysis of *Trichomonos gallinae* in Racing Pigeons at Mosul City, Iraq

**Mohammed H. Altememy and Mohammed G. Saeed***

Department of Pathology and Poultry Diseases, College of Veterinary Medicine, University of Mosul, Mosul, Iraq
***Corresponding Author:** Mohammed G. Saeed, **E-Mail:** mgsaeed@uomosul.edu.iq

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## ABSTRACT

This is the first study in Mosul to use genetic sequencing technology to diagnose and document the type, strain, and genotype of *Trichomonos gallinae* in racing pigeons. It was distinguished by the geographical sequence of Mosul, Iraqi city. Thirty isolates of *T. gallinae* were chosen from a total of 56 that had been molecularly characterized to examine the extent to which these isolates matched in terms of genetic sequencing. The DNA from the *T. gallinae* parasite was extracted, and the master mix for all of the polymerization reaction components was created based on the needed quantities of the reaction components for each sample. The acquired sequences were matched to known sequences in databases to determine the *trichomonos species* parasite and strain responsible for the infection. The results of the DNA sequencing examination revealed that after the polymerase chain reaction amplification products were sent to Macrogen, Korea, to determine the genetic variation of the local strains, the products of the small subunit rRNA-Gene and the reaction product of bp 194 of the *Trichomonos gallinae* parasite were sent to the National Centre for Biotechnology Information, NCBI Gen Bank, for recording. Based on the small partial ribosomal RNA according to blast in GenBank **of** the NCBI, the percentage of match in the genetic sequence was 100% between the genetic sequence in Mosul and the genetic sequences in Brazil and Portugal. France, Spain, Iran, Poland, Prague, Hungary, Australia, and the United States are among the countries involved. The Trichomonas gallinae genetic sequence in racing pigeons from Mosul has been discovered for the first time in the GenBank database, revealing a 100% match with other countries' sequences. This discovery reveals the pathogen's worldwide dissemination and interconnection, aiding in the development of effective diagnostic procedures, preventive measures, and targeted treatments. The discovery also emphasises the need for cooperation in monitoring and regulating the spread of the infection, supporting a collaborative strategy against avian diseases.

***Keywords:*** GenBank, PCR, Pigeons, T. gallinae.

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## INTRODUCTION

Trichomoniasis in birds is a rapidly deadly disease characterized by caseous lesions of the upper digestive system in infected birds. The rock-pigeon *Columba* is an old-world bird that is thought to be the sole source of *T. gallinae* **(Lawson, 2010).** Trichomoniasis is a potentially fatal disease that has been observed in numerous parts of the world and affects many birds, including hawks, owls, doves, pigeons, and other wild birds **(Saikia, *et al.,* 2023).**

The condition is characterized by severe inflammation and necrosis of many tissues, and the upper GI (gastrointestinal) tract (pharynx, mouth, and crop). Infected birds frequently die from malnutrition due to diminished foraging capacity, respiratory failure due to lesions obstructing the trachea, sepsis due to secondary infection, or failure of visceral organs after disseminated infection **(Amin *et al,,* 2014; Quillfeldt *et al.,* 2018).** *T. gallinae* was found in 85% of Coppernet's hawks in Tucson, Arizona, and trichomoniasis was the major cause of death in chicks **(Real *et al.,* 2000).**

*Gallinae* infection revealed a wide range of virulence. Sequencing of 58S- ribosomal RNA (rRNA) and the inner regions surrounding transcribedspacersr 1 and 2 (ITS 1, ITS2) from Tetratrichomonas spp. revealed unexpected diversity **(Gerhold *et al.,* 2008; , Grabensteiner *et al.*, 2010; Collántes-Fernández *et al.,* 2018)** in highly pathogenic isolates. The presence of 16 unique monophyletic groups within the genus was found, with the majority of these monophyletic groupings being host-specific **(Nováková *et al.*, 2009; Escalante *et al.,* 2022**).

ITS1, 58S, and ITS2 rRNA sequences from 24 T. gallinae isolates from pink pigeons and Madagascar turtle doves (Streptopelia picturata) on the island of Mauritius, on the other hand, revealed no single nucleotide polymorphisms, and the sequences were identical for *T. gallinae* in a rock bath in the United States **(Grabensteiner *et al.,* 2010).** The molecular approach is based on the internal transcription of DNA gaps **(Schoch *et al.,* 2012).**

The DNA-sequencing analysis technique is one of the most important techniques of genetic engineering, as it made it possible to identify the sequence of nucleotides of a specific piece of DNA with high accuracy, and there is no doubt that this technique added a new dimension to molecular biology and revealed many secrets about the DNA molecule. It was also feasible to determine the nature and properties of several of the constituent genes **(França *et al.,* 2002; Al Junid *et al.,* 2008; Mardis, 2017).**

The goal of this study was to use Gene Sequencing technology to analyze the genetic sequence of *T. gallinae* and determine the type and strain of Trichomonos parasite that is responsible for infection in racing pigeons from isolates of infected pigeons obtained from different regions of the city of Mosul in Iraq. Polymerase chain reaction (PCR) was also used to characterize the parasite at the molecular level.

## MATERIALS AND METHODS

### Cases investigation

Trichomoniasis cases in domesticated homing pigeons Columba livia, specifically Racing Homer pigeons, were studied in random locations in Mosul, including periodic visits to local marketplaces dedicated to selling homing pigeons and organized visits to pigeon breeders' residences. From October 2022 to March 2023, there will be 2561 birds. Through macroscopic and microscopic inspection of the oral macroscopic lesions, which are in the form of white to yellow necrotic foci of cheesy colour with an unpleasant odour, 30 birds out of 56 recorded a positive result, and the macroscopic examination included each of the oral cavity, the pharynx, and the respiratory tract's entrance.

### Sample collection and preservation

Gaseous necrotic lesions were sampled from various locations in the infected birds, including the oropharyngeal cavity, oesophagus, crops, and liver, as well as tissues from the same organs. They were placed in Eppendorf tubes and frozen at -20 degrees Celsius.

### Extraction of DNA

The DNA of the parasite *Trichomonos gallinae* was isolated using a DNA preparation kit. The goal of this stage is to separate the Trichomonos parasite's genetic material. (DNeasy Tissue Extraction Kit; QIAGEN, Valencia, California, USA).

### Prepare the PCR master mix

The Add Bio master mix kit was used to prepare the master mix for all PCRs, according to the needed quantities of reaction components for each sample, and these tests confirmed the presence of the parasite in the samples **(Felleisen ,1997).**

### Programming a thermocycler for the purpose of genetic sequencing

After preparing the PCR tubes, they were placed in a Thermocycler T 100 TM Thermal cycler with a polymerase chain reaction programme (Bio-Rad, USA), and then the tubes were removed from the device and placed in the refrigerator for 4–8 m until electrophoresis was performed to detect the process products. DNA amplification and sequence of specific regions of the parasite's DNA, such as the small ribosomal RNA gene.

### Agarose gel electrophoresis

After the migration process was completed, the gel was extracted and placed in the Gel Documentation System to identify the amplification products, and photographs of the results were saved. To use bioinformatics tools and databases to analyse the given genomic sequences (**Ghatak *et al.,* 2013).** This study could shed light on the Trichomonos parasite's genetic features, connections with known strains, and perhaps evolutionary links.

## RESULTS

### DNA sequencing

The polymerase chain reaction amplification products were delivered to Macrogen, Korea, to explore the genetic variation of local strains, while the small-subunit rRNA gene products and the reaction product of bp 194 were sent to the *Trichomonos gallinae* parasite. The collected results were then analysed and submitted for recording to the NCBI GenBank.

### Polymerase chain reaction technology

DNA isolated from oropharyngeal cavity lesions was electrophoretically separated on an agarose gel. Path M Marker has a length of 100 bp. Lanes 1-11 represent RNA, while lane 12 represents a negative control (Fig. 1).

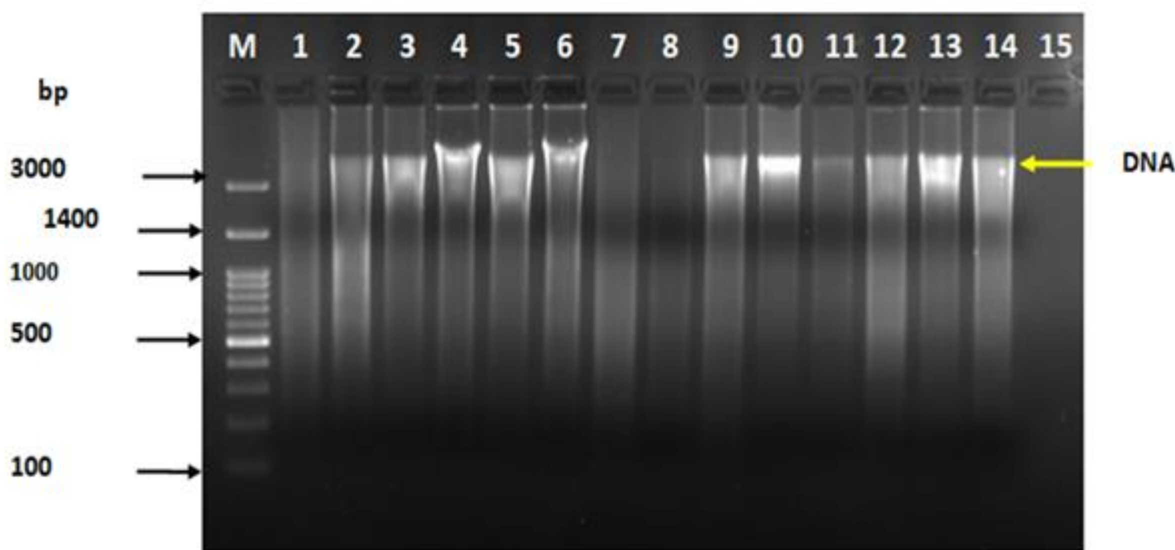


Fig. 1: The results of agarose gel electrophoresis of pest DNA. Track M: The marker symbolises 100 bp in size. Lanes 1–11 represent RNA, while lane 12 represents the negative control.

The agarose gel electrophoresis of the PCR results for the detection of Trichomonos spp. is shown in Figure 2. TSSU-F and TSSU-R primers were used. Path M Marker has a length of 100 bp. Lanes 1–6 are positive samples with a size of 335 bp, and lane 7 is the negative control (Fig 2).

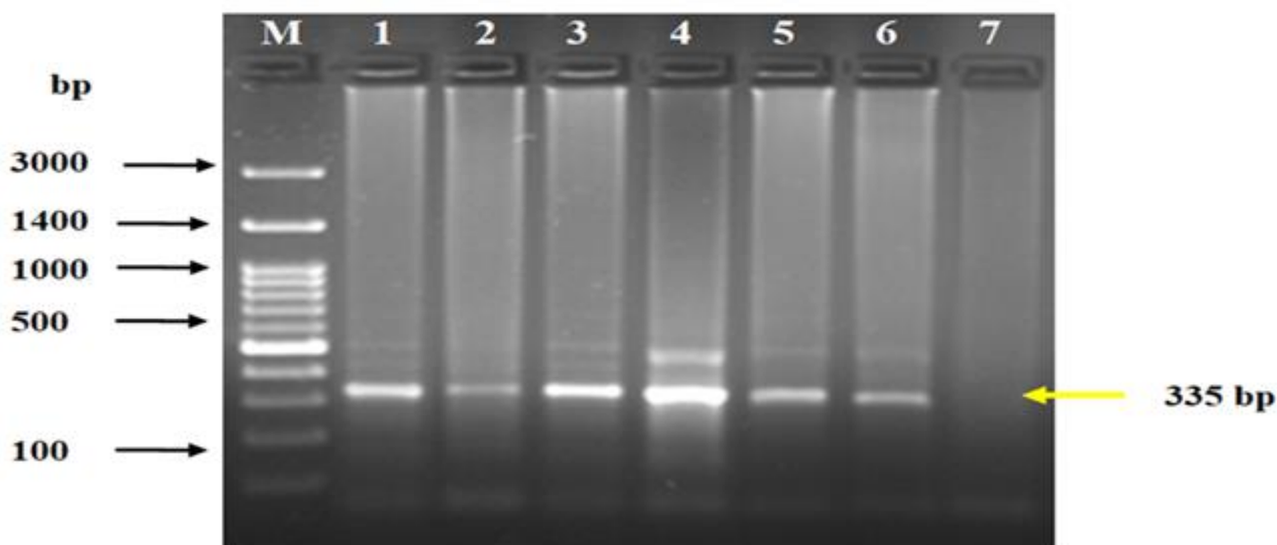


Fig. 2: PCR product electrophoresis in agarose gel for detection of Trichomonos spp. Pathway M: The marker reflects the size of 100 bp. Lanes 1–6 are positive samples with a size of 335 bp, and lane 7 is the negative control

Figure 3 depicts the agarose gel electrophoresis of PCR results for Trichomonos spp. detection. Primers - TN3-F and TN4-F were used. Path M Marker has a length of 100 bp. Lanes 1–6 are positive samples with a size of 149 bp, while Lane 7 is the negative control (Fig.3).

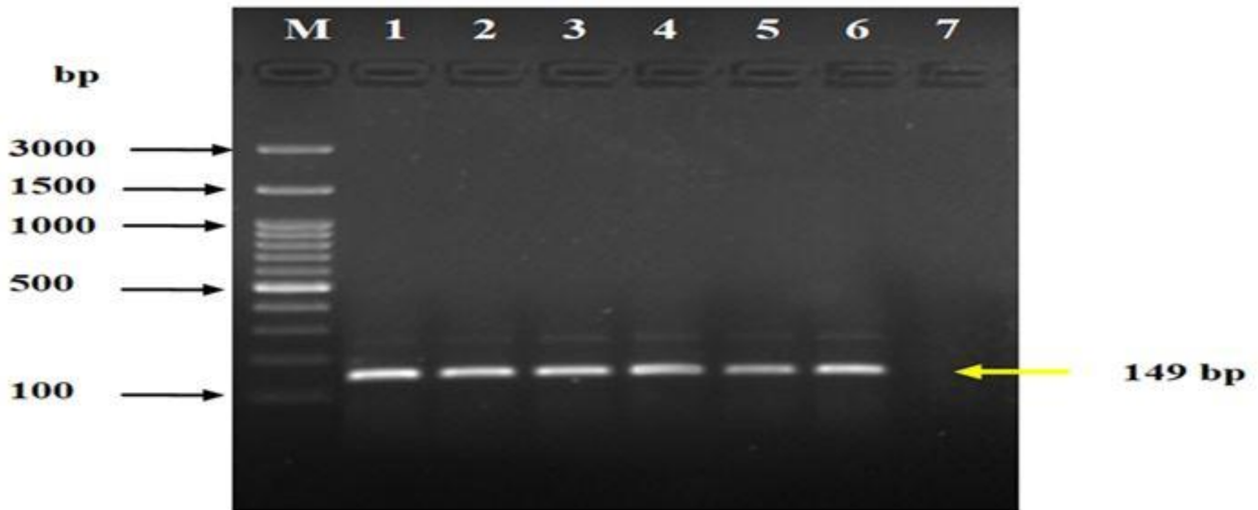


Fig. 3: PCR product electrophoresis results on agarose gel for detection of Trichomonos spp. Pathway M: The marker signifies a length of 100 bp. Lanes 1–6 are positive samples with a size of 149 bp, while lane 7 is the negative control.

As shown in Fig. 4 depicts the identification of the query *Trichomonos gallinae* and its alignment with the NCBI GenBank.

**Trichomonas gallinae isolate Carcara1 small subunit ribosomal RNA gene, partial sequence**

Sequence ID: MN630564.1 Length: **188** Number of Matches: **1**

**Range 1: 35 to 186** GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 275 bits(304) | 5e-76 | 152/152(100%) | 0/152(0%) | Plus/Plus |

```
Query  1    AATCCCTAACGTAGTTGGGATTGACGTTTGTAATCAGCGTCATGAACCAGGAATCCCTTG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35   AATCCCTAACGTAGTTGGGATTGACGTTTGTAATCAGCGTCATGAACCAGGAATCCCTTG  94

Query  61   TAAATGTGTGTCAACAACGCACGTTGAATACGTCCCTGCCCTTTGTACACACCGCCCGTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95   TAAATGTGTGTCAACAACGCACGTTGAATACGTCCCTGCCCTTTGTACACACCGCCCGTC  154

Query  121  GCTCCTACCGATTGGATGACTCGGTGAAATCA  152
            ||||||||||||||||||||||||||||||||
Sbjct  155  GCTCCTACCGATTGGATGACTCGGTGAAATCA  186
```

Fig. 4: indicate *Trichomonos gallinae* identification and alignment with NCBI Gen Bank .

Table 1 shows the percentage of *Trichomonos gallinae* distribution based on partial small ribosomal RNA according to nblast in the NCBI Gen Bank, where the percentage of match in the genetic sequence reached 100% between the genetic sequence in Mosul and the genetic sequences in Brazil and Portugal, France, Spain, Iran, Poland, Prague, Hungary, Australia, and the United States.

Table 1: Percentage distribution of *Trichomonos gallinae* based on partial small subunit ribosomal RNA according to nblast in Gen Bank of NCBI

| Sample ccession Number | Identified Organism | Query Cover % | Identic Number % | Gen Bank Accession Number | Country Identification |
|---|---|---|---|---|---|
| OR002127 | *Trichomonos gallinae* | 100 | 100 | MN630564 | Brazil |
| | | 100 | 100 | MK932772 | Portugal |
| | | 100 | 100 | MK172847 | France |
| | | 100 | 100 | KX514379 | Spain |
| | | 100 | 100 | KX353946 | Iran |
| | | 100 | 100 | KU954105 | Poland |
| | | 100 | 100 | KM095107 | Prague |
| | | 100 | 100 | ON631565 | Hungary |
| | | 100 | 100 | KM246609 | Spain |
| | | 100 | 100 | FN433484 | Austria |
| | | 100 | 100 | EU215375 | USA |
| | | 100 | 100 | EU215374 | USA |
| | | 100 | 100 | EU215373 | USA |

The maximum likelihood approach in the MEGA11 programme and bootstrap analysis (Fig. 5) were used to compare sequences to reference databases, create phylogenetic trees, and determine the relationship of strains defined by strains known from other geographic locations.

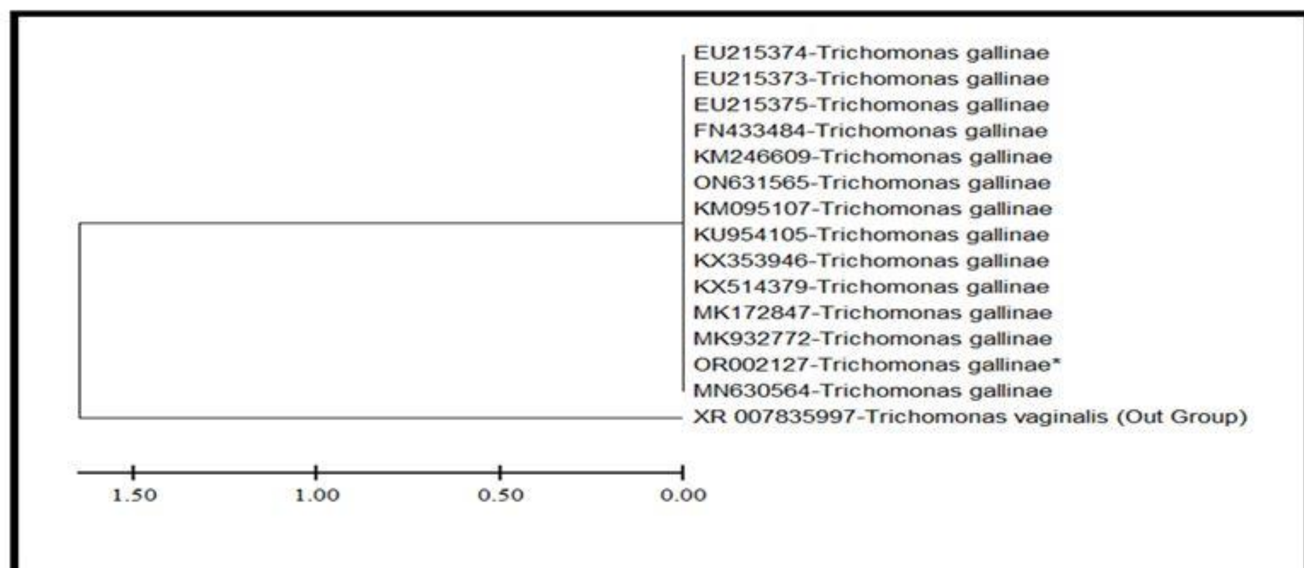


Fig. 5: Iraq (Mosul*) phylogenic tree of *Trichomonos gallinae*. The phylogenic tree was built in MEGA11 software using the Maximum Likelihood approach and bootstrap analysis with 1000 re-samplings according to the Tamura-Nei model.

## DISCUSSION

Because microscopy is difficult to explain the distinction between Trichomonos spp., Polymerase Chain Reaction (PCR) and DNA sequencing were used to identify trichomoniasis molecularly in racing pigeons in Mosul (**Rentera-Sols *et al.*, 2020; Mohamed *et al.*, 2023; Saikia *et al.*, 2023).**

To amplify and identify an organism's DNA, specific primers that target its genetic material can be utilized **(Sint *et al.*, 2012; Ye *et al.*, 2012).** By comparing the resulting sequence to known

sequences in databases, DNA sequencing validates the organism's identity.

Using genetic sequencing techniques, we validated the diagnosis of trichomoniasis and identified the type and strain of the Trichomonos parasite responsible for illness in homing pigeons in Mosul. The genomic sequence of *Trichomonos gallinae* (MMT1 small subunit ribosomal RNA gene) was reported for the first time in the GenBank database (www.ncbi.nlm.nih.gov/ Gen Bank /)(24).

Searches for the ribosomal RNA gene sequence of *T. gallinae* strains using the subspecies name and other identifying information. The sequence of the subunit small ribosomal-RNA gene varies between *T. gallinae* strains and isolates. To get the sequence of a short ribosomal RNA gene from a specific strain of *T. gallinae* **(Lawson *et al.,* 2011; , Martínez-Díaz *et al.,* 2015, Chen *et al.,* 2022).**

These findings revealed a perfect match between various strains from around the world, including Brazil, Portugal, France, Spain, Iran, Poland, Prague, Hungary, Australia, and the United States. One reason for this genetic match could be the transfer of parasitic illnesses by migrating birds. As sharing diseased or infected wild birds in water and fodder is one of the sources of parasitic infection with *Trichomonos species* **(Tsiodras *et al.,* 2008).** Furthermore, the nature of the racing pigeon, which may fly up to 1000 km per day, could be one of the reasons for the existence of this match in the genetic sequence from neighbouring areas to Mosul **(Wikelski *et al.,* 2015).**

Several studies have been conducted to determine the prevalence of trichomoniasis in birds. A microscopy study in India found *T. gallinae* in 26.85% of domestic pigeons **(Al-Hasnawy and Rabee, 2023).** A microscopic study in Iran Throughout the year, researchers examined the oropharyngeal cavity, crops (oesophagus), droppings (cloaca), and conjunctival swabs from several species of birds belonging to different orders and discovered that the total number of positive samples was 23.7%, distributed from the upper digestive tract to the eyes and respiratory tract **(Borji *et al.,* 2011).**

One study found Trichomonos genetic diversity in domestic animals and The incidence of Trichomonos is substantially higher in domestic pigeons, with a variety of strains detected in birds in Saudi Arabia, confirming that the genotypes of ribotypes A and C are frequent **(Albeshr and Alrefaei,, 2019).** Another recent study verifies this genetic variety by discovering four strains of T. gallinae in Saudi Arabian falcons, namely C,A, II, and KSA11 **(Grabensteiner *et al.,* 2010).**

Gallinae may be divided into at least two species, according to the findings of a study done in the United States. Based on ITS sequences, one group had significant nucleotide identity to T. gallinae sequences in GenBank, whereas the second group was more closely related to T. vaginalis (98%) than T. gallinae (92%). Based on ITS sequences, one group had a high nucleotide identity to *T. gallinae* sequences in GenBank, whereas the second group was more closely linked to *T. vaginalis* (98%) than *T. gallinae* (92%). *T. vaginalis* isolates share 95% commonality with *T. gallinae* and *T. tenax isolates*, while *T. gallinae* and *T. tenax* isolates share 92% identity. A sequence study of a subgroup of 5.8S-ITS isolates' 18S rRNA and tubulin genes confirms this **(Gerhold *et al.*, 2008; Grabensteiner *et al.,* 2010).**

This study discovered that trichomoniasis is common in the birds of Mosul, using the genetic sequence mentioned in the Gen-Bank database (www.ncbi.nlm.nih.gov/ Gen Bank /). Because trichomoniasis in pigeons can have serious consequences for public health and bird performance, adequate preventative and treatment methods must be implemented to limit the spread and impact of illness within pigeon populations **(Santos *et al.,* 2020).** *Comparing the acquired sequences to those from other places or lineages can provide* useful insights into Trichomonos parasite genetic diversity and probable transmission routes. Comparative research can help us understand the epidemiology and evolutionary trends of *Trichomonos* infection in homing pigeons **(Conrad *et al.,* 2012; Ramsubeik *et al.,* 2023).**

More future investigations with researchers specializing in avian illnesses or molecular diagnostics will be required to improve the diagnostic method for Trichomoniasis in pigeons. Furthermore, awareness efforts for bird breeders should be performed to alert them of the infection and its effects on their birds.

## CONCLUSION

The discovery of the T. gallinae genetic sequence in racing pigeons from Mosul, which was reported for the first time in the GenBank database, opens up exciting opportunities for future research and international collaboration. The creation of a phylogenetic tree that revealed a 100% match with genetic sequences from other countries emphasises the pathogen's worldwide dissemination and interconnection. This discovery sheds light on the

epidemiology and transmission patterns of T. gallinae, which could aid in the development of more effective diagnostic procedures, preventive measures, and targeted treatments to safeguard racing pigeons and other bird species around the world. Furthermore, the discovery of a genetically identical strain in different places highlights the necessity of cooperative efforts in monitoring and regulating the spread of this infection, supporting a collaborative strategy in the battle against avian diseases. As researchers explore deeper into T. gallinae's genetic traits, new avenues for understanding its pathogenicity and host interactions may emerge, perhaps leading to novel solutions for bird health management and conservation.

**Competing interes**

There is no conflict of interests of any sort between authors or elsewhere.

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