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Clinical survey and molecular detection of *Trichomonas gallinae* infection of domestic pigeons (*Columba livia domestica*) in Al-Qadisiyah Province, Iraq

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Abstract

The present study encompasses two aspects: monitoring the typical clinical features of trichomoniasis among domestic pigeons (*Columba livia domestica*) in Al-Qadisiyah Province, Iraq, and molecular typing of the etiological agent. The typical clinical features of trichomoniasis, including glutinous plaques that create yellow, necrotic, cheese-like masses, were verified in a sample of fifty pigeons with a frequency of occurrence of 100% (n=50/50). Microscopic examination also revealed a frequency of occurrence among pigeons of 100% (n=50/50). The molecular typing of the causative agent was performed using the 18S rRNA sequence analysis approach with the universal Trichomonas ITS1-5.8S rRNA-ITS2 specific primer through conventional PCR. Among the ten pigeons tested, the molecular typing revealed that *Trichomonas gallinae* was the causative agent of trichomoniasis with a frequency of occurrence of (n=10/10). The ITS1-5.8S rRNA-ITS2 nucleotide sequences of the ten strains of *Trichomonas gallinae* were submitted to GenBank under the following accession issues: OM367997, OM457006, OM457005, and OM456998. These findings underscore the indispensable need for implementing high preventive standard measures against avian trichomoniasis to better control this disease.

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Introduction

Avian trichomoniasis is a life-threatening protozoan illness caused primarily by the pathogen *Trichomonas gallinae*, which infects birds through the upper respiratory tract. This parasite affects a vast variety of birds worldwide, including pigeons, chickens, turkeys, and other fowl (1,2). *Trichomonas gallinae* infection can be asymptomatic or lethal, with common signs such as anorexia, vomiting, ruffled feathers, diarrhea, dyspnea, dysphagia, weight loss, and increased thirst (3-6). *Columba livia domestica* is considered the primary host of *T. gallinae*, which helps elevate the disease's propagation. The chief target of this parasite is young pigeons. While death is one potential outcome of trichomoniasis, asymptomatic trichomoniasis is another scenario in which there are no signs of illness, which can frequently occur (7). *T. gallinae* is considered highly virulent and can induce proliferative findings in the oropharynx that cause the mortality in a non-immunizing bird (8). Trichomoniasis occurs when adult pigeons feed their squabs or when using feeders and water (9,10). The standard method for identifying *T. gallinae* is through microscopic and morphological examination, although this method is time-consuming and requires fresh materials and a specific procedure (11,12). In the search for alternative methods, the internal transcribed spacers (ITS) of ribosomal DNA have shown potential as a genetic marker for discriminating between various Trichomonas species implicated in avian trichomoniasis using a polymerase chain reaction (PCR) approach (13-18).

Additionally, sequence analysis has shown the co-existence of heterogenic species of trichomonads not only in various fowl populations but also within the same host species. This study aims to monitor the prevalence of avian trichomoniasis among pigeons in Al-Qadisiyah Province, Iraq, using a molecular ITS-r RNA approach, in conjunction with clinical manifestations of trichomoniasis and visualizing the etiological agent under a light microscope.

Materials and methods

Ethical approval

All the authors of the present work ensure that all procedures of our experiment were performed under the Ethical Norms approved by the scientific board of College of Veterinary Medicine, University of Al-Qadisiyah (committee approval number 1314 in 18/10/2022).

Sampling of domestic pigeons

A total of 50 domestic pigeons, scientifically known as *Columba livia domestica*, were enrolled in the present study. The study was conducted in Al-Qadisiyah Province, Iraq, and the sampling duration was almost one year, from September 2020 to September 2021. Wild birds were collected through a pest control program authorized locally using cage traps in urban public parks (19).

Examination of clinical features of trichomoniasis in pigeons

A smear of wet mount from the oral cavity/crop of pigeons was prepared on a slide and stained with Giemsa for *T. gallinae* (20,21). Additionally, a gross macroscopic examination of white, caseous lesions or stomatitis was indicative of trichomoniasis.

Staining method of *T. gallinae*

Each sample was examined under a light microscope at magnifications of x100 and x400. The sample was considered negative if there was no evidence of *T. gallinae* within 72 hours of incubation. In this investigation, three staining methods were employed: Giemsa, Gram, and Fields. The *T. gallinae*-smeared slides were dried for 15-20 minutes at room temperature before staining with Gram stain using a standardized four-step staining procedure with Atom Scientific kits. The dried slides were treated with methanol for 30 seconds before staining with Giemsa stain (22). The slides were then air-dried for 15-20 minutes at room temperature, rinsed with distilled water, and air-dried again. Microscopic slide examination was conducted at magnifications of x100 and x400. For staining parasites with the Fields stain, Sivanandam and Mak's approach was followed. Smeared slides containing *T. gallinae* were dried at room temperature for 15-20 minutes before adding eight drops of Field's stain B and sixteen drops of Field's stain A to the smear. The slides were then gently shaken for fifteen seconds before rinsing for two seconds under a strong spray of water. All slides were then dried at room temperature and investigated under magnifications of x100 and x400.

Isolation of genomic DNA

Genomic DNA was isolated from ten samples collected from ten pigeons in the study. The DNA was extracted from tongue lesions using the GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific™, USA), following the manufacturer's instructions (Figure 1). The concentration of the genomic DNA from the lesion samples was estimated using the NanoDrop™ 2000/2000c Nanspectrophotometer (ThermoFisher Scientific™, USA). The purity of the genomic DNA was evaluated using the Ab260/Ab280 ratio. A ratio of Ab260/Ab280 ≥ 1.8 indicates good DNA purity. The integrity of the genomic DNA was analyzed using agarose gel electrophoresis and visualized under a UV-transilluminator.

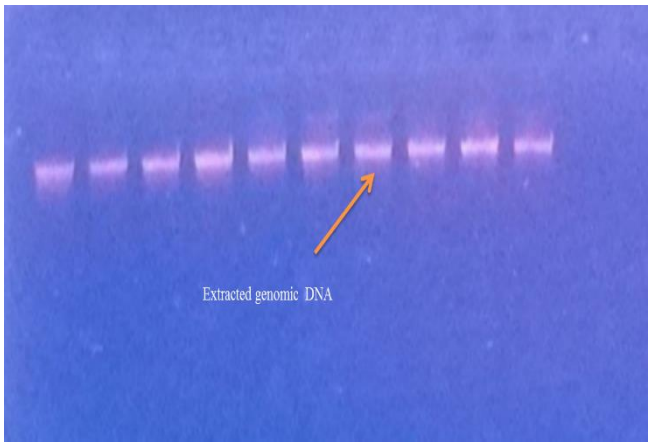

Figure 1: Electrophoresis of DNA extraction.

Amplification of ITS1- 5.8S rRNA-ITS2 gene

The ITS1-5.8S-ITS2 gene was amplified from ten out of fifty samples using ITS1-5.8S rRNA-ITS2 specific primers for the Trichomonas genus. The following primer set was used in this study: Fw-ITS1-5.8S rRNA-ITS2-Trico: 5'-TGCTTCAGTTCAGCGGGTCTTCC-3' and Rv-ITS1-5.8S rRNA-ITS2-Trico: 5'-CGGTAGGTGAACCTGCCGTTGG-3' (23,24). 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 350 bp. 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 (25). 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 nucleotide sequences in correlation to closely related ITS1-5.8S rRNA-ITS2 parasites (Figure 2).

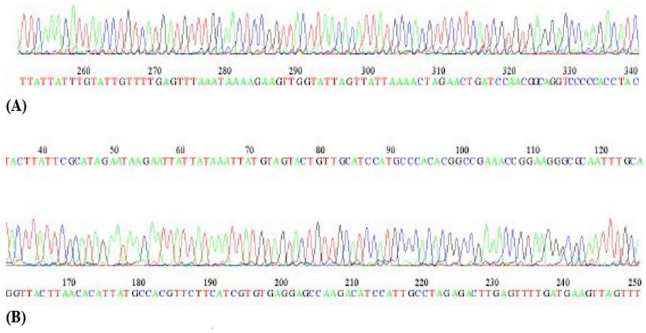

Figure 2: A and B waves of sequencing for sequence No. 1 of *18s rRNA.*

Results

Clinical manifestation of trichomoniasis in pigeons

In acute forms, there were intermediate signs in affected fowl, and death happened quite suddenly. In other forms, infected pigeons lack appetite, have low weight gain, look ruffled, dull, and have difficulties in movement. Papillae abrasion on the palatal flaps is a remarkable indicator of infection. White, glutinous plaques create yellow, necrotic, cheese-like masses on the choanae, tongue, or mucous membrane of the pharynx, as well as occasional moisture around the beak. Moreover, a foul smell can be inhaled from the affected region. Some fowls may experience difficulty breathing and swallowing due to the accumulation of cheesy material in the oral cavity and trachea (Figure 3). Diarrhea observed. Death occurs throughout 21 days of infection. Greenish liquid or cheesy deposits are seen in the oral cavity and crop, and these substances may exude from the beak.

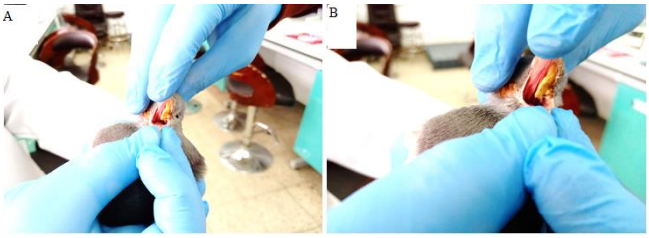

Figure 3: Clinical manifestation (show plague in the oropharynx) of Trichomoniasis in pigeons. A: view 1. B: view 2.

Microscopic examination of *T. gallinae*

The wet mount smear test revealed that 100% of the studied pigeons (n=50/50) were positive for *T. gallinae*. The wet mount preparation showed that *T. gallinae* had a prominent axostyle at the posterior end of the parasites (Figure 4). Additionally, the organism exhibited a pyriform shape with four anterior flagella and one recurrent flagellum attached to an undulating membrane. The giemsa stain stained the cytoplasm and the nucleus with light purple and dark purple, respectively, and provided excellent visualization of the flagella. In contrast, the Field stain stained the nucleus of *T. gallinae* with dark pink and the cytoplasm with pink. However, the Gram stain was able to stain the cytoplasm and the nucleus with light pink and dark pink, respectively.

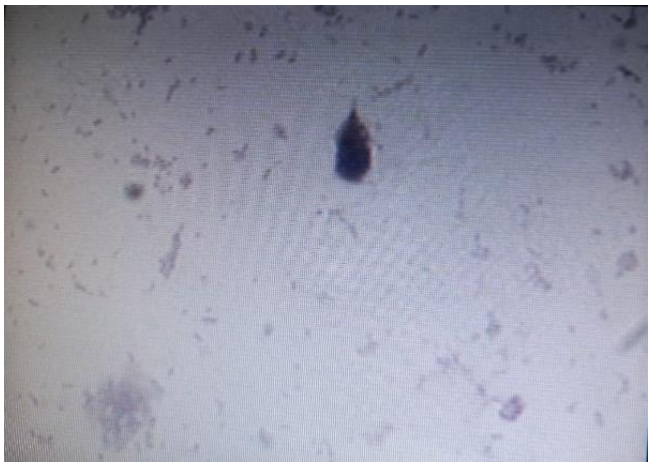

Figure 4: Photo of a light microscopic field showing *T. gallinae*. Giemsa, 100x.

Molecular identification of avian trichomoniasis etiological agent among pigeons

A PCR product of 350 bp was successfully amplified using the primer set described in the materials and methods section. Ten samples were randomly selected from the fifty samples enrolled in this study, and the PCR product (Figure 5).

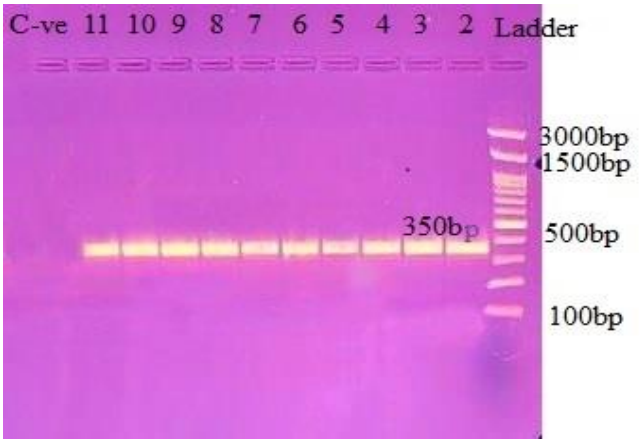

Figure 5: Agarose gel electrophoresis (1%) showing the PCR product of *ITS1-5.8S rRNA-ITS2* gene from 10 samples randomly selected. Lane M: DNA ladder. Lanes 1-10: PCR product of *ITS1- 5.8S rRNA-ITS2 gene* of 350 bp from 10 randomly selected samples.

Molecular identification using ITS1-5.8S rRNA-ITS2 confirmed the etiological agent of Trichomoniasis in pigeons as *T. gallinae*. The ten randomly selected samples out of fifty were found to carry *T. gallinae* with a frequency of occurrence of 100%. The nucleotide sequences of four strains of *T. gallinae* were listed in GenBank under the accession numbers OM367997, OM457006, OM457005, and OM456998. These four strains were named *T. gallinae* isolate AQ1, AQ2, AQ3, and AQ4, respectively. The phylogenetic tree, organized by CLC Sequence Viewer 8.0, is presented in Figure 6. The four isolates of *T. gallinae* identified in this study were distantly related to each other and also distantly related to some ITS1-5.8S rRNA-ITS2 sequences of *T. gallinae* retrieved from GenBank, which represent different *T. gallinae* genotypes (Figure 6).

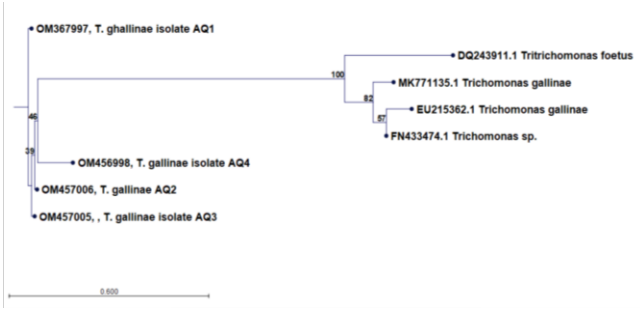

Figure 6: Neighbor-Joining phylogenetic tree organized by CLC Sequence Viewer 8.0 clarifying the genetic relatedness among the four *T. gallinae* identified in this study (*T. gallinae* isolate AQ1, AQ2, AQ3, and AQ4) and other ITS1-5.8S rRNA-ITS2 nucleotide sequences of *T. gallinae* retrieved from GenBank. Numbers on branches indicate the bootstrapping values.

Discussion

In the present study, the clinical manifestations of trichomoniasis in birds were consistent with the results of Narcisi *et al.* (26), who noted weight loss, ruffled feathers, vomiting, dyspnea, dysphagia, and diarrhea. The clinical signs were also consistent with those observed by Amin *et al.* (5), who reported the collection of greenish fluid or white fibrinous material and the possibility of death (5). These findings are also in agreement with those of Mohamed *et al.* (27) and El-Khatam *et al.* (28).

However, the results of the microscopic examination of *T. gallinae* are consistent with Mehlhorn *et al.* (1) and Tasca and Carli (29), who showed that Trichomonas gallinae trophozoites vary in shape from oval to pyramidal, and the organism has four free flagella. Moreover, the results of Bon Durant and Honigberg (30) showed that the organism has four anterior flagella, no free posterior flagellum, an axostyle that protrudes posteriorly, and a well-developed undulating membrane (30).

However, Bunbury *et al.* (31) demonstrated that growth media for trichomonads detection is more sensitive than wet mount preparations, where numerous media support the growth of *Trichomonas gallinae* (32).

The results of the molecular identification of avian Trichomoniasis agree with the study of Fadhil and Faraj (33), which used PCR and specific primers for the 18S ribosome gene to detect a high incidence of infection in pigeons (33). Additionally, the results agree with Alrefaei (34).

Although only 10 out of 50 samples were randomly selected to detect the presence of the etiological agent *T. gallinae*, the prevalence of the pathogen was 100% (n=10/10) based on the successful PCR amplification using the ribosomal-internal transcribed spacer primer set specific for *T. gallinae*. This indicates a prevalence of about 20%. In the city of Mosul, Iraq, the prevalence of trichomoniasis was 16% (n=16/100) (35), while in the city of Kirkuk, Iraq, the frequency of occurrence of trichomoniasis among pigeons was 49.26% (36). In this study, the ITS1-5.8S rRNA-ITS2 molecular approach was employed to confirm the identity of the etiological agent of Trichomoniasis in pigeons. This molecular approach has the potential to identify *T. gallinae* among suspect pigeons and provides valuable support to the clinical features of the disease. Although slightly expensive, it is mandatory to confirm the identity of the pathological agent of trichomoniasis among infected pigeons with clear manifestations. Based on our data, this molecular approach is highly recommended for conducting large-scale epidemiological studies on the prevalence of trichomoniasis among pigeons. However, in the context of rapid diagnosis on a small, randomized sample, clinical manifestations are often sufficient.

The importance of this study lies in the widespread distribution of ecto and endoparasites in our country. For example, many species of Moniezia have been found in

Mosul city (37), Anaplasma spp. has been identified in small ruminants (38), and *Theileria annulata* strains have been detected in calves (39). Moreover, ducks and geese are infected with *Eimeria truncate* (40). *T. gallinae* infects the upper alimentary tract, causing avian trichomoniasis. However, various species of trichomonas are classified as pathogenic and affect health of the host (41).

Conclusion

The high prevalence of *T. gallinae* among pigeons in this study, in comparison to other studies previously conducted in other countries or within other provinces in the country, urgently necessitates the indispensable need for proper housing, disease management, and feeding to be carried out perfectly by the owners to help ensure a better reduction in the prevalence of trichomoniasis and, hopefully, eradicate the etiological agent *T. gallinae*.

Acknowledgments

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Conflict of interest

No conflict.

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المسح السريري والتحري الجزيئي عن الإصابة بداء المشعرات في الحمام المحلي في محافظة القادسية، العراق

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الخلاصة

تشمل الدراسة الحالية جانبين وهما مراقبة العلامات السريرية النموذجية لداء المشعرات بين الحمام المنزلي في محافظة القادسية في العراق بالإضافة الى معرفة التوصيف الجزيئي للعامل المسبب للمرض. تم إجراء التوصيف الجزيئي للعامل المسبب باستخدام نهج تحليل تسلسل 18S rRNA باستخدام برايمر خاص لداء المشعرات ITS1-5.8S rRNA-ITS2 من خلال اختبار السلسة البولميرية التقليدي. لوحظت العلامات السريرية النموذجية لداء المشعرات في العينة المعطاة المكونة من خمسين حمامة وبنسبة ١٠٠٪ (العدد = ٥٠/٥٠). علاوة على ذلك، أن الفحص المجهري اظهر تكرار حدوث المرض بين الحمام بنسبة ١٠٠٪ (ن = ٥٠/٥٠). من ناحية أخرى، أظهر التصنيف الجزيئي الذي تم إجراؤه على ١٠ من كل ٥٠ طير أن العامل المسبب لداء المشعرات هو المشعرة الطيرية مع تكرار حدوث (ن=١٠/١٠). تم إيداع تسلسل النوكليوتيدات ITS1-5.8S rRNA-ITS2 للسلالات العشر من المشعرة الطيرية في بنك الجينات تحت أرقام الانضمام التالية: OM367997وOM457006 و OM457005 و.OM456998 من شأن البيانات الحالية أن تدعم الحاجة لتطبيق معايير وقائية عالية ضد داء المشعرات للطيور من أجل تحسين السيطرة على داء المشعرات.