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Original article

PREVALENCE OF *TRICHOMONAS GALLINAE* WITH MOLECULAR CHARACTERISATION AND PHYLOGENETIC ANALYSIS IN DOMESTIC PIGEONS USING ITS1-5.8s rRNA-ITS2 GENE IN ALBORZ PROVINCE, IRAN

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Summary

Ayati, R., N. Taiefi Nasr-Abadi, Z. Momeni & S. S. R. Shojaei, 2023. Prevalence of *Trichomonas gallinae* with molecular characterisation and phylogenetic analysis in domestic pigeons using ITS1-5.8s rRNA-ITS2 gene in Alborz province, Iran. *Bulg. J. Vet. Med.,* **26**, No 1, 65–72.

Avian trichomonosis is an important parasitic disease throughout the world caused by the protozoan *Trichomonas gallinae*, commonly seen in pigeons and wild birds. Lesions in the upper gastrointestinal tract (beak and crop) are complications of this disease. Currently, the diagnosis of this organism is made using laboratory methods including direct smear, culture medium and molecular methods. The aim of the present study was to survey the prevalence of *T. gallinae* in pigeons of Alborz province, Iran through molecular and phylogenetic identification using ITS1-5.8s rRNA-ITS2 gene. A total of 87 samples were collected from domestic pigeons from May to September 2019. The samples were taken directly from the mouth and larynx using an oral swab. Out of 87 collected samples, 28 (32.18 %) were positive using direct smear, culture and polymerase chain reaction (PCR) methods. Based on the results, the size of the amplification product of this gene was 372 base pairs. The results of this study were analysed using a phylogenetic tree and Neighbour-Joining (NJ) method. The present study showed high prevalence of *T. gallinae* in pigeons. Two types of *T. gallinae* genotypes, A and B, were found in pigeons. Also, the phylogenetic analysis of ITS1-5.8s rRNA-ITS2 sequences from positive samples, showed high coverage with sequences present in the GenBank.

Key words: Alborz province, ITS1-5.8s rRNA-ITS2 gene, phylogenetic analysis, prevalence, *Trichomonas gallinae*

INTRODUCTION

Trichomonas gallinae is an amitochondrial anaerobe which is considered a flagellated protozoan (Levine, 1985). *T. gallinae* infects mainly digestive and respiratory systems of birds, especially pigeons, and is spread globally (Forrester & Foster, 2008; Amin *et al*., 2012). This disease among pigeons is known as canker. The formation of necrotic lesions and cheese-like injuries and inflammations in the

mouth prevent swallowing and causes respiratory failure, which leads to death (Stockdale *et al*., 2015; Fadhil & Faraj, 2019). Pathogenesis in birds depends on parasite strains; the symptoms of this disease include yellowish-green foetid discharge from the mouth, diarrhoea, emaciation, severe weakness and death (Sansano-Maestre & Garijo-Toledo, 2009). *T. gallinae* is transmitted between birds through feeding squabs by parents, consuming infected water and food, mating behaviours and bathing in polluted water, and among birds of prey and carnivorous birds – by consuming infected birds (Stabler, 1954; Lemahieu & Dhond, 1977). Diagnosis of the organism is possible on the basis of lesions, clinical symptoms, necropsy, microscopy of direct smear of the parasite, inoculation in parasite-specific culture media and molecular methods (Levi *et al*., 1977; Fouts & Kraus, 1980). Usually, PCR and PCR-related methods are considered sensitive and reliable methods for genetic studies in microorganisms' molecular epidemiology. In microorganisms, the rRNA gene cluster includes consecutive repetitions of three exon regions: 5.8s, 18s, and 28s. The two intron regions of ITS and IGS give useful information for taxonomy phylogenetic descriptions of genetic diversity in the *Trichomonadidae* family (Hillis & Dixon, 1991; Dimasuay & Rivera, 2013).

Genotypes of *T. gallinae* have been identified worldwide, such as genotype A and B in China (Feng *et al*., 2018), genotype A in Spain (Sansano-Maestre & Garijo-Toledo, 2009), genotype B in Germany (Stenkat *et al*., 2013). In Iran, *T. gallinae* genotypes A and B were recognised in Tehran province (Arabkhazaeli *et al*., 2020). Several studies have been performed regarding trichomoniasis in Iran, but few attempts have been made for genetic characterisation of this parasite.

The importance of trichomoniasis and the lack of previous comprehensive studies concerning phylogenetic distribution and diversity motivated the necessity of conducting such a study in Alborz province. Therefore, the present study aimed to survey the prevalence and genotypes of *T. gallinae* in Alborz, Iran using ITS1-5.8s rRNA-ITS2 gene.

MATERIALS AND METHODS

Samples collection

A total of 87 samples from domestic pigeons in Alborz Province, Iran, were collected directly from the mouth and larynx using oral swabs from May to September 2019. The samples were transferred to the Parasitology Lab of Karaj Azad University, Faculty of Veterinary Medicine and examined through the direct smear method.

Culture medium

Samples of *T. gallinae* were cultured in 15 mL Diamond's (TYM) medium (Narcisi *et al*., 1991) with 10% inactivated foetal bovine serum, antibiotics (100 µg/mL ceftriaxone and 50 µg/mL ciprofloxacin) and fungicides (2.5 µg/mL amphotericin B) for three days at 37 ^{o}C (Levi *et al*., 1977). After 72 hours of growth and reproduction, the culture media were examined under a microscope. The sediment containing parasites was then used for DNA extraction.

DNA extraction

The culture medium was washed three times in PBS solution, then centrifuged at 9000 rpm for 2 min. To extract samples, DNA extraction kit of blood and tissue

Table 1. The nucleotide sequence of primers targeting ITS1-5.8s rRNA-ITS2 gene of *Trichomonas gallinae*

Gene	Primer sequence 5′-3′	Product size	Annealing temperature
ITS1-5.8s rRNA-ITS2	F: TGC TTC AGT TCA GCG GGT CTT CC	372 bp	60 °C
	R: CGG TAG GTG AAC CTG CCG TTG G		

(Sinnagene Company, Iran) was used. Samples were stored frozen at –20 °C until analysis.

PCR

The ITS1-5.8s rRNA-ITS2 gene was selected as the target sequence for DNA amplification with primer pairs shown in Table 1 (Felleisen, 1977). The size of amplified ITS1-5.8s rRNA-ITS2 products was 372 base pairs. The PCR was performed as described, with minor modifications as explained below. The 25 µL reaction mixture contained 5 µL pattern DNA, 12.5 Master Mix (manufactured in Sinnagene Company), 1 µL TFR1 primer; 1 µL TFR2 primer and 5.5 µL deionised water. PCR began with initial denaturation for 10 min at 95 °C, then followed 35 cycles, including 30 sec at 95 °C, 30 sec at 60 °C, and 1 min at 72 °C. The final extension was done for 10 min at 72 °C. Amplification products were analysed by electrophoresis through 1.5% (w/v) agarose gel in 1× TBE buffer.

Sequence analysis

A part of the positive sample PCR product was sent to confirm molecular identification accuracy for gene sequencing to Pishgam Company, Iran. The sequences were edited and aligned using ClustalW and compared with reference sequences from GenBank. The ITS1-5.8s rRNA-ITS2 gene region sequences were analysed using MEGA software v. 7.0 and the obtained data were compared with GenBank sequences. Local bootstrap probability was calculated from 1,000 replications.

RESULTS

Prevalence of T. gallinae in pigeons

In this study, 28 out of the 87 *T. gallinae* samples analysed were positive to culture in TYM medium and PCR, demonstrating the prevalence of this protozoan in 32.18% of domestic pigeons. All of the 28 *T. gallinae*-positive samples detected by microscopic examination were confirmed as positive by the PCR assay. Furthermore, PCR amplification using primers TFR1 and TFR2 successfully amplified the ITS1-5.8s rRNA-ITS2 sequences of all isolates as shown by the band size of approximately 372 bp (Fig. 1).

Phylogenetic analyses of ITS1/5.8S/ITS2 sequences

To evaluate the genetic diversity among *T. gallinae* isolates in this study, multiple alignments were performed with the isolates previously registered in the GenBank. Multiple sequences alignment of the ITS1-5.8s rRNA-ITS2 gene showed five variable nucleotides sites at positions of 46, 108, 124, 231 and 246 (Fig. 2). Analysis of the complete 5.8S rRNA gene and its two flanking ITS1 and ITS2 se-

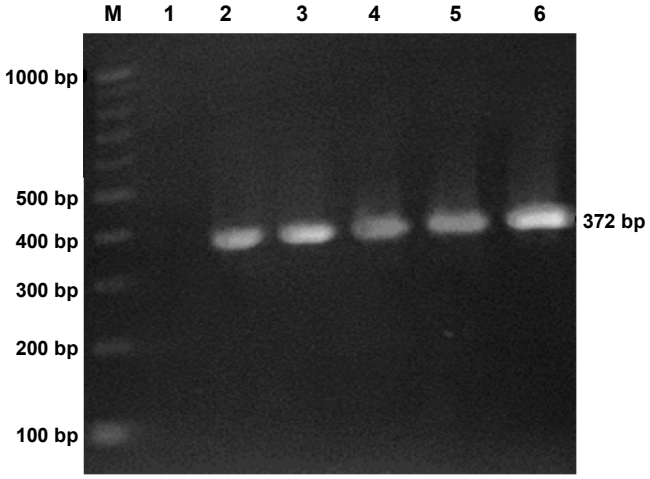

Fig. 1. PCR products targeting the ITS1-5.8s rRNA-ITS2 gene (372 bp) for *T. gallinae* in 1.5% agarose gel. M: 100 bp DNA marker; lane 1: negative control; lanes 2–6: *T. gallinae* positive samples.

quence data specified two *T. gallinae* genotypes. Genotypes A and B of *T. gallinae* were identified by sequencing and the phylogenetic tree was constructed, showing that the I ITS1/5.8S/ITS2 gene isolated from samples had a high coverage to the sequences in the GenBank (Fig. 3). The obtained nucleotide sequence from the ITS1-5.8s rRNA-ITS2 gene was sent and registered for five isolates in GenBank with accession numbers MT133584, MT133574, MT133573, MT133881, and MT133800.

DISCUSSION

Avian trichomoniasis is an important parasitic disease caused by the protozoan *T. gallinae,* which causes granulomatous lesions, oesophageal obstruction, and death (Mesa *et al*., 1961; Narcisi *et al*., 1991). The variable ITS region sequences have useful information for phylogenetic classification. They are much less conserved than the actual genes, making them ideal candidates for intraspecies and intragenus comparisons (Commar *et al*., 2007). According to reports, the prevalence of *T. gallinae* in pigeons is 37% to 85% in Iran (Borji *et al*., 2011). The present study has reported a prevalence of this parasite of 32.18% e.g. similar to the values found in Spain (Villanua *et al*., 2006; Sansano-Maestre & Garijo-Toledo, 2009).

Other countries reported a prevalence of *T. gallinae* from 5.5% to 95% (Sansano-Maestre & Garijo-Toledo, 2009). Weather changes, geography, the season, resistance, nutrition, age, sampling, or pigeons' sexual cycle may explain the differences in this disease's prevalence (Saleem *et al*., 2008). The alignment of five sequences from this study with other isolates registered in GenBank revealed several nucleotide differences in ITS regions from 5.8s rRNA gene, which was less conserved, demonstrating the high homology of this gene with other existing sequences. In order to investigate with which of the registered sequences in Gen-

Fig 2. Multiple sequences alignment of the ITS1-5.8s rRNA-ITS2 gene of the *T.gallinae* in this study compared with the reference sequence (No. MH733822) in GenBank.

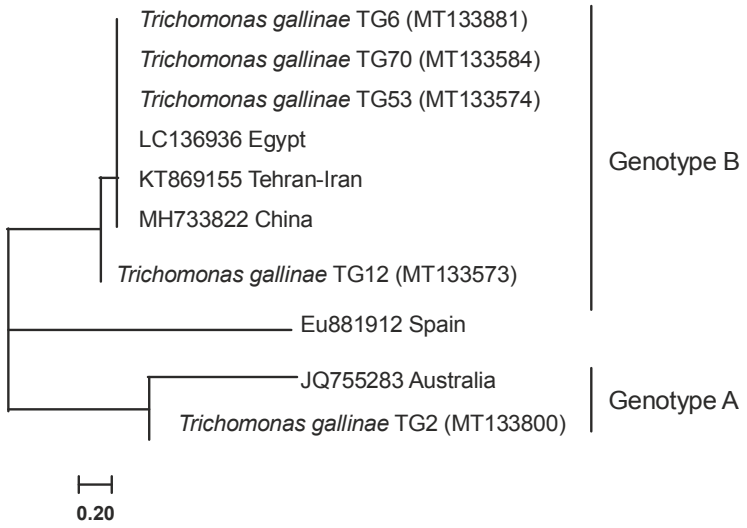

Fig. 3. Phylogenetic tree for *T. gallinae* isolates ITS1-5.8s rRNA-ITS2 compared to isolates from other parts of the world. The neighbour-joining algorithm was conducted using bootstrapping 1000.

Bank the studied isolates had more similarity, the above sequences were aligned with standard sequences existing in GenBank. According to the phylogenetic tree, this study showed two genotypes: A and B. On the basis of the drawn phylogenetic tree, four isolates had 98% similarity with each other, and only the MT133880 isolate was different from other isolates.

Regarding the similarity of isolates with registered isolates in GenBank, the highest similarity was found out with LC136936 (Egypt), T869155 (Tehran-Iran), MH733822 (China) and EU881912 (Spain) isolates from genotype B and the most significant difference – with JQ755283 (Australia) isolate which was of genotype A. Mutation or environmental factors may cause the difference between separated sequences and existing GenBank sequences. Their similarities show that all of them have one single origin. Therefore, separate sequences in this study showed a similar single-nucleotide polymorphism in the ITS region with other registered sequences in GenBank. The present study performed molecular characterisation and phylogenetic analysis to diagnose *T. gallinae* infection in domestic pigeons, based on the ITS sequence as a genetic marker. Therefore, it is suggested to compare sequences from other genome-related regions to isolates in this study to identify phylogenetic similarity and differences in other isolates. Also, a molecular epidemiology survey in other areas of Iran is recommended to identify genetic diversity in *T. gallinae* isolates.

CONCLUSIONS

In conclusion, the present survey is the first to report the prevalence and genotypes of *Trichomonas gallinae* in domestic pigeons in Alborz, Iran. The prevalence of infection was 32.18 %, and two genotypes (A and B) were found for *T. gallinae* among domestic pigeons.

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