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RESEARCH PAPER

Molecular Prevalence of Three Chicken Gastrointestinal Tract Pathogens and Phylogenetic Characterization of *Tetratrichomonas gallinarum* in Türkiye

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Abstract

Several species of parasites infect chickens and can cause economic losses. *Histomonas meleagridis*, its intermediate host *Heterakis gallinarum*, and *Tetratrichomonas gallinarum* are the most common parasites of poultry. The current study aimed to determine the molecular prevalence and phylogenetic relationships of *H. meleagridis*, *He. gallinarum*, and *T. gallinarum* in chickens in Central Anatolia Region of Türkiye. For this, a total of 100 fecal specimens from chickens were analyzed by using a PCR assay targeting the 18S rRNA gene region of *H. meleagridis* and *T. gallinarum* and the mtDNA COI gene region of *He. gallinarum*. PCR analysis identified *T. gallinarum* in 11 (1.1%) specimens, whereas other species were not found in all the examined specimens. Positive specimens were detected only in backyard chickens. Sequence analyses identified two novel genotypes named TRTgal1 and TRTgal3, and one known genotype (TRTgal2) of *T. gallinarum*. The TRTgal2 genotype was found to be identical to the human genotype (AY247746) and the four avian genotypes. Phylogenetic analyses of the obtained genotypes clustered in separate monophyletic clades. Our study provides the first data on *T. gallinarum* in chickens in Türkiye and contributes to public health with the identified zoonotic genotype and knowledge of the epidemiology of this parasite.

Introduction

Poultry plays an important role in meeting animal protein needs, with short production cycles and low ecological requirements (Marangon and Busani, 2007). The poultry breeding is faced with diseases such as necrotic enteritis, coccidiosis and avian influenza that cause adverse economic effects. (Galarneau et al., 2020). Histomoniasis, mostly known as blackhead disease, caused by *Histomonas meleagridis*, represented by losses in turkeys with pathological lesions in the caeca and liver (Grabensteiner and Hess, 2006). In chickens, histomoniasis lesions are often confined to the caeca and associated with less severe production losses and some mortality (Esquenet et al., 2003). Hu et al. (2006) reported that the transmission dynamics of histomoniasis in chickens differ from

turkeys. In general, infections are transmitted by intermediate hosts such as mainly *Heterakis gallinarum*, an enteric nematode parasite that primarily affects turkeys and chickens. However, some reports suggest that transmission also occurs without vectors (Hu and McDougald, 2003). *Heterakis* worms are small, white, and thin, and found mostly in the ceca of birds, and several species infect poultry (McDougald, 2020). *Heterakis gallinarum* infections may cause weight loss in heavy infections, like enteric nematodosis. Moreover, the damage of *He. gallinarum* infection may increase depending on co-infection with histomoniasis (Cupo and Beckstead, 2019). *Tetratrichomonas gallinarum* is a widespread enteric flagellate that can colonize the digestive tract of galliform and anseriform

birds (Cepicka et al. 2005). The pathogenicity of this species is controversial (Amin et al., 2011). *T. gallinarum* is considered nonpathogenic but diarrhea and inflammation of the caecum have been reported in chukar partridges (Wichmann and Bankowski, 1956), mockingbird (Patton and Patton S, 1996), white pelican (Burns et al., 2013), and chickens (Landman et al., 2016). The first infection with *T. gallinarum* was described in chickens in 1911 (Martin and Robertson, 1911). Therewithal, *T. gallinarum*-like organisms were isolated from human patients in 2005 (Cepicka et al., 2005).

Molecular methods such as polymerase chain reaction and DNA sequencing have been successfully used for species identification and phylogenetic relationships (Cepicka et al., 2010). Different gene regions such as ITS1/5.8S/ITS2, 18S rRNA, and protein-coding genes are commonly used to describe the phylogenetic relationships of the species (Grabensteiner et al., 2010). Analysis of 18S rRNA gene is a good marker for species identification and characterization of trichomonads (Delgado-Viscogliosi, et al., 2000; Dimasuay and Rivera, 2013).

Studies on the current epidemiological status and molecular characterization of these three species in Türkiye are limited. Therefore, we aimed to determine the molecular prevalence, phylogenetic characterization of *H. meleagridis*, *He. gallinarum*, and *T. gallinarum* in backyard chickens and laying hens in Kirsehir province, Türkiye.

Materials and Methods

Collection of fecal samples

A total of 100 fresh fecal droppings from chickens, including 80 backyard and 20 laying hens, were collected, and placed in plastic individual containers, and labeled. Ethics approval was not required. All samples were stored at -20 °C until DNA extraction.

DNA extraction and PCR amplification

Genomic DNA (gDNA) extraction was carried out from each fecal sample by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The final nucleic acids were precipitated in a 50 µL elution buffer and stored at -20 °C. The DNA quality of each isolate was assessed by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Waltham, USA). All gDNA samples were examined with PCR analyses to determine the *H. meleagridis* and *T. gallinarum* by amplifying the partial sequence of the 18S rRNA gene region and *He. gallinarum* mtDNA COI gene region. Primer pairs used in PCR analyses are shown in Table 1. A total of ~25 ng of gDNA of each sample was included in the ready to use PCR Master Mix (Dream Taq Hot Start Green PCR Master Mix, Thermo Scientific, USA) in a final reaction

volume of 25 µL. The same cycling conditions for the PCR analyses were used as the references given in Table 1. The PCR amplifications were performed in a Sensoquest Labcycler (SensoQuest, Germany). Positive controls of each species and nuclease-free water as a negative control were used in all analyses. PCR products were electrophoresed on 1.5% agarose gel and visualized using the gel documentation system (Fig. 1).

Sequencing and phylogenetic analysis

All PCR positive amplicons were purified by using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced in both directions via Sanger sequencing (BMLabosis, Ankara, Türkiye). The raw sequence reads (~500 bp) were checked, aligned and assembled using Geneious Prime 2020.0.3 (<https://www.geneious.com>) software to create consensus sequences. Final sequences (469-490 bp) were compared with reference sequences in the GenBank database by BLAST analyses (Fig. 2). The phylogenetic analyses of the identified genotypes were performed by the Maximum Likelihood (ML) method with the HKY genetic distances model in MEGA X (Kumar et al., 2018). The best DNA model was determined according to the Akaike Information Criterion (AIC) algorithm by using jModeltest v.0.1.1. (Posada, 2008). Branch support was assessed with 1000 bootstrap replicates. For phylogenetic analyzes, the twenty-four *T. gallinarum* isolates from China, Philippines, France, Austria, Czechia, and Germany that had been found from a variety of hosts, including chickens, humans, and lemur, and had been registered in the Genbank database were employed. *Tetratrichomonas gallinarum* 18S rRNA gene region partial sequences obtained in the study were deposited in the GenBank database under accession numbers OP379566-68.

Results

Molecular prevalence of *H. meleagridis*, *He. gallinarum* and *T. gallinarum* in chickens

In the present study, 100 specimens were examined. *Tetratrichomonas gallinarum*-positive samples were found in 11 (1.1%), whereas *H. meleagridis* and *He. gallinarum* were not detected. All positive specimens were collected from backyard chickens.

Nucleotide Sequence and phylogenetic analyses of *T. gallinarum*

Owing to sequence analysis of the partial 18S rRNA region of 11 *T. gallinarum* isolates, two novel genotypes (TRTgal1 and TRTgal3) and one (named as TRTgal2 by us) known genotypes were characterized.

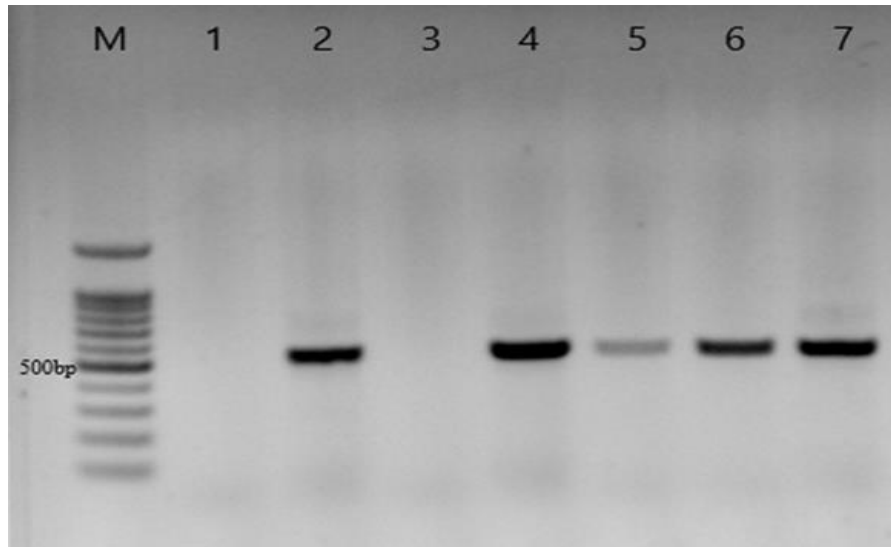


Figure 1. Some *T. gallinarum* positive samples on agarose gel. M: Marker (100bp), 4-7: Positive samples, 3: Negative samples, 2: Positive control, 1: Negative control

Table 1. Primer pairs were used in this study.

Species	Primers	Sequences	Amplicon size (bp)	References
<i>H. meleagridis</i>	Hmf	5'-GAAAGCATCTATCAAGTGGAA-3'	574	Grabensteiner and Hess, 2006
	Hmr	5'-GATCTTTTCAAATTAGCTTTAAA-3'		
<i>He. gallinarum</i>	P1	5'- ATATCCTCTAGGTGGTAAGCTCTTG-3'	696	Gu et al. 2018
	P2	5'-AGCCCCAAATAAAAACCTTCAAACACAC-3'		
<i>T. gallinarum</i>	Tgf	5'-GCAATTGTTTCTCCAGAAGTG-3'	526	Grabensteiner and Hess, 2006
	Tgr	5'-GATGGCTCTCTTTGAGCTTG-3'		

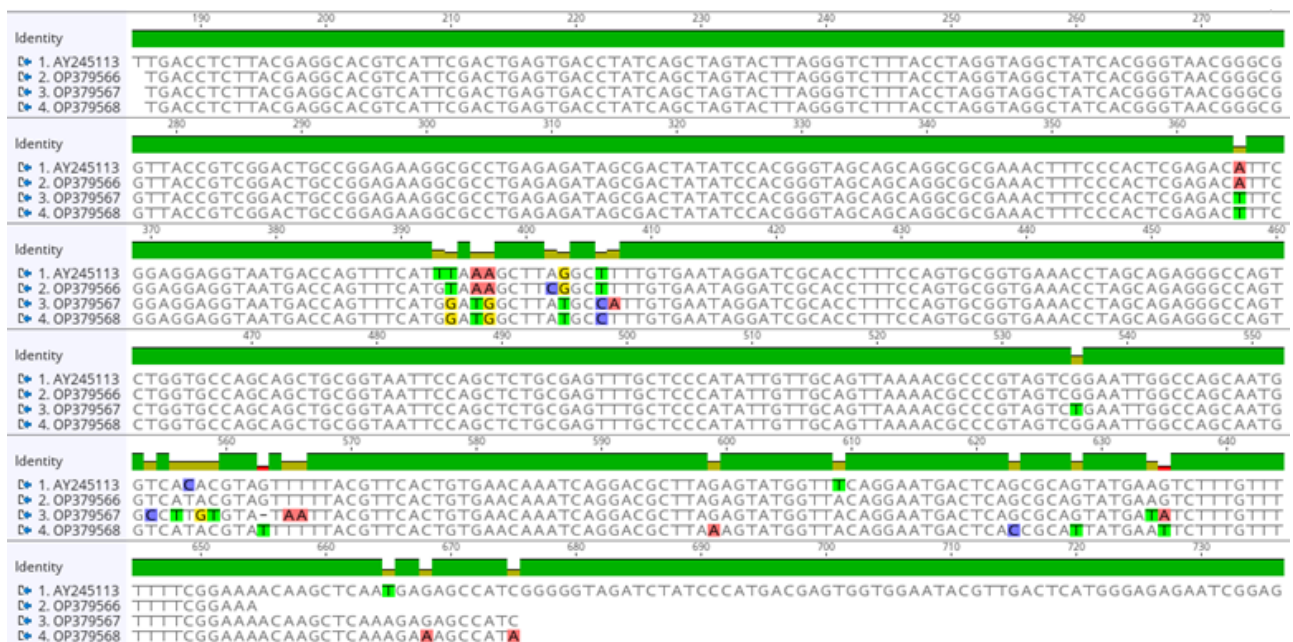


Figure 2. Sequence variation in the 18S rRNA region of *T. gallinarum* isolates. Polymorphic sites are represented by color

The identified genotypes contain 12-17 variable sites among them. The TRTgal2 (OP379567) isolate was found to be identical to isolates identified from duck (JX565083, JX565081), human (AY247746), turkey (AJ920324) and swan (AY245111). The molecular prevalence and identified genotypes with GenBank accession number are presented in Table 2.

Phylogenetic analyses of *T. gallinarum* 18S rRNA gene region clearly separated all the isolates in two monophyletic clades (53%). The first clade comprised of the genotypes from different hosts and countries while the second clade comprised of mainly chicken genotypes is shown in Fig. 3.

Table 2. Molecular prevalence, identified genotypes and GenBank accession number of *T. gallinarum*

<i>Tetratrichomonas gallinarum</i>					
Location	No. of samples	No. of positive	Prevalence (%)	Identified genotypes	GenBank Acc. No.
Kirsehir	100	11	11	TRTgal1 (n=4)	OP379566
				TRTgal2 (n=3)	OP379567
				TRTgal3 (n=4)	OP379568



Figure 3. Phylogenetic relationships between *T. gallinarum* genotypes obtained in this study (in red) and known genotypes previously reported from different countries and hosts. The tree was constructed using Maximum Likelihood analyses of 18S rRNA gene partial sequences. Numbers at the branches indicate bootstrap values (1000 replicates). The *Blastocystis* sp. genotype identified from chicken (MW093219) is used as an out group

In phylogenetic analysis, the TRTgal1 and TRTgal3 genotypes differed from the subgroup E (AY245113) in 4 and 17 nucleotides, respectively, and TRTgal2 genotype is identical to subgroup B1 (AY245111) of *T. gallinarum* reported by Cepicka et al. (2005).

Discussion

Backyard chickens are often found in outdoors where biosecurity is poor, and they have close contact with each other, wild birds, other animals (especially turkeys), and humans. This makes them susceptible to sources of infection. In this study, the molecular prevalence of *H. meleagridis*, *He. gallinarum*, and *T. gallinarum* was investigated in backyard chickens and laying hens. We were unable to detect *H. meleagridis* and its intermediate host *He. gallinarum* in any of the specimens. In contrast, some reports of histomoniasis in chickens have been published in different countries. (Patra et al., 2013; van der Heijden and Landman 2011; Grafl et al., 2011; Nguyen et al., 2015). Das et al. (2021) reported in an experimental study that an average of one hundred histomonads were excreted from 1 g of feces and three to four thousand histomonads were excreted within 24 hours from chickens infected with *H. meleagridis*. In the same study, the average daily egg excretion varied from five hundred to two thousand from a bird infected with *He. gallinarum*. Grabensteiner and Hess (2006) investigated PCR specificity for identification of *H. meleagridis* and *T. gallinarum* in tissue samples from infected birds and reported that PCR assays (using the same primers as in our study) demonstrated to be a useful and sensitive diagnostic tool for identification of *H. meleagridis* and *T. gallinarum*. In this context, it can be presumed that the studied chickens were not infected with *H. meleagridis* and *He. gallinarum*.

Cepicka et al. (2005) analyzed 29 isolates of *T. gallinarum* sequences and divided them into five groups (A-E) with eleven subgroups and suggested that groups A and B predominate in domestic fowls. In addition, human tetratrachomonads were assigned to groups A and B, hypothesizing that infections in humans are transmitted by avian genotypes or host-specific genotypes. In this study, the TRTgal2 genotype (OP379567) was found to be identical to the human genotype (AY247746) and the avian genotypes including the group B. In this perspective, it can be suggested that human tetratrachomoniasis was originate from avian genotypes. However, this assumption needs to be supported by the detection of more common genotypes. The TRTgal1 and TRTgal3 genotypes were exhibited highest identities of 96.9% to 99.1% to the genotypes reported in chicken in Czechia, China, Iran, and Philippines. This similarity is probably due to high gene flow with migratory hosts or

neglected submission of sequences to genetic databases and low sampling. Novel genotypes are likely to be identified with studies that include new and large numbers of samples.

In conclusion, our results contribute to the molecular characterization and transmission dynamics of *T. gallinarum*. Moreover, to our knowledge, this is the first report on molecular characterization of *T. gallinarum* genotypes in chickens in Türkiye. Considering the zoonotic potential of *T. gallinarum*, chickens could pose a risk for public health. To better understand the molecular epidemiology, zoonotic potential, and transmission dynamics of *T. gallinarum*, further studies in a larger number of species with large-scale sampling are required.

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