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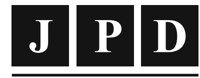
ORIGINAL ARTICLE



Occurrence of *Tetratrichomonas gallinarum* (Trichomonadida: Trichomonadidae) in chicken feces from Lorestan Province, Western Iran

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Abstract Species of *Tetratrichomonas gallinarum* and *Trichomonas gallinae*, are found in the digestive tract of birds. To investigate trichomonads of a lower intestinal tract of native chickens, we examined 72 feces samples from the different life stages of fowl species in Khorramabad County, Lorestan Province, Western Iran. Using microscopical examination, out of 72 collected samples, 26 (31.6%) bird feces samples were found infected with a trichomonad similar to *T. gallinarum*. A 527-bp fragment of 18S rRNA gene of *T. gallinarum* was amplified by PCR. The 18S constructed phylogeny indicates two different clades within *T. gallinarum,* suggesting that commensal *T. gallinarum*-like of the bird hosts may be the reason for the genetic divergence in *T. gallinarum* species complex.

Keywords Trichomonad · Chicken · Bird's feces · PCR · Phylogenetic tree · Iran

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Introduction

The family Trichomonadidae (Parabasalia: Trichomonadea: Trichomonadida), are divided into several genera based on the number of their anterior flagella such as *Trichomonas*, *Tetratrichomonas* and *Pentatrichomonas* which have 3, 4 and 5 anterior flagella, respectively (Cepicka et al. 2010). More than 100 species of trichomonad have been reported; most of them living within internal organs of birds and mammals as the commensal organisms (Crucitti 2013). Totally, two species can colonize within the alimentary tract of domestic birds so-called trichomonads, including *Tetratrichomonas gallinarum* and *Trichomonas gallinae* (Amin et al. 2014a). Two *Trichomonas* and *Tetratrichomonas* are closely related genera cluster with each other, phylogenetically (Viscogliosi et al. 1993). Species of both genera, including *T. gallinarum* and *T. gallinae*, are found in the digestive tract of birds (Hess and McDougald 2013). *Tetratrichomonas gallinarum* (Martin and Robertson 1911) (= *T. pullorum*, *T. gallinarum*) with a worldwide distribution can infect a large variety of gallinaceous birds, such as chicken, turkey, guinea fowl, quail, and chukar partridge (BonDurant and Honigberg 1994). This flagellate protozoa has been described from the intestine, ceca, and cloaca of fowls (Pearl et al. 1915). Generally, *T. gallinarum* is considered to be non-pathogenic. Although an enterohepatitis with liver lesions have been ascribed to this organism, no confirmation of pathogenicity has come from experimental infection (Hess and McDougald 2013; Seneviratna 1969). Diarrhoeas and typhlitis, an inflammation of the caecum may be seen in bird species due to infections with *T. gallinarum* (Mehlhorn 2016; Richter et al. 2010). In this regard, Liebhart et al. (2014) reported a circulation of a virulent *T. gallinarum* strain in reared red-legged

partridges. Some *T. gallinarum*-like organisms from the oral cavity and lower respiratory tract of human patients have been isolated (Cepicka et al. 2005). Infection of bird is acquired by ingestion of trichomonads in contaminated feed or water of bird host (Kaufmann 1996). Infected birds excrete live parasites 2 days after infection (Amin et al. 2014b). Transmission of low/none pathogenic *T. gallinarum* from infected to in-contact birds has been shown as the rapid transmission route of *T. gallinarum* resulting in the establishment of a latent infection (Amin et al. 2011). The aim of the present study is to identify *T. gallinarum* using traditional methods (wet mount, staining, and culture) and to confirm species identification using PCR and sequencing of 18S ribosomal RNA fragment in Lorestan Province, Western Iran.

Materials and methods

Sampling

The present study was conducted at 10 different farms in Khorramabad County, Lorestan Province, Western Iran (Fig. 1) in April to October of 2017. During the warm months (late spring, whole summer, and earlier autumn), 72 feces samples originating from different life stages of native egg fowl, *Gallus gallus* (native Lori race) including hen, rooster, and chicken were collected (Table 1). The breeding system of poultries was restricted to free organic type in farms around the human buildings. Collection was done by aid a pair tongue depressors. According to the importance and accessibility of different life stages of chickens, 57, 13, and 2 cases of hen, rooster, and chicken were examined in terms of excreted feces, respectively. From every farm, up to 10 birds were included in the present study. To the viability of trophozoites within chicken's feces, the samples were transported to the laboratory at an optimum temperature (37.5 °C) without any specialized processing.

Wet mount, staining, and culture method

Wet mount smears were prepared and examined under a light microscope (Olympus CX21, Japan) for detection of positive samples via trichomonad motility observation. Next individual positive samples was used for culturing of flagellate organisms. Trichomonads were cultured in a modified RPMI medium. Almost 1 g of stool sample was mixed in 10 ml of warm physiological saline, and after passing through the 400 lm sieve centrifuged in 3000*g*, the supernatant was discarded, and the sediment was incubated to 5 ml of RPMI-1640 medium supplemented with inactivated horse serum and an amount of starch without the use of antibiotics at 38 °C, so it was investigated using the microscope for three consecutive days at 24, 48, and 72 h. Trichomonads were subcultured every second or third days if needed. Finally, for rudimentary morphological detection, dry mount and methanol fixed smears were stained by 20% Giemsa for flagellum number counting of trichomonads. Since at least two closely related and almost morphologically indistinguishable species of *T. gallinae* and *T. gallinarum* are present in the gastrointestinal tract of birds, we examined samples for PCR to confirm trichomonad species identification.

Fig. 1 Map of Khorramabad County as the study area located in Lorestan Province, Western Iran

Table 1 Origin of feces samples from different localities in Khorramabad County

Locality	Host (different sexual stages)			Coordinates (N/E)
	Hen	Rooster	Chicken	
Badrabad	1		2	33°25′35.96″ 48°16′52.73″
Ghaleh Sangi	3	1	1	33°33′41.68″ 48°17′53.86″
Gile Varan	8			33°27′46.60″ 48°18′9.36″
Kahriz	8		2	33°28′36.74″ 48°23′52.36″
Kamalvand-e Parvaneh	4		2	33°28′55.79″ 48°25′52.33″
Miangelal	7			33°25′59.99″ 48°17′59.34″
Robat-e Namaki	4		1	33°36′28.51″ 48°18′13.20″
Sarab-e Yas	9			33°25′51.15″ 48°20′38.08″
Sarcheshmeh-e Kamalvand	7		3	33°28′41.26″ 48°26′34.35″
Telori-e Soflá	6	1	2	33°25′21.17″ 48°18′54.79″
Total	57	2	13	

PCR amplification and sequencing

DNA was extracted using phenol–chloroform according to our previous laboratory protocol setup by Hosseini-Chegeni et al. (2017). Primer pairs Tgf: 3′- GCA ATT GTT TCT CCA GAA GTG -5′ and Tgr: 3′- GAT GGC TCT CTT TGA GCT TG -5′ for 18S rRNA gene of *T. gallinarum* (Grabensteiner and Hess 2006) and TrichhydF: 3′- GTT TGG GAT GGC CTC AGA AT -5′ and TrichhydR: 3′- AGC CGA AGA TGA TGA CGA AT -5′ for hydrogenosomal Fe-hydrogenase gene of *T. gallinae* (Lawson et al. 2011) were carried out in PCR reactions. PCR performed using a thermal cycler (Corbett®, Australia) based on a touchdown temperature profile by 3 min at 94 °C, 11 X (45 s at 94 °C, 50 s at 60–50 °C, 60 s at 72 °C), followed by 24 X (45 s at 94 °C, 50 s at 45 °C, 60 s at 72 °C), and 3 min at 72 °C. PCR for each 25 µl final volume reaction was done using 12.5 µl RedMaster PCR 2X (Sinaclon®, Iran), 1 µl from each forward and reverse primers (10 pM), 4 µl gDNA template (100 ng/µl), and 6.5 µl sterilized distilled water. The negative and positive PCR controls were reaction tubes without sample (H_2O) and a *T. gallinarum* DNA sample that was sequenced in this study (MG022093). The PCR products were visualized by 1% agarose gel electrophoresis and the desired bands were purified using GF-1 Gel DNA Recovery Kit (Vivantis®, Malaysia). Finally, the purified PCR products were submitted to a third-party service provider for sequencing (Faza-Biotech® Inc., Iran).

Phylogenetic analysis

A sequence of this study was manually checked using FinchTV® software (www.geospiza.com) to correct any sources of error or ambiguities if present. Homologies with the available sequence data in GenBank were checked using BLAST analysis. Finally, the sequence was submitted to GenBank and an accession number was assigned. In the next step, the sequence was aligned using SeaView4 software (Gouy et al. 2010), and the genetic distances among the sequences were calculated using Maximum Composite Likelihood (MCL) model in the MEGA7 software (Kumar et al. 2016). Afterward, in order to construct a phylogenetic tree of *Tetratrichomonas*, the aligned sequences of GenBank and a single sequence of this study were analyzed using BEAST® software (version 2.4.8) (Drummond et al. 2012) based on the Bayesian Inference (BI) method. This method employs Markov Chain Monte Carlo (MCMC) algorithms for Bayesian phylogenetic inference. For this purpose, 14 taxa (including a sequence of the present study as well as the comparable GenBank data sequences as in- and out-group forms were submitted from different areas and hosts of the world) were used for constructing 18S rRNA phylogenetic tree. The constructed clades of phylogenetic trees were arranged and labelled based on posterior probability support value greater than 99% as well as, the genetic distance within- and between the clade members. A *Ditrichomonas* taxon was examined as an out-group in phylogenetic tree.

Results

In this study, out of 72 collected samples, 26 (36.1%) of bird feces were found to be infected with a trichomonad form similar to *T. gallinarum* using microscopical examination (Fig. 2). All these 26 positive cases were grown within the culture medium, successfully.

PCR and phylogenetic tree

In this step, 26 microscopically positive samples were performed for molecular work. A 527-bp fragment of 18S rRNA gene of *T. gallinarum* was amplified by polymerase chain reaction (PCR) but the target gene of *T. gallinae* was not amplified in PCR reactions. Two samples from Telori-e Soflá and Ghaleh Sangi were PCR positive for *T. gallinarum* target 18S rRNA gene fragment. One of three PCR amplified samples was sequenced, successfully. BLAST analysis showed 98–99% similarity between the 18S rRNA sequence of this study with *T. gallinarum* 18S sequences submitted from the Czech Republic (HQ149971, AY245113), Philippines (JX565084). Also, it showed a 95–96% similarity with *T. gallinarum* 18S sequences submitted from Austria (AJ920324, HM162407), Czech Republic (AF124608, AY245106-7, AY245110-1), and Germany (JN619423). Moreover, a sequence of this study showed a 92–93% similarity with *T. gallinarum* 18S sequences deposited in GenBank (EU215372-4, HG008106, KM095107, KM246603-9, KX353936-46, KX514373, KX514379, and KX584001). An accession number (MG022093) was assigned to a sequence of this study by GenBank. A phylogenetic tree was constructed using BEAST software including in-group (sequences included in BLAST analysis) and out-group taxa (Fig. 3). The 18S constructed phylogeny indicated two different clades within *T. gallinarum* sequences deposited in GenBank. No intraspecies variation in terms of genetic distance was found within two clades, although 6% genetic distance difference was found between two clades.

Discussion

Prevalence of *T. gallinarum*

Tetratrichomonas gallinarum is known as one of the most important protozoan organisms colonizing caecum of chickens described by Martin and Robertson in 1911. Order Trichomonadida can be a major agent of infection in poultry in Lorestan Province, Western Iran (Badparva and Kheirandish 2017). The less studied intestinal parasitic birds while, the human parasites studies have been sufficiently studied in Lorestan Province (Badparva et al. 2014a, b). In the present study, *T. gallinarum* was detected in bird feces in western Iran. It was then confirmed with PCR amplification and sequencing of a partial 18S rRNA gene fragment. This work can be considered as the first study in this area. In this study, out of 72 collected samples, 26 (31.6%) bird feces were found infected with a trichomonad form similar to *T. gallinarum* using microscopical examination. However, the pathogenicity of *T. gallinarum*, which is reportedly somewhat controversial, was detected in various bird species. Thus, it has to be considered as an important commensal bird flagellate (Richter et al. 2010; Liebhart et al. 2014; Cepicka et al. 2005; Grabensteiner and Hess 2006; Hess et al. 2006; Dimasuay and Rivera 2013; Hauck et al. 2010). Hauck et al. (2010) showed that *T. gallinarum* has a similar incidence in chicken and turkey flocks. They report that organism was found in 27.4% of investigated samples from

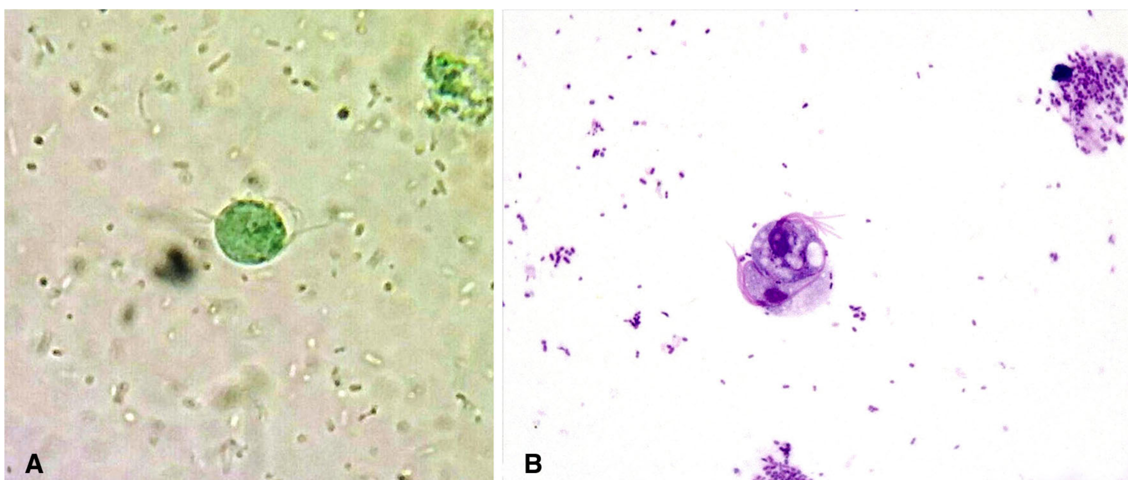

Fig. 2 Light microscopic photo related to an isolated trichomonad from wet mount (**a**) and Giemsa stained (**b**) smear

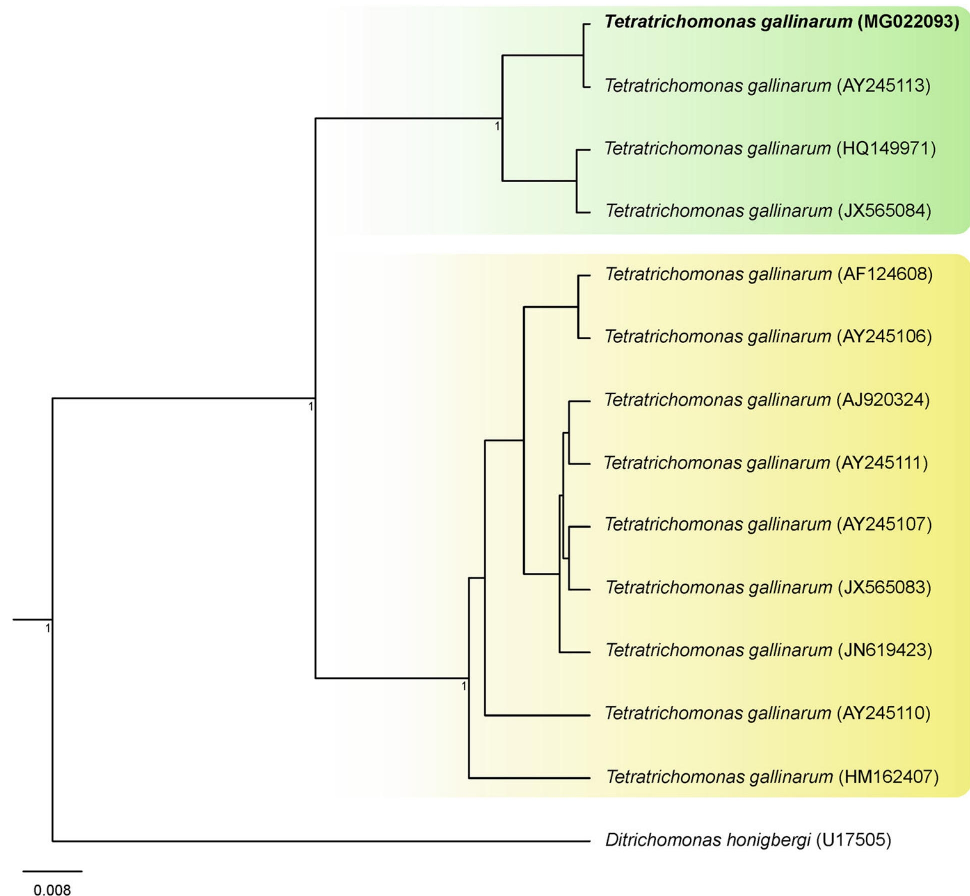

Fig. 3 The phylogenetic relationship of *Tetratrichomonas* taxa derived from analysis 18S rRNA sequence data based on Bayesian Inference (BI); phylogenetic trees indicating two *Tetratrichomonas* clades (yellow and green boxes); numbers below the nodes denote the posterior probability value in BI analyses (1000 replicates). Taxa are arranged as species name following GenBank accession number are given in the parenthesis. Taxon of the present study is denoted as bold. *Ditrichomonas* was examined as an out-group in the phylogenetic tree (color figure online)

flocks. PCR assays were established as a sensitive diagnostic tool for the direct detection and differentiation of *H. meleagridis*, *T. gallinarum,* and *Blastocystis* spp. the nucleic acid in organ samples of infected turkeys and chickens regardless of the geographic origin (Grabensteiner and Hess 2006). Most samples that were considered positive in the culture and by microscopy did not amplify in the PCR. However, PCR is not without limitations, false-negative and false-positive test results can occur. The infection status of the animal feces from which the sample was derived. 30–40 PCR cycles may still not be sufficient to amplify the target DNA. Since, we aimed to differentially diagnosed *T. gallinarum*, *T. gallinae* and *Histomonas meleagridis* (data not shown) by conventional PCR, so universal trichomonad primers were not used.

Phylogenetic consideration

BLAST analysis showed a 98–99% similarity between the sequence of this study with *T. gallinarum* 18S sequences submitted from the Czech Republic and the Philippines and a 95–96% similarity with *T. gallinarum* 18S sequences submitted from Austria, Czech Republic, and Germany. Unlike morphological similarity, the sequence of this study showed a 92–93% similarity with *T. gallinarum* 18S sequences deposited in GenBank. Thus, *T. gallinae* sequence is quite distinct from that of another avian trichomonad, i.e., *T. gallinarum*, in terms of genetic distance (da Silva et al. 2007). In the present study, phylogenetic tree indicated two different clades within *T. gallinarum* sequences with 6% genetic distance difference between two clades. Origin of sequences of one clade (clade comprising a sequence of this study) includes chicken (AY245113, JX565084, and MG022093) and, interestingly, a lemur (HQ149971), as a non-bird host. In addition, sequences of other clade were isolated from duck (AF124608, AY245107, AY245110, HM162407, and JX565083), turkey (AJ920324, AY245106), swan (AY245111), and goose (JN619423). Genetic heterogeneity of *T. gallinarum* found in this study was confirmed by RAPD and sequencing of 16S rRNA, 5.8S rRNA, *ITS1*, and *ITS2* genes (Cepicka et al. 2005). Cepicka et al. (2005) examined polymorphism and genetic relationship among 29 isolates of *T. gallinarum* obtained from eight bird species and five *T. gallinarum*-like isolates from the oral cavity and lower respiratory tract of human patients. Furthermore, a close relationship was found between the human tetratrichomonad strains with common avian

species *T. gallinarum* (Kutisova et al. 2005). However, according to a traditional concept, *T. gallinarum* is specific to avian hosts (Kleina et al. 2004). Various *T. gallinarum* isolates within the avian hosts with the origin of duck and chicken were reported identical with the similarity of 99.9–100% (Dimasuay and Rivera 2013), suggesting that commensal *T. gallinarum*-like the bird hosts may be the reason of genetic divergence in *T. gallinarum* species complex. Further studies, however, are needed to clarify this issue.

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Authors' contribution EB designed the study. SB, AH-C and EB collaborated to the laboratory assays and the manuscript writing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval I hereby declare all ethical standards have been respected in preparation of the submitted article. This research is registered under the approval number A-101378-1 of Lorestan University of Medical Sciences.

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