

MOLECULAR CHARACTERIZATION OF *TETRATRICHOMONAS GALLINARUM* IN DOMESTIC TURKEYS (*MELEAGRIS GALLOPAVO*) IN VAN PROVINCE, TURKEY

Adnan Ayan^{1*}, Ozlem Orunc Kilinc², Jamal Muhammad Khan³, Ali Bilgin Yilmaz⁴, Ayse Sona Karakus⁵, Mohammed Mebarek Bia⁶, Gurkan Akyildiz⁷, Dongmin Lee⁶

¹Department of Genetics, Faculty of Veterinary Medicine, Van Yuzuncu Yil University, Van, Turkey;

²Özalp Vocational School, Van Yuzuncu Yil University, Van, Turkey;

³Department of Parasitology Cholistan University of Veterinary and Animal Sciences Bahawalpur, Pakistan;

⁴School of Health, Van Yuzuncu Yil University, 65080, Van, Turkey;

⁵Department of Parasitology, Faculty of Veterinary Medicine, Van Yuzuncu Yil University, 65080, Van, Turkey;

⁶Department of Parasitology, Parasite Research Center and Parasite Resource Bank, School of Medicine, Chungbuk National University, Korea;

⁷Genkord Health and Biotechnology Services Inc., 34212, Istanbul, Turkey;

*Corresponding Author Adnan AYAN, e-mail: adnanayan@yyu.edu.tr;

Received November 2019; Accepted January 2020; Published March 2020;

DOI: <https://doi.org/10.31407/ijeess10.207>

ABSTRACT

Tetratrichomonas gallinarum is a protozoan parasite of various galliform birds causing a type of enterohepatitis termed tetratrichomoniasis. During the year 2018-2019 an outbreak of unspecified granulomatous diseases, characterized by the sudden deaths of young turkeys were observed in 18 turkey farms in the Van province of Turkey. Three hundred eighty fecal samples were obtained from the infected farms and 42 tissue samples (liver and caecum) collected from the dead turkeys were examined under light microscope and through molecular characterization. The necropsies of dead turkeys revealed granulomatous necrotic foci in the caecum and liver. A number of 125 (32.89%) fecal samples and all of the 42 (100%) tissue samples were found positive for *T. gallinarum* by molecular characterization. The sequence analysis for both fecal and tissue samples were confirmed with *T. gallinarum* (MH935510) those showed 97.9% similarity on the 'Basic Local Alignment Search Tool'. This is the first molecular characterization report of *T. gallinarum* in Turkey.

Keywords: *Tetratrichomonas gallinarum*, Molecular characterization, Turkey

INTRODUCTION

There are two major parasitic agent that cause granulomas in turkeys; *Tetratrichomonas gallinarum* and *Histomonas meleagridis*. There are also certain bacterial diseases that cause granuloma, where it was reported that the granulomas are not observed only in the liver and caecum, but also the other organs, (Landman et al. 2016).

Tetratrichomonas gallinarum is a trichomonad flagellate protozoan, most commonly found in the cecum of galliform and anseriform birds (Burns et al. 2013). It was first described in 1911 (Martin and Robertson, 1911) and was named *Trichomonas gallinarum* (Allen, 1940) and renamed *T. gallinarum* (Honigberg, 1963).

T. gallinarum is commonly observed in the ceca of gallinaceous birds. It is often mixed with *Histomonas meleagridis*. Standard methods such as the analysis of cecal smear used in the diagnosis of *H. meleagridis* lead to its confusion with *T. gallinarum* or to negative diagnoses (Hauck et al. 2010). *Tetratrichomonas* species that are doubtful in various bird species were reported. *Tetratrichomonas gallinarum* in chickens, turkeys, and quails, *T. anseris* in geese, *T. anatis* in ducks, *T. phasiani* in pheasants, *T. corvus* in crows, and *Tetratrichomas* sp. species in budgerigars are reported with certainty (Friedhoff, 1991). *Tetratrichomonas* species in different bird species were not the same and these were host-specific, however this view was challenged by other researchers, who managed to create successful experimental infections in anseriform and galliform birds and utilized molecular typing (Hegner, 1929; Kulda et al. 1974; Cepicka et al. 2005).

The pathogenicity of *T. gallinarum* is controversial, and it may vary based on the host (Richter, 2010). It was reported that it leads to lethal enterohepatitis in turkeys (Hadley, 1916). Cepicka et al. (2005) indicated that the pathogenicity and virulence of *T. gallinarum* varies based on the genotype and claimed that there are strains of this species that lead to cryptic diseases. Other parasitic diseases such as *Eimeria* and *Histomonas* and bacterial infections such as or *Escherichia coli* and the youth of the host increase the pathogenicity of *Tetratrichomonads* (Norton, 1997; McDougald, 2005; Richter et al. 2010).

By using conventional diagnosis methods such as the cecal smear technique, it was difficult to differentiate between *T. gallinarum* and *H. meleagridis*, which often lead to false diagnoses (Hauck, 2010). Based on this granulomatous disease by *T. gallinarum*, this study was conducted to investigate about the reason of mortality observed in turkey farms by using molecular methods from Van province in Turkey.

MATERIALS AND METHODS

Sample collection and macroscopic examination: The study was conducted in Van province in Turkey, which is located in Eastern part of Turkey (Fig. 1).

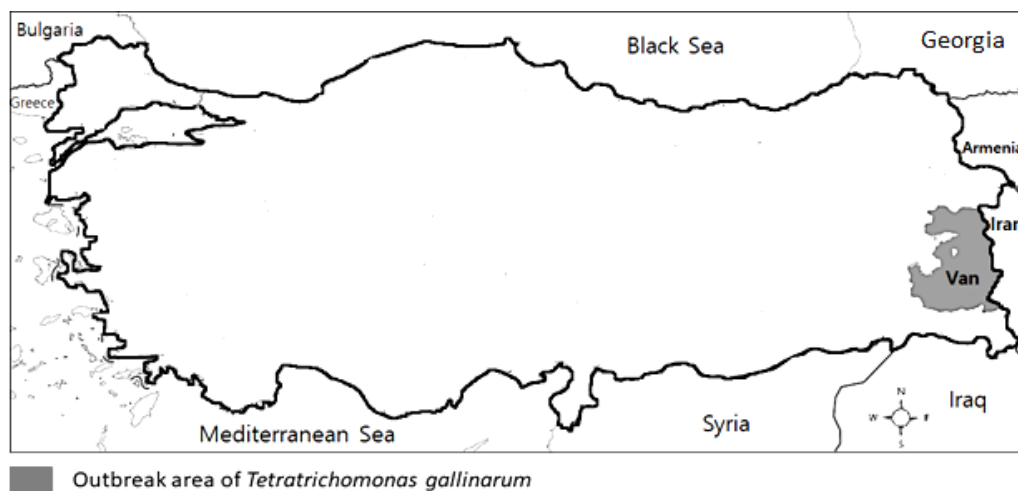


Figure 1. Map of Turkey showing the outbreak area of avian granuloma in Van province by *Tetratrichomonas gallinarum*. In 2018-2019, an epidemic illness that led to mortality in 18 turkey farms, especially among young animals, was observed at a turkey farm in Van province, Turkey.

The rationale for this specific area was due to sudden death observed in 18-turkey farms occurred during 2018 to 2019. About 1.0 x 0.5 cm sized tissue of liver and caecum was collected from each of 21 turkeys died suddenly and

a number of 380 fecal samples were randomly collected from infected turkey farms irrespective of their age, sex, and growth status. The samples were transferred to the laboratory and were kept at +4°C for extraction of DNA.

Molecular examination

DNA extraction

Tissue samples of liver and caecum were processed for the extraction of DNA using 'Nucleic Acid Extraction Kit' (GF-1, Cat: GF-TD-050, Vivantis), while fecal samples were processed using 'Mini-stool kit' (QIAampFast, Cat. 51604, Qiagen, Germany) for the extraction of DNA according to the manufacturer's instructions. The extracted DNA was identified as 18S SSU rRNA gene for *Tetratrichomonas* and *Histomonas*.

PCR

The 18S SSU rRNA gene region from *Tetratrichomonas* spp. and *Histomonas* spp. was amplified using HIS5F (5'-CCTTTAGATGCTCTGGGCTG-3') and HIS5R (5'-CAGGGACGTATTCAACGTG-3') primers (Huber et al. 2005). For this purpose, DNase/RNase free distilled water (Gibco Thermo Fisher Scientific, Waltham, MA USA), 1X Buffer (SolisBioDyne, Tartu, Estonia), 0.75 mM MgCl₂ (SolisBioDyne, Tartu, Estonia), 120 uM dNTP (SolisBioDyne, Tartu, Estonia), 10 pmol Forward primer, 10 pmol Reverse primer, 0.5 U Taq DNA polymerase (SolisBioDyne, Tartu, Estonia) and 4-100 ng DNA samples were used to obtain the final volume of 25 µL. The reaction was evidenced in an Eppendorf Master cycler® brand automatic Thermalcycler device. The reaction phases included 15.0 minutes pre-denaturation at 95°C, 1.0 min denaturation for each cycles at 95°C, 1.0 min annealing at 59°C, 1.0 min elongation at 72°C and 40 cycles were conducted for 5.0 min during final elongation cycle at 72°C (Huber et al. 2005). For gel electrophoresis, 1.5% agarose gel was prepared and stained with Safe-T-Stain. Subsequently, PCR products were run in the agarose gel and images were obtained with the gel-imaging device (Syngene bio imaging system).

DNA sequence and phylogenetic analysis

The study was conducted with a PCR method that targeted the *T. gallinarum* 18S SSU rRNA gene. The sequencing was conducted in PCR products ABI 3130 (Applied BioSystems) genetic analyzer with bidirectional BigDye™ terminator sequence kit (Ver 3.1 Applied BioSystems, Foster City, CA) and HIS5F and HIS5R primers. As a result of the sequencing procedure, approximately 181 bp 18S rRNA sequences were obtained. The sequence analysis and alignment of the sequences were performed with the Bioedit software (Hall, 1999). National Center for Biotechnology Information (NCBI) data was used for primer blast and for similarity studies. The resulting gene sequence was named according to the GenBank terminology and was recorded with MH935510 accession number. The smallest Bayesian Information Criterion (BIC) value was determined with the Jukes-Cantor+Gamma distribution (JC+G) method by modeling it in the MEGA version 7 software (Kumar et al. 2016) in order to determine the relationship between the sequences of the protozoa in trichomonadidae family obtained from the GenBank and the present sequences study. To observe the phylogenetic relationships between individuals, a phylogenetic tree was plotted with Maximum Likelihood method and the divergence rates was estimated using pairwise fixation index (Fst) distance matrices.

Ethical considerations

All the procedures involved in the study were approved by the local ethics committee of Van Yuzuncu Yil University, Van, Turkey vide letter No. VAN YUHADYEK/2018/07 dated 26 July 2018.

RESULTS

Macroscopic observation: In the present study, the disease-causing agents were isolated from turkey farms of Van province in Turkey, where the mortalities were observed. Clinical signs i.e. asthenia and fatigue were observed in the symptomatic birds. These symptoms were more common among young turkeys as compared to the adults. None of the birds exhibited the blackhead appearance. The post-mortem examination of the birds showed multifocal granulomatous necrosis foci in both of liver and caecum samples. Furthermore, caecum content of the turkeys was

sanguine, and the caecum wall was thick (Fig. 2A). Yellow and cream-colored miliary lesions were observed in the liver tissue (Fig. 2B).

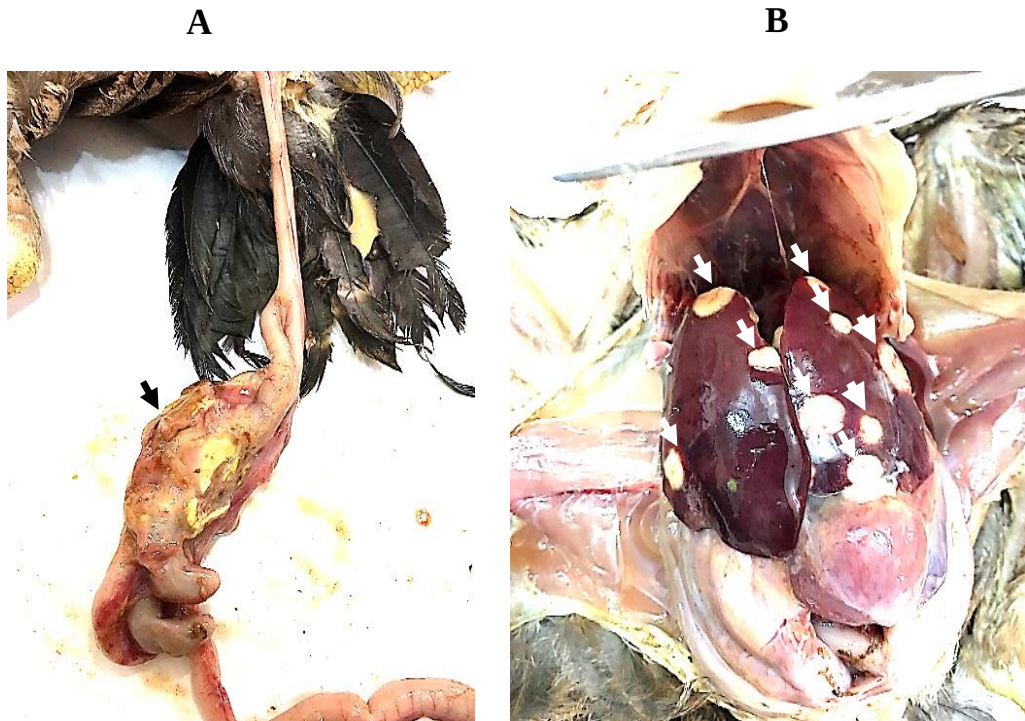


Figure 2. Gross lesions in visceral organ of domestic turkeys (*Meleagris gallopavo*) causing by *Tetratrichomonas gallinarum*. A) Bloody and inflamed caecum. B) Multifocal granulomatous necrosis on the liver.

Molecular detection and phylogenetic analysis of *Tetratrichomonas gallinarum*: Molecular analysis were conducted in both feces and tissues samples. PCR demonstrated that 181 bp fragments were obtained from 32.89% of the total number of fecal samples (125/380). All of the liver and cecum tissues (42) were infected with *Tetratrichomonas* spp. A randomly picked product of PCR from each of liver and cecum tissues, showed 100% similarity between the liver and fecal genes sequence. Alignment analysis resulted a similarity of 97.9% to the *T. gallinarum* identified in rooster from Philippine (JX565083, JX565085) and of goose from Germany (JN619423) when compared with them. The sequence of the liver tissue sample was registered in Gen-Bank with an accession No. MH935510 and alignment with the existing sequences from Philippine, Germany, and Czech in the Rooster, goose and chicken, registered in Gen-Bank with accession No. JX565085, JX565083, JN619423, AY245113, respectively. In addition, five *Trichomonas* spp. isolated from various regions (China, Czech, Iran, Philippine and USA) and hosts (Human, dog, pigeon and dove) registered in GenBank with KM603351, KX977495, KX353940, JX943581, EU215370, respectively. As an out-group, *Histomonas meleagridis* was also included. The phylogenetic tree constructed by the Maximum Likelihood method showed two clades supported by 1000 bootstrap (Fig. 3A). The first clade showed the genus *Tetratrichomonas* group that include with the sequence isolated from the liver tissue sample (MH935510). The divergence rates based on the analysis of the pairwise fixation index (Fst) values/percentage. Identification indicated that our samples showed similarity with *T. gallinarum* and resulted in p-distance in 0.022-0.046 and 95.6-97.9% respectively, (Fig. 3B).

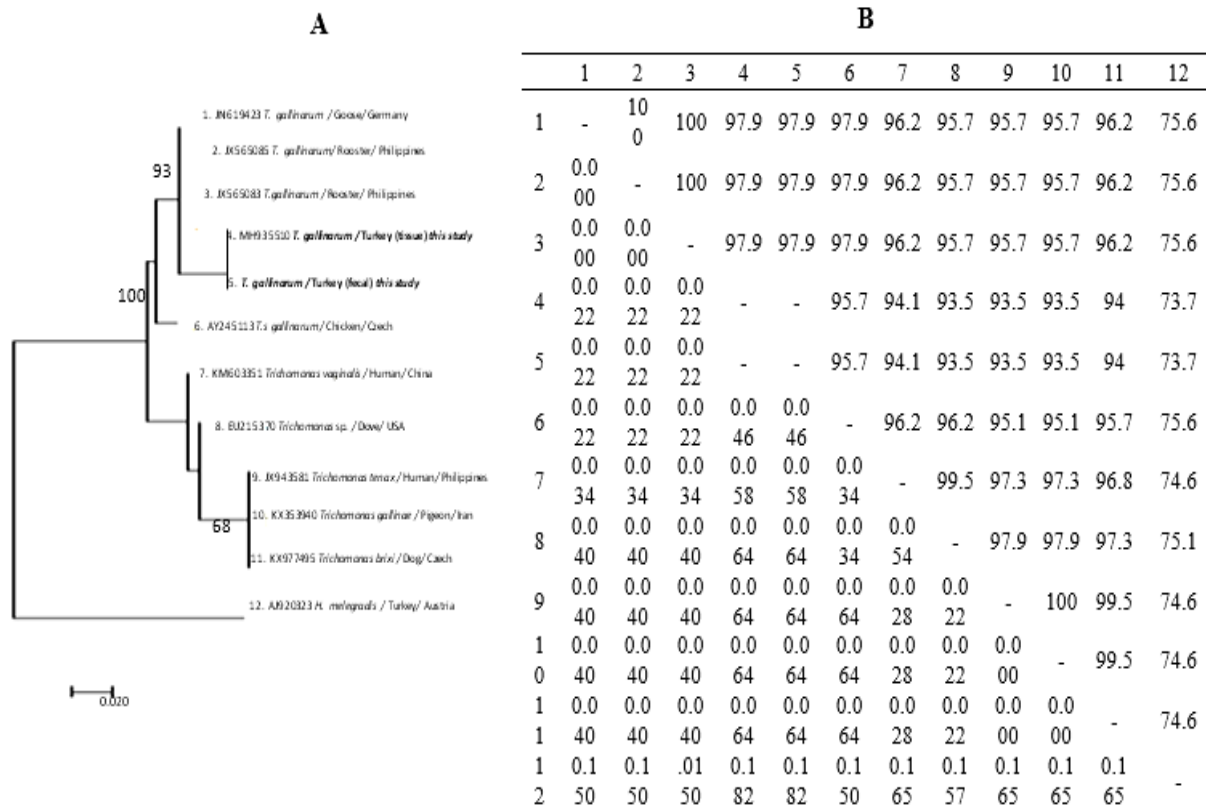


Figure 3. Molecular characterization analyses for *Tetratrichomonas gallinarum* found in Domestic Turkey (*Meleagris gallopavo*) from Turkey.

- A). Maximum Likelihood tree of *Tetratrichomonas gallinarum* from Turkey in Van province in Turkey, constructed under a Jukes and Cantor plus gamma (JC+G) distribution site model of DNA substitution with variance estimation (1000 Bootstrap replication), based on 18S SSU rRNA gene sequences(181 bp) and *H. meleagridis* as an outgroup. Scale bar indicates for every 100 nucleotides two nucleotides changes (0.02 substitutions per base pair).
- B). Pairwise fixation index (Fst) for *T. gallinarum* populations in different hosts from Turkey and other countries using nucleotide data of the 18S SSU rRNA gene and the percent of nucleotide sequence identity.

Discussion

Tetratrichomonas gallinarum is a flagellated protozoan prevalent in the digestive system of different poultry species around the world. Since this protozoan is often accompanied by *H. meleagridis* in mixed infections, the diagnosis may be inconsistent. Kemp and Reid, (1965) were the first to address this case. The agents may be present in the lesions; however, the identification of flagella and wavy membranes is not a reliable with conventional smears and histopathological examinations. Therefore, advanced diagnostic applications such as In situ hybridization (ISH) and PCR were introduced and are extremely sensitive and reliable in diagnosis. These methods are particularly significant in the diagnosis of factors that cause similar pathologies. The 18S rRNA gene sequence and internal transcribed regions 1-2 (ITS1-5.8SrRNA-ITS2) locus analyses conducted after the traditional PCR provide definite results in the diagnosis of *T. gallinarum* (Liebhart et al. 2014).

Methods based on the nucleotide sequence of rRNA allow precise and accurate diagnosis of different agents with flagella. (Grabensteiner and Hess, 2006). Grabensteiner and Hess, (2006) and Hauck et al. (2010) argued that molecular methods were essential for the diagnoses of *T. gallinarum* and *H. meleagridis*. In the present study both conventional and molecular methods were used. No definitive diagnosis was obtained in smear examination; however, the agent was identified with the sequence analysis of the DNA samples obtained from the samples examined with PCR method.

To amplify the 18S SSU rRNA gene region, HIS5F (50-CCTTTAGATGCTCTGGGCTG-30) and HIS5R (50-CAGGGACGTATTCAACGTG-30) primers, previously used by Huber et al. (2005) were used in the present study. According to the above-mentioned researchers, these primers can be used in the diagnosis of *Histomonas* and *Tetratrichomonas*. The identification of the agents is performed based on the bp size of the amplified gene region. In the present study, 181 bp wide bands that demonstrated the presence of *T. gallinarum* in the augmented gene region were determined with PCR method.

There are controversial views on *T. gallinarum* pathogenesis, the most important reason being that the protozoon is mostly accompanied by other protista such as *Blastocystis* and *Histomonas* (Tyzzer, 1919; Allen, 1936). The studies, which argued that *T. gallinarum* was not a pathogen, claimed that there could be a problem in the ethology of the agent or there could be mixed infections (Delappe, 1957; Kemp and Reid, 1965; Kulda et al. 1974). In a study conducted by Amin et al. (2011), it was argued that *T. gallinarum* was not a pathogen. Furthermore, they reported that they did not observe any clinical symptoms and lesions in the organs in studies conducted with *T. gallinarum* infected turkeys and pathogen free chickens. Also, in a study conducted on *Trichomonas gallinae* and *T. gallinarum* cell cultures, Amin et al. (2012) reported that the cytotoxic action of *T. gallinarum* was lower than that of *T. gallinae*. In contrast, there are studies that reported pathogen lesions induced by *T. gallinarum* infection in chickens, turkeys, ducks, partridges, mockingbirds, ibis and pelicans (Allen, 1936; Wichmann and Bankowski, 1956; Patton and Patton, 1996; Richter et al. 2010; Burns et al. 2013; Laing et al. 2013; Liebhart et al. 2014). A significant study that reported *T. gallinarum* as a pathogen was conducted by Cepicka et al. (2005). Cepicka et al. (2005) investigated the genetic correlation between *T. gallinarum* isolates isolated in human oral cavities and twenty-nine *T. gallinarum* isolates obtained from eight bird species. They categorized the *T. gallinarum* isolates obtained from birds in five groups between A and E and reported that the isolates obtained from humans and the isolates in A2-B2 avian subgroups were genetically identical and these were both pathogens and zoonoses. Also, the same authors argued that *T. gallinarum* could alter biological characteristics and further detailed studies should be conducted on *T. gallinarum*. Kutisova et al. (2005) identified *T. gallinarum* isolates in the samples obtained from the bronchi and oral cavities of individuals with various lung problems. They suggested that *T. gallinarum*-like tetratrichomonads were zoonoses and had a broad host spectrum. In the present study, the mortality factors in turkeys in Van region using various parasitic methods and only *T. gallinarum* was identified in the turkeys. Based on the study findings, we believe that *T. gallinarum* is pathogenic and may induce lethal typhlo hepatitis.

Tetratrichomonas gallinarum is located in the distal digestive system (caecum and rectum). Similar to *H. meleagridis*, it induces inflammation and lesions in the liver and caecum. Allen, (1941) reported differences between *T. gallinarum* and *H. meleagridis* lesions. *T. gallinarum* lesions were cheesy, granular and cream colored, especially the lesions on liver surface were observed as well-limited crests on the liver surface. Landman et al. (2016) investigated a outbreaks of granuloma disease with high mortality in chicken farms in the Netherlands in 2013. As a result of that study, granuloma-like liver and necrosis foci in cecum were observed in all necropsy samples. In the PCR analysis conducted on the samples in that study, *Blastocystis* sp. and *T. gallinarum* agents were identified. They investigated the pathogenicity of these agents by culturing these agents and inoculating them to pathogen free hens and reported that *T. gallinarum* induced liver granulomas and lesions in the caecum and led to mortality. In the present study, dense cheesy granular and cream-colored necrosis foci were observed in the necropsy of the samples obtained from turkey farms where mortalities were observed and the findings were consistent with those reported by Landman et al. (2016) and Allen, (1941).

Tetratrichomonas gallinarum was not only symptomatically more or less similar with *H. meleagridis*, but also the mixed infection with *H. meleagridis* and *T. gallinarum* reported to occur frequently (Richter et al. 2010; Landman et al. 2019). Several researches argued that molecular methods were essential for the differential diagnoses of *T. gallinarum* and *H. meleagridis* (Cepicka et al. 2005; Grabensteiner and Hess, 2006; Clarke et al. 2017). Therefore, advanced diagnostic applications such as in-situ hybridization (ISH) and PCR were introduced, those were extremely sensitive and reliable for the confirmatory diagnosis of infection (Stoeck and Epstein, 2003; Hussain et al. 2015; Landman et al. 2016).

In the present study, *T. gallinarum* was identified by using the DNA sequence analysis of the obtained samples, examined by PCR method at the target of the 18S SSU rRNA gene. Regarding the recent research tool, the 18S SSU rRNA gene seems to be a good marker for species identification and characterization of trichomonads and frequently used for phylogenetic analysis (Dimasuay and Rivera, 2013). The 18S SSU rRNA gene sequence and internal transcribed regions 1-2 (ITS1-5.8S rRNA-ITS2) locus analysis conducted after the traditional PCR provide

definite results in the diagnosis of *T. gallinarum* (Liebhart et al. 2014). Furthermore, method based on the nucleotide sequence of 18S SSU rRNA confirmed the precise and accurate diagnosis of different agents with flagella. Phylogenetically, the *T. gallinarum* species reported in this study in Turkey was most closely related to *T. gallinarum* species from Philippine, Germany, and Czech with high bootstrap values (93%) (Cepicka et al. 2005; Hauck et al. 2010; Amin et al. 2011; Dimasuay and Rivera, 2013). The presence of 18S SSU rRNA gene showed 97.9% homology to the reference sequence of *T. gallinarum*. Moreover, the analyzed sequences showed 95 to 99% similarities indicated the level of its relationship of species to the genus. In present study, the sequence data could be novel diversified references for the species of *T. gallinarum* for further studies.

CONCLUSION

Presently identified a tetratrachomonosis in domestic turkeys by using molecular approach and to our best knowledge, it is the first study conducted for identifying and characterization of *T. gallinarum* in Turkey. In the present study, a high prevalence of *T. gallinarum* infection was recorded in domestic turkeys occurred in the province of Van in Turkey. Furthermore, all the analyzed samples were not infected with Histomoniasis and the other protozoan based granulomatous pathogen factors. In addition, the results confirmed that domestic turkeys develop clinical disease similar to that described previously in other gallinaceous birds infected by *T. gallinarum*. However, further investigation in the domestic turkey (*Meleagris gallopavo*) birds with more detailed molecular, epidemiological, and economical aspects of *T. gallinarum* may be performed in future.

Acknowledgments. We are grateful to Dr. Seongjun Choe and the members of the Parasite Research Center in Chungbuk National University School for their kind advice and all who have collaborated with this research.

REFERENCES

1. Allen EA, (1936) A pentatrachomonas associated with certain cases of enterohepatitis or "blackhead" of poultry. Transactions of the American Microscopical Society 55, 315-322;
2. Allen EA, (1940) A redescription of *Trichomonas gallinarum* Martin and Robertson, 1911, from the chicken and turkey. Proceedings of the Helminthological Society of Washington 7, 65-68;
3. Allen EA, (1941). Macroscopic differentiation of lesions of histomoniasis and trichomoniasis in turkeys. Am J Vet Res 2:214-217;
4. Amin A, Liebhart D, Weissenböck H, Hess M, (2011) Experimental infection of turkeys and chickens with a clonal strain of *Tetratrachomonas gallinarum* induces a latent infection in the absence of clinical signs and lesions. Journal of Comparative Pathology 144, 55-62;
5. Amin A, Bilic I, Berger E, Hess M, (2012) *Trichomonas gallinae*, in comparison to *Tetratrachomonas gallinarum*, induces distinctive cytopathogenic effects in tissue cultures. Veterinary Parasitology 186, 196-206;
6. Burns RE, Braun J, Armien AG, Rideout BA, (2013) Hepatitis and splenitis due to systemic tetratrachomoniasis in an American White pelican (*Pelecanus erythrorhynchos*). Journal of Veterinary Diagnostic Investigation 25(4), 511-514;
7. Cepicka I, Kutišová K, Tachezy J, Kulda J, Flegr J, (2005) Cryptic species within the *Tetratrachomonas gallinarum* species complex revealed by molecular polymorphism. Veterinary Parasitology 128, 11-21;
8. Clarke LL, Beckstead RB, Hayes JR, Rissi DR, (2017). Pathologic and molecular characterization of histomoniasis in peafowl (*Pavo cristatus*). Journal of veterinary diagnostic investigation 29, 237-241;
9. Delappe IP, 1957. Effect of inoculating the chicken and the turkey with a strain of *Trichomonas gallinarum*. Exp Parasitol 6:412-417;
10. Dimasuay KGB, Rivera WL, (2013) Molecular characterization of trichomonads isolated from animal hosts in the Philippines. Veterinary Parasitology 196, 289-295;
11. Friedhoff K, Kuhnigk C, Müller I, (1991) Experimental infections in chickens with *Chilomastix gallinarum*, *Tetratrachomonas gallinarum*, and *Tritrichomonas eberthi*. Parasitology Research 77, 329-334;

12. Grabensteiner E, Hess M, (2006) PCR for the identification and differentiation of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and *Blastocystis* spp. *Veterinary Parasitology* 142, 223-230;
13. Hall TA, (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. in *Nucleic acids symposium series*. [London]: Information Retrieval Ltd., 1979-2000;
14. Hauck R, Balczulat S, Hafez HM, (2010) Detection of DNA of *Histomonas meleagridis* and *Tetratrichomonas gallinarum* in German poultry flocks between 2004 and 2008. *Avian Diseases* 54, 1021-1025;
15. Hegner R, (1929) The infection of parasite-free chicks with intestinal protozoa from birds and other animals. *American Journal of Epidemiology* 10, 33-62;
16. Honigberg B, (1963) Evolutionary and systematic relationships in the flagellate order Trichomonadida Kirby. *The Journal of Protozoology* 10, 20-63;
17. Huber K, Chauve C, Zenner L, (2005) Detection of *Histomonas meleagridis* in turkeys cecal droppings by PCR amplification of the small subunit ribosomal DNA sequence. *Veterinary Parasitology* 131, 311-316;
18. Hussain I, Jaskulska B, Hess M, Bilic I, (2015) Detection and quantification of *Histomonas meleagridis* by real-time PCR targeting single copy genes. *Veterinary Parasitology* 212, 382-388;
19. Kemp RL, Reid WM, (1965) Pathogenicity studies on *Trichomonas gallinarum* in domestic poultry. *Poultry Science* 44, 215-221;
20. Kulda J, Suchánková E, Svoboda S, 1974. Studies on pathogenicity of *Tetratrichomonas gallinarum* in mice and turkey poults. *Acta Veterinaria Brno* 43, 53-64;
21. Kumar S, Stecher G, Tamura K, (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33, 1870-1874;
22. Kutisova K, Kulda J, Cepicka I, Flegr J, Koudela B, Teras J, Tachezy J, (2005) Tetratrichomonads from the oral cavity and respiratory tract of humans. *Parasitology* 131, 309-19;
23. Laing ST, Weber ES, Yabsley MJ et al., 2013. Fatal hepatic tetratrichomoniasis in a juvenile Waldrapp ibis (*Geronticus eremita*). *J Vet Diagn Invest* 25:277-281;
24. Landman WJ, Molenaar RJ, Cian A, Van der Heijden HMJF, Viscogliosi E, (2016) Granuloma disease in flocks of productive layers caused by *Tetratrichomonas gallinarum*. *Avian Pathology* 45, 465-77;
25. Landman WJM, Gantois N, van Eck JHH, van der Heijden HMJF, Viscogliosi E, (2019) *Tetratrichomonas gallinarum* granuloma disease in a flock of free range layers. *Veterinary Quarterly* 39, 153-160;
26. Liebhart D, Neale S, Garcia-Rueda C, Wood AM, Bilic I, Wernsdorf P, Jaskulska B, Hess M, (2014) A single strain of *Tetratrichomonas gallinarum* causes fatal typhlohepatitis in red-legged partridges (*Alectoris rufa*) to be distinguished from histomonosis. *Avian Pathology* 43, 473-80;
27. Martin CH, Robertson M, (1911) Further observations on the caecal parasites of fowls, with some reference to the rectal fauna of other vertebrates. *Quarterly Journal of Microscopical Science*, 57, 53-81;
28. Norton R, (1997) Pathogenicity of a strain of *Trichomonas gallinarum* in turkeys and its possible interaction with cecal coccidia. *Avian Diseases* 41, 670-675;
29. Patton CS, Patton S, (1996) *Tetratrichomonas gallinarum* encephalitis in a mocking bird (*Mimus polyglottos*). *Journal of Veterinary Diagnostic Investigation* 8, 133-137;
30. Richter B, Schulze C, Kämmerling J, Mostegl M, Weissenböck H, (2010) First report of typhlitis / typhlohepatitis caused by *Tetratrichomonas gallinarum* in three duck species. *Avian Pathology* 39, 499-503;
31. Stoeck T, Epstein S, (2003) Novel eukaryotic lineages inferred from small-subunit rRNA analyses of oxygen-depleted marine environments. *Applied and Environmental Microbiology* 69, 2657-2663;
32. Tyzzer EE, (1919) Developmental phase of the protozoan of the "blackhead" in turkeys. *The Journal of Medical Research* 40, 1-30;
33. Wichmann RW, Bankowski RA, (1956) A report of *Trichomonas gallinarum* infection in chukar partridges (*Alectoris graeca*). *The Cornell Veterinarian* 46, 367-369;