Kafkas Univ Vet Fak Derg
24 (4): 539-543, 2018
DOI: 10.9775/kvfd.2018.19488

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

Prevalence and Molecular Characterization of *Trypanosoma* spp. in Domestic Geese *(Anser anser domesticus)* from the North-East Anatolia Region of Turkey [1] [2]

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[1] This study was supported by Scientific Research Project Coordination Office of Kafkas University with the Project *No: 2015-TS-62*
[2] *Presented in 20th National Parasitology Congress (with International Participation) as an oral presentation, Eskişehir-Turkey, 25-29 September 2017 and published in Abstract Book*

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Article Code: KVFD-2018-19488 Received: 22.01.2018 Accepted: 24.04.2018 Published Online: 24.04.2018

How to Cite This Article

Taşçı GT, Vatansever Z, Parmaksızoğlu Aydın N, Ölmez N, Akça A, Arslan MÖ, Mor N, Sarı B: Prevalence and molecular characterization of *Trypanosoma* spp. in domestic geese *(Anser anser domesticus)* from the North-East Anatolia Region of Turkey. *Kafkas Univ Vet Fak Derg,* 24 (4): 539-543, 2018. DOI: 10.9775/kvfd.2018.19488

Abstract

Avian trypanosomiosis is a protozoan disease transmitted by blood-sucking arthropods belonging to the Simuliidae, Culicidae, Ceratopogonidae, Hippoboscidae, and Dermanyssidae. *Trypanosoma* spp. are diagnosed by using microscopic and/or molecular methods. In this study, a total of 400 domestic geese blood samples were examined using nested PCR with the primers that amplify a gene fragment of 18S rRNA of *Trypanosoma* spp. The molecular prevalence of *Trypanosoma* spp. was determined as 50.25%. 18S rRNA amplicons from the two positive isolates were sequenced with the PCR primers. The obtained sequences from the two isolates exhibited 99.7% identity to each other. Sequence and blastn analyses revealed that two isolates were highly identical (99.7-100%) to the published 18S rRNA sequences of *T. thomasbancrofti, T. gallinarum* and *T. avium* from *Xanthomyza phrygia* (regent honeyeater) and *Gallus gallus* (the red junglefowl). This study provides the first molecular data on *Trypanosoma* generations in poultry from Turkey. The presence of *Trypanosoma* spp. was also firstly identified in domestic geese *(Anser anser domesticus)* in the world by molecular tools and genetic characterization of a short fragment of the 18S rRNA gene region has been achieved.

Keywords: *Domestic geese, Kars, Molecular characterization, Trypanosoma spp.*

Türkiye'nin Kuzeydoğu Anadolu Bölgesinde Kazlarda *(Anser anser domesticus) Trypanosoma* spp. Prevalansı ve Moleküler Karakterizasyonu

Öz

Kanatlı trypanosomiosisi, Simuliidae, Culicidae, Ceratopogonidae, Hippoboscidae ve Dermanyssidae ailelerinde bulunan kan emen artropodlar tarafından bulaştırılan bir protozoan enfeksiyonudur. *Trypanosoma* türleri mikroskobik ve/veya moleküler yöntemlerle teşhis edilebilir. Bu çalışmada, *Trypanosoma* türlerinin 18S rRNA gen fragmanını çoğaltan primerler kullanılarak nested PCR ile toplam 400 kazın kan örneği incelenmiştir. *Trypanosoma* spp. prevalansı %50.25 olarak tespit edilmiştir. İki pozitif izolattan elde edilen 18S rRNA amplikonlarının PCR primerleri ile sekans analizi yaptırılmıştır. İki izolattan elde edilen sekanslar birbirleri ile %99.7 oranında identiklik göstermiştir. Sekans ve blastn analizleri, bu iki izolatın *Xanthomyza phrygia* (regent honeyeater) ve *Gallus gallus*'tan (kırmızı junglefowl) izole edilen *T. thomasbancrofti, T. gallinarum* ve *T. avium*'un 18S rRNA dizilimlerine çok benzer olduğunu (%99.7-100) ortaya koymuştur. Bu çalışma Türkiye'de kanatlılarda yayılış gösteren *Trypanosoma* türleriyle ilgili ilk moleküler verileri sağlamaktadır. Ayrıca *Trypanosoma* türlerinin evcil kazlarda *(Anser anser domesticus)* varlığı dünyada ilk olarak tespit edilmiş ve 18S rRNA gen bölgesinin kısa bir parçasının genetik karakterizasyonu sağlanmıştır.

Anahtar sözcükler: *Evcil kaz, Kars, Moleküler karakterizasyon, Trypanosoma spp.*

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INTRODUCTION

Avian trypanosomes (Trypanosomatidae, Kinetoplastidae) are widespread worldwide and transmitted by blood-sucking arthropods belonging to the Simuliidae, Culicidae, Ceratopogonidae, Hippoboscidae, and Dermanyssidae [1-5]. *Trypanosoma* spp. are well adapted for various ecosystems from oceanic islands to the countries with cold climates where avian hosts and vectors are present [6-8].

To date, in spite of poorly taxonomical development, more than 100 avian *Trypanosoma* species have been described and named [6-8]. Trypanosomiosis is caused by the species of *T. avium* Danilewsky, 1885, *T. calmettei* Mathis and Leger, 1909 and *T. gallinarum* Bruce, Hamerton, Bateman, Mackie and Bruce, 1911 in geese. *T. avium* and *T. gallinarum* species are common in Canadian geese, many avian species in Europe and chickens in Africa, and also *T. calmettei* is seen in ducks in Southeast Asia. But there are no clinical signs in domestic geese. *Trypanosoma* spp. can be diagnosed by detecting trypomastigote forms in blood and/or bone marrow smears and using PCR based methods [1,8-12]. However, due to nucleated erythrocytes of avian species DNA may contain primarily host DNA leading to reduce efficiency of PCR amplification of the target parasite gene. Therefore, in order to optimize DNA amplification by using ribosomal genes, a nested PCR protocol was developed [12].

Due to the broad host range and wide geographical distribution, many studies have been focused on [5,8,9,12-14] the prevalence of trypanosomiasis in wild birds but the epidemiological parameters of avian trypanosomiasis has not yet been investigated in Turkey.

The aim of this study was therefore to determine the prevalence and molecular characterization of *Trypanosoma* spp. by nested PCR in domestic geese *(Anser anser domesticus)*.

MATERIAL and METHODS

Study Area and Collection of Blood Sample

Having obtained ethics approval from Kafkas University-Animal Experiments Local Ethics Committee (Aproval No: KAÜ-HADYEK: 2014-052) and the approval of the farmers, a total of 400 blood samples were collected, using an insulin syringe, from domestic geese (*Anser anser domesticus*) which are grown for nutrition purposes when they were slaughtered in November 2015 in Kars province (40°36'04.82"N, 43°05'50.83"E), Northeastern Turkey.

Extraction of gDNA and PCR

Blood samples were extracted according to the commercial DNeasy kit protocol (Zymo Research, Quick-gDNA Blood Mini Prep, USA). The extracted gDNA was stored at -20°C until analysis. Concentrations of DNA samples were measured (Qubit Fluorometric Quantitation, Invitrogen, Life Technologies) to optimize the amount of gDNA used in the PCR master mix. Genomic DNA was used in nested PCR reactions to amplify 18S rRNA fragments. The primers [S762 (5'-GACTTTTGCTTCCTCTAWTG-3') and S763 (5'-CATATGCTTGTTTCAAGGAC-3')] were used in the first step of nested PCR. The cycling profile conditions were as follows: initial denaturation at 95°C for 5 min, followed by 5 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 30 sec, and extension at 65°C for 1 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 sec, extension at 72°C for 1 min, and then a final extension at 65°C for 10 min. A total volume of 25 µL reaction mixture containing 8.5 µL nuclease-free water, 12.5 µL master miks (Mytaq, Bioline), 1 µL of each primer and 2 µL of template DNA was used. Two mL of the first PCR reaction products were used as the template for the second PCR. The primers [S755 (5'-CTACGAACCCTTTAACAGCA-3') and S823 (5'-CGAAYAACTGCYCTATCAGC-3')] were used in the second step of nested PCR. The reaction conditions were as follows: initial denaturation at 96°C for 3 min, followed by 35 cycles of denaturation at 96°C for 30 sec, annealing at 58°C for 1 min, extension at 72°C for 30 sec, and then a final extension at 72°C for 7 min [12]. Positive control blood samples were supplied from Prof. Dr. Gediminas VALKUINAS (Institute of Ecology, Nature Research Centre, Vilnius, Lithuania) and nuclease free water was used as negative controls to check for contamination in each PCR. PCR products were run out on a 1.5% agarose gel using 0.5X TBE and visualized by an ethidium bromide stain under ultraviolet light. A 326-bp fragment of PCR products were accepted positive.

Sequence and Phylogenetic Analysis

In order to confirm PCR results and to explore the phylogenetic relationships, two PCR products were chosen and purified by excising from agarose gel, using a commercial kit (High Pure PCR Product Purification Kit, Roche Life Science). The amplified 18S rRNA target fragments were sequenced in both directions, using S-755 and S-823 primers for *Trypanosoma* spp. (Sentegen, Turkey). Sequences were oriented, edited, and aligned with Geneious 10.2.3 software [10] to produce a single consensus. Intra- and interspecific genetic diversity was determined with MEGA version 7 by using the Kimura two-parameter (K2P) distance model [15,16].

The obtained consensus sequences compared at 301 bp with sequences in the GenBank database using the Basic Local Alignment Search Tool (NCBI website) [17]. The characterized isolates were deposited in GenBank with the accessions MG593843 and MG593844. Phylogenetic reconstructions were performed by Bayesian (BA) inference. The best-fit DNA-substitution model for BA analyses based on the Akaike information criterion (AIC) algorithm was selected by using jModel test v.0.1.1 [18]. The BA analyses were run in MrBayes version 3.2.6 [19] and

PhyML [20] through the plugin available with Geneious 10.2.3 software [21].

RESULTS

Among the 400 examined domestic geese blood samples, 201 were found positive for *Trypanosoma* spp. by Nested PCR and the mean prevalence of *Trypanosoma* spp. was determined as 50.25%.

The partial 18S rRNA gene region (301 bp) of two isolates (KAU-Gtryp1, KAU-Gtryp2) from the positive samples were sequenced and the final consensus of the isolates were deposited in GenBank with accessions MG593843 and MG593844. The characterized isolates within *Trypanosoma* genus provides the first genetic data from domestic geese. Pairwise analyses of the KAU-Gtryp1 and KAU-Gtryp2 genotypes revealed %0.03 genetic difference between the isolates. The phylogenetic analyses of the isolates were shown on BA tree in *Fig. 1*. Blastn and multiple alignment analyses of the obtained *Trypanosoma* spp. KAU-Gtryp2 isolate indicated that this isolate was identical (100%) with the isolates *T. thomasbancrofti* and *T. avium* reported from *Xanthomyza phrygia* in Australia (KT728396, KT728395, KT728394, KT728402) and *T. gallinarum* reported from *Gallus gallus* in Uganda (DQ676828, DQ676827, DQ676826). *Trypanosoma* spp. KAU-Gtryp1 isolate was also determined to close (99.7%) with the isolates indicated above and presented a novel haplotype.

DISCUSSION

Goose breeding is one of the source of income with the highest number of domestic geese from Kars province in Northeastern Region of Turkey. Not to use any medicines for preventive purposes, domestic geese breeding is performed by traditional methods instead of scientific methods in Kars province [22,23].

Trypanosoma spp. are found in some species of birds

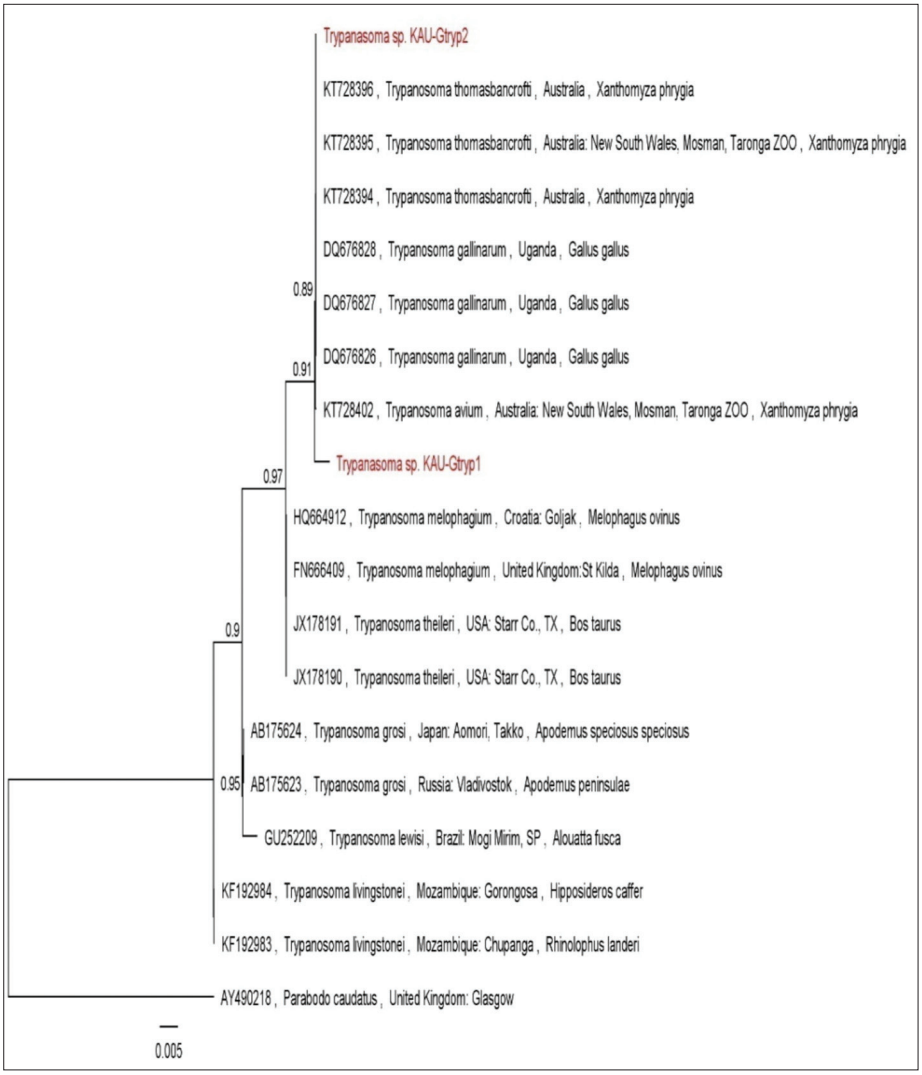

Fig 1. BA tree based on partial 18S rRNA sequences deposited in GenBank and our original data (red character) for *Trypanosoma* species. BA posterior probability values are shown before the nodes. *Parabodo caudatus* was used as outgroup taxa. Bars represent 0.1 substitutions per site

occasionally [6,12,14,24]. But there is no data about the prevalence of *Trypanosoma* spp. in domestic geese around the world. This is the first study to determine the prevalence of trypanosomes in domestic geese.

Limited studies on *Trypanosoma* infections in avian species have generally focused on passerines or raptor birds. In a study, the overall prevalence was determined as 31% [12]. Using microscopy, the prevalence of *Trypanosoma* spp. was found as 7.3% (consisting of 121 species from 21 families and 8 orders) in West African Nations of Cameroon, Equatorial Guinea and Ivory Coast [25], and (68 species from 15 families and 4 orders) 11.4% in Uganda [26], 36.4% in blood samples collected from Europe, Africa, and North America [27], 18.0% of American robins and 16.9% of house sparrows in Chicago, USA [14], 60.0% in Northern Goshawk [28]. In another study, 26.1% (40/153) of yellow-breasted chats were found infected with trypanosomes [29]. *Trypanosoma* spp. prevalence was ranged between 1.9 and 87.2% in Eurasian sparrow hawks *(Accipiter nisus)* and common buzzards *(Buteo buteo)* [24]. The prevalence of avian trypanosomes was 51.3% in Cameroon and Ghana [13].

In our study, since the parasitemia in host peripheral blood and the sensitivity of microscopy are very low [6,28], we did not use microscopical examination of blood smears. Among the 400 examined gDNA from the domestic geese, 201 samples were found positive for *Trypanosoma* spp. by nested PCR. It is expected that due to prolonged exposure and seasonally increasing occurrence of vectors, birds have a greater probability and higher parasite prevalence of infection [24]. In the direction of this knowledge, this may be the reason why we found a high rate of infection in domestic geese.

For the molecular characterization of *Trypanosoma* lineages found in the research area, sequence analyses were conducted on partial 18S rRNA sequences from two positive isolates. Two lineages were determined by sequence analyses and a mean of 0.03% genetic distance was found between the two isolates under these lineages. *Trypanosoma* spp. KAU-Gtryp2 isolate was found to be identical (100%) with the isolates *T. thomasbancrofti* and *T. avium* reported from *Xanthomyza phrygia* in Australia and *T. gallinarum* reported from *Gallus gallus* in Uganda. *Trypanosoma* spp. KAU-Gtryp1 isolate was also highly identical (99.7%) to the above-mentioned isolates. This isolate also designated as a new haplotype. While Sehgal *et al*.[12] found eight trypanosome lineages in African rainforests, only *T. avium* was detected by Oakgrove *et al*.[30]. Although the phylogenetic resolution of the targeted 326 bp region of 18S rRNA successfully explore the lineage diversity trypanosome lineages in African rainforests [12], our findings revealed that this region is not enough to resolve species delimitation of trypanosomes in order anseriformes.

In this study, the first molecular data has been provided on *Trypanosoma* generations spreading in poultry in Turkey, the presence of *Trypanosoma* spp. was firstly identified in domestic geese (*Anser anser domesticus*) by molecular tools in Turkey and genetic characterization of a short fragment of the 18S rRNA gene region has been achieved. We concluded that sequence characterization with 18S rRNA nested primers amplifying 301 bp region was insufficient to resolve genetically based generations. In order to be able to establish the phylogenetic structures of the isolates we determined in the outbreaks, it has been found that there is a need for sequence characterization and phylogenetic analysis with a longer fragment of the gene region of interest or different gene regions such as mitochondrial genes (eg COX1).

Most trypanosomes, especially *T. avium*, which is transmitted by blackflies (Diptera, Simuliidae), *T. bennetti*, whose vector is unknown, and *T. corvi*, which is transmitted by hippoboscid flies (Diptera, Hippoboscidae) were isolated from raptor birds [4,24,31-34]. Therefore, more detailed studies on avian trypanosomiasis, especially on the condition of the disease in the vectors, will solve questions about avian host-parasite relationships.

Acknowledgements

We thank to Prof. Dr. Gediminas VALKUINAS (Institute of Ecology, Nature Research Centre, Vilnius, Lithuania) for the positive control blood samples and his suggestions. We also thank to the members of Erciyes University, Faculty of Veterinary Medicine, Parasitology Department and Assist. Prof. Dr. Cem Ozic (Kafkas University, Faculty of Medicine) for their help.

Conflict of Interests Statement

The authors declare that there is no conflict of interests regarding the publication of this article.

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