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First Molecular Report of *Rickettsia aeschlimannii* in Water Buffalo (*Bubalus Bubalis*); with a Note of Phylogenetic Analyses of Ompa and Ompb Genes

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The importance of vector-borne pathogens has increased in recent years and molecular studies have revealed the presence of these pathogens in new hosts in different parts of the world. Türkiye is one of the important countries where vector-borne pathogens are reported in different hosts. Although there are many studies investigating vector-borne pathogens in cattle, sheep, and goats, there is paucity of data on the presence and distribution of these pathogens in water buffaloes. In this study, it was aimed to investigate Rickettsia species in buffaloes by molecular methods for the first time in Türkiye. In this study, 364 water buffalo blood samples obtained from seven distinct in Sivas province were researched for *Rickettsia* species using genus-specific PCR assay. Moreover, the partial parts of the *ompA* and *ompB* genes of the positive sample were sequenced for species identification and to perform phylogenetic analyses. DNA sequence analysis results showed that the sample identified in the study was infected with *Rickettsia aeschlimannii*. The obtained DNA sequence belonging to *ompA* and *ompB* genes were submitted to GenBank and accession numbers were for *ompA* gene PQ197208 and *ompB* gene PQ197209. The BLASTn analyses of both genes revealed that high nucleotide similarities (98.93-100% for *ompA* and 98.39-100% for *ompB*) were present between our *Ri. aeschlimannii* isolate and *Ri. aeschlimannii* isolates present in the GenBank. *Rickettsia aeschlimannii* is one of the most important vector-borne pathogens causing infection in humans. This is the first molecular report of *Ri. aeschlimannii* in water buffalo in Türkiye and the world. This result indicated that the agent circulates in buffaloes. However, large-scale molecular studies are still needed to better understand the contribution of buffaloes to the epidemiology of *Ri. aeschlimannii*.

Key Words: *Water buffalo, Rickettsia aeschlimannii, DNA sequence, Türkiye*

Su Mandalarında (*Bubalus Bubalis*) *Rickettsia aeschlimannii*'nin varlığına dair İlk Moleküler Raporu; Ompa ve Ompb Genlerinin Filogenetik Analizlerine İlişkin Bir Not

Son yıllarda vektör kaynaklı patojenlerin önemi artmış olup, moleküler çalışmalar dünyanın farklı bölgelerindeki yeni konaklarda bu patojenlerin varlığını ortaya koymuştur. Türkiye, vektör kaynaklı patojenlerin farklı konaklarda bildirildiği önemli ülkelerden biridir. Sığır, koyun ve keçilerde vektör kaynaklı patojenleri araştıran birçok çalışma olmasına rağmen, bu patojenlerin mandalardaki varlığı ve dağılımı hakkındaki veriler yetersizdir. Bu çalışmada, Türkiye'de ilk kez mandalarda *Rickettsia* türlerinin moleküler yöntemlerle araştırılması amaçlanmıştır. Bu amaçla, Sivas ilinde yedi ayrı bölgeden alınan 364 manda kan örneği, soy-spesifik PCR yöntemi kullanılarak *Rickettsia* türleri açısından araştırılmıştır. Ayrıca pozitif örnekteki *ompA* ve *ompB* genlerinin kısmi parçaları tür teşhisi ve filogenetik analizler yapmak amacıyla sekanslanmıştır. Çalışmada tespit edilen örneğin DNA dizi analizi sonuçları *Rickettsia aeschlimannii* ile enfekte olduğunu göstermiştir. Elde edilen *ompA* ve *ompB* genlerine ait DNA dizileri GenBank'a yüklenmiş olup sırasıyla PQ197208 ve PQ197209 erişim numaraları alınmıştır. Her iki genin BLASTn analizleri, *Ri. aeschlimannii* izolatımız ile GenBank'ta bulunan *Ri. aeschlimannii* izolatları arasında yüksek nükleotid benzerliklerinin (*ompA* için %98,93-100, *ompB* için %98,39-100) bulunduğunu ortaya koymuştur. *Rickettsia aeschlimannii*, insanlarda enfeksiyona neden olan en önemli vektör kaynaklı patojenlerden biridir. Bu, Türkiye ve dünyada su mandalarında *Ri. aeschlimannii*'nin ilk moleküler tespitidir. Bu sonuç, etkenin mandalarda sirküle olduğunu ve konak görevi gösterdiğini ortaya koymuştur. Ancak, mandaların *Ri. aeschlimannii* epidemiyolojisine katkısını daha iyi anlamak için hala büyük ölçekli moleküler çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: *Su mandası, Rickettsia aeschlimannii, DNA dizisi, Türkiye*

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Introduction

Rickettsia species are obligate intracellular bacterial pathogens, and these are classified in the *Rickettsia* genus placed Rickettsiaceae family. *Rickettsia* species are grouped into four major branches typhus group rickettsiae, spotted fever group (SFG) rickettsiae, *Rickettsia canadensis,* and *Rickettsia bellii* group by researchers according to biological and clinical symptoms (1). Rickettsiosis, caused by *Rickettsia* species, is recognized as one of the oldest vector-borne diseases, and the disease has been reported in different parts of the world (1-4).

The spotted fever group *Rickettsia* (SFGR) species are mostly transmitted by hard ticks (Ixodidae), besides hard tick species, these are also spread by soft ticks *(*Argasidae) and other blood-feeding arthropods (1, 5, 6). The spotted fever group *Rickettsia* species have worldwide distributions, and these species may lead to infection in domestic and wild animals and humans (1, 6). The spotted fever group *Rickettsia* species often cause non-specific clinical symptoms in hosts, such as eschar, local lymphadenopathy on the tick-bite site, headache, fatigue, pyrexia, muscle pain (localized or generalized), anorexia, and nausea. The clinical symptoms show up in five to seven days after tick-bite. The course of the disease may change according to the species that caused the infection, the time of diagnosis, and the immune status of the patient (1, 2, 7-9).

Different identification methods (staining, culture, serological, and molecular) are used for the detection of *Rickettsia* species in the hosts (5). Staining (Giemsa, Diff-Quik, and acridine orange stains) and culture (cell, embryonated eggs, and lab animals) can be used for the detection of pathogens, but these methods have low sensitivity and specificity, moreover, culture methods take a long time and are laborious (5). Serological methods (Weil-Felix, indirect immunoperoxidase (IIP), complement fixation, immunofluorescent assay (IFA), and enzyme-linked immunosorbent assay (ELISA), etc.) are preferred by researchers for the detection of *Rickettsia* species in hosts. However, these methods have several disadvantages, such as less sensitivity and specificity, laborious to perform, and cross-reaction with other *Rickettsia* species (5, 10). Compared to other techniques, molecular techniques have many advantages over other identification techniques, like high sensitivity and specificity and identification from different clinical samples (10). Molecular techniques provide contributions to understanding the epidemiology of *Rickettsia* species, identifying different *Rickettsia* species in various hosts that never reported them, and reporting novel *Rickettsia* genotypes/species. Therefore, researchers have recommended the use of these techniques in studies on *Rickettsia* species (1, 5, 10).

Türkiye is a suitable country for many vector arthropods thanks to its climate and vegetation diversity. Many vectors and vector-borne pathogens have been detected in the country so far (11-19). *Rickettsia* species have been also researched in the country and *Rickettsia aeschlimannii* (*Ri. aeschlimannii*), *Ri. raoultii, Ri. slovaca, Ri. africae, Ri. helvetica, Ri. massiliae, Ri. felis, Ri. conorii* subsp. *conorii*, *Ri. sibirica mongolitimonae, Ri. hoogstraalii, Ri. monacensis, Candidatus* Rickettsia barbariae *Candidatus* Rickettsia goldwasserii, and *Candidatus* Rickettsia vini have been reported in different hosts (20-28). However, literature research showed that no data on *Rickettsia* species among water buffalo herds in Türkiye. This study aimed to investigate the *Rickettsia* species in buffaloes in Sivas province using molecular methods and to reveal the phylogenetic characteristics of the identified species by DNA sequence analysis.

Materials and Methods

Research and Publication Ethics: All procedures done in this work involving animals were in accordance with the ethical standards approved by the Sivas Cumhuriyet University Animal Experiments Local Ethics Committee (Approval number: 09.02.2021–65202830–050.04.04–495).

Study Area and Materials: Türkiye consists of seven geographical regions: Marmara, Aegean, Mediterranean, Central Anatolia, Black Sea, Eastern Anatolia, and South Eastern Anatolia. Sivas province is located in the Central Anatolia region and the intersection between Central Anatolia, Black Sea, and Eastern Anatolia regions (Figure 1). The city is the second-largest province in the country with a geographical area of approximately 28,400 km^2. Sivas is an important region for water buffalo breeding due to its large pasture lands.

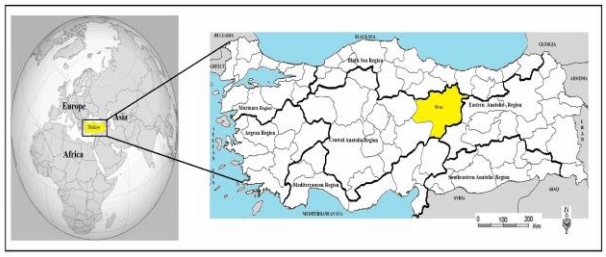

Figure 1. Location of Türkiye in the world maps, and location of sampling site in the Türkiye map.

In this study, DNA belonging to 364 water buffalo blood samples was used. These materials were collected by Sahin et al. (18) for the other study, and stored in appropriate conditions in the research laboratory. Detailed information about the samples can be found in the study by Sahin et al. (18). Briefly, these blood samples were obtained from 119 buffalo herds in seven districts (Sivas city center, Koyulhisar, Zara, Yildizeli, Sarkisla, Susehri, and Ulas) of Sivas province (Table 1).

Tablo 1. Age, sex and location of water buffalo blood samples

		Number of Animals
Age	< 1	133
	1–3	165
	> 3	66
Gender	Female	178
	Male	186
Locations	Sivas center	115
	Yıldızeli	49
	Zara	47
	Şarkışla	46
	Ulaş	41
	Suşehri	43
	Koyulhisar	23
Total		**364**

Molecular Research of Rickettsia spp. in Water Buffalo Blood Samples: The gDNAs were screened in terms of Rickettsia species with PCR assay using 190-70 (5'-ATGGCGAATATTTCTCCAAAA-3') and 190.701 (5'-GTTCCGTTAATGGCAGCATCT-3') amplified to ompA gene (29).

PCR assay was performed to a total volume 25 µL, including 10× PCR buffer (Invitrogen™, Carlsbad, USA), $MgCl_2$ (50 mM) (Invitrogen™, Carlsbad, USA), 200 µM of each dNTP (Cat.No.: R0181, Thermo Scientific™, Lithuania), 1 µL (10 pmol/µL) of each of the primers, 1.25 U of Taq DNA polymerase (5U/µL) (Ref.No.: 100021276, Invitrogen™, Carlsbad, USA), 2.5 µL template DNA, and DNase-RNase-free sterile water.

The thermal cycling protocol used for PCR was 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension of 5 min at 72°C. The resulting PCR products were loaded onto a 1% agarose gel and then electrophoresed at 90 volts for 60 minutes. After electrophoresis, the agarose gel was stained by ethidium bromide for 20 min and visualized with a UV transilluminator for specific amplicons.

Preparation of the Samples for DNA Sequence Analyses: The positive sample detected in this work was sequenced for species identification and phylogenetic analyses. For these purposes, PCR assays were done to amplify *ompA* using primers *190-70 and 190-701* and *ompB* gene with the primers 120-2788 and 120-3599 (29, 30). In these PCR assays, the EZ-FD PCR High Fidelity DNA polymerase kit (Cat.No.: 9K-005-0019, BioBasic) was used, and the PCR was done with reagents supplied by manufacturers and in a total volume of *25µL, including* 10× EZ-FD Reaction buffer with 25mM $MgCl_2$ (Lot.No.: O807R0K, BioBasic), 200 µM of each dNTP (Lot.No.: R22256RoX, BioBasic), 1 µL (10 pmol/µL) of each of the primers, 1.25 U of EZ-FD PCR High Fidelity DNA Taq polymerase (2.5U/*µL)* (Lot.No.: Q6A00120KF, BioBasic), 2.5 µL template DNA, and DNase-RNase-free sterile water. After PCR assays, PCR products were loaded into the agarose gel and checked in terms of appropriate amplicon sizes, and then, these products were sent to DNA sequence analyses.

DNA sequence analyses were performed in the commercial company (BM Labosis, Ankara). Before DNA sequence analyses, all PCR products were purified with the HighPrepTM PCR Clean-up System (Cat. No.: AC-60005, MagBio) following the manufacturer's instructions. The sequence data were checked with FinchTV (version 1.4.0) software (Geospiza Inc., Seattle, Washington, USA) for chromatogram qualities, and nucleotides that had poor chromatogram qualities were trimmed. The consensus sequences were determined using MEGA-11 software (31). The identified consensus sequences were deposited to GenBank, and accession numbers were determined.

The maximum likelihood method was used to construct phylogenetic trees using MEGA-11 software (31) to reveal the genetic variation between the *Rickettsia* species identified in this study and the Rickettsia species in GenBank. Before constructing the phylogenetic tree, it was determined that the best algorithm to be used in the phylogenetic tree of related pathogens was the Tamura-3 parameter model (32) using the Find Best-Fit Substitution Model in MEGA-11 and this algorithm was used in the phylogenetic tree. Bootstrap analysis (1,000 repetitions) was performed.

Results

In this study, 364 water buffalo blood samples were screened for *Rickettsia* species using PCR assay, and one (0.27%) water buffalo was found to be infected with *Rickettsia* sp. (Figure 2). Species identification of *Rickettsia* species in the study was performed with DNA sequence analyses, and *Ri. aeschlimannii* was identified. These nucleotide sequences were uploaded to the GenBank and accession numbers were taken for *ompA* (PQ197208) and *ompB* (PQ197209) genes.

The *ompA* gene sequence analyses of the positive sample revealed 98.93-100% nucleotide similarities between *Ri. aeschlimannii* isolate identified in this study and *Ri. aeschlimannii* isolate present in the GenBank. In addition, our *Ri. aeschlimannii* isolates had 100% nucleotide identities with *Ri. aeschlimannii* identified in *H. marginatum* larvae (MG920564) from Türkiye, in *H. lusitanicum* (MH532238) from Italy, in *H. marginatum* (MT793816) from Lithuania, in *H. impeltatum* (HQ335157) from Egypt, and in goat (OR248871) from Ghana.

The BLASTn analyses of the consensus sequence of the *ompB* gene showed that there were 98.39-100% nucleotide resemblance between our *Ri. aeschlimannii* isolate and *Ri. aeschlimannii* isolate identified in various parts of the world. Moreover, 100% nucleotide identities were seen *Ri. aeschlimannii* isolate determined in the study and *Ri. aeschlimannii* isolate detected in *H. marginatum* (MK215218) from Germany, *D. reticulatus* (OR000446) from Poland, and *H. rufipes* (OR734630) from Kenya.

The phylogenetic trees based on *ompA* (Figure 3A) and *ompB* (Figure 3B) genes revealed our *Ri. aeschlimannii* isolate placed with the same clade with *Ri. aeschlimannii* isolates reported in different parts of the world and positioned in different branch from other *Rickettsia* species.

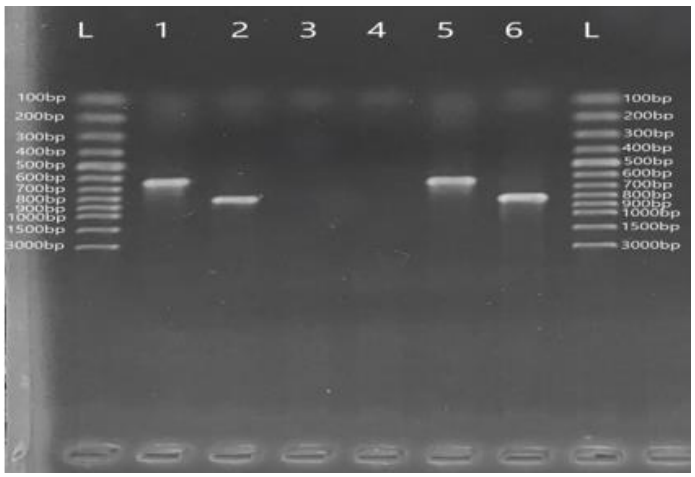

Figure 2. Agarose-gel electrophoresis of water buffalo *Rickettsia* species. L. Ladder, 1. *Ri. aeschlimannii* positive sample (*ompA*, gene), 2. *Ri. aeschlimannii* positive sample (*ompB*, gene), 3-4. Negative control, 5. *Ri. aeschlimannii* positive water buffalo sample (*ompA*, gene), 6. *Ri. aeschlimannii* positive water buffalo sample (*ompB*, gene)

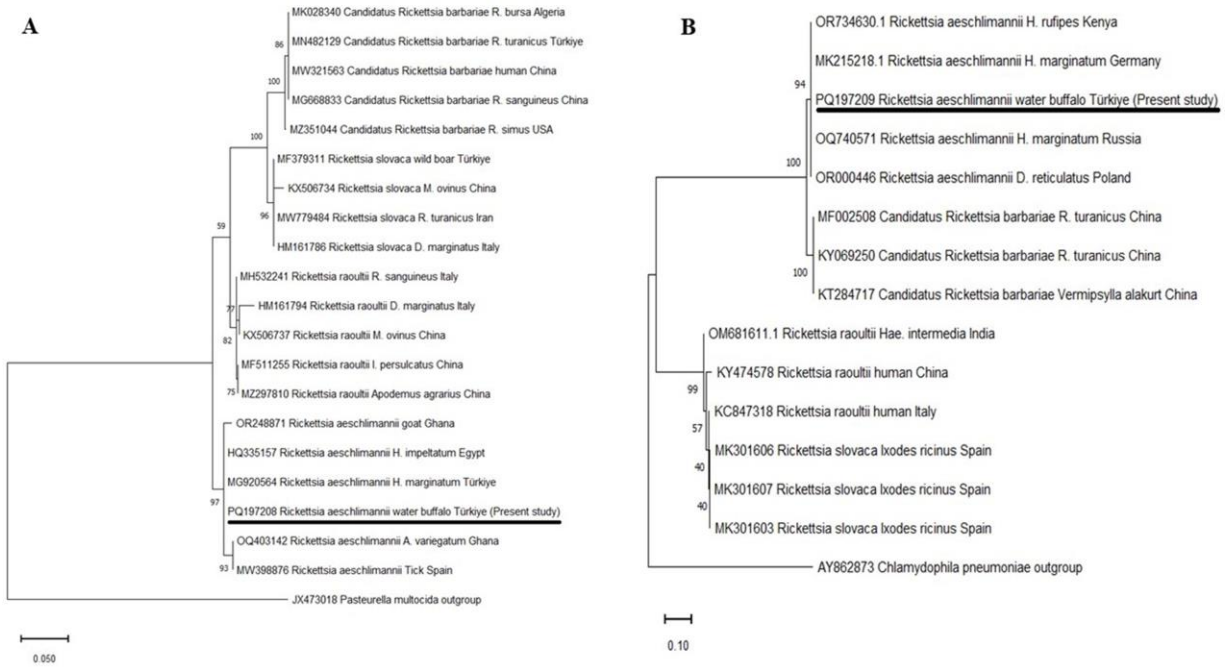

Figure 3. Phylogenetic tree based on the *ompA* (A) and *ompB* (B) genes sequences of *Rickettsia* species using the maximum likelihood method. *Rickettsia* species identified in this study were marked with underlined. Numbers at the nodes represent the bootstrap values with 1,000 replicates. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-3 parameter model (32). *Pastorella multocida* and *Chlamydophila pneumoniae* were used as an outgroup in the phylogenetic trees

Discussion

The importance of vector-borne pathogens increases year by year due to different reasons, such as climate change, deforestation, and expanded human settlement towards nature. Studies show that millions of people and animals live in areas at risk of vector-borne diseases (33, 34). Türkiye has a unique location, as the country is situated between the continents of Europe and Asia. This location makes the country a bridge for diseases to cross from one continent to another. In addition, Türkiye has a variety of climates suitable for the

survival of many vector species (17). Therefore, many studies have been conducted to understand the epidemiology, prevalence, and distribution of vector-borne pathogens in Türkiye and various vector-borne pathogens have been identified in the country, especially among domestic animals (17). Water buffaloes are important domestic ruminants in Türkiye, but the number of studies conducted to investigate vector-borne pathogens in buffaloes in the country is quite low. Only four molecular studies were performed on these animals, in these studies, *Anaplasma capra* (18), *Anaplasma phagocytophilum*-like 1 (19), *Mycoplasma wenyonii*, *Candidatus* Mycoplasma haemobos (36), and *Theileria buffeli* (35) were identified, to date. According to the literature review, no study presents on *Rickettsia* species in water buffalo in Türkiye. In this study, *Rickettsia* species were investigated in water buffalo herds for the first time and phylogenetic analyses of the detected *Rickettsia* species were performed.

In the last two decades, many *Rickettsia* species, some of them novel species, have been identified among various hosts and their vectors in the different parts of the world using molecular-based techniques (1, 4). These studies also showed that *Rickettsia* species have been circulated in domestic ruminants, and DNA of these pathogens was detected in blood samples of cattle, sheep, and goats (37-39). Serological studies were performed in water buffalo, and antibodies against *Rickettsia* species were detected in these animals (40). In this study, 364 water buffalo blood samples were researched and one sample was found to be infected with *Rickettsia* species. This is the first molecular detection of *Rickettsia* DNA in a water buffalo blood sample. Our work and study performed by Pangjai et al. (40) demonstrated that water buffaloes may be exposed to *Rickettsia* species. The prevalence and distribution of vector-borne pathogens such as *Rickettsia* may vary according to the climatic characteristics of the study regions, the common vector species in the sampling regions, the specificity and sensitivity of the diagnostic methods used in the study, and the number of animals included in the study and their ages. In addition, since new *Rickettsia* species and strains have been detected in different hosts in recent years (1, 4), it is thought that studies should be carried out to determine the epidemiological risk factors of these pathogens.

DNA sequence analyses have been done for different purposes such as the correction of PCR results, revealing phylogenetic analyses and genetic diversity of species, identification of novel species or genotypes of pathogens, and discovery of new host or epidemiological areas of pathogens in the studies (1, 14, 18). The DNA sequence of one *Rickettsia* species identified in the work was done by targeting *ompA* and *ompB* genes. These sequence results revealed that *Ri. aeschlimannii* was circulated in a water buffalo herd in Sivas province. The *ompA* (98.93-100%) and *ompB* (98.39-100%) gene sequences showed that our *Ri. aeschlimannii* isolate had high nucleotide similarities with *Ri. aeschlimannii* isolates identified in different hosts from various parts of the world. With this study *Ri. aeschlimannii* was detected for the first time in water buffalo using PCR and DNA sequence analyses. Although *Ri. aeschlimannii* was detected in a water buffalo in the work, large-scale molecular studies involving buffaloes and their ectoparasites in different countries are needed to understand the contribution of these animals to the epidemiology of *Ri. aeschlimannii*.

Rickettsia aeschlimannii was identified firstly in ticks (*H. marginatum*) from Morocco (41). Five years later, this pathogen was reported in humans returning to France from Morocco using serological and molecular techniques (42). In the same and following years, *Ri. aeschlimannii* was detected in humans from South Africa (43), and Greece (8). In these studies, clinical manifestations such as necrotic vesicular symptoms on the ankle, high fever, and maculopapular skin rash around the tick-bite sites were reported in patients, and these patients were treated with doxycycline for one week (8, 42, 43). To date, several studies have been performed, and *Ri. aeschlimannii* was reported in different hosts (*Meriones shawi,* camel, human*, Hyalomma marginatum, Rhipicephalus sanguineus etc.)* from various parts of the world (4). In this study, the DNA of *Ri. aeschlimannii* was detected for the first time in a water buffalo blood sample both in Türkiye and world. In Türkiye, *Ri. aeschlimannii* was identified in different cities from multiple hosts like *Hyalomma* spp. collected from cattle (27), hares (26), and humans (23, 25), *H. marginatum* from wild boars (26), cattle (23), goats (28), sheep (28), and humans (20, 22, 23, 25), *H. aegyptium* from human (20, 23, 25), and hedgehogs (24), *H. excavatum* from humans (25), *Rhipicephalus* spp. from goats (27), *Rhipicephalus bursa* from humans (20, 25), *R. turanicus* from humans (25), cattle (28), and sheep (23), *Haemaphysalis parva* from human (25), *Hae. punctata* from human (25), *Hae. sulcata* from humans (25), *Dermacentor marginatus* from humans (25), and *Ixodes ricinus* from humans (25). Above-mentioned studies revealed *Ri. aeschlimannii* is carried by multiple tick species in Türkiye. Sivas is the second biggest city in Türkiye, and different tick species, some of them vectors of *Ri. aeschlimannii,* circulate among domestic and wild animals in the city (44-46). In studies conducted by Altay et al. (45, 46) several tick-borne pathogens were reported in the ticks collected from cattle and sheep. Considering that *Ri. aeschlimannii* is a zoonotic pathogen transmitted by ticks, it is thought that buffalo breeders and people living in the region should take the necessary precautions against the pathogen.

In conclusion, *Ri. aeschlimannii* was reported for the first time with this study in a water buffalo. This molecular report showed that buffaloes, like other domestic ruminants, are hosts/reservoirs for vector-borne zoonotic pathogens. Vector-borne pathogens have a wide range of pathological effects on human and animal health. In addition, the economic loss due to these pathogens can reach billions of dollars, and studies showing the current prevalence and distribution of vector-borne pathogens among hosts are needed to reduce this economic loss.

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