MOLECULAR INVESTIGATION OF SOME BACTERIA (COXIELLA BURNETII, MYCOPLASMA HAEMOCANIS, CANDIDATUS MYCOPLASMA HAEMATOPARVUM, WOLBACHIA) IN RHIPICEPHALUS SANGUINEUS TICKS IN SIIRT PROVINCE, TURKEY

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ABSTRACT

Ticks harbor the largest diversity of microorganisms, ranging from viruses, prokaryotes, and eukaryotes. *Rhipicephalus sanguineus* ticks are the most common ticks worldwide. Although dogs are the main host of this tick species, it has been reported that it also infests humans in various parts of the world. This study aimed to examine some bacteria (*Coxiella burnetii, Mycoplasma haemocanis, Candidatus Mycoplasma haematoparvum, Wolbachia*) in *Rhipicephalus sanguineus* ticks sampled from dogs. In this study, 350 tick samples collected from 85 dogs in Siirt province were determined to be *Rhipicephalus sanguineus* ticks. *Coxiella* DNA was detected in 3 (0.85%) out of 350 ticks using Nested PCR (687 base pairs). None of the samples were found to contain *Mycoplasma haemocanis, Candidatus Mycoplasma haematoparvum*, and Wolbachia DNA. A partial sequence of the IS1111 gene region was registered in GenBank with OM472143 accession numbers. Considering the zoonotic nature of the Q disease, it is very important for dog owners and related institutions to periodically spray animals against ticks, and to take any other necessary precautions. More samples are needed to determine the Mhc, CMhp, and Wolbachia prevalence.

**Keywords:** *C. burnetii, Mycoplasma haemocanis, Candidatus Mycoplasma haematoparvum, Rhipicephalus sanguineus, Siirt*
INTRODUCTION

Ticks, after mosquitoes, are the most prevalent arthropod vectors, capable of spreading the widest range of infections. For this reason, the detection of microorganisms carried by ticks is an important issue for human or animal health (Plantard et al., 2012). Ticks are responsible for the transmission of a variety of infections that infect both people and animals, including bacteria, helminths, protozoa, and viruses (Dantas-Torres, 2010; Ayan et al., 2019).

Q fever is an important highly infectious zoonotic disease caused by an obligate intracellular gram-negative bacterium called Coxiella burnetii (Leulmi et al., 2016; Rezaei et al., 2018; Ma et al., 2020). C. burnetii can survive in outdoor environments for long periods since it is resistant to many physical and chemical factors (Maurin and Raoult, 1999; Kalender, 2001). A wide range of reservoirs exists for the disease that consists of domestic and wild mammals, birds, and arthropods (Rezaei et al., 2018; Tukur et al., 2019). Rodents, birds, and rabbits play an important role as reservoirs, but cattle, sheep, and goats are the primary reservoirs that are related to potential human infection (Webster et al., 1995; Ma et al., 2020). Animals acquire the infection by direct contact with diseased material, or through ticks (Kalender, 2001; Rezaei et al., 2018). Infections caused by C. burnetii in animals are largely asymptomatic, but coxiellosis is known to cause decreased fertility, abortions, infertility, retained placenta, weak newborns, and perinatal deaths in ruminants (Woldehiwet, 2004; Cantas et al., 2011; Ma et al., 2020). People who come into contact with animals (such as veterinarians, and slaughterhouse workers) are at high risk (Kılıç, 2017). In humans, Q fever may occur subclinically, with no clinical signs of an acute or chronic disease that can cause life-threatening conditions or death (Cooper et al., 2011). The most important clinical signs in humans are high fever and severe headaches (Kalender, 2001). Individuals who come into contact with infected asymptomatic animals, especially at the time of bearing, can become infected (Rezaei et al., 2018). While Microagglutination (MA), Complement Fixation (CF), Indirect Fluorescent Antibody (IFA), and Enzyme-Linked Immunosorbert Assay (ELISA) tests are used for the serological diagnosis of the disease (Kalender, 2001), the PCR method is successfully used as a molecular method (Rezaei et al., 2018).

Hemotropic Mycoplasmas (Haemoplasmas) are epierythrocitic parasites of mammals that are small, pleomorphic, cell wall-deficient, facultative intracellular bacteria in the group of non-cultured mycoplasma species (Sykes et al., 2005; Barker et al., 2010; Sababoglu et al., 2021). These bacteria cause asymptomatic intravascular infections in domestic and wild animals, however, they are not regarded particularly harmful (Maggi et al., 2013).

The disease agent can be determined by Giemsa's staining of blood smears in the form of small coccoids, rings, or strings on the erythrocyte membrane, or they can be found free in the plasma (Lumb, 2001; Hosseini et al., 2017). Although the pathogenic potential of hemotropic mycoplasmas as a cause of human disease is unknown, these zoonotic pathogens may constitute a greater public health threat than is currently recognized (Maggi et al., 2013). Two types of hemotropic mycoplasma have been identified that infect dogs, which are Mycoplasma haemocanis (Mhc) and Candidatus Mycoplasma haemotarparum (CMhp) (Messick, 2004; Rosanna et al., 2020; Sababoglu et al., 2021). Mhc infection usually causes clinically significant anemia only in splenectomized or immunocompromised dogs, although latent infections can still cause subclinical anemia (Messick, 2004; Barker et al., 2010). CMhp was first described in association with anemia in a splenectomized dog undergoing chemotherapy for leukemia (Barker et al., 2010). The infection is characterized by
fatigue, depression, loss of appetite, weight loss, and anemia and it can cause death (Lumb, 2001; Hosseini et al., 2017).

Wolbachia is classified within the Rickettsiales order and is obligate-intracellular bacteria transmitted by a wide range of arthropods (Chao et al., 2021). Wolbachia was first detected in the ovaries and testicles of the mosquito Culex pipiens. Wolbachia is so frequent and omnipresent that some studies have estimated they have infected almost half of the earth-based arthropods, and more than half of the insects overall (Yildirim et al., 2013; Chao et al., 2021). While their involvement with mosquitos has been well-established, their presence in ticks or tick-transmitted pathogens is not well understood (Chao et al., 2021).

Wolbachia have been detected in several studies in ticks (Hartelt et al., 2004; Tijsse-Klasen et al., 2011).

The objectives of this study were to examine some bacteria (Coxiella burnetii, Mycoplasma haemocanis, Candidatus Mycoplasma haematoparvum, Wolbachia) in Rhipicephalus sanguineus ticks sampled from dogs.

MATERIALS AND METHODS

Study area and Ticks Collection
Tick samples in this study were collected from 85 dogs in Siirt province. After the dogs were inspected, the ticks were collected into separately labeled 25 mL containers containing 70% alcohol and taken to the laboratory.

Tick Morphology and DNA separate
The detection of ticks was carried out by the method reported by Walker et al. (2000) and Estrada-Peña et al. (2004). Before DNA extraction, each sample was washed with 70% ethanol. Then, the ticks were taken into individual tubes and subjected to freezing and thawing processes. Ticks inside the tubes were crushed using a sterile glass rod. For DNA isolation, the Invitrogen PureLink™ Genomic DNA Mini Kit was used according to the manufacturer suggestion. The obtained DNA was stored at -20 °C until further analysis.

Detection of Coxiella burnetii
PCR was performed to amplify the IS1111 gene region of Coxiella burnetii of 687 bp. IS1111 is a multicopy transposon with a highly increased sensitivity for the detection of C. burnetii. The primers, Tran1 (5’TATGTATCCACGTAGCCAGTC-3’) forward and Tran2 (5’-CCCAACAAACCTCTTATTTC-3’) reverse were used as previously described (Mares-Guia et al., 2014). 200 µM dNTPs, 1.5 mM MgCl2, 6 pmol forward and reverse primers, 0.1 U Taq Polymerase, and 10X PCR buffer (500 mM Tris-HCl, pH 8.8, 160 mM (NH4)SO4 and 0.1% Tween®20), Nuclease Free Water, and 4 µL DNA were used in a 25 µL master mix. The reaction was created by pre-denaturation for 15 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, bonding period at 60°C for 30 s, elongation period at 72°C for 1 min, and a final elongation period of 7 min at 72°C. The reaction was performed on a Kyratec Gradient Thermal cycler. The prepared 1.5% agarose gel was stained with RedSafe Nucleic Acid Staining Solution. The PCR products were then run on an agarose gel and the images were recorded with the gel imaging device (Syngene bioimaging system).

Detection of Mycoplasma haemocanis (Mhc)
The primers, 5’-GAAAATCAGGGCCATAATGACGC-3’ forward and 5’-ACCTGTCACTCGATAACCTCTAC-3’ reverse were used to amplify the 309 bp 16S rRNA gene region of Mycoplasma haemocanis (Mhc). PCR reaction and Temperature cycling conditions were adapted according to Torkan et al. (2014).

Detection of Candidatus Mycoplasma haematoparvum (CMhp)
The primers, 5’-ACGAAAGTCACATTGAAGAATGC-3’ forward and 5’-TATCTACGATTCCACCCGCTAC-3’ reverse were used to amplify the 328 bp 16S rRNA gene region
of Candidatus Mycoplasma haematoparvum (CMhp). PCR reaction and Temperature cycling conditions were adapted according to Torkan et al. (2014).

Detection of Wolbachia
The primers, 5’-TGGTCCAATAAGTGATG AAGAAACTAGCTA-3’ forward and 5’- AAATTTAAAGCTACTCCAGCTTCTGCA C-3’ reverse were used to amplify the 590 bp to 632 bp wsp gene region of Wolbachia (Zhou et al., 1998; Simsek and Ciftci, 2016). PCR reaction and Temperature cycling conditions were adapted according to (Zhou et al., 1998). Primer pairs, target gene, and PCR product sizes are present in Table 1.

Sequence and Phylogenetic Analysis:
The QiAquick PCR Purification Kit (QIAGEN, Germany) was used to purify the PCR product according to the manufacturer’s instructions. The same primers used in PCR amplification were utilized to sequence purified PCR products in both directions. Applied Biosystems’ ABI 3100 Genetic Analyzer Automated Sequencer (Applied Biosystems, USA) was used to run the sequencing operations, which used ABI PRISM BigDyeTM terminator cycle sequencing kits (Applied Biosystems, Foster City, USA). The sequences were assembled and edited using Bioedit software (version 7.2). Molecular and evolution genetic analysis (MEGA X) software was used to accomplish multiple sequence alignment. Distances between sequences were calculated automatically using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993; Kumar et al., 2018).

Ethical Approval
This study was approved by the Siirt University Animal Experiments Local Ethics Committee (Approval no: 2021.01.15).

RESULTS
An overall of 350 ticks were collected and identified down to the species level using morphological analysis. All ticks were identified as Rhipicephalus sanguineus species. Coxiella DNA was detected in 3 (0.85%) out of 350 ticks using the Nested PCR method (687 base pairs) (Fig.1). Mycoplasma haemocanis (Mhc), Candidatus Mycoplasma haematoparvum (CMhp), and Wolbachia DNA were not detected in any of the samples. The phylogenetic tree was constructed with the Maximum Likelihood (MCL) method, using the DNA sequences (Fig.2). The statistics of the obtained phylogenetic tree were evaluated with 1000 repetitive bootstrap analyses. Partial sequences of the IS1111 gene region were registered in GenBank with OM472143 accession numbers. Legionella pneumophila (DQ897170.1) was selected as the out-group. The partial sequence of C. burnetii from this study was compared to the sequence available in the GenBank with BLAST search. The partial C. burnetii sequence obtained in this study showed 100% homology to Coxiella burnetii strain Coxi-IR-FM-112 insertion sequence IS1111A transposase gene, and Coxiella burnetii isolate goat_614 transposase gene, respectively. It was also found that the sequence obtained from this study had 99.84% similarity to Coxiella burnetii strain Coxi-IR-FM-101 insertion sequence IS1111A transposase gene, and Coxiella burnetii isolate Coxi-SM2/Iraq transposase gene, respectively.

Legends
Table 1. Species-specific primers used in the study, and their genome sizes

Figure 1. 16S rRNA amplification of C. burnetii in ticks using PCR. Lanes M: Marker, N: Negative control, P: positive control, Lanes 45,46,47 represent C. burnetii (687 bp).

Figure 2. Phylogenetic tree of C. burnetii with IS1111 partial sequences using the Maximum Likelihood method and Tamura-Nei model. Evolutionary analyses were conducted in MEGAX. The nucleotide sequence determined in this study is indicated in the black dot. Legionella pneumophila was used as an out-group.
Table 1: Species-specific primers used in the study, and their genome sizes.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Specificity (%)</th>
<th>Primary</th>
<th>Sequence (5’-3’)</th>
<th>Product Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS1111</td>
<td><em>C. burnetii</em></td>
<td>Forward</td>
<td>5’-TATGTATCCAC CGTAGCCAGTC-3’</td>
<td>687</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-CCCCAACAAACAC TCTCATT-3’</td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td><em>Mhc</em></td>
<td>Forward</td>
<td>5’-GAAAATAGG CATAATGACGC-3’</td>
<td>309</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-ACCTGTCACCTGATAACCTCTAC-3’</td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td><em>CMhp</em></td>
<td>Forward</td>
<td>5’-ACGAAAATGTCTAG GAGCA A TA C-3’</td>
<td>328</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-TATCTACGCAA TCAGCTAC-3’</td>
<td></td>
</tr>
<tr>
<td>wsp</td>
<td><em>Wolbachia</em></td>
<td>Forward</td>
<td>5’-TGGTC AAGTAGTGA A AAAACTA GCTA-3’</td>
<td>590-632</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-AAAAATACGTCAC GCTAC-3’</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

One of the most prominent vectors along with mosquitos, especially considering zoonotic diseases, are the ticks. Due to their hematophagous nature, they are involved in transmitting numerous pathogens between animals and/or humans (Leulmi et al., 2016). Ticks host the widest range of microorganisms, ranging from viruses, prokaryotes, and eukaryotes (Plantard et al., 2012). R. sanguineus ticks are the most common type of ticks worldwide. Although dogs are the main host of this tick species, it has been reported that it also infests humans in various parts of the world (Chao et al., 2021).

Q fever is a zoonotic infectious disease with a worldwide presence, caused by the obligate intracellular bacterium Coxiella burnetii (Andoh et al., 2013; Oskam et al., 2017). More than 40 types of ticks have been associated with C. burnetii and other Coxiella species to date (Oskam et al., 2017; Khalili et al., 2018).

Different prevalence has been reported by studies conducted in different parts of the world. Nine of 164 (5.5%) ticks in the Philippines (Ybañez, 2014), 5 of 209 (2.4%) tick pools in Italy (Satta et al., 2011), 1 of 8 (12.5%) tick pools in Iran (Khalili et al., 2018), 1 of 24 (4.17%) R. sanguineus ticks in Egypt (Loftis et al., 2006), 26 of 44 (59%) R. sanguineus ticks in Malaysia (Watanabe et al., 2015) and all of 199 R. sanguineus ticks (100%) in Australia (Oskam et al., 2017) were determined to be positive. In a study conducted by Andoh et al. (2013) in Japan, all 261 ticks involved in the study were found to be negative concerning C. burnetii.

Even though there are seroprevalence studies in Turkey for the diagnosis of Coxiella burnetii, the number of studies performed on ticks is quite limited. It was reported that 46.15% and 1.89% positivity were detected in Denizli and Ankara provinces, respectively, in two studies (Capin et al., 2013).

Coxiella burnetii positivity was detected in 3 (0.85%) of 350 ticks examined in this study. The results of this study are similar to the results of the study conducted by Capin et al. (2013) and Satta et al. (2011). Differences between the findings may be due to geographical location, different climates, sample size, sampling period, tick species, number and stage of infected ticks, and availability of appropriate reservoirs and methods.

The PCR method has long been accepted as a highly sensitive and accurate determination process for C. burnetii in a wide range of sample types. The method offers some advantages compared to classical serological methods where the determinations can only be performed retrospectively and in a limited fashion (Capin et al., 2013). In this study, the PCR method was used for the detection of agents in ticks. The partial C. burnetii sequence obtained in this study showed 100% similarity to C. burnetii strain registered to GenBank from Iran (KP719175.1, KP719174.1, KP719165.1), and Brazil (JF972643.1).

Hemoplasmosis is an infection caused by hemotropic mycoplasmas and R. sanguineus ticks are reported to be a possible vector for hemoplasmosis. Studies show that there is a significant relationship between the presence of R. sanguineus and hemoplasma infection (Willi et al., 2007; Wengi et al., 2008). R. sanguineus type ticks play an important role in the transmission of canine hemoplasmas and are reported to be found in arid regions of Turkey (Aydin et al., 2015).

In a study conducted in Diyarbakır, it was reported that the Mhc rate was 26.2% and the CMhp rate was 6.7% (Aktas and Ozubek, 2017). In the study carried out by the same researchers in different provinces of Turkey the Mhc rate was 4.5% and the CMhp rate was 4.3% determined (Aktas and Ozubek, 2018). In a study conducted by Sababoglu et al. (2021) in Adana, it was reported that 8
(2.56%) of 312 ticks were positive for CMhp, while all samples were negative for Mhc.

Wolbachia bacteria can be found as endosymbionts in insects, arachnids, crustaceans, and filarial nematodes (Yetişmiş et al., 2018). Some studies report as high as 65% infection rates for insects with Wolbachia (Yıldırım et al., 2013). In a study conducted in Taiwan, it was reported that the rate of the agent in Rhipicephalus sanguineus ticks was 46.1% (Chao et al., 2021).

The examined ticks were found to be negative in terms of Mhc, CMhp, and Wolbachia as a result of this study. The reason for the negative result obtained from the study might depend on the tick species, the number of ticks involved with the study, and/or the sampling environment.

CONCLUSION

The data obtained from this study shows that dog-infesting ticks can be infected by C. burnetii. Considering the zoonotic nature of Q disease, it is very important for dog owners and related institutions to periodically drug animals against ticks and take any other necessary precautions. Efforts should be focused on understanding the role and epidemiological significance of dogs and infected ticks, especially for human Q fever, which can be a life-threatening disease. More tick samples are needed to determine the Mhc, CMhp, and Wolbachia status in Siirt province.

ACKNOWLEDGMENTS

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COMPETING INTERESTS

Authors state no conflict of interest.

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