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Original Article

Molecular Identification of *Salmonella* spp. in Ticks Isolated from Domestic Animals in Zanjan Province

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Abstract
Salmonella is a Gram-negative Bacteria that is commonly found in most environments and organisms and is a causative agent of disease. *Salmonella* spp. is one of the most common foodborne illnesses. Salmonellosis is a common infectious disease in humans and animals that manifests with gastrointestinal or hepatic symptoms and can lead to various clinical symptoms such as diarrhea in infants, fetal abortion, orchitis, pneumonia, and septicemia. In the current study, 412 hard ticks were classified and identified to investigate Salmonella spp. based on diagnostic keys. In total, 412 hard ticks, including 208 *Hyalomma* species and 204 *Rhipicephalus* species, were identified. The samples were divided into 82 pools according to the tick genus, and DNA was extracted from the ticks. Pathogens transmitted by ticks were diagnosed using PCR, and samples were examined for the presence of *Salmonella* spp. bacteria. In the study, a total of 208 *Hyalomma* tick samples and 204 *Rhipicephalus* tick samples were collected and were separated by gender in pools of five. Out of these, 51 male pools and 30 female pools were identified. *Rhipicephalus* ticks had 27 male and 14 female pools, while *Hyalomma* ticks had 24 males and 16 female pools. The study found that 8 out of 40 (20%; 95% Cl: 10.5%-34.76%) *Hyalomma* ticks, and 12 out of 41 (29.27%; 95% Cl: 17.61%-44.48%) *Rhipicephalus* ticks were carriers of the pathogens, indicating that these pathogens can be transmitted by different species of hard ticks. Ticks and tick-borne diseases are a significant public health concern worldwide.
Keywords: PCR, *Salmonella* spp., Ticks, Zanjan province

Introduction

The family *Enterobacteriaceae* are Gram-negative bacteria found in the digestive systems of humans, animals, plants, insects, water, soil, and decaying matter. Since the natural habitat of this family is the intestines of humans and animals, they are also referred to as enteric bacteria (Brown et al., 2005; Orkun et al., 2014; Gruenberg et al., 2018). Some members of this family, such as *Salmonella*, *Yersinia*, and *Shigella*, are true pathogens, while others, like some strains of *Escherichia coli*, *Proteus*, and *Klebsiella*, are part of the normal flora of humans and animals, and only cause disease under specific conditions (Parola and Raoult, 2001).

Salmonellosis is a common infectious disease between humans and animals that manifests with gastrointestinal or liver symptoms and can lead to various clinical symptoms, including diarrhea in infants, fetal abortion, orchitis, pneumonia, and septicemia (Yagoub et al., 2005). Various types of *Salmonella* spp. play a role in such infections. Non-

typhoidal *Salmonella* spp. infections have been reported to cause urinary tract infections in immunocompromised human patients. In general, salmonellosis is caused by two species of *Salmonella*, *S. enterica* and *S. bongori* (Allerberger et al., 1992; Gordon et al., 2008; Teklu and Negussie, 2011).

Ribosomal RNA gene 16 Svedberg (*16SrRNA* gene) exists in all bacteria and has a constant common region among all bacterial species. The inclusive primers amplify the constant region in the 16 Svedberg ribosomal RNA gene sequence (*16SrDNA*), which is constant in all bacteria (Olwoch et al., 2007). Hard ticks are found in Asia, Europe, and Africa, and there are 702 different species across 14 genera. Ticks are the second most widespread pathogen vectors worldwide, followed by mosquitoes. The diseases caused by ticks are increasingly threatening human and animal health, besides causing economic losses (Enferadi et al., 2023). They are important because they can spread diseases to animals and humans, such as East Coast fever, *Anaplasmosis*, *babesiosis*, and rickettsiosis (Barker and Murrell, 2004; Olwoch et al., 2007). Ticks feed on blood and can transmit various harmful microorganisms, causing economic losses. In Iran, there is a diverse range of tick species, each capable of transmitting different infectious agents that lead to diseases like Lyme disease (Prescott, 2002; Enferadi et al., 2023). This study aimed to identify *Salmonella* spp. in hard ticks collected from domestic animals in Zanjan.

Materials and methods

Study area

Zanjan province is one of the 31 provinces of Iran (Figure 1), whose capital is the city of Zanjan. It is a mountainous province of approximately 22,000 km^2 of land located in Iran's Region 3. The livestock population of Zanjan province is 2,366,411 units. According to the data for the first half of this year, the number of sheep and lambs in the livestock sector of Zanjan province is 657,820. This figure is equivalent to the number of livestock units in another department. In terms of goats, the province has 162,322 heads. (Kalantari et al., 2021).

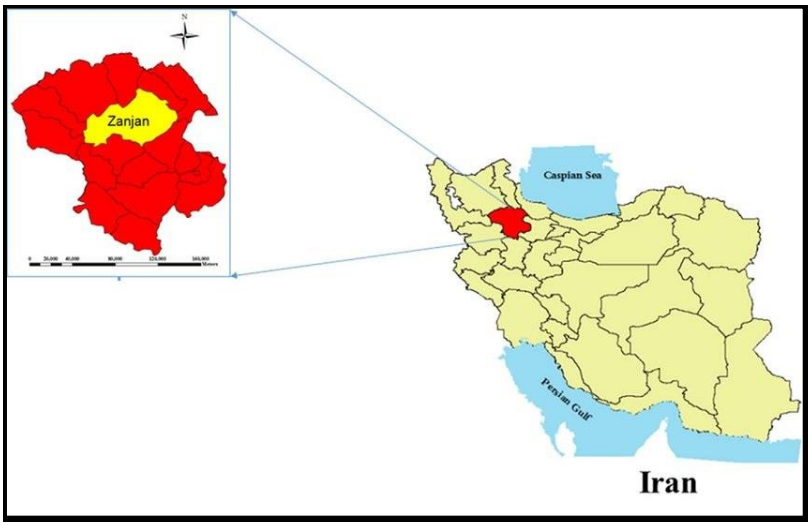

Fig. 1. Map of the Zanjan region in Iran where ticks samples were collected.

Sample collection

A total of 412 ticks were collected from sheep and goats in various livestock environments in the Zanjan region. The tick samples were fixed on 96% ethanol and transferred to the parasitology laboratory of the Faculty of Veterinary Medicine,

the University of Urmia (Iran). After identifying the genus and species of the ticks using diagnostic keys under a microscope, they were transferred to the microbiology department of the Faculty of Veterinary Medicine. The DNA extraction followed by PCR were performed to identify the *Salmonella* spp. genus in the ticks.

DNA extraction

To extract DNA from tick samples, after air drying the ticks, the samples were completely ground with a scalpel and transferred to 2 mL micro tubs. Then, a DNA extraction kit from tissue and blood (Biotechnology Company, Tehran, Iran) was used. To ensure the extraction method and evaluate the DNA concentration, 10 samples were randomly selected, and their absorbance was read at 260 nm using NanoDrop 2000 (Thermo Scientific, USA). The ratio of 260/280 (DNA/protein) was also determined.

Polymerase Chain Reaction (PCR)

The *16SrRNA* gene was used to identify the *Salmonella* spp. genus. The primers were designed using Amplifx version 1.5.4 software (France). The list of primer pairs and PCR temperature programs are provided in Table s1 and 2, respectively.

Table 1. *Salmonella 16S rRNA* gene primers sequence

Gene	Primer name	Primer sequence	The length of the piece
16SrRNA	F	ATTTCTCACGCCAGGATTTG	641
	R	GATCGGCAAAGGTTAGGTCA	

Table 2. Program and temperature cycles of *16SrRNA* gene of *Salmonella (*PCR conditions C/ of S).

Gene	First Denaturation	Denaturation	Annealing	Extension	Final Extension
16SrRNA	5mine/72℃	1mine/72℃	1mine/63℃	1mine/96℃	5mine/96℃

The PCR reaction was performed in a total volume of 25 µL, containing 4 µL of template DNA, 1 µL of each primer, and 12.5 µL of red master mix (Ampliqon, Denmark), and the total volume was completed with sterile distilled water. The thermal cycling and PCR program were defined in a thermal cycler (Quanta Biotech, UK) according to Table 2. The PCR products were electrophoresed on a 2% agarose gel containing a safe stain (Labnet, ENDURO, USA) and then visualized using Genius Gel Documentation (Syngene Bio-Imaging, UK) (Figure 2).

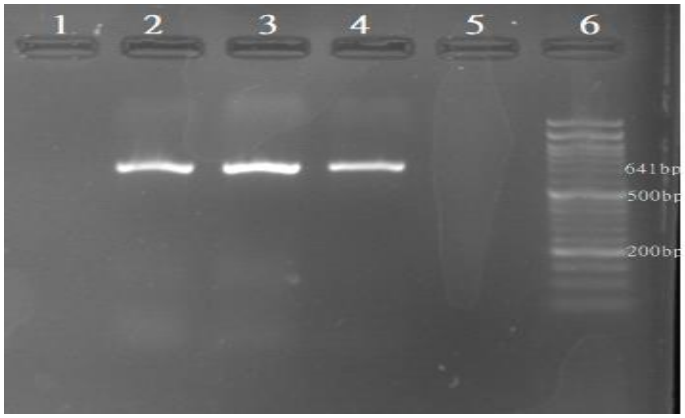

Fig. 2. Electrophoresis image of *Salmonella 16srRNA* gene in agarose gel, six marker wells (50bp), wells 1 and 5 are negative samples, wells 2 to 4 are positive samples.

Phylogenetic analysis results

The nucleotide sequences of each species were aligned for diversity positions in the same direction. The sequences were uploaded to NCBI to search for the most similar reference sequences, and the COI position was determined using

BLAST available in NCBI. The COI nucleotide sequences belonging to the *Salmonella* spp. genus in the gene bank were used for phylogenetic analysis. The alignment was manually edited to eliminate any alignment errors using Clustal W and exported as MEGA 11 (France) and FASTA format files. All nucleotide sequences obtained were deposited in GenBank with assigned accession numbers. Then, the phylogenetic relationship was constructed using the maximum likelihood (ML) method through molecular evolutionary genetics analysis software MEGA version 11. The DNA sequence polymorphism analysis was estimated using MEGA 11 version 7.0.1 and Blastn software to determine nucleotide diversity.

Data analysis

The obtained data were calculated based on the confidence interval of 95%. It calculates the lower and upper limits of the 95% confidence interval for a proportion (Table 3).

Results

In Zanjan Region, 412 hard ticks from animals like sheep and goats were collected. The ticks belonged to two different groups - 208 were *Hyalomma* species and 204 were *Rhipicephalus* species. A microscope was used to identify ticks by examining their external features such as festoons, uterine channels, anal shields, lateral shields, and scutum color (Table 3). The study collected a total of 412 ticks, 208 of which were *Hyalomma* ticks and 204 were *Rhipicephalus* ticks. These ticks were separated into pools of five, with 27 male and 14 female pools for *Rhipicephalus* ticks and 24 male and 16 female pools for *Hyalomma* ticks. In total, 51 male pools and 30 female pools were collected. After performing PCR, it was shown that 9 out of 27 male pools and 3 out of 14 female pools were positive for *Salmonella* spp. in *Rhipicephalus* ticks, while six male and two female pools were positive for *Salmonella* spp. in *Hyalomma* ticks. The percentage and 95% confidence interval of the presence of *Salmonella*-positive bacteria are presented in Table 3.

Discussion

The species of *Hyalomma* and *Rhipicephalus* ticks cause irritation, stress, and blood loss in their hosts (Brown et al., 2005). While a few ticks are generally not harmful to animals, a large number of ticks (dozens or hundreds) can cause significant harm, such as weight loss, reduced fertility, and decreased milk production. Some tick species can also spread diseases to animals and humans, and since they feed on various hosts during their life cycle, they have a higher chance of acquiring and transmitting pathogens (Brown et al., 2005).

The *Rhipicephalus* species of ticks are the most commonly found around the world, and they are known to spread diseases to humans, cattle, and sheep. They can be found both in urban and rural areas and can live in human dwellings. These ticks are active in tropical, subtropical, and temperate regions (Dantas-Torres, 2010). They are an important group of arthropod vectors, transmitting various disease-causing microorganisms like viruses, bacteria, and protozoa to both wild and domestic animals, as well as humans (Uilenberg, 1995; Jongejan and Uilenberg, 2004). In our study, *Salmonella* spp. bacteria were commonly isolated from *Rhipicephalus* and *Hyalomma* ticks. This is consistent with the findings of Mohanad and Moaed (2012), who studied ticks collected from sheep in Basrah, Iraq, and found that they were contaminated with *Enterobacteriaceae* bacteria (Kirecci et al., 2015).

Another study conducted by Kirecci et al. (2015) showed that Bacillus species were the most common pathogens isolated from *Hyalomma* ticks collected from turtles (Kirecci et al., 2015). In a study conducted by Orkun et al. (2014), researchers collected a total of 169 ticks from people in different parts of Ankara, Turkey. They found that the most commonly collected tick species were *Hyalomma* and *Rhipicephalus* ticks (Orkun et al., 2014).

Table 3. The results of contamination of tick samples collected from the surface of goats and sheep in the Zanjan region.

Ticks					
Number of ticks	**Ticks species**	**No.**	**pool**	**Genus**	***16S rRNA*** (95%Cl)
141	***Rhipicephalus sanguineus***	98	20	Male	7/20(35%) 7 (n=20; 35%; 95%Cl: 18.12%-56.71%)
		43	9	Female	2/9(22.22%) 2 (n=9; 22.22%; 95%Cl: 6.32%-54.74%)
63	***R.turanicus***	37	7	Male	2/7(28.57%) 2 (n=7; 28.57%; 95%Cl: 8.22%-64.11%)
		26	5	Female	1/5(20%) 1 (n=5; 20%; 95%Cl: 3.62%-62.45%)
56	***Hyaloma asiaticum***	33	6	Male	1/6(16.66%) 1 (n=6; 16.67%; 95%Cl: 3.01%-56.35%)
		23	4	Female	0/4(0.0%) 0 (n=4; 0%; 95%Cl: 0%-48.99%)
82	***H. aegyptium***	51	10	Male	4/10(40%) 4 (n=10; 40%; 95%Cl: 16.82%-68.73%)
		31	6	Female	2/6(33.33%) 2 (n=6; 33.33%; 95%Cl: 9.68%-70%)
70	***H. anatolicum***	41	8	Male	1/8 (12.50%) 1 (n=8; 12.5%; 95%Cl: 2.24%-47.09%)
		29	6	Female	0/6(0.0%) 0 (n=6; 0%; 95%Cl: 0%-39.03%)
Total		260	51	Male	15/51(29.41%) 15 (n=51; 29.41%; 95%Cl: 18.71%-43%)
		152	30	Female	6/30(20%) 6 (n=30; 20%; 95%Cl: 9.51%-37.31%)
		412	81		21/81(25.92%) 21 (n=81; 25.93%; 95%Cl: 17.63%-36.41%)

The current study collected a total of 412 hard ticks, consisting of 208 *Hyalomma* and 204 *Rhipicephalus* ticks from sheep and goats in the Zanjan region. The results showed that *Rhipicephalus* ticks had the highest contamination with *Salmonella* spp. *Salmonella* spp. bacteria, and also, male ticks were more contaminated with *Salmonella* spp. than female ticks, possibly due to their higher frequency of blood feeding and greater host diversity (Williams et al., 2005). In a four-year surveillance study conducted in the United States, 66,000 tick species were identified, and *Rickettsia*, an important pathogenic bacterium, was found in these ticks (Merten and Durden, 2000; Orkun et al., 2014).

Our study has revealed that *Hyalomma* and *Rhipicephalus* ticks are capable of transmitting *Salmonella*, a bacterium that can cause illness in humans and animals. We conducted molecular identification using PCR and sequencing, and the results showed that the genetic makeup of the *Salmonella* strains was most similar to those of *Salmonella enterica subsp. enterica*, *Salmonella enterica subsp. enteritidis, and Salmonella enterica subsp. typhimurium*. This was confirmed through phylogenetic tree analysis. (Parola and Raoult, 2001; Chandra et al., 2007; Dantas-Torres, 2010).

Some previous studies revealed how hard ticks can transmit diseases to humans, such as tick paralysis, Lyme disease, and Crimean-Congo hemorrhagic fever virus (CCHV), among others. Such studeis did not investigate viruses or non-culturable pathogens, but they found *Salmonella* spp. in these ticks, which can cause gastroenteritis and other infections in humans (Stromdahl and Hickling, 2012; Reifenberger et al., 2022).

With the increasing prevalence of zoonotic diseases and global climate change, tick-borne illnesses are becoming more dangerous, and preventive measures should be taken (Parola and Raoult, 2001; Dietrich et al., 2011; Palomar et al.,

2021). The results of this study are consistent with a previous one conducted by Heaney in 2012, which emphasized the importance of ticks in transmitting diseases to humans and animals at all stages of their life cycle (Heaney and Cannon, 2012). Firstly, they can feed on an infected host animal and ingest the pathogen into their blood. Secondly, ticks can transmit the pathogen through their eggs for most diseases, meaning that the tick mother can pass the disease-causing agent to her offspring. Thirdly, simultaneous feeding or feeding by multiple ticks on one host can cause infection. Therefore, ticks can transmit diseases at any stage of their life cycle. Each time they take a blood meal, humans are at risk of contracting the disease (Parola and Raoult, 2001; Sili, 2017).

Conclusion

Hard ticks are the most dangerous arthropods that endanger the health of vertebrates or threaten them, and they can transmit the greatest diversity of pathogens to both humans and animals. Hard ticks can transmit many pathogenic bacteria to vertebrates, among which the most important are the bacteria of the Enterobacteriaceae family. In this study, contamination with the *Salmonella* spp. a genus in hard ticks (*Hyalomma* and *Rhipicephalus* genera) was identified and sequenced using PCR.

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Conflict of Interests

The authors declare no conflict of interest.

Ethical approval

Not applicable.

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