Molecular identification and characterization of Rickettsia spp. and other tickborne pathogens in cattle and their ticks from Birjand, Iran

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Abstract:

Introduction

Tick-borne pathogens (TBPs) pose significant threats to both animal and human health worldwide. Among these pathogens, *Babesia*, *Theileria*, and *Rickettsia* species are of particular concern because of their prevalence in cattle and their potential zoonotic impact.

Objective

The objective of this study was to investigate the prevalence of major tick-borne pathogens in cattle and their associated tick species collected from different geographical locations in eastern Iran using molecular approaches.

Materials and Methods

This cross-sectional study was conducted from January to June 2024. Blood samples were collected from 100 heads of cattle, and ticks were simultaneously removed from the animals in the study areas. A total of 95 ticks were morphologically identified, including *Hyalomma marginatum* (42%, n = 40), *Dermacentor marginatus* (36.8%, n = 35), and *Rhipicephalus sanguineus* (21%, n = 20). Both cattle blood samples and tick specimens were screened for tick-borne pathogens using molecular techniques.

Results

The most frequently detected pathogen in cattle blood samples was *Rickettsia* spp., identified in 5% (n = 5) of the samples, followed by *Theileria annulata* detected in 4%. Overall, 7.3% (7/95) of the tick DNA pools tested positive for protozoan pathogens. Sequencing analysis revealed infection of ticks with *Rickettsia conorii* (3%), *Babesia bovis* (3%), and *Theileria annulata* (1%).

Conclusion

The results of this study demonstrate the presence of multiple tick-borne pathogens in cattle and their associated tick species in eastern Iran. These findings emphasize the need for continuous surveillance and the implementation of

appropriate control measures to reduce the risk of tick-borne diseases in animal populations and potentially in humans

Keywords: Cattle, Polymerase chain reaction, Tick-borne pathogens, Iran

1. Introduction

The blood-feeding behavior of many arthropods, especially ticks and fleas, allows them to act as important vectors for a wide range of viral, bacterial, and protozoan pathogens that affect both animal and human health [1]. Some microorganisms can survive within ticks throughout their developmental stages through transstadial transmission, which supports their continued presence in tick populations [2]. Tick-borne diseases are a major concern for public and veterinary health worldwide. Among the different tick genera involved in pathogen transmission, Rhipicephalus species are particularly important due to their broad geographic distribution and their ability to carry several infectious agents. In Iran, cattle are commonly infested with Rhipicephalus ticks, which may contribute to the transmission of pathogens of veterinary relevance as well as those with zoonotic potential [3].

Recent studies have indicated that ticks can transmit a variety of microorganisms, including Ehrlichia, Anaplasma, hemotropic Mycoplasma, Babesia, and Theileria species [4]. Infected animals may develop clinical outcomes ranging from subclinical infection to severe or fatal disease. The use of molecular diagnostic methods has greatly improved the detection of tick-borne pathogens. Traditional diagnostic techniques, such as microscopic examination and serological tests, often show limited sensitivity and specificity, particularly when pathogen levels are low or when mixed infections are present [5]. In comparison, molecular methods such as polymerase chain reaction (PCR) and quantitative PCR (qPCR) provide more reliable detection and allow accurate identification of closely related pathogens [5]. However, information on the prevalence and diversity of tick-borne pathogens in cattle and their associated ticks in Iran is still limited [6]. Therefore, the present study aimed to investigate the occurrence of major tick-borne pathogens in cattle and their associated ticks in Iran using molecular methods.

2. Materials and methods:

2.1.Study area

The study was conducted from January to June 2024 in different locations in eastern Iran. The research was carried out at the Islamic Azad University, Science and Research Branch, Iran. The cattle included in the study had no history of deworming or treatment with ectoparasiticides.

2.3. DNA extraction

Genomic DNA was extracted from blood and tick samples using a Blood DNA Extraction Kit and the G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology, South Korea), respectively, according to the manufacturer's instructions. The extracted DNA samples were stored at −20 °C until further analysis. A total of 95 tick specimens were selected for DNA extraction. To minimize the possibility of amplifying host blood DNA, visibly engorged ticks were excluded from molecular analysis.

Prior to DNA extraction, ticks were removed from ethanol and air-dried at room temperature. Each tick was then placed in a sterile Petri dish and bisected using a sterile blade. One half of each specimen was used for DNA extraction, while the remaining half was preserved for future analyses. Molecular identification and confirmation of tick species were performed by PCR amplification and sequencing following the methodology described by Latrofa et al. (2013) [9]. The quality and concentration of the extracted DNA were evaluated using agarose gel electrophoresis and a NanoDrop spectrophotometer.

2.4. PCR amplification

Polymerase chain reaction (PCR) was used to detect tick-borne pathogens (TBPs) in DNA samples obtained from cattle and ticks. Positive PCR products were subsequently subjected to DNA sequencing for accurate pathogen identification. Primers targeting conserved genomic regions of Rickettsia spp. and Babesia/Theileria spp. were used in the assays. Details of the target genes, primer sequences, PCR conditions, and corresponding references are provided in Table 1.

Following amplification, PCR products were analyzed by agarose gel electrophoresis to confirm the presence of expected DNA fragments. A 1.5% agarose gel was prepared, and DNA bands were stained using ethidium bromide or

SYBR Safe. Five microliters of each PCR product were mixed with loading dye and loaded into the gel wells. Electrophoresis was performed at 100 V for 30–45 min, and the bands were visualized under ultraviolet light. The sizes of the amplified products were determined by comparison with a DNA ladder to confirm pathogen identity.

Primer sequences and PCR conditions used for tick species, Rickettsia spp., Babesia/Theileria spp. identification.

Assay	**Target**	**Primer**	**size**	**References**
Rickettsia spp.	16S rRNA	**TAAGGAGGTAATCCAGCC** **CCTG GCTCAGAACGAA**	1482	(23)
Babesia spp.	18S rRNA	**AATACCCAATCCTGACACAGGG** **TTAAATACGAATGCCCCCAAC**	408	(24)
Theileria	18S rRNA	**GAGGTAGTGACAAGAAATAA** **CAATA TCTTCGATCCCCTAACTTTC**	390 430	(25)
Mitochondrial 16S ribosomal DNA	16S rDNA	**CTGCTCAATGATTTTTTAAATTGCT** **GTGG TTACGCTGTTATCCCTAGAG**	350 450	(24)

2.5.Sequencing

Following the successful amplification of target genes through PCR, the resulting PCR products were purified using a commercial DNA purification kit (e.g., Qiagen QIAquick PCR Purification Kit) to remove excess primers, nucleotides, and enzymes. The purified DNA was then subjected to Sanger sequencing, which was performed by a commercial sequencing facility. To confirm the identity of the sequenced products, the obtained sequences were subjected to a Basic Local

Alignment Search Tool (BLAST) search against the National Center for Biotechnology Information (NCBI) database.

2.6.Ethical Considerations

All sampling procedures were conducted in accordance with ethical guidelines for animal research. Informed consent was obtained from wners prior to blood collection, and all efforts were made to minimize discomfort and stress to the animals during the sampling process.

3- RESULT

From the total 95 ticks collected, 40 (42 %) were morphologically identified as *Hyalomma marginatum*. and 35 (36.8%) as *Dermacentor marginatus*, and 20 (21%) *Rhipicephalus sanguines*. A total of two *H. marginatum*, five *D. marginatus*, and three *R. sanguines* were further studied on 16S rDNA mitochondrial genes for molecular identification. From the sequence analysis Table 2 of the 16S rDNA mitochondrial gene of the two *H. marginatum* ticks, both showed to be identical, sharing 99.5 identity with *H. marginatum* (PP937568.1) from Egypt. The 16S rDNA analysis of the *D. marginatus* ticks, showed that all five were identical, sharing 100 identity with an Kazakhstan *D. marginatus* (OR486023.1). Analysis for the 16S rDNA mitochondrial gene of three ticks also showed that all were identical, sharing 98.9% with *R. sanguines* (MK732015.1) from Portugal. From the total of 95 ticks studied, three (3.1%) were found positive for rickettsiae using the 16srRNA assay, all in *R. sanguines* ticks. Further characterization of the 16srRNA sequences showed an identity between 99.8 with *R. conorii* (NR_074480.2). When screening for *Babesia* genera using the 18S rRNA gene only three tick (*H. marginatum*) (3.1%) amplified a product of the expected size. The analysis of 18S rRNA sequence obtained showed 99.4% identity with *Babesia bovis* (OL583948.1). When screening for *Theileria* genera using the 18S rRNA gene only one tick (*R. sanguineus*) (1%) amplified a product of the expected size. The analysis of 8S rRNA sequence obtained showed 99.6% identity with T. *annulata* (MF287930.1). Regarding blood specimens, five sample was found positive for rickettsiae, by using the 16SrRNA PCR assays. From the 100 blood specimens tested for *Theileria* protozoan parasites four (4%) presented amplified products of the expected size.

The amplified sequences of rickettsiae and *Theileria* were same as the tick isolated. GenBank accession numbers of tick sequences obtained in this study are:

PV490755 (*Hyalomma marginatum*), PV490754 (*Dermacentor marginatus)*, PV490756 (*Rhipicephalus sanguines*). GenBank accession numbers of *Rickettsia* sequences obtained in this study is: PV490810 (R. conorii). GenBank accession numbers of *Theileria* sequences obtained in this study is: PV490808. GenBank accession numbers of *Babesia* sequences obtained in this study is PV490811. (Table 2).

Table2.Tick-borne pathogens present in cattle blood and tick specimens

	R. conorii	***B. bovis***	***T. annulata***
Cattle blood	5	0	4
Adult ticks	3	3	1

4- DISCUSSION

In the present study, molecular methods were employed to detect *Rickettsia*, *Babesia*, and *Theileria* species in blood samples obtained from apparently healthy cattle as well as from their associated tick specimens. Morphological examination revealed that cattle in Iran were predominantly infested with *Hyalomma marginatum*, *Dermacentor marginatus*, and *Rhipicephalus sanguineus*. The findings showed that 10% of cattle with heavy tick infestations were positive for tick-borne pathogens (TBPs), indicating a notable level of pathogen exposure among animals in the study area. This prevalence is slightly higher than that reported in a previous Iranian study, which documented a TBP infection rate of 8% [10]. Among the detected agents, *Rickettsia conorii* was the most frequently identified pathogen, being found in 5% of cattle blood samples and 3% of pooled tick DNA samples. This result differs from earlier reports in Iran that described a considerably higher overall prevalence of *Rickettsia* spp. (24.9%; 95% CI: 20.28–29.52) [11]. The pronounced level of tick infestation observed in the current study suggests a high degree of environmental contamination in the sampled regions, which likely increases the risk of cattle exposure to infected ticks, as also noted by previous studies [12]. These observations emphasize the need for effective control measures directed at both livestock and their ectoparasites [13,14]. The 18S rRNA gene of *Theileria* and *Babesia* was detected exclusively in *R. sanguineus* and *H. marginatum* ticks collected from cattle, respectively. Partial sequencing of the 18S rRNA gene from positive tick samples demonstrated a high degree of sequence similarity with *Theileria annulata* and *Babesia bovis* sequences available in the GenBank database. Both *T. annulata* and *B. bovis* are well-known tick-borne pathogens affecting cattle and other domestic ruminants, including sheep and goats, as well as wild ruminant species [15]. Theileriosis and babesiosis are among the most important parasitic diseases of livestock, causing substantial economic losses worldwide [16,17]. Previous investigations have examined the distribution of bovine theileriosis in eastern and northern regions of Iran and have also reported molecular characterization and phylogenetic analysis of the 18S rRNA gene from *Theileria* and *Babesia* isolates recovered from domestic animals in these areas [18]. Molecular techniques, particularly PCR amplification followed by DNA sequencing, are widely accepted as reliable methods for epidemiological studies and phylogenetic analysis of tick-borne pathogens, especially piroplasmids [19]. In the present study, PCR targeting the 18S rRNA gene was applied for the detection of *Theileria* and *Babesia* DNA. Broad-range PCR assays directed at this gene, together with partial sequencing, have previously enabled the identification of several known and novel *Babesia* and *Theileria* species [20]. In contrast, the relatively low prevalence of *B. bovis* observed in this study is comparable with findings from Egypt and Tunisia, where prevalence rates of 8.0% [21] and 3.0% [22] were reported, respectively.

Unlike previous studies conducted in Iran, which reported a prevalence of babesiosis in cattle up to 42% [23]., no cases were observed in the present study. In the present study, the prevalence of *Babesia spp* in ticks collected from cattle being 3% while being zero in the cattle. This discrepancy could be due to several reasons: The infected ticks did not successfully transmit the *Babesia* parasite to the cattle during the feeding process; The cattle may have had some level of immunity against

Babesia that prevented them from becoming infected even when exposed to infected ticks and the tick population collected for the study may not have been actively feeding on the cattle, leading to a lower likelihood of transmission [24].

CONCLUSION

This study verifies the presence of multiple vector-borne pathogens (VBPs) in cattle in east of Iran, likely due to their significant exposure to arthropod vectors like ticks. While the implementation of regular preventive measures may be hindered by a lack of financial resources, public-private partnerships could enhance efforts to reduce the risk of VBP transmission, particularly those of zoonotic significance. This study concludes that R. sanguineus and H. marginatum are the primary vectors responsible for babesiosis and theileriosis in east of Iran.

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Ethical approval

All animal owners were interviewed for sampling and consents were taken. This study has been approved by the Research Ethics Committee at Azad University IR.IAU.SRB.REC.1403.330. All authors read and approved the final manuscript.

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Study concept and design:P.T

Acquisition of data:,M.S

Analysis and interpretation of data: S.SH &R.S

Drafting of the manuscript: S.SH &R.S Critical revision of the manuscript for important intellectual content: R.S

Statistical analysis: M.S, P.T

Administrative, technical, and material support:P.T **Data Availability Statement:**

The data that support the findings of this study are available upon request from the corresponding author

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