Molecular Identification of *Rickettsia* Species in Ticks Infesting Cattle, Sheep, and Goats from Kerman, Southeastern Iran: Application of Real-Time PCR for Epidemiological Surveillance

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Abstract

Background: *Rickettsia* spp. represent emerging zoonotic pathogens transmitted by arthropods such as ticks, posing considerable implications for public health and livestock management. Although there is growing acknowledgment of their significance in adjacent areas, data from southeastern Iran are still scarce.

Methods: One hundred ticks were collected from cattle, sheep, and goats in April to June 2025 in Anbarabad, Kerman Province. Genomic DNA extraction was performed, and samples were analyzed for *Rickettsia* spp. utilizing SYBR Green-based real-time PCR that targets the *gltA* gene. Amplification profiles and melt curve analysis were utilized to confirm the sensitivity and specificity of detection.

Results: *Rickettsia* DNA was detected in 15% of ticks overall, with infection rates of 16% in ticks from cattle and 14% in ticks from sheep and goats. These findings confirm the presence of *Rickettsia* spp. in mixed-livestock systems in Anbarabad, highlighting the circulation of these pathogens across different host species. The observed infection rates suggest a consistent level of exposure among ticks parasitizing various livestock, reinforcing the need for continued surveillance and control measures in such endemic settings.

Conclusions: This study represents one of the few molecular examinations of Rickettsia species conducted in southeastern Iran, offering essential foundational information for future research endeavors. The discovery of a moderate presence of ticks in both cattle and small ruminants underscores the ongoing risk of pathogen spread within mixed-livestock systems, which may facilitate transmission between species. The use of advanced molecular techniques, such as sequencing, is essential for enhancing species-level resolution, clarifying complex epidemiological patterns, and guiding targeted public health strategies in regions where diseases are prevalent.

Keywords: Epidemiological surveillance, *gltA*, Real-time PCR, *Rickettsia* spp., Zoonotic risk factors

1. Introduction

Zoonotic illnesses, which are diseases transmitted by arthropod vectors, are a major concern for public health officials worldwide [1]. Zoonotic diseases arise from a diverse array of microorganisms, including bacteria, viruses, and protozoa, which possess the capability to cross the species barrier that distinguishes humans from animals [2]. Among the zoonoses transmitted by vectors, tick-borne illnesses stand out due to their extensive geographic distribution, intricate ecological dynamics, and their potential to induce severe health issues in both humans and domestic animals. The recent decades have seen a resurgence of tick-borne illnesses, highlighting the critical necessity for enhanced monitoring, diagnostic tools, and preventive measures to mitigate the impact of these diseases on public health and livestock productivity [3].

The genus *Rickettsia* comprises Gram-negative, obligate intracellular bacteria that are tiny in size. They are classified under the order *Rickettsiales* [4]. Endothelial cells are often the target of infection by these microbes, which are conveyed to vertebrate hosts by arthropod vectors such as fleas, ticks, and lice. A number of groups, such as the Spotted Fever Group (SFG), the Typhus Group (TG), and the Scrub Typhus Group (STG), have been established within the genus on the basis of the genetic and antigenic traits that determine them [5]. These categories not only signify evolutionary connections but also align with the manifestations of the illness, the involved vectors, and the geographical spread of the disease.

Rickettsial infections, which are commonly known as rickettsioses, are zoonotic diseases that are capable of causing a wide variety of clinical symptoms in humans. These symptoms may vary from minor illnesses similar to the flu to difficult situations that are potentially fatal [6]. It is particularly well-known that the Spotted Fever Group is responsible for generating high fever, rash, lymphadenopathy, and, in rare instances, vascular problems. *Rickettsioses* have the potential to cause severe morbidity and death if they are not effectively diagnosed and treated in a timely manner. When it comes to animals, particularly cattle and small ruminants, these illnesses often go undetected, which creates a concealed hazard to public health owing to the possibility of zoonotic transmission [7].

Brown dog ticks, also known as *Rhipicephalus sanguineus sensu lato*, are a species of tick that may be found all over the globe, including in both tropical and temperate locations. In spite of the fact that it is most often seen in dogs, it is capable of infecting a wide variety of animals, including humans [8]. In addition, *R. sanguineus* is capable of transmitting some infections, including *Ehrlichia canis*, *Babesia vogeli*, and *Rickettsia conorii* [9].

The pathogenesis of *Rickettsia* begins with the invasion of endothelial cells by bacteria, which is then followed by the multiplication of the bacteria inside the cells themselves and their transfer via the circulation [10]. This process causes vasculitis and damage to blood vessels that are tiny to medium in size, which

contributes to the systemic symptoms that are noticed in hosts who are infected. Despite the fact that vertical transmission among tick populations and exposure to contaminated arthropod excrement have also been identified, the most common mode of transmission is via the bite of infected ticks [11]. The management and monitoring of rickettsial illnesses are extremely difficult to accomplish due to the intricacy of the interaction between the host, the vector, and the pathogen [6].

The unclear early symptoms of *rickettsial* infection, which often seem to be similar to those of other febrile illnesses, make it challenging to identify the infection [12]. There are diagnostic methods that are not sensitive enough for early diagnosis, such as serological tests, bacterial cultures, and immunofluorescence. These methods need specialized laboratory facilities. A further limitation of these approaches is that they are often more beneficial during the convalescence period; hence, their use is restricted to circumstances that demand a prompt diagnosis. It is of the utmost importance to make use of molecular methods such as Real-Time Polymerase Chain Reaction (RT-PCR) since these techniques are sensitive, specific, and may offer results in a short period of time. In situations when there is a low pathogen load, RT-PCR is able to correctly identify *Rickettsia* DNA regardless of the kind of sample or the location of the sample. The assessment of zoonotic hazards is very necessary in rural regions because of the close relationship that exists between people and animals, as well as the prevalence of tick infections. In this study, *Rickettsia* species will be characterized by real-time PCR to identify *Rickettsia* species from ticks that are caught on cattle, sheep, and goats in Anbarabad in the country of Kerman.

2. Methods and Materials

2.1. Collection of ticks and study area

Anbarabad, a site targeting cattle, sheep, and goats, is located in Kerman Province, southeast Iran. During April to June 2025, when tick activity is highest in the region, ticks were collected from naturally infested, untreated animals. A sterile forceps was used to carefully remove individual ticks from their host and place them immediately in microtubes containing 70% ethanol using sterile forceps. 100 ticks were collected and stored at -20°C until molecular analysis (50 from cattle, 50 from sheep and goats).

2.2. Tick sterilization and washing

All tick specimens were surface sterilized to ensure that external contamination was not present. Two washes of sterile phosphate-buffered saline (PBS) were applied after immersion in 70% ethanol for five

minutes. We constructed a laminar flow cabinet under aseptic conditions for air drying ticks using sterile filter paper.

2.3. Extraction of DNA

Genomic DNA was extracted from whole ticks by homogenizing and digesting them enzymatically. After grinding each tick, proteinase K was introduced into the lysate buffer. This method ensured the complete lysing of the ticks. The purification of DNA was conducted after its extraction through the phenol-chloroform method, followed by precipitation using ethanol. Following the resuspension of the DNA pellet in 30 µL of TE buffer, it was subsequently stored at -20°C and allowed to freeze. To assess the concentration and purity of DNA, a NanoDrop spectrophotometer was employed.

2.4. Real-Time PCR for Rickettsia spp. Identification

Rickettsia species were detected using a SYBR Green-based real-time PCR assay targeting the citrate synthase gene (gltA) (Table 1). 20 µL of SYBR Green Master Mix (BioFACT, South Korea), 0.3 µL of forward and reverse primers (10 pmol/ µL), 2.5 µL of extracted DNA, and 6.9 µL of nuclease-free water were used in the reaction. Following an initial denaturation at 95°C for 5 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds were performed. To assess the specificity of the amplification, a melt curve analysis was conducted at the end of the run.

Table 1. Primers used for amplification of the gltA gene in Rickettsia spp.

Primer Name	Sequence (5'→3')	Target Gene	Amplicon
SizePanRick-f	ATAGGACAACCGTTTATTT	citrate synthase (*gltA*)	70 bp
PanRick-R	CAAACATCATATGCAGAAA	citrate synthase (*gltA*)	70 bp

2.5. Positive and Negative Controls

In each qPCR experiment, *Rickettsia* DNA was utilized as a positive control, while nuclease-free water acted as a no-template control to verify the validity of the assay. The specificity of detection was evaluated by analyzing the amplification profiles of the test samples alongside the melting temperatures (Tm) of the positive control.

2.6. Examination of Data

Analyzing amplification curves and melting curves was performed using the proprietary software provided by the qPCR platform. A positive sample is determined by its quantification cycle (Cq) value and a single peak in its melt curve matching the positive control. The prevalence of *Rickettsia* species for each host species and geographical location was determined by comparing the number of positive samples to the total number of samples.

3. Results

Validation of real-time PCR assays for *Rickettsia* species targeting the *gltA* gene was conducted. The validation process involved conducting serial dilutions of positive control DNA, ranging from 10^{-1} to 10^{-3}. The consistent amplification of the test yielded average quantification cycles (Cq) of 34.28, 38.74, and 40.93 for each dilution level, indicating that the assay exhibited a suitable degree of sensitivity (Figure 1). The absence of an amplified product in the template-free control indicates that contamination was not present. All positive dilutions exhibited a melting peak, and all replicates demonstrated consistent melting temperatures (Tm) (Figure 2).

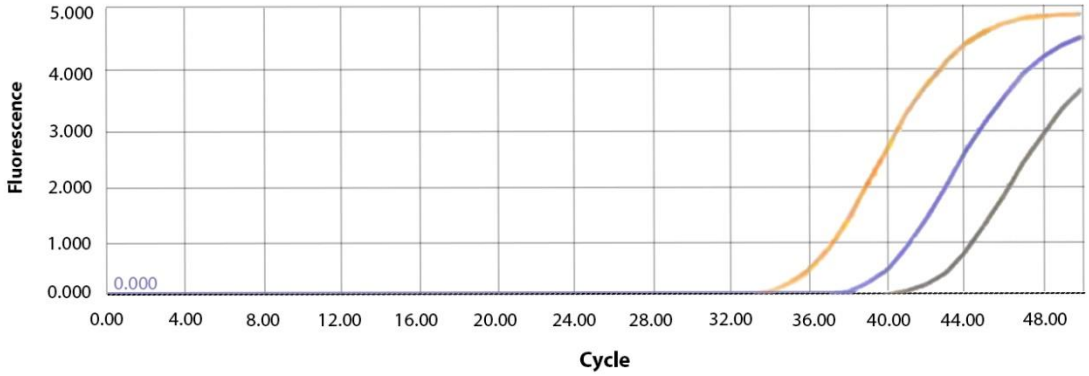

Figure 1: Amplification curves for serial dilutions of *Rickettsia* positive control DNA using SYBR Green-based qPCR.

Figure 2 illustrates the fluorescence amplification curves corresponding to a 10-fold serial dilution of positive control DNA, demonstrating assay sensitivity and dynamic range. The consistent increase in Cq values across dilutions confirms the linearity of the reaction and optimization of qPCR conditions.

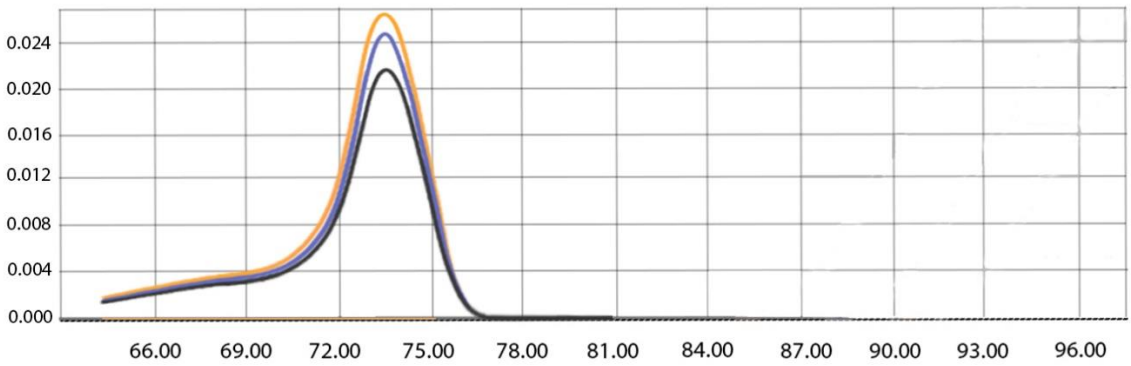

Figure 2: Melt curve analysis showing specificity of amplification in positive and negative samples.

Distinct single-melt peaks were observed in positive control samples, with no amplification peaks detected in negative controls. This confirms the analytical specificity of the SYBR Green-based qPCR assay targeting the *gltA* gene of *Rickettsia* spp.

3.1. Identification of *Rickettsia* DNA in Tick Samples from Cattle

Among the total fifty samples, 8 were positive for *Rickettsia* spp amplification, accounting for 16%. A positive control result was consistent with the Cq values and melting peaks. A total of 84% of the samples were classified as negative due to nonspecific melt profiles or no amplification (Table 2, Figure 3, and Figure 4).

Table 2. Frequency of positive and negative samples for *Rickettsia* spp. among 50 cattle tested specimens

Categories	**Positive**	**Negative**
Count (Total = 50)	8	42
Frequency (%)	16%	84%

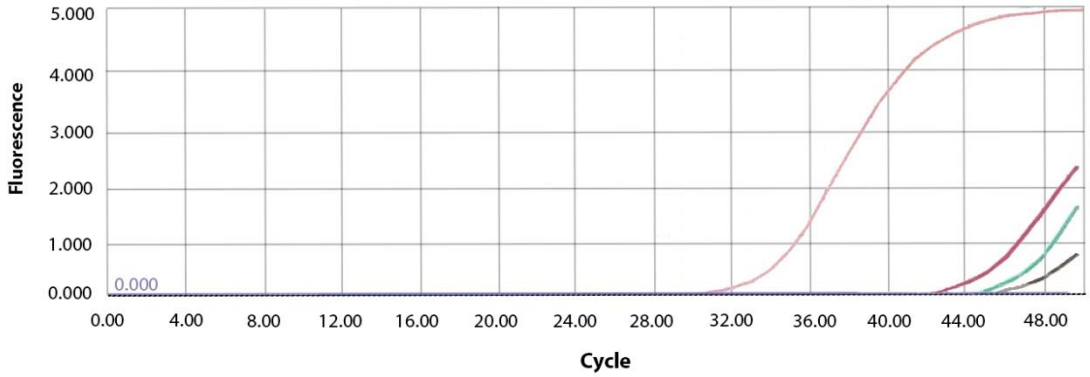

Figure 3: qPCR amplification curves of tick samples from sheep and goats.

Eight positive samples showed distinct Cq signals; the remaining samples were negative with flat baseline curves.

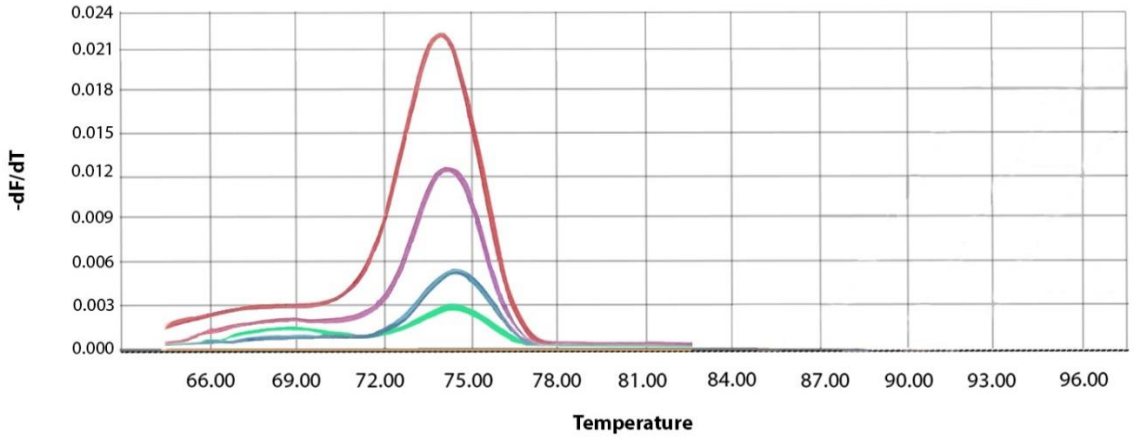

Figure 4: Melt curve analysis of selected positive tick samples from cattle.

Single, sharp melt peaks were observed in all positive samples, confirming the specificity of amplification and absence of primer-dimer or nonspecific products.

3.2. Detection of *Rickettsia* DNA in Tick Samples from Sheep and Goats

A total of 50 tick samples obtained from sheep and goats in Anbarabad County revealed the presence of seven *Rickettsia* DNA sequences. Seven samples are presented, which constitute fourteen percent of the entire total. The profiles of the melting curves indicated that the amplification was indeed specific. Furthermore, the amplification curves obtained from these samples exhibited exponential growth phases that corresponded with those of the positive control. Regarding the remaining 43 samples, which account for 86% of the total, there was no observed amplification signal or generic melting behavior (Table 3, Figure 5, and Figure 6).

Table 2. Frequency of positive and negative samples for *Rickettsia* spp. among 50 Sheep and goat specimens

Categories	Positive	Negative
Count (Total = 50)	7	43
Frequency (%)	14%	86%

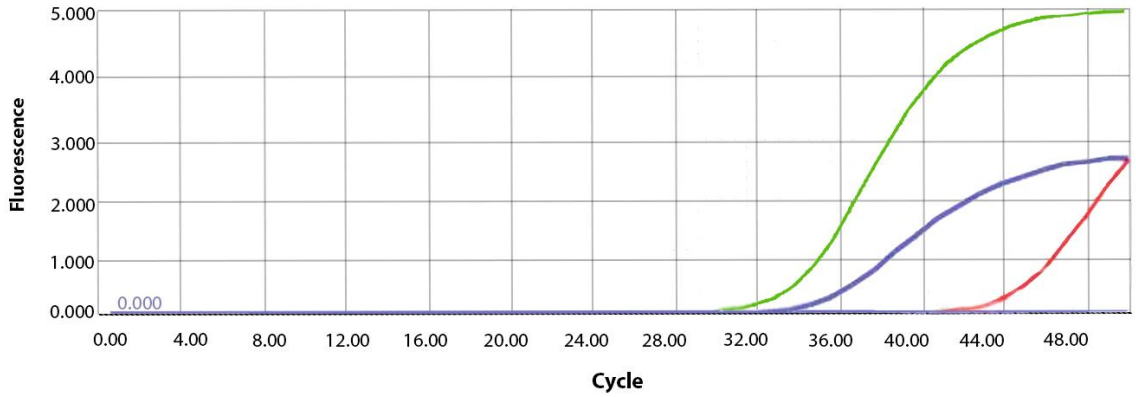

Figure 5: qPCR amplification curves of tick samples from sheep and goats.

Seven positive samples showed distinct Cq signals; the remaining samples were negative with flat baseline curves.

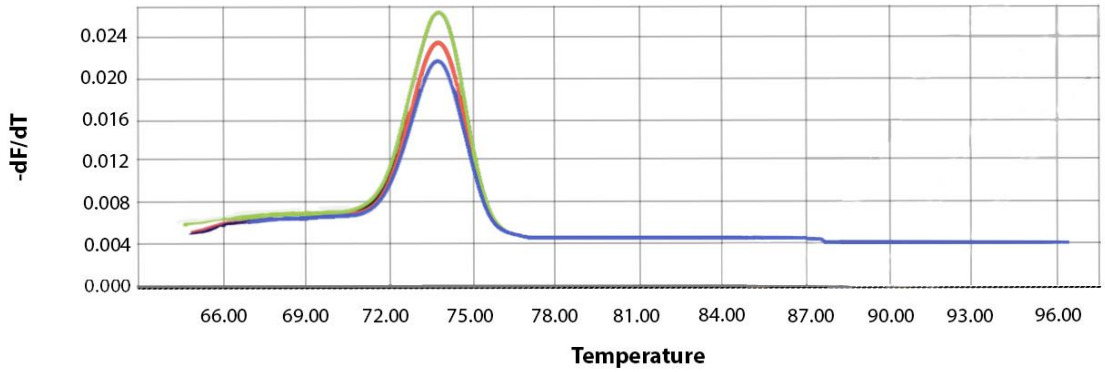

Figure 6: Melt curve analysis of selected positive tick samples from sheep and goats.

Single, sharp melt peaks were observed in all positive samples, confirming the specificity of amplification and absence of primer-dimer or nonspecific products.

4. Discussion and Conclusion

A total of one hundred tick specimens were collected from cattle, sheep, and goats in Anbarabad County, which is located in the southeast of Iran. The purpose of this research was to determine the prevalence of *Rickettsia* species. We were able to identify *Rickettsia* DNA in ticks from cattle as well as small ruminants by using a real-time PCR test that was based on SYBR Green and targeted the *gltA* gene. It was found that out of the fifty ticks that were collected from cattle, eight samples (16%) were positive, whereas forty-two samples (84%) were negative. Ticks taken from sheep and goats (n = 50) generated 7 positives (14%), whereas 43 negatives (86%). The validation of all favorable findings was achieved through melt curve analysis, which demonstrated that specific single-peak profiles matched those of the positive control. The identification of Rickettsia DNA in ticks gathered from cattle and small ruminants suggests that the pathogen is actively circulating in the area, highlighting the importance of implementing monitoring systems that include multiple species. Notably, the variation in infection rates among the host groups, recorded at 14% and 16%, was quite minimal.

Molecular research conducted in Iran indicates that the incidence of *Rickettsia* species varies significantly from one region to another. Approximately fifty percent of the cases are reported from Khuzestan, with *R. aeschlimannii*, *R. massiliae*, and *R. conorii* frequently identified during the infection. The disease exhibits a moderate incidence in northern provinces, with Golestan at 29.7%, and both Mazandaran and Gilan

provinces around 25%, as documented by Ghasemi et al. [13] in 2022. The presence of ecological conditions conducive to tick vectors may account for this phenomenon. The study presented by Ghavami et al. in 2024 [14] indicates that the prevalence rate in Zanjan stands at 6.44 percent. The total prevalence observed in this research is 15% in Anbarabad, with 16% in cattle ticks and 14% in small ruminant ticks. This aligns effectively with mid-range endemic areas, providing a sound estimate within the local epidemiological context.

There is a relatively high frequency of this condition in Pakistan and Turkey, both of which are geographically close to Iran, and the topic has been extensively researched. Ghafar et al. (2021) [15], reported that Pakistan has 54 different species of ticks, the majority of which are *Haemaphysalis*, *Hyalomma*, and *Rhipicephalus*. In the summer months, crossbred young calves exhibit elevated parasite loads compared to older cattle. The connection between these strains and Turkish isolates suggests a likelihood of their dissemination beyond national boundaries. In Turkey, a prevalence of up to 25 percent has been recorded, with *R.* aeschlimannii, *R. slovaca*, and *R. raoultii* detected in ticks from both livestock and wildlife [16]. These findings highlight the significant epidemiological relevance of Pakistan and Turkey for Iran. In both countries, there is a genetic and epidemiological connection, which contributes to the occurrence of this disease. Both countries are prone to the disease, and there may be a genetic link between them that explains its occurrence.

A variety of *Rickettsia* species are responsible for significant tick-borne diseases, with their prevalence influenced by ecological conditions and the specific species of vectors involved. According to Orkun et al. (2014) [16], the strains of *R. helvetica* most frequently reported in Europe include *R. conorii*, *R. slovaca*, and *R. helvetica*. Depending on the species of tick, infection rates range from 2% to 30% in Europe. Zhang et al. (2024) [17] performed a comprehensive synthesis of more than 65 studies conducted in sub-Saharan Africa, demonstrating that *R. africae* stands out as the most prevalent tick species identified in this area. The occurrence of tick-borne pathogens (TBP) in cattle was documented at 52.2% as per the findings of Tawana et al. (2022) [18]. In contrast Cossu et al. (2024) [19] reported that the prevalence of *Rickettsia* in ticks stood at 18.4%.

Several studies conducted in Iran indicate that *Rickettsia* prevalence differs markedly based on host species, geography, and tick genus. Qorbani et al. conducted a survey, which employed both the ELISA method and *gltA*-targeted quantitative polymerase chain reaction (qPCR) method (2024) [20], akin to ours, revealing the presence of *R. aeschlimannii* and *R. conorii* in ticks collected from cattle in Zarand and Jiroft. This validated the regional distribution of these zoonotic agents. Our research builds upon their previous work by presenting host-specific infection rates with a differentiation based on a larger sample size. The current

research employed SYBR Green-based real-time PCR, empowering the accurate identification of *Rickettsia* DNA. Furthermore, the amplification profiles demonstrated reliability, exhibiting minimal background noise, and the melt curve validation remained consistent. As a result of its many advantages, this technology makes it ideal for field-based surveillance programs because of its high-throughput screening capabilities and reduced risk of cross-contamination compared to traditional PCR and nested techniques. Although the method does not achieve resolution at the species level without sequencing, it offers a reliable initial step in the pathogen screening process. There have been a number of studies conducted in Iran, such as Ghasemi et al. in 2017 [13], Mostafavi et al. 2022 [21], Ghavami et al. 2024 [14], and Qorbani et al. 2024 [20], that have used sequencing of *ompA* or *gltA* amplicons in order to identify *Rickettsia* to the species level. When it comes to mapping the zoonotic risk of certain *Rickettsia* lineages in Iranian cattle, the incorporation of such genetic confirmation into future investigations will be very necessary.

Rickettsia species and public health detection in livestock-associated ticks are amplified in regions where other high-risk tick-borne diseases, such as Crimean-Congo Hemorrhagic Fever (CCHF), are co-endemic. It has been reported that the CCHF virus has been detected in *Hyalomma* ticks and livestock throughout Iran, including Kerman Province, such as Jiroft. Farhadpour et al. [22] (2016) reported 6.6% and 4.5% in the south of Iran. In contrast, Champour et al. [23] reported the 4 varieties of CCHF species in northeastern Iran, as opposed to southeastern Iran, where Kerman is located, with *Hyalomma dromedarii* as the most predominant detected tick species in camels, suggesting that *Hyalomma* and *Rhipicephalus* may be the main vectors of the CCHF virus. Interestingly, *Rickettsia*'s primary vector is *Rhipicephalus,* known as the brown dog tick. CCHF and *Rickettsia* infection often share similar clinical features like fever, malaise, and headache, making it difficult to differentiate them. Tick-borne diseases are especially prevalent among herders, veterinarians, and abattoir workers, who may be detected using differential molecular diagnostics. The need for multiplex PCR panels that detect *Rickettsia,* CCHFV, and other tick-borne pathogens is particularly acute in southeastern Iran. *Rickettsia* DNA is found in cattle (16%) and small ruminants (14%), possibly due to similar tick species that parasitize them. There may be subtle differences due to different grooming behaviors, immune responses, and preferences for vectors. To demonstrate the similarities between *Rickettsia* and CCHFV, we conducted similar location studies and provided a comparative figure that enabled us to better understand their similarities (Figure 7).

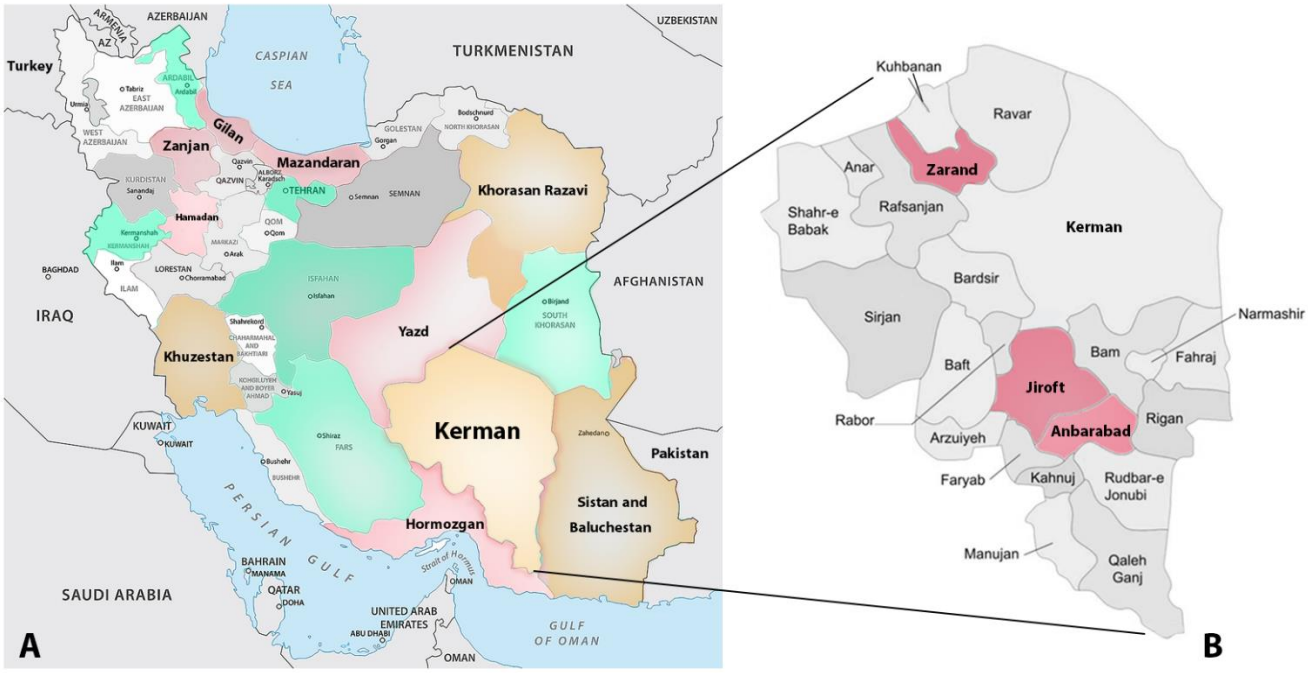

Figure 7: Iranian provinces that have reported *Rickettsia* and Crimean-Congo Hemorrhagic Fever (CCHF) in the past 10 years (2015-2025).

A: The provinces highlighted in red represent the regions affected by *Rickettsia*, while the green areas show the locations of CCHF reports in Iran. Interestingly, the major overlap between them, the orange provinces, indicates a similar host origin for these two diseases.

B: The current research in Anbarabad, located in Kerman province, on cattle, sheep, and goats (n=100, red highlighted), indicates the prevalence of *Rickettsia* in this region. Zarand is also reported to be affected by *Rickettsia* [24].

Although the locations of the two groups are separate and have a distance from each other, a common grazing environment and tick vector likely contributed to the detection of *Rickettsia* DNA in cow ticks (16%) and small ruminant ticks (14%) in the study area. In regions with tick-borne diseases, integration of multiplex PCR panels could improve surveillance precision and public health preparedness. Anbarabad has a warm climate, which favors tick vectors, which contribute to pathogen spread during the long grazing period. A high-risk ecological zone requires sensitive molecular tools, as well as improved diagnostic capabilities, such as sequencing, in Iranian livestock systems, to better understand pathogen diversity, transmission dynamics, and appropriate care and support.

In endemic areas such as southeastern Iran, the clinical similarities between spotted fever group *Rickettsia* infections and CCHF create a significant diagnostic challenge. Both pathogens are transmitted by Hyalomma ticks and present with initial clinical manifestations, including acute fever, cephalalgia, myalgia, and general malaise, which complicates differentiation in the absence of laboratory confirmation. *Rickettsial* infections often present with symptoms such as skin rashes, necrosis of tissues, and lymphadenopathy. Symptoms associated with CCHF may often be disregarded or incorrectly diagnosed during the evaluation process. This diagnostic uncertainty can lead to delays in appropriate treatment, unnecessary isolation of patients, or improper care. The prompt identification of rickettsial infections holds significant importance, as opposed to CCHF, these infections exhibit a swift response to antibiotic treatment, with doxycycline being the preferred pharmacological agent and showcasing considerable effectiveness when administered without delay [25]. Consequently, medical practitioners operating in endemic regions ought to uphold a heightened level of vigilance for *Rickettsia* species in individuals exhibiting fever, rash, and a background of tick exposure, especially when CCHF laboratory evaluations are either negative or awaiting results. From a public health perspective, awareness initiatives and updated clinical protocols are crucial to provide medical practitioners, veterinary professionals, and health officials with decision-making tools that include both CCHF and rickettsioses in differential diagnostic assessments. Improving molecular monitoring and integrating multiplex diagnostic systems that can simultaneously detect *Rickettsia*, CCHFV, and multiple tick-borne pathogens will reduce diagnostic uncertainty. The timely initiation of doxycycline in suspected cases, supported by epidemiological and clinical evidence, may prevent complications, reduce mortality rates, and enhance trust in healthcare systems.

This study accurately identified a species of Rickettsia. In livestock systems, it is essential to implement targeted tick surveillance due to the presence of ticks in southeastern Iran. The use of sensitive molecular diagnostics across various host species revealed a moderate yet regionally consistent prevalence of infection. These findings will enhance epidemiological maps in Anbarabad, Kerman Province, emphasizing the critical importance of accurately identifying tick-borne pathogens for public health objectives. To effectively address emerging zoonotic threats in high-risk ecological environments, the implementation of sophisticated diagnostic tools and strategies centered on host genes will be essential.

Acknowledgments

The authors would like to express their gratitude to the Kerman University research deputy.

Authors' contribution

Conceptualization: [S.S., A.H., M.K.], …; Methodology: [All Authors], …; Formal analysis and investigation: [All Authors], …; Writing - original draft preparation: [All Authors]; Writing - review and editing: [All Authors], …; Funding acquisition: [M.K.], …; Supervision: [M.K., A.M]. All authors checked and approved the final version of the manuscript for publication in the present journal

Ethics

This study has been approved by the Ethics Committee of Kerman University of Medical Sciences. The Ethic approval code is IR.KMU.AEC.1404.063

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

The authors confirm that they did not receive any financial assistance for the research, authorship, and/or publication of this article

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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