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Molecular Identification of *Coxiella burnetii* and *Anaplasma ovise* in Ticks Isolated from the Livestock of Fars Province, Iran

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Abstract

TICKS are significant vectors for transmitting pathogens to humans and animals. This study aimed to identify *Coxiella burnetii* and *Anaplasma ovise* in ticks from livestock and domestic animals in Fars province, south of Iran. Between 2021 and 2022, 4,104 animals (sheep, goats, cows, camels, and dogs) were examined, collecting 3,169 hard ticks. DNA extraction followed by conventional PCR using gene-specific primers was performed. The study identified three genera with the following species: *Rhipicephalus sanguinius* (32.70%), *Rhipicephalus turanicus* (12.51%), *Hyalomma marginatum* (7.5%), *Hyalomma asiaticum* (7.9%), *Hyalomma anatolicum* (34.25%), and *Dermacentor marginatus* (0.16%). The prevalence of *A. ovise* was 18.97%, while *C. burnetii* was found at 22.43%. These pathogens were detected in sheep (25.4%), goats (13.5%), and camels (1.7%). Approximately half of the isolated ticks from livestock were infected with one of these pathogens, thus, this study emphasizes the importance of surveillance and targeted interventions to manage tick populations and reduce the incidence of related zoonotic diseases.

Keywords: Tick-Borne Diseases, Anaplasmosis, Q Fever, Ixodidae, Iran.

Introduction

Ticks are key vectors for transmitting pathogens to livestock and humans, particularly in tropical and subtropical regions [1, 2]. Ticks are key vectors for transmitting pathogens to livestock and humans, particularly in tropical and subtropical regions [3].

Tick-borne diseases (TBDs) significantly impact livestock worldwide, affecting production and related products [4]. *Coxiella burnetii* a small, Gram-negative coccobacillus and obligate intracellular bacterium, causes Q fever, a highly infectious zoonotic disease. According to 16S rRNA sequence analysis, *C. burnetii* is classified in the Gama-subdivision of Proteobacteria, within the Legionellales order and Coxiellaceae family [5]. *Anaplasma ovis*, another tick-borne obligate intracellular bacterium, causes ovine anaplasmosis, primarily affecting the erythrocytes of sheep and goats, though it can also be found in wild ungulates like roe deer and red deer. Reported infections of *A. ovis* in humans are rare [6].

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Global studies indicate that ticks are reservoirs for various common pathogens affecting both humans and animals , including *Anaplasma, Coxiella burnetii, Rickettsia spp.,* and *Ehrlichia spp.* [7]. As livestock are primary hosts for ticks, this significantly contributes to the prevalence of TBDs [8]. Anaplasmosis, an emerging vector-borne disease caused by *Anaplasma* species, can infect both domestic animals and humans, resulting in acute or non-clinical infections [9]. In livestock, it causes symptoms such as high fever, anorexia, weight loss, and decreased milk production [8-12]. *Anaplasma* species have also been detected in horses and dogs [13].

C. burnetii is a zoonotic pathogen transmitted by ticks to humans and animals, causing Q fever and potential latent infections. Symptoms of *C. burnetii* infection can range from acute and asymptomatic to chronic, potentially leading to meningoencephalitis in humans [14, 15]. In livestock and domestic animals, *C. burnetii* can result in perinatal infections and bronchopneumonia [16, 17].

Identifying pathogens that cause zoonotic diseases in animals and humans is crucial for effective control measures. Ticks are significant vectors for pathogens shared between humans and animals. Tick contamination of livestock can impact the animal husbandry industry, leading to various challenges and losses [18]. Therefore, identifying pathogens in ticks that infect livestock helps develop strategies to combat and control these pathogens [19]. Fars province in Iran, where animal husbandry is a primary occupation, was the focus of this study aimed at detecting *C. burnetii* and *A. ovis* in ticks from livestock and domestic animals using molecular techniques.

Material and Methods

Study Area

Tick specimens were randomly collected from various herds across twelve districts in Fars Province, Iran, including Eghlid (30.8932° N, 52.6893° E), Farashband (28.8588° N, 52.0952° E), Fasa (28.9484° N, 53.6376° E), Ghirokarzin (28.3559° N, 52.9600° E), Kazeroon (29.6186° N, 51.6478° E), Larestan (27.6628° N, 54.3223° E), Marvdasht (29.8788° N, 52.8067° E), Shiraz (29.5926° N, 52.5836° E), Sepidan (30.0451° N, 52.2762° E), Mohr (27.6059° N, 52.7456° E), Firuzabad (28.8445° N, 52.5714° E), and Abadeh (31.1627° N, 52.6483° E) (Figure 1).

Tick Samples

Ticks were collected in Fars Province from March 2021 to October 2022 across 12 districts and 36 villages in four geographical regions, primarily from livestock hosts. The districts included Shiraz, Firuzabad, Farashband, Qir-o-Karzin, Eghlid, Marvdasht, Kazerun, Abadeh, Fasa, Larestan, Mohr, and Sepidan (Figure 1). In each village, three pens were randomly checked for ticks—external ectoparasites—under safety conditions (masks, gloves, and overalls) for two hours at sunset. The livestock sampled included sheep, goats, cows, dogs, and camels, with a consistent sampling standard applied across all locations. Each village's latitude and longitude were recorded using Global Positioning System (GPS).

Morphological Identification of Ticks

The collected tick specimens were transported to the entomology laboratory of Shiraz University of Medical Sciences Faculty of Health while maintaining the cold chain and were identified to the species level using a stereo microscope (Olympus, China) and a recognized identification key.

Primer design, DNA Extraction and PCR assay

Two pairs of primers for detecting *Coxiella* (IS1111 gene) and *Anaplasma* (msp4 gene) were designed based on sequences available in GeneBank. The primers are FCoxilla: GCAAACAGGATTAGACC and RCoxilla: GCGATTACTAGCGATTCC for *Coxiella*, and FAnaplasma: GTYARRGGCTAYGRCAAGAG and RAnaplasma: AGTRAACTGGTAGCTWATYCCA for *Anaplasma*. All specimens were surface sterilized twice with 70% ethanol and then washed twice with sterile water to remove debris and animal hairs before being crushed in 1.5 mL Eppendorf® tubes. In this study, ١٠ ticks weighing approximately ٤٠ mg were pooled and homogenized for DNA extraction. Genomic DNA (gDNA) was extracted using a tissue DNA extraction kit (Sambio, Taiwan) following the manufacturer's protocol and stored at −20 °C until needed.

The 20 µL PCR reaction mixture consisted of 1 µL genomic DNA (100-200 ng), 1 µL each of forward and reverse primers (400 pmol), 9 µL double-distilled water (DDW), and 8 µL PCR Master Mix (Amplicon, 2X). The thermocycling program included an initial step at 94ºC for 5 minutes, followed by 40 cycles of 94ºC for 30

seconds, 53ºC for 30 seconds, and 72ºC for 45 seconds, concluding with a final extension at 72ºC for 10 minutes. DDW was used as controls for both negative and positive PCR reactions. The PCR products were analyzed on a 1% agarose gel and visualized using Gel Documentation (InGenius 3, China).

DNA Sequencing and Phylogenetic Analysis

PCR products showing the expected size band were amplified in four replicates and analyzed on a 2% agarose gel. The bands were excised from the gel, purified using a DNA Gel extraction kit (Kit-EX6151), and sequenced with specific primers by Iranian Pishgam Company. The phylogenetic tree was constructed using the MEGA 6.0 bioinformatics program. Sequences of relevant samples from NCBI were retrieved and converted to a FASTA file using Gene Runner. The expected PCR product of the identified *C. burnetii* was sequenced, and the nucleic acid results were edited with G Prime software. Similar sequences from the gene bank were extracted and aligned using the ClustalW method, and the phylogenetic tree was created using the maximum likelihood (ML) method in MEGA 6.0, with a bootstrap value of 1000 for all methods.

Results

This study identified three genera and the following species: *Rhipicephalus sanguinius* (32.70%), *Rhipicephalus turanicus* (12.51%), *Hyalomma marginatum* (7.5%), *Hyalomma asiaticum* (7.9%), *Hyalomma anatolicum* (34.25%), and *Dermacentor marginatus* (0.16%). The prevalence of *A. ovise* infection was 18.97%, while *C. burnetii* was 22.43%. These pathogens were found in sheep (25.4%), goats (13.5%), and camels (1.7%). The most common ticks captured were *Hy. anatolicum* (34.25%), *Rh. sanguineus* (32.70%), and *Rh. turanicus* (12.51%). Table 1 offers comprehensive and detailed information regarding the various ticks that were collected, organized specifically by city. This table serves as a valuable resource for understanding the distribution and prevalence of ticks across different locations.

PCR analysis of 580 tick samples revealed that 240 were positive for the pathogens *A. ovise* and *C. burnetii*, resulting in a prevalence rate of 41.4%. Specifically, 18.97% of the positives were attributed to *A. ovise* and 22.43% to *C. burnetii*, with their nucleic acid sequences registered in the gene bank under accession numbers PP355842 and PP848948.1 in 2024. Both *C. burnetii* and *A. ovise* were found in *Hy. marginatum* and *D. marginatus*, while only *C. burnetii* was present in *Hy. anatolicum*, and only *A. ovise* was found in *Hy. asiaticum*. These pathogens were not detected in other tick species (Table 2).

The investigation of *C. burnetii* and *A. ovise* prevalence in ticks, based on livestock type, revealed infections only in ticks collected from sheep, goats, and camels. The highest infection rates were found in sheep (62.5%) and goats (33.41%). No infections were detected in cattle or dogs, while only *C. burnetii* was found in camels (Table 3).

Sequence analysis was conducted on *A. ovis* samples isolated from ticks, and the resulting sequence was submitted to GenBank under accession number PP355842. Blast analysis revealed 94% to 100% homology with *A. ovis* strains. Phylogenetic tree data indicated that *A. ovis* was categorized in a branch corresponding to the endemic specimens from their host and the specific geographical region (Fig 2).

Sequence analysis was conducted on *C. burnetii* samples isolated from ticks, and the resulting sequence was submitted to GenBank under accession number PP355843. Blast analysis revealed 94% to 100% homology with *C. burnetii* strains. Phylogenetic tree data indicated that *C. burnetii* was categorized in a branch corresponding to the endemic specimens from their host (Fig 3).

The distribution map of *A. ovis* and *C. burnetii* was created using GIS software, revealing similar pollution distribution in the western province. Tick infestation with *A. ovis* was noted in the Eghlid region, whereas *C. burnetii* was not detected (Fig 4).

Discussion

The tick species identified during screening included *Hy. anatolicum*, *Hy. asiaticum*, *Hy. dromedarii*, *Hy. marginatum*, *Rh. sanguineus*, *Rh. turanicus*, and *D. marginatus*, affecting both livestock and domestic animals. The most frequently caught species were *Hy. anatolicum*, *Rh. sanguineus*, and *Rh. turanicus*. Additionally, the prevalence of infection was 18.97% for *A. ovis* and 22.43% for *C. burnetii*. A study by Chaligiannis et al. (2018) in Greece identified *D. marginatus*, *H. parva*, *H. sulcata*, *H. punctata*, *Ixodes gibbosus*, *Rh. sanguineus*, and *R. bursa* in

livestock, highlighting the diversity of tick species across different regions globally [19].

Overall, the dominant and active tick species on livestock vary across different regions of the world. In Iran, the predominant ticks belong mainly to the genera *Hyalomma* and *Rhipicephalus*, with varying distributions throughout the country [20]. The presence of these tick species as reservoirs for pathogens is crucial. The infection rates in ticks revealed a prevalence of 18.97% for *A. ovis* and 22.43% for *C. burnetii*.

In the study by Chaligiannis et al. (2018) in Greece, the prevalence of *A. ovis* in domestic animals was 20.1%, while *C. burnetii* had a prevalence of 17.9% [19]. In the study of Wallménius et al. (2011) found that 0.7% of *Ixodes ricinus* ticks in Sweden were infected with *Anaplasma*, but no *C. burnetii* infections were detected [21]. Dahmani et al. (2016) reported that 7.9% of *R. bursa* ticks isolated from sheep in France were infected with *A. ovis* [22]. Tomanovic et al. (2013) observed co-infections in ticks of the species *D. reticulatus*, *H. concinna*, and *I. ricinus* in Serbia, including *A. ovis*, *A. phagocytophilum*, *B. canis*, *B. burgdorferi, C. burnetii, Rickettsia helvetica*, and *R. monacensis* [23].

The findings of this study indicate that the prevalence of *C. burnetii* infection in Fars province was higher than that of *A. ovis*, differing from trends observed in other regions. There was an increase in *C. burnetii* infections, highlighting the need for heightened awareness due to its role in Q fever. It is crucial to consider the potential for spreading this pathogen in Fars province. While the prevalence of *A. ovis* is also significant and could contribute to anaplasmosis, *C. burnetii* deserves particular attention.

The investigation of pathogen prevalence by reservoir type revealed that *A. ovis* and *C. burnetii* infections were present in sheep, goats, and camels. In Germany, Benjamin Ulrich Bauer and colleagues (2023) found that the prevalence of *Anaplasma spp.* antibodies in sheep was 47.2%, while *C. burnetii* antibodies were present at 3.7% [24]. Similarly, a study by Alessandra et al. (2012) in Italy reported *A. ovis* prevalence in sheep at 82.9% and in goats at 74.9% [25].

A. ovis can cause anaplasmosis, leading to severe symptoms in sheep, particularly in those with compromised health [25]. In a study by Chaligiannis et al. (2018) in Greece, *A. ovis* and *C. burnetii* were found in ticks from sheep, goats, and dogs [19]. Khamesipour et al. (2018) reviewed *A. ovis* and *C. burnetii* in Iran, reporting *C. burnetii* prevalence in cattle ticks at 0.8-22.3%, in goats at 22.4-22.78%, and in sheep at 19.5-36%. The pathogen was also present in dogs (7.7-11%) and camels (28.7%). Additionally, *A. ovis* was detected in cattle, sheep, and goats[26].

In Iran, the prevalence of *A. ovis* among sheep ranged from 5% to 87.4%, in goats from 22.3% to 63.7%, and in cattle from 1% to 22.2%27]]. *A. ovis* and *C. burnetii* have been found in various livestock, including cattle, sheep, and goats, across different regions of Iran, particularly in Fars province. These animals are crucial to Iran's animal husbandry sector, and contamination with these pathogens can expose them to diseases, negatively impacting the industry.

This can lead to an increased spread of diseases caused by these pathogens, such as Q fever and anaplasmosis in both humans and animals. Sequence analysis of the *Coxiella* isolate was conducted [28], resulting in the creation of a phylogenetic tree based on the IS1111 transposon gene with bootstrap support of 1000. Notably, this tree did not indicate distinct branches related to host type or geographical region of the samples. Similar findings were reported by Y. Kilicoglu et al. (2020) in their study of *C. burnetii* [29].

Conclusion

In Fars province, research has shown that *Hyalomma* and *Rhipicephalus* are the predominant species of ticks found on livestock. Approximately half of these ticks carry infections of *A. ovis* and *C. burnetii*, with evidence of these pathogens present in ticks collected from various hosts, including sheep, goats, and camels. The widespread presence of these infections among livestock underscores the critical need to closely monitor and address the diseases that these pathogens can cause in both humans and animals. Therefore, it is essential to take appropriate control and preventive measures to mitigate the risks associated with these infections. This highlights the importance of understanding the transmission dynamics and implementing effective strategies to safeguard the health of both animals and the human population that may come into contact with them.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This research has been approved by the Medical Research Ethics Committee of Shiraz University of Medical Sciences (Ethic Code: IR.SUMS.AEC. 1402.025).

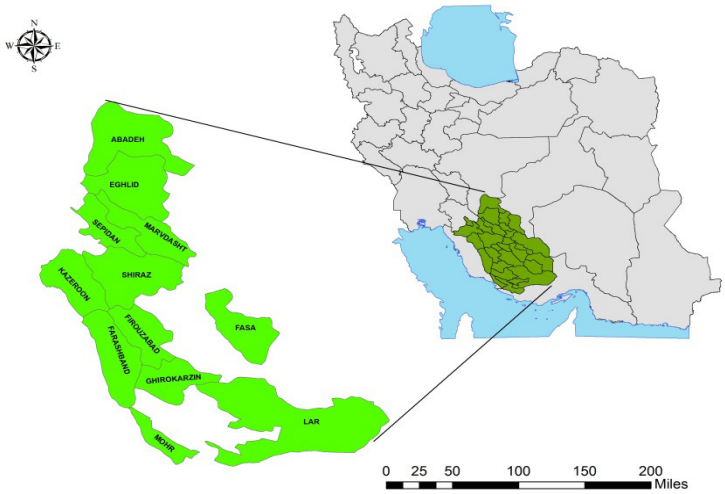

Fig. 1. Counties in Fars Province chosen for the molecular identification of *C. burnetii* and *A. ovise* in ticks from livestock in Fars Province, Iran.

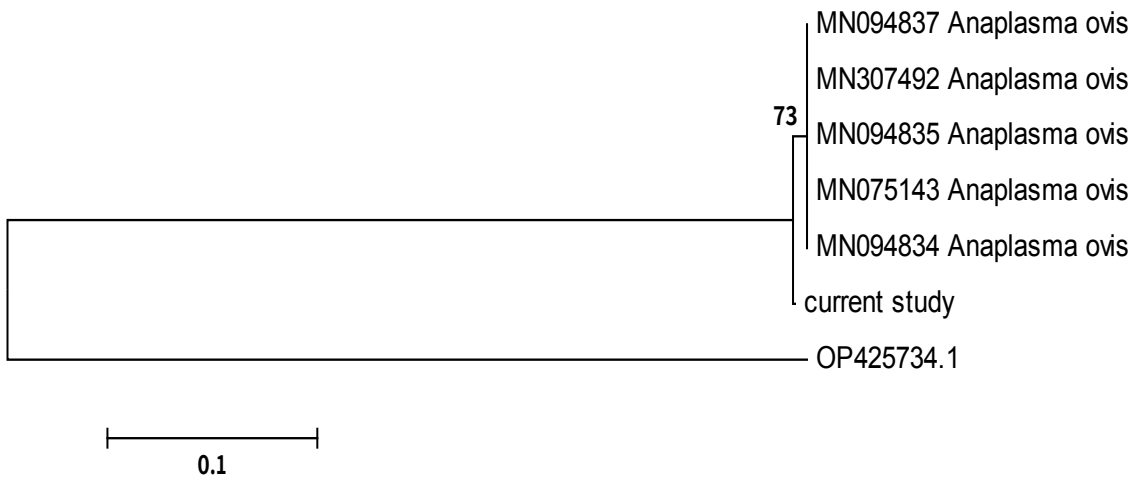

Fig. 2. Phylogenetic tree constructed from the nucleotide sequence of the major surface protein-like gene of *A. ovis* from Iran and other countries. Sequences were aligned using ClustalW, and the tree was generated with the MEGA 6.0 package and a bootstrap value of 1000 replicates. *A. marginale* (GenBank accession: OP425734.1) served as the outgroup for tree rooting.

TABLE 1. Percentage abundance of tick species identified across 12 districts in Fars province, south of Iran

Tick Species	Shirazn N (%)	Firoozabad N (%)	Ggirokarzin N (%)	Farashband N (%)	Larestan N (%)	Mohr N (%)	Fasa N (%)	Eghlid N (%)	Abadeh N (%)	Marvdasht N (%)	Sepidan N (%)	Kazerun (%) N	Total N (%)
Hy. Anatolicum	90(8.30%)	80(7.38%)	(1.38%) 15	70(6.45%)	443(39.94%)	159 (14.66%)	45(4.15%)	33(3.04%)	34(3.136%)	0	51(4.70%)	64(5.90%)	1084 (34.25)
Hy. Asiaticum	56(22.4 %)	27 (10.80 %)	99(39.6%)	0	0	0	68(27.2%)	0	0	0	0	0	250 (7.90)
Hy. Dromedarii	20(12.65%)	47(29.74%)	0	0	0	25(15.82%)	0	0	0	0	44(27.84%)	22(13.92%)	158 (4.99)
Hy. Marginatum	18(7.59%)	22(9.28%)	17(7.17%)	0	0	0	86(36.28%)	5(2.10%)	0	0	51(21.51%)	38(16.03%)	237 (7.49)
Rh. Sanguineus	131(12.65%)	72(6.95%)	186(17.97%)	42(4.05%)	0	0	0	65(6.28%)	287(27.72%)	111(10.72%)	136(13.14%)	5(0.48%)	1035 (32.70)
Rh. Turanicus	0	0	0	0	0	0	0	248(62.62%)	62(15.65%)	86(21.71%)	0	0	396 (12.51)
D.marginatus	0	0	0	0	0	0	0	0	0	0	5(100%)	0	5 (0.16)
Total	315	268	317	112	433	184	199	351	383	197	277	129	3165 (100)

TABLE 2. Results of the PCR assay conducted on tick samples from the livestock of Fars Province, Iran

Ticks	Screened (pools)	*A. ovise* (+ve pools)	C. burnetii (+ve pools)	(+ve pools)
Hy.anatolicum	16	*-	11	11
Hy.Asiaticum	14	6	-	6
Hy.dromedarii	4	-	-	-
Hy.Marginatum	6	4	1	5
Rh.sanguineus	11	-	-	-
Rh.Turanicus	5	-	-	-
D.marginatus	2	1	1	2
Total (+ve) tick samples	58	11	13	24

*Each pools= 10 specimens, (+ve) = Positive, *- = Negative

TABLE 3. Infection rates of ticks from livestock and the number of positive tick pools for targeted zoonotic pathogens in Fars province .

Hosts	Total No. of Tick Pools (N)	*A. ovise* (%)	*C. burnetii* (٪)	Total No. of (+ve)Tick Species of Domestic Animals (%)
Sheep	28	8	7	15
Goat	23	3	5	8
Cow	3	*-	-	-
Camel	2	-	1	1
Dog	3	-	-	-
Zoonotic pathogens overall infection rate (%)	59	11	13	24

*Each pools= 10 specimens, *- = negative for the pathogen; +ve = positive ticks for the pathogen

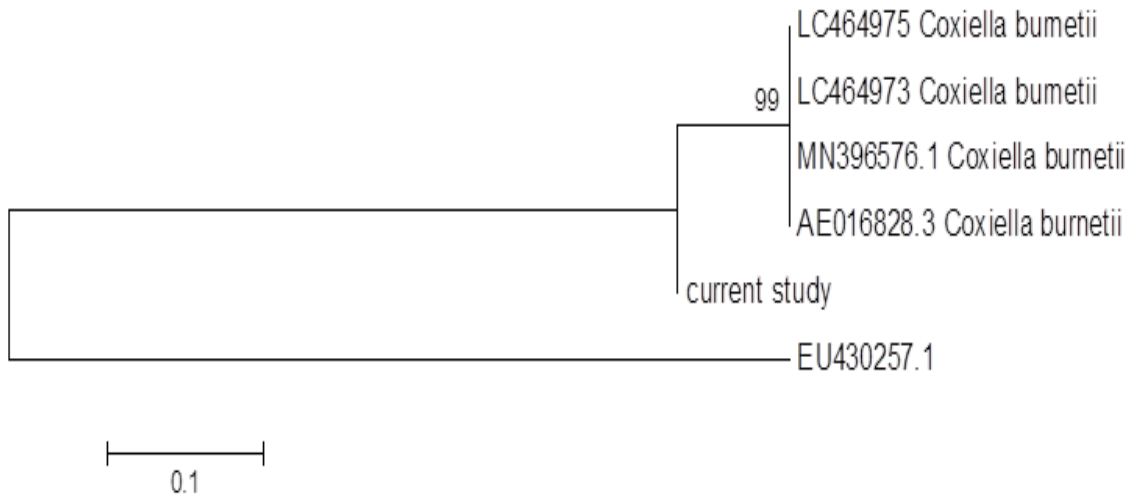

Fig. 3. Phylogenetic tree constructed from the nucleotide sequence of the IS1111 gene of C. burnetii from Iran and other countries. Sequences were aligned using ClustalW, and the tree was generated with the MEGA 6.0 package and a bootstrap value of 1000 replicates. Coxiella endosymbiont (GenBank accession: EU430257.1) served as the outgroup for tree rooting.

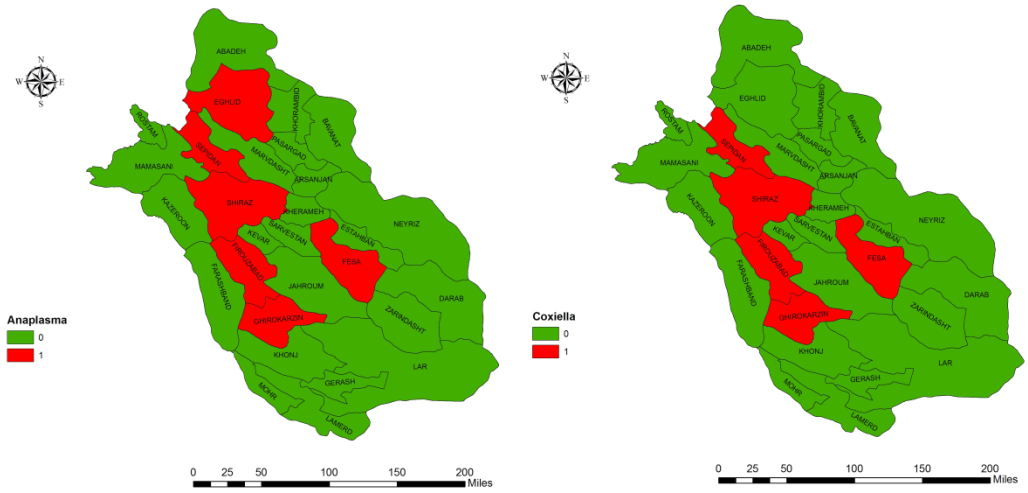

Fig. 4. Distribution of *C. burnetii* and A. *ovis* identified from the livestock of Fars Province, Iran by GIS software
0 = negative for the pathogen 1= positive ticks for the pathogen

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