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Molecular Epidemiology and Risk Factors Assessment of *Anaplasma* spp. on Dairy Cattle in Southwest of Iran

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Abstract

The present study was carried out to determine *Anaplasma* species and potential risk factors associated with molecular prevalence of *Anaplasma* spp. among dairy cattle in southwest of Iran. A total of 88 samples out of 200 generated an expected amplicon of 866 bp from *Anaplasma marginale* msp4 gene. Six samples that were identified as A. *marginale* gave also positive results for A. *phagocytophilum* 16S rRNA gene with specific nested polymerase chain reaction (nPCR). The multivariate analysis of risk factors revealed that the cattle of mountain regions were significantly (p=0.0001) at higher risk as compared to the plain regions. Cattle <1 year age and the latitude 32-33°C were significantly at lower risk (p<0.01). The cattle with low milk yield were significantly (p=0.002) at lower risk. Low hygienic farms were significantly (p=0.011) at higher risk as compared to good and normal hygienic farms. Distance from other farms (<1Km) was another important risk factor which showed significant association with the occurrence of *Anaplasma* infection (p=0.021). The results of this study can be used in strategic planning for prevention and control of bovine anaplasmosis in dairy cattle in the southwest of Iran.

Keywords: *Anaplasma,* cattle, Iran, molecular epidemiology, risk factors

Introduction

Anaplasmosis, theileriosis, and babesiosis are the most important tick-borne diseases in dairy cattle of tropical and subtropical regions of the world (Kocan et al., 2015). In cattle, anaplasmosis caused by different genres of *Anaplasma* (Rickettsiales: *Anaplasmataceae*) including *Anaplasma marginale* (*A. marginale*), *A. phagocytophilium*, *A. central* and *A. bovis* (Kocan et al., 2015).

Bovine anaplasmosis caused by *A. marginale* is the most prevalent and pathogenic forms of the disease in Iran. In affected animals, anemia, icterus, fever, abortion, lethargy weight loss and death are the most prevalent clinical signs of the disease. Infected animals with *A. marginale* remain as carriers throughout their lifetime. Under some circumstances such as stress or other predisposing factors can induce anaplasmosis in carriers

or persistent infected animals (Kocan et al., 2010; Noaman and Bastani, 2016).

A. phagocytophilum is a wide range host organism and can cause tick borne fever in dairy cattle and the other ruminants. The disease is characterized by fever, abortion, reduced fertility, reduced milk yield, leukopenia and inclusions in circulating neutrophils. The infections commonly have not any observable symptoms except combined with other pathogens. *A. phagocytophilum* is also considered as a zoonotic agent and can cause human granulocytic anaplasmosis (Bakken and Dumler, 2015; Noaman et al., 2016). Giemsa-stained blood smears and serological tests like competitive enzyme-linked immunosorbent assay and immunofluorescent antibody have been used widely in epidemiological researches. However, they have not sufficient sensitivity and specificity for the determination of early infections, true negative and carrier animals (Aubry and Geale,

2011; Noaman and Shayan, 2010a). Polymerase chain reaction (PCR), nested polymerase chain reaction (nPCR) and restriction fragment length polymorphism based on the 16S rRNA and major surface proteins are capable to identify low levels of *Anaplasma* spp. in persistent infected animals (Noaman, 2013a ; Quiroz-Castaneda et al., 2016).

Several factors such as type of livestock, breed, sex, age, milk yield, herd size, interaction with wildlife, stress management, pasture type, presence of vectors, ecological and climatic conditions and socio-economic factors may play important roles in the epidemiology of anaplasmosis. However, in different regions, there are different risk factors associated with the presence of anaplasmosis (Amorim et al., 2014; Atif, 2015).

The diversity of climate in Iran can cause the diversity of tick species and subsequently, tick-borne diseases (Noaman, 2012; Noaman et al., 2017; Walker, 2014). Four *Anaplasma* genres including *A. marginale*, *A. centrale*, *A. phagocytophilium* and *A. bovis* have been recognized in Iranian cattle based on molecular assays (Noaman, 2013b; Noaman et al., 2009; Noaman and Shayan, 2009; Noaman and Shayan, 2010b). Khuzestan province is located in the southwest of Iran with tropical climate where the tick-borne diseases (especially anaplasmosis) are important in livestock. In Iran, anaplasmosis has been usually detected in blood smears using traditional Giemsa staining. However, this method only suitable in acute anaplasmosis and has no ability in detection of carrier animals and epidemiological studies. The goals of this study were to recognize the *Anaplasma* species in cattle using molecular method and to assess the risk factors affecting the epidemiology of *Anaplasma* spp. in tropical region of Iran.

Materials and Methods

Study area
The province of Khuzestan is located in the southwest of Iran, borders Iraq and the Persian Gulf and occupies an area of 63.213 km^2. It is located between 48°E and 49.5°E longitudes and between 31°N and 32°N latitudes (Figure 1). Topographic elevations in the province vary between zero and 3740m (above MSL). The climate of this area varies from arid to humid. The northern parts of the province experience cold weather, whereas the southern parts have tropical climate. Summer season is from April to September, and winter is from October to March. The annual mean of maximum summer temperatures in the province is about 50°C (in July), and annual mean of minimum winter temperature is 9°C (in December). The average annual rainfall is 150-256 mm in the south and 995-1100 mm in the north, and about 70% of annual rainfall events occur from February to April. The annual evaporation is 2000-4000mm (Zarasvandi et al., 2011). Figure 1 shows the geographical situation of Khuzestan province in Iran.

Sampling
From 21 June 2010 to 20 December 2016, a total 200 blood samples were collected from healthy dairy cattles of Khuzestan province based on multistage random sampling method. Sampling was carried out in 22 countries including: Andimeshk, Dezful, Shosh, Gotvand, Anika, Shoshtar, Masjed-soleiman,
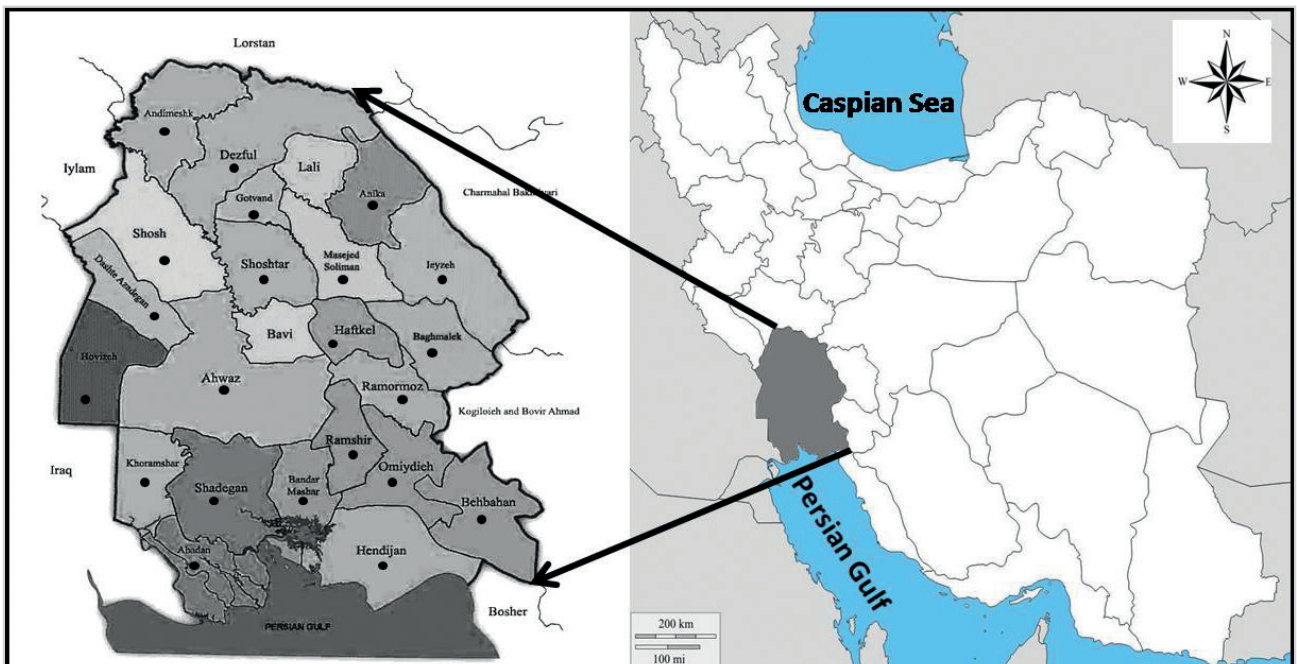

Figure 1. Geographical location of Khuzestan province in southwest of Iran. Sampled counties for *Anaplasma* spp. infection prevalence assessment are denoted by black circles

Leyzeh, Baghmalek, Haftkel, Ramhormoz, Ramshir, Dasht-Aza-deghan, Ahwaz, Hoveizeh, Omidiyeh, Behbahan, Hendijan, Mahshahr, Shadegan, Abadan, Khoramshahr (Figure 1). Sample size was estimated based on a prevalence of 15%, a confidence level of 95%, and a precision of 0.5. In addition, a personal interview was conducted via a standardized questionnaire on farm management. Use of the chemical acaricides and kind of vectors (Tick/Mosquito) were recorded according to the farmer's statements.

The variables of climate, altitude, latitude, season, farm type, hygiene, vectors, use of acaricide, distance from other farms, farm density, race, age, sex and milk yield were recorded for each animal. Blood samples were taken from the jugular vein of each animal using vacuum tube containing the anticoagulant Ethylene Diamine Tetra-Acetic acid (EDTA), (Ava Co., Tehran, Iran). The blood samples were stored at -20˚C until DNA extraction.

DNA extraction

DNA was extracted using the DNA isolation kit [Molecular Biology System Transfer, Iran] according to the manufacturer's instructions. The qualification analysis was determined using spectrophotometer (Varian Medical Systems, Palo Alto, CA, USA) at wavelength of 260 and 280 nm. The purification of the extracted DNA was conducted by OD260/OD280 ratio. The quantification analysis of the extracted DNA was performed using 1.5% agarose gel electrophoresis.

PCR and specific nPCR

For the identification of all *Anaplasma* species, a first PCR was used to amplify almost a 1468 bp fragment of the 16S rRNA gene containing the hyper variable (V1) region. The first PCR was performed using the universal primers fD1 and Rp2, in 50 μL total volume including one time PCR buffer, 1.25 U Taq Polymerase (Cinnagen, Iran), 0.4 μM of each primer, 0.2mM of each

dATP, dTTP, dCTP and dGTP (Cinnagen, Iran), 1.5mM $MgCl_2$ and approximately 100-500 ng extracted DNA in automated thermocycler (Bio-Rad T100, Bio-Rad Laboratories Inc., CA, USA) using the following program: 5 min incubation at 95°C to denature double strand DNA, 40 cycles of 45 s at 94°C (denaturing step), 45 s at 55°C (annealing step) and 1.5 min, at 72°C (extension step) (Weisburg et al., 1991).

Specific internal primer sets targeting the V1 region of the 16S rRNA were used to detect *A. bovis* and *A. phagocytophilum* (Barlough et al., 1996; Kawahara et al., 2006). Specific nPCR reactions were performed directly with 1 μL of the primary PCR product separately. The nPCR for *A. bovis* and *A. phagocytophilum* was performed in 25 μL total volume.

The nPCR for detecting *Anaplasma centrale* (Amori strain) was performed as described by Inokuma et al. (2001).

The *A. marginale msp4* gene was amplified by MSP45/MSP43 primers as reported previously by de la Fuente et al. (2002) in a 25 μL volum PCR. The PCR and nPCR products were analyzed on 2% agarose gel in 0.5 times Tris-Borate-EDTA buffer and visualized using ethidium bromide (Merck, Darmstadt, Germany) and UV-transilluminator (Vilber Lourmat, Marne-la-Vallee Cedex, France). The primers are listed in Table 1.

The PCR products were purified with a MBST Gel extraction Kit (MBST, Tehran, Iran) and submitted for sequencing to Pishgam Biotech Co. (Tehran, Iran). The PCR product was sequenced three times in one direction. The *A. marginale* and *A. phagocytophilum* 16S rRNA gene sequences were deposited to GenBank under accession numbers MG757665 and MG768969, respectively.

Categorization and classification of evaluated risk factors

Risk factors were categorized and classified as Climate (Mountain, Plain), Altitude (500-1000, <500), Latitude (32-33, <31),

Table 1. PCR and n-PCR tested including primers, annealing, cycling conditions and PCR product length

Name of primer	Publication references and Accession No. in GenBank	Nucleotid sequences	Annealing temp (C°)	No. of cycles	PCR-product
fD1	Weisburg et al., 1991	5`AGAGTTTGATCCTGGCTCAG 3`	55	40	1468 bp
Rp2	AF414399	5'ACAGCTACCTTGTTACGACTT3'			
Anaplasma phagocytophilum sense	Barlough et al., 1996	5'GTCGAACGGATTATTCTTTTATAGCTTGC 3'	50	35	926 bp
Anaplasma phagocytophilum Antisense	M73220	5'CCCTTCCGTTAAGAAGGATCTAATCTCC 3'			
Anaplasma bovis sense	Kawahara et al., 2006	5'CTCGTAGCTTGCTATGAGAAC3'	55	35	551 bp
Anaplasma bovis Antisense	U03775	5'TCTCCCGGACTCCAGTCTG3'			
Anaplasma centrale (Amori strain) sense	Inokuma et al., 2001	5'CAAATCTGTAGCTTGCTACGGA3`	54	35	403 bp
Anaplasma centrale (Amori strain) Antisense	AF283007	5' GAGTTTGCCGGGACTTCTTCT 3'			
MSP45	de la Fuente et al., 2002	5'GGGGAGCTCCTATGAATTACAGAGAATTGTTTAC3'	56	35	866 bp
MSP43	AF393742	5'CCGGATCCTTAGCTGAACAGGAATCTTGC3'			

PCR: Polymerase Chain Reaction

Season (Fall, Summer), Farm type (Semi-Industrial, Traditional), Hygiene (Good, Low, Normal), Vectors (Mosquito, Tick), Use of acaricide (No, Yes), Distance from other farms (<1Km, >5Km), Farm density (High, Low, Normal), Race (Hybrid, Native), Age (<1 Year, 1-3 Years, 3-5 Years, >5 Years), Sex (Female, Male), Milk yield (High, Low, Normal, Without).

Statistical analysis

A multiple logistic regression was performed for analyzing risk factors by using Statistical Package for Social Services (SPSS Inc, Chicago, USA) version 18.0. Chi-square (χ^2) test was used to compare the variable factors in the cattle infected with *A. marginale* and *A. phagocytophilum*. A p value less than 0.05 was considered statistically significant.

Results

A total of eighty-eight samples out of two hundred examined generated an expected amplicon of 866 bp from *A. marginale msp4* gene. Following the first PCR for amplifying the 16S rRNA gene of all *Anaplasma* species, positive samples were examined by specific nPCR for detection of *A. phagocytophilum*, *A. bovis* and *A. centrale* (Amori strain). Six of eighty-eight positive samples were giving positivity for *A. phagocytophilum* with nPCR. No samples generated an expected amplicon of *A. bovis* and *A. central* in specific nPCR. The overall prevalence of *A. marginale* and *A. phagocytophilum* infections were 44% and 3% respectively. All infected cattle with *A. phagocytophilum* were also involved with *A. marginale*.

Multivariate analysis of risk factors revealed that cattle of mountain regions were significantly (p<0.0001; OR=1.18) at higher risk as compared to plain regions. Significant association was found among different ages (p<0.002). Cattle <1 year age was (p<0.02; OR=605.3) at lower risk as compared to 1-3, 3-5 and >5 year age. Significant association was found between different latitude (p<0.01), i.e. the latitude 32°-33° (p<0.003; OR=30.48) was at lower risk as compared to <31°. Cattle with low milk yield were significantly (p<0.002; OR=175.86) at lower risk as compared to high, normal and without milk yield.

Low hygienic farms were significantly (p<0.011; OR=0.013) at higher risk as compared to good and normal hygienic farms. Distance from other farms (<1Km) was another important risk factor which showed significant association with the occurrence of *Anaplasma* infection (OR=66.18, p=0.021) (Table 2). There was no significant association between altitude, season, farm type, vectors, use of acaricide, farm density, race and sex with the occurrence of *Anaplasma* infection.

The Chi-square test output showed that the *A. marginale* prevalence was significantly higher (p=0.006) in cattle at latitude <31° as compared to the latitude 32°-33°. The prevalence of *A. marginale* was higher (p<0.0001) in fall as compared to that in summer. Farms with normal hygienic level had significantly higher (p=0.0001) prevalence as compared to those in other hygienic levels.

Table 2. Multivariate analysis of risk factors associated with *Anaplasma* spp. in Khuzestan province, Iran

| Category | Level | Total N | Anaplasma spp. Positive | | | 95% Confidence Interval for Odds ratio | | |
			Count	Row Total N %	p value	Odds ratio	Lower Bound	Upper Bound
Climate	Mountain	20	12	60.0	0.0001	1.18	4.49	3.12
	Plain	180	76	42.2	-	-	-	-
Latitude	32°-33°C	56	16	28.6	0.003	30.48	3.18	291.33
	<31°C	144	72	50.0	-	-	-	-
Hygiene	Good	10	2	20.0	0.995	3.86	.000	.c
	Low	144	50	34.7	0.011	.013	.000	.372
	Normal	46	36	78.3	-	-	-	-
Distance from other farms	<1Km	196	86	43.9	0.021	66.18	1.90	2296.47
	>5Km	4	2	50.0	-	-	-	-
Age	<1Year	16	4	25.0	0.002	605.30	9.64	37981.46
	1-3Years	56	26	46.4	0.057	32.95	.905	1200.66
	3-5Years	46	26	56.5	0.096	.30	.077	1.23
	>5Years	82	32	39.0	-	-	-	-
Milk yield	High	24	12	50.0	0.162	19.33	.305	1225.55
	Low	80	28	35.0	0.002	175.86	6.51	4744.49
	Normal	24	12	50.0	0.824	.732	.047	11.40
	Without	72	36	50.0	-	-	-	-

c. Floating point overflow occurred while computing this statistic. Its value is therefore set to system missing.

The presence of mosquito vectors in farm was found to be significantly associated to the prevalence of *A. marginale* infection (p=0.002) than presence of tick vectors. Farms with acaricide treatment showed significantly a higher (p=0.007) prevalence of *A. marginale* infection as compared to other farms. No significant association was found between prevalence of *A. marginale* infection and climate, altitude, farm type, distance from other farms, farm density, race, age, sex and milk yield (p>0.05).

The highest prevalence of *A. phagocytophilum* was observed (p<0.0001) in fall as compared to summer significantly.

Farms with normal hygienic level had a higher (p<0.0001) prevalence of *A. phagocytophilum* infection as compared to other farms (good and low hygienic level).

The higher infection rates of *A. phagocytophilum* were observed in the farms with normal density (p=0.004) than the farms with low or high density.

Farms with acaricide treatment showed significantly higher (p=0.042) prevalence of *A. phagocytophilum* infection as compared to other farms. No significant association was found between prevalence of *A. phagocytophilum* infection and climate, altitude, latitude, farm type, vectors, distance from other farms, race, age, sex and milk yield (p>0.05).

Discussion

The present study is the first molecular epidemiology in Iran to estimate the overall prevalence for *Anaplasma* spp. and recognize risk factors significantly associated with highly infected animals. Since dairy cattle breeding in Khuzestan province is more common in the shape of semi-industrial and traditional type dairy farms, samples were collected from these farms. Molecular results showed that *Anaplasma* species were frequent and widely distributed in Khuzestan province of Iran. In another study, overall molecular prevalence for *Anaplasma* spp. has been recorded at 38.7% of cattle in the central region of Iran (Noaman et al. 2009).

In the climate category, cattle in mountain regions where the elevation is between 735-482 m above the sea level and average temperature is between 26.9°C-31.8°C, had 1.18 times higher positivity than cattle in the plain regions where the elevation is between 0-307 m above the sea level and average temperature is between 30.3°C-41.2°C. It can be predicted that microclimate in mountainous area is more suitable than plain areas for plant growth, cattle breeding and tick proliferation. Therefore, the presence of tick-borne disease agents such as *Anaplasma* species in mountainous cattle is more likely in these areas compared to plain areas (Dantas-Torres, 2015). Studies in other geographical regions (Central and South America) have also revealed that the tick borne diseases are detected at a higher altitudes (mountain) than where the diseases was present in the recent past (plain) (Estrada-Peña and Salman, 2013; Milner and van Beest, 2013).

Clinical signs of anaplasmosis mainly appear in cattle older than one year. However, cows of all ages are susceptible to anaplasmosis (Aubry and Geale, 2011; Atif, 2015; Kocan et al., 2010). The present study revealed that the group of cattle under one year age was at a lower risk compared to other age groups. The good level of protective colostral immunity and lower exposure to *Anaplasma* spp. vectors have impact on the lower risk of anaplasmosis in this age group. Relation of anaplasmosis with age in this study was supported by the finding of other researchers in Brazil, Bangladesh, India and Pakistan (Amorim et al., 2014; ; Atif et al., 2013; Rahman et al., 2015; Sharma et al., 2015).

The latitude 32°-33° was at lower risk when compared to <31° for *Anaplasma* spp. infections. The higher infection rate was seen in cattle of Ramshir, Omidiyeh, Behbahan, Hendijan, Mahshahr, Shadegan, Abadan and Khoramshahr at latitude <31°. Global warming may have different impact in the epidemiology of vector-borne diseases such as anaplasmosis. It has been demonstrated that the changes in climate including temperature levels can cause changes in the geographic distribution of ticks and other vectors in new latitudes (Milner and van Beest 2013). The *A. marginale* prevalence in cattle was significantly higher at latitude <31° than 32°-33°. The findings show that, vectors survived better at this latitude. The information on geographical latitude incidence patterns is important for the practitioners in planning the control. Since the disease occurred mainly in the southern parts of the province, vectors and transmission pattern in this region should be identified.

The present study confirmed that low yielding cattle have significantly lower risk when compared to high-yielding, moderate-yielding and non-milkers, in parallel with the results of da Silva and da Fonseca (2014). In another study, da Silva and da Fonseca (2014) found association between milk yield and seroprevalence for *A. marginale* in cattle. They observed that dairy cattle with higher milk production had 0.78 times chance to be more seropositive than animals with lower milk production. They suggested that lactation stress along with per parturient hormonal changes have some impact on immunosuppression status in animals and maintenance of anaplasmosis (da Silva and da Fonseca, 2014).

It may be expectable that farms in an isolated area and far from other farms are at very low risk of disease transmission. The present study showed that the farms with less than one km distance to each other played a main role as a risk factor which had significant association with the occurrence of anaplasmosis. There is no confirmed report about association between "distance between farms" and infection with *Anaplasma* species. To our knowledge, this is the first study that found "distance between farms" is an important risk factor of anaplasmosis.

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Farms with good hygienic level had significantly lower prevalence than those in other hygienic levels. Previous studies indicated the hygienic management was one of the potential risk factors for anaplasmosis (Kispotta et al., 2017; Sajid et al., 2014).

There was any relation with the prevalence of anaplasmosis and the use of acaricide in the present study. The results of this paper disagree with Atif et al. (2013) who observed a significant relation between the moderate acaricide application within 60-90 days and seroprevalance to *A. marginale* in cattle.

Da Silva and da Fonseca (2013) observed a significant association between high animal density and high prevalence of anaplasmosis. In the current study, we found no evidence to suggest that farm density was associated with the prevalence of anaplasmosis.

Tick infestation is identified as an important risk factor which has significant association with the occurrence of *Anaplasma* infection (Atif et al., 2013; Costa et al., 2013; da Silva et al., 2014; da Silva and da Fonseca, 2014; Rahman et al., 2015;). Use of chemical acaricides and kind of vectors (Tick/Mosquito) were recorded according to the farmer's statements. Usually, acaricide spraying on the body of the cattle is a simultaneous method in case of the presence of the tick or mosquito on the skin of the cattle and thus, in these farms the livestock has been exposed to pathogens. This probably explains the higher prevalence of *A. marginale* in herds with acaricide treatment.

Only cattle in the rural areas of Gotvand and Shoshtar cities were infected by *A. phagocytophilum.* The climatic conditions in these areas are different from those in other Khuzestan zones. These areas have a lower average temperature and less than 100 meters altitude above sea level. The six positive cases were from traditional small scale cattle farms and pasture grazing was the main feed source. In pasture grazing feeding, the cattle have a great likelihood of tick infestation, so indoor housing hygiene and acaricide treatment do not have a great impact on control of tick-borne diseases in indoor housing.

Although season, race, sex, and vectors were reported as risk factors by Sajid et al. (2014) and Rahman et al. (2015), there was no significant association between these factors and infections with *Anaplasma* species.

Conclusion

The present study shows that in Khuzestan province the tropical region of Iran, infections were caused by *Anaplasma* spp. and the prevalence of anaplasmosis was 44%. The mountain regions, age, latitude, milk yield, farm hygiene and distance from other farms are the major risk factors associated with molecular prevalence to *Anaplasma* spp. in dairy cattle in Khuzestan, Iran. It can be a guide to strategic control programs for anaplasmosis in this area. There was no significant association between altitude, season, farm type, vectors, use of acaricide, farm density,

race and sex with the occurrence of *Anaplasma* infection. Further studies are needed on the identification of biological and mechanical vectors of *Anaplasma* species in this region.

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