Original Article

Prevalence, Risk Factors, and Molecular Epidemiology of *Anaplasma phagocytophilum* in Sheep Raising in Khuzestan Province, Iran

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ABSTRACT

Background: *Anaplasma* sp. is a blood protozoon that causes economic damage to the livestock industry. Therefore, studying this disease's epidemiology and distribution pattern in different regions is essential.

Objectives: This study aimed to investigate the variety of infections of the *Anaplasma* sp. in the sheep population of Khuzestan Province in Iran.

Methods: A total of 200 sheep blood samples were randomly collected and examined using specific nested polymerase chain reaction (nPCR) based on the *16S rRNA* gene.

Results: The prevalence of *Anaplasma phagocytophilum* was 17%, and infected sheep had no clinical signs. The effective risk factors in the spread of infection in Khuzestan Province include sheep aged 3-5 years, low sanitation, high-density farms, use of acaricides in the field, and hot season ($P \leq 0.05$). There was no significant association between the occurrence of *A. phagocytophilum* infection and variables of altitude, farm type, vectors, distance from other farms, and sex.

Conclusion: Since the infection often has no clinical symptoms, identifying the risk factors and epidemiology is essential to develop control and prevention planning.

Keywords: *Anaplasma phagocytophilum*, Nested-PCR, Risk factors, Sheep, 16S rRNA

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Introduction

The genus *Anaplasma* belongs to the family Anaplasmataceae and order Rickettsiales. The important species of this genus in ruminants are as follows: *Anaplasma marginale*, *Anaplasma centrale*, *Anaplasma ovis*, *Anaplasma bovis*, and *Anaplasma phagocytophilum* (common species between humans and livestock). They are transmitted through hard ticks and cause anaplasmosis in ruminants (Abdullah et al., 2020; Kocan et al., 2015; Noaman, 2019). Anaplasmosis has resulted in a lot of losses for the livestock industry around the world so that in the United States, the annual losses in the cattle industry are estimated to be 300 million dollars, and in Latin America, the losses are estimated to be 800 million dollars (da Silva et al., 2018). In Iran, bovine anaplasmosis is a significant disease that causes a lot of damage to the livestock industry. Still, sheep anaplasmosis usually does not cause severe illness, and only in some cases does the sheep exposed to stress or contributing factors show clinical signs of anaplasmosis (Noaman et al., 2016; Noaman et al., 2017). The infections commonly have no observable symptoms, but anemia, icterus, fever, lethargy, and weight loss are rarely seen in infected animals (Stuen et al., 2009; Stuen et al., 2010).

Microscopic examination of Giemsa-stained blood slides can only confirm acute anaplasmosis and fails to diagnose persistent infection and disease reservoirs (Aubry et al., 2011; Tabrizchi et al., 2023). In serological diagnosis methods, the differentiation of *Anaplasma* sp. is impossible due to cross-species interactions; these methods lack sensitivity and reliability compared to molecular tests. To identify *Anaplasma* sp. in infected animals, polymerase chain reaction (PCR), nested polymerase chain reaction (nPCR), and restriction fragment length polymorphism (RFLP) is used (Atif, 2015).

Additionally, many studies have been conducted on the molecular identification of *Anaplasma* spp. and the differentiation of species in Iran so that *A. marginale* (Noaman, 2013; Noaman & Shayan, 2010a; Noaman & Bastani, 2016), *A. phagocytophilum* (Jalali et al., 2013; Noaman & Shayan, 2009), *A. bovis* (Noaman & Shayan, 2009), *A. ovis* (Jalali et al., 2013; Noaman, 2012), and *A. centrale* of amorphous strain in cattle and sheep have been reported in Iran (Noaman, 2012).

Considering the economic losses caused by the disease and its unnoticeable clinical symptoms in sheep, it is necessary to investigate the risk factors in the distribution of infection in different areas for its control and prevention. Despite the distribution of different species of hard mite in Khuzestan Province, Iran, this study aimed to report the molecular identification of *A. phagocytophilum* in sheep. We intended to determine the environmental and risk factors involved in its prevalence in southwestern Iran, which has a tropical climate.

Materials and Methods

Sample collection

A random sampling of 200 sheep from semi-industrial and traditional farms in Khuzestan Province (hot and humid climate in the southwest of Iran: 31° 32′ 73″ N, 48° 69′ 40″ E) was conducted in 22 cities with two climate types: mountain and plain regions. Five milliliters of blood were taken from the jugular veins of apparently healthy sheep and collected in tubes containing an anticoagulant (EDTA), which was sent to the laboratory in ice at 4°C.

DNA extraction

According to the manufacturer's instructions, genomic DNA was extracted using an extraction kit (MBST Iran). The purification of the extracted DNA was conducted by OD260/280 ratio.

PCR and nested PCR:

The *Anaplasma* sp. all primer was used; the nucleotide sequence was found in all Anaplasma species (Table 1). The first product amplified the *16S rRNA* gene (1468 bp) of the *Anaplasma* sp. The PCR solution was prepared based on the following instruction and with a final volume of 25 µL: 2.5 µL of DNA, 2.5 µL of PCR 10X buffer, 0.75 µL of $MgCl_2$ solution at a concentration of 50 µM, 0.5 µL of dNTP at a concentration of 10 µM, 0.5 µL of each primer at a concentration of 20 µM, 0.5 µL of Taq DNA polymerase at a concentration of 5 U/µL, and 17.625 µL of distilled water. After preparing the solutions, frequent DNA amplification was conducted: primary denaturation step at 95°C for 5 minutes, denaturation step at 94°C for 45 seconds, primer connection step at 55°C for 45 seconds, chain lengthening step at 72°C for 90 seconds. Each step was conducted for 35 cycles and then examined in 1.5% agarose gel of electrophoresis with ethidium bromide staining. Specific internal primer sets targeting the V1 region of the 16S rRNA (962 bp) were used to detect *A. phagocytophilum*. Specific nPCR reactions were performed directly with 1 µL of the primary PCR product separately. The nPCR for

Table 1. PCR and n-PCR tested with primers, accession No. in GenBank and PCR product length

Primer	Accession No. in GenBank	Nucleotide Sequence	PCR-Product
Anaplasma all sense	AF414399	5`AGAGTTTGATCCTGGCTCAG3` 5'ACAGCTACCTTGTTACGACTT3	1468 bp
A. phagocytophilum	M73220	5'GTCGAACGGATTATTCTTTATAGCTT-GC3' 5'CCCTTCCGTTAAGAAGGATCTA-ATCTCC3'	926 bp

A. phagocytophilum was performed in 25 µL total volume (Kawahara et al., 2006). The nested-PCR solution, with a total volume of 20 µL, was prepared as follows: 0.5 µL of the sample (from primary PCR), 2 µL of PCR 10X buffer, 0.6 µL of $MgCl_2$ solution at a concentration of 50 µM, 0.4 µL of dNTP at a concentration of 10 µM, 0.4 µL of each primer at a concentration of 20 µM, 0.1 µL of Taq DNA polymerase at a concentration of 5 U/µL, and 15.6 µL of distilled water. After preparing the solutions, the frequent DNA amplification was conducted under the following program: primary denaturation step at 95˚C for 5 minutes, denaturation step at 94˚C for 45 seconds, primer connection step at 56˚C for 45 seconds, and chain lengthening step at 72˚C for 45 seconds. Also, each step was conducted for 35-40 cycles and then was examined in 1.5% agarose gel of electrophoresis with ethidium bromide staining.

Statistical analysis

The chi-square (χ^2) test was used to compare the variables: Climate, altitude, season, farm type, hygiene, distance from other farms, and farm density. Factors such as vectors (mosquito, tick), use of acaricide, age, and sex were performed for analysis by using SPSS software (SPSS Inc, Chicago, USA), version 18 in the sheep infected with *A. phagocytophilum* (P≤0.05).

Results

After DNA extraction, 200 sheep blood samples from Khuzestan Province, located southwest of Iran, were amplified with the *16S rRNA* gene (1468 bp) in the primary PCR with *Anaplasma* sp. Of 200 sheep, 154 samples (77%) were positive for infection with *Anaplasma* sp. detected by PCR (Figure 1).

Nested PCR was used to confirm the detection of *A. phagocytophilum*. The amplification of primary PCR products with this primer pair produced a 926 bp, entirely consistent with the size of the expected product (Figure 2). Of 200 samples, 34(17%) were positive for *A. phagocytophilum* with nPCR. As a result, the prevalence of *A. phagocytophilum* infections in sheep in Khuzestan Province was 17%.

Based on the statistical analysis of distribution risk factors (Table 2) of infection in different regions, it was determined that factors such as age, hygiene, farm density, use of acaricide, and season were significantly influential in the prevalence of anaplasmosis. The prevalence of *A. phagocytophilum* was significant in sheep aged 3-5 years (P=0.027). Low hygienic farms were significantly affected (P=0.001) compared to good and normal hygienic farms. In the statistical study of the use of acaracide on the farm, all herds that did not use it were infected (P<0.0001). Farms with high

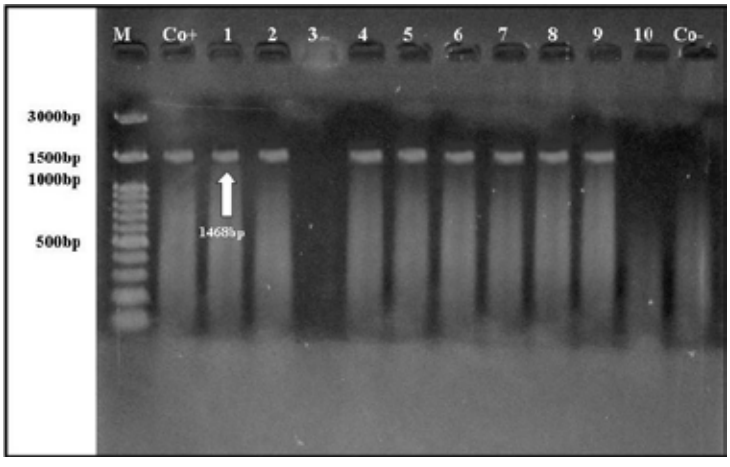

Figure 1. 1 to 10 amplified DNA samples with 1468 bp Anaplasma primer

Co+: Anaplasma positive control, Co-: Anaplasma negative control, M: Marker (bp100).

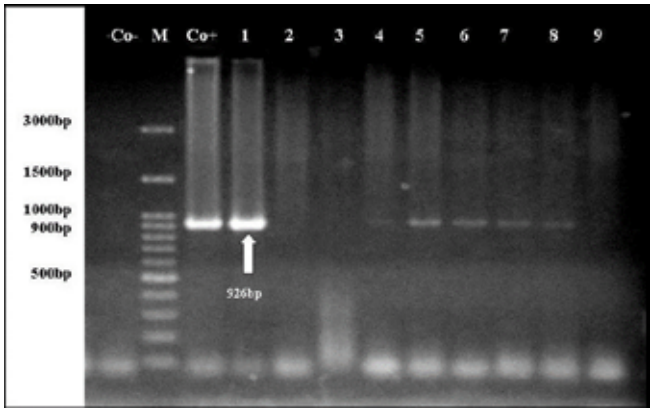

Figure 2. Nested-PCR product amplified with a specific primer A. phagocytophilum (926 bp)

Co+: A. phagocytophilum positive control, Co-: A. phagocytophilum negative control, M: Marker (bp100).

density were significantly infected (P<0.0001) compared to low-density farms. The warm season was another factor that showed a statistically significant difference in the prevalence of infection (P=0.32). There was no significant association between the occurrence of *A. phagocytophilum* infection and the variables of altitude, farm type, vectors, distance from other farms, and sex (Table 2).

Discussion

A. phagocytophilum is recognized as an emerging tick-borne pathogen that is important for animals and humans (Atif, 2016; Rar et al., 2021). Various tick vectors and reservoir hosts are responsible for this pathogen's geographic distribution and emergence (Woldehiwet, 2010). Epidemiological studies have demonstrated that climate changes and temperature levels can lead to changes in the geographic distribution of ticks and, as a result, the diseases they transmit (Atif, 2016; Stuen et al., 2013). The absence of clinical signs of ruminants infected with *A. phagocytophilum* highlights the need for molecular techniques to discriminate this species from other species. In determining the genus *Anaplasma* sp., it has been shown that the *16SrRNA* gene is highly capable of detecting the genus (Atif, 2015). Various genes, such as *gltA*, *msp4*, and *groEL*, have been used to detect *A. phagocytophilum* in sheep (Kang et al., 2011). The molecular results presented the high frequency (19.4%) of *Candidatus Anaplasma* camelii in camels in the south of Iran (Moradi et al., 2021). In this study, the presence of *Anaplasma* sp. was determined in 77% of samples collected from sheep by PCR based on 16SrRNA. Positive samples were genetically characterized by *A. phagocytophilum* and 34(17%) of 154 positive samples gave positivity with nPCR based on the V1 region of the 16S rRNA (962 bp).

The epidemiological study of risk factors of *A. phagocytophilum* in the region showed that factors such as age, hygiene, farm density, use of acaricide, and season play essential roles in the prevalence of the infection. Infection was more common in sheep raised over 3 years of age in poor hygienic conditions and high-density farms. There was also a significant difference in the infection prevalence in herds that did not use acaricide. The warm season has also been another important factor in the prevalence of infection in the region.

In the study of the distribution of infection of *A. phagocytophilum* in cattle in different regions of Iran with various climatic conditions, it was found that the highest infection has been reported in the Caspian zone (33%) located in the north of Iran (Noaman, 2020). The zone has high rainfall, high humidity, vast forests, and grasslands, which are suitable conditions for the growth and reproduction of ticks and, as a result, the development of tick-borne diseases. In Northern and Central Europe, the tick-borne disease usually appears from early April until mid-November. However, most cases are seen between the middle of May and the middle of June due to suitable conditions for the growth and reproduction of vector ticks. Although in this study there is no statistically significant difference in latitude and longitude of different regions, research on tick-borne diseases such as *Anaplasma* sp. has shown that prevalent in mountainous areas are more likely compared to plain areas (Khaki et al., 2015; Noaman, 2020; Dantas-Torres, 2015).

Another factor in increasing the prevalence of *Anaplasma* sp. is poor hygiene and old facilities on the farm, which are good places to increase ticks and tick-borne diseases. In the study of anaplasmosis infection in Pakistan, the prevalence was higher (26.05%) on farms where cleaning was done weekly than on farms cleaned once or twice daily (Shaukat et al., 2019).

Table 2. Analysis of risk factors associated with *A. phagocytophilum* in sheep in Khuzestan Province, Iran

Factors		*A. phagocytophilum* No. (%)		P
		Positive	Negative	
Climate	Mountain	4(12.5)	28(87.5)	0.460
	Plain	30(17.9)	138(82.1)	
Altitude (500-1000 m)	1000-500	4(14.3)	24(85.7)	0.680
	<500	30(17.4)	142(82.6)	
Longitude (48-50°)	48-50	34(17.0)	166(83.0)	0.000
Latitude (32-33°<31°)	32-33	16(22.2)	56(77.8)	0.140
	<31	18(14.1)	110(85.9)	
Season	Cold	2(5.3)	36(94.7)	0.032
	Warm	32(19.8)	130(80.2)	
Hygiene	Good	4(66.7)	2(33.3)	0.001
	Low	22(20.0)	88(80.0)	
	Normal	8(9.5)	76(90.5)	
Vectors	Tick	28(19.7)	114(80.3)	0.109
	Mosquito	6(10.3)	52(89.7)	
Use of acaricide	Yes	0(0)	54(100.0)	0.000
	No	34(23.3)	112(76.7)	
Distance from other farms	<1 km	30(16.1)	156(83.9)	0.232
	1-5 km	4(28.6)	10(71.4)	
Farm density	High	18(36.0)	32(64.0)	0.000
	Low	16(10.7)	134(89.3)	
Age (y)	>1	0(0)	16(100.0)	0.027
	1-3	10(12.8)	68(87.2)	
	3-5	24(22.6)	82(77.4)	
Sex	Female	28(17.7)	130(82.3)	0.598
	Male	6(14.3)	36(85.7)	

All age groups of lambs are of epidemiological importance for maintaining *A. phagocytophilum* in tick populations. In the present study, the infection in sheep over 3 years of age had a statistically significant difference with other ages. In research by Noaman and Moradi (2019), the prevalence of *A. phagocytophilum* infections was 3% in dairy cattle in Southwest Iran. Regarding the risk factors of the infection epidemiology, cattle <1 year of age, with low milk yield, and low hygienic farms were significantly at lower risk, versus cattle raised in mountain regions, which were significantly at higher risk.

In this study, the blood smears were taken from 160 cattle, 391 sheep, and 385 goats and examined for various *Anaplasma* spp. The results showed that 19.37% of cattle were infected with *A. marginale*, 80.3% of sheep, and 38.92% of goats were infected with *A. ovis*. In a study by Jalali et al. (2013), the contamination of Ahvaz sheep in Iran was reported to be 33%. The species detected by RFLP revealed that all PCR-positive samples were *A. ovis*. A mixed infection with *A. marginale* was seen in 50% of *Anaplasma* sp. infected samples. According to the review of Soosaraei et al. (2020), the most prevalent infection among *Anaplasma* spp. was related to A. ovis and A. marginale infection rates, and the lowest prevalence to *A. phagocytophilum*. Noaman and Shayan (2009), in a study on *Anaplasma* sp. in cattle around Isfahan City, Iran, showed that out of 150 extracted DNA samples, 58(38.67%) were positive for *A. marginale* in primary PCR, semi-nested PCR, and RFLP-PCR. In a study performed by Noaman and Shayan (2010b) on sheep in Isfahan Province based on the *16S rRNA* gene, it was found that 33% of the samples were positive for PCR-RFLP. In the above research, no clinical signs were recorded in PCR-positive sheep. In the analysis of blood smears of livestock, no inclusion was observed in neutrophils, indicating that the measured livestock are vectors. The results of this research are consistent with the research conducted. The report of genotyping and phylogenetic analysis of *Anaplasma capra* in Europe, in domestic, endemic, and wild ruminants, shows the wide host range previously described for this species in Asian countries (Jouglin et al., 2022). Noaman (2020), in an epidemiological study of infection in different parts of Iran cattle, showed that the highest prevalence was found in the Caspian zone (18%) North of Iran, followed by Central (16.8%), Zagros (16%), and Persian-Gulf zone (3%). Prevalence rates of *A. marginale*, *Theileria ovis*, and *Theileria lestoquardi* in sheep in Pakistan were 07%, 06%, and 1.2%, respectively. Phylogenetic analysis revealed that these isolates were closely related to what was found in Iran (Tanveer et al., 2022).

To control diseases such as anaplasmosis, with its zoonotic potential and ticks-born nature, it is necessary to study the critical risk factors in the epidemiology of the infection. Based on the present study and other research, the temperature during the season, mountain climate, farm sanitary conditions, use of acaricides, long-term breeding, and increasing herd age are risk factors that contribute to the prevalence of infection and should be considered in the design controlling and prevention programs.

Conclusion

The present study shows that in Khuzestan Province the tropical region of Iran and the prevalence of anaplasmosis was 17% with no clinical sign. It can be a guide to strategic control programs for anaplasmosis in this area. Further studies are needed on the identification of biological and mechanical vectors of Anaplasma species in this region.

Ethical Considerations

Compliance with ethical guidelines

The animal study was reviewed and approved by Ethical Committee of Razi Vaccine & Serum Research Institute. Written informed consent was obtained from the owners for the participation of their animals in this study.

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Authors' contributions

Conceptualization and supervision: Vahid Noaman; Methodology, investigation, data collection and data analysis: Vahid Noaman and Razieh Heidari, Writing: All authors.

Conflict of interest

All authors declared no conflict of interest.

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مطالعه پژوهشی

بررسی شیوع، عوامل خطر و اپیدمیولوژی مولکولی آناپلاسما فاگوسیتوفیلوم در گوسفندان استان خوزستان، ایران

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چکیده

زمینه مطالعه: آناپلاسما فاگوسیتوفیلوم یک تک یاخته خونی است که به صنعت دامداری آسیب اقتصادی وارد می‌کند، بنابراین مطالعه اپیدمیولوژی و الگوی توزیع بیماری در مناطق مختلف حائز اهمیت است.

هدف: این مطالعه باهدف بررسی انواع عفونت‌های قارچ Anaplasma sp در جمعیت گوسفندان استان خوزستان انجام شد.

روش کار: برای بررسی آلودگی به آناپلاسما فاگوسیتوفیلوم در جمعیت گوسفندان استان خوزستان، در مجموع ۲۰۰ نمونه خون گوسفند به‌طور تصادفی جمع‌آوری شد. شیوع آلودگی پس از استخراج DNA نمونه‌های خون با استفاده از واکنش زنجیره‌ای پلیمراز (Nested-PCR) براساس ژن 16S rRNA ارزیابی شد. همچنین عوامل مؤثر در پراکندگی بیماری در منطقه مورد تحلیل آماری قرار گرفت.

نتایج: شیوع آناپلاسما فاگوسیتوفیلوم ۱۷ درصد بود و گوسفندان آلوده علائم بالینی نداشتند. بررسی آماری عوامل خطر مؤثر در شیوع عفونت در استان خوزستان شامل سن ۳ تا ۵ سال، مزارع با بهداشت کم، تراکم بالا، استفاده از کنه‌کش‌ها در مزرعه و فصل گرما از عوامل تعیین‌کننده بود (P≤۰/۰۵). ارتباط معنی‌داری بین ارتفاع، نوع مزرعه، ناقلین، فاصله از مزارع دیگر و جنسیت با بروز عفونت آناپلاسما فاگوسیتوفیلوم وجود نداشت.

نتیجه‌گیری نهایی: ازآنجایی‌که عفونت اغلب علائم بالینی ندارد، بنابراین شناسایی عوامل خطر مؤثر بر اپیدمیولوژی عفونت در توسعه برنامه‌ریزی کنترل و پیشگیری از بیماری مهم است.

کلیدواژه‌ها: آناپلاسما فاگوسیتوفیلوم، واکنش زنجیره‌ای پلیمراز، 16S rRNA، عوامل خطر، گوسفند

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