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Prevalence, Risk factors and Molecular Epidemiology of *Anaplasma phagocytophilum* in Sheep 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 the study of epidemiology and distribution pattern of disease in different regions is important.

OBJECTIVES: To investigate the variety of infection to 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 *A. phagocytophilum* was 17% and infected sheep had no clinical signs. The statistical study of the effective risk factors in the spread of infection in Khuzestan province includes age 3-5 years, farms with low sanitation, high density, use of acaricides in the field, and hot season were significant determinants (($P \leq 0.05$). There was no significant association between altitude, farm type, vectors, distance from other farms, and sex with the occurrence of *A. phagocytophilum* infection.

CONCLUSIONS: Since the infection often has no clinical symptoms, so identifying the risk factors affecting the epidemiology of the infection is important in developing control and prevention planning of the disease.

KEYWORDS: *Anaplasma phagocytophilum*, Nested-PCR, Risk Factors, Sheep, 16S rRNA

Introduction

The genus *Anaplasma sp.* is classified in the family Anaplasmataceae and order Rickettsiale. The important species of this genus in ruminants are as follows: *Anaplasma marginale, Anaplasma centrale, Anaplasma ovis, Anaplasma bovis, Anaplasma phagocytophilum* (common species between humans and livestock), which are transmitted through hard ticks and cause Anaplasmosis in ruminants (Abdullah *et al., 2020;* Kocan *et al., 2015;* Noaman *et al., 2019*). Anaplasmosis has led to a lot of losses for the livestock industry in the world so that in the United States, the annual losses in the cattle are estimated to be 300 million dollar, and in Latin America, the losses are estimated to be 800 million dollars (da Silva *et al., 2018).* In Iran, the bovine Anaplasmosis is an important disease that causes a lot of damage to the livestock industry, but sheep Anaplasmosis usually does not cause severe disease, and only in some cases, the sheep exposed to stress or contributing factors show clinical signs of Anaplasmosis (Noaman *et al., 2016 & 2017*). The infections commonly have not any observable symptoms but anemia, icterus, fever, lethargy weight loss, and rarely seen in infected animals (Stuen *et al., 2009& 2010*).

Microscopic examination of Giemsa-stained blood slides can be used to confirm acute anaplasmosis and cannot be used to diagnose persistent infection and disease reservoirs (Aubry *et al., 2011;* Tabrizchi *et al., 2023*). In serological diagnosis methods, the differentiation of *Anaplasma sp* species is not possible due to cross-species interactions, these methods lack sensitivity and reliability compared to molecular tests. To identify *Anaplasma* spp. in infected animals, Polymerase chain reaction (PCR), nested polymerase chain reaction (nPCR), and restriction fragment length polymorphism (RFLP) had been used (Atif, *2015*).

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

Considering the economic losses and the absence of clinical symptoms of the disease in sheep, it is necessary to investigate the risk factors in the distribution of infection in different areas for control and prevention. Despite the distribution of different species of hard mite in Khuzestan province, the aim of this study is to report the molecular identification of *A.*

phagocytophilum in sheep and to determine environmental factors and risk factors in its prevalence in southwestern Iran, which has a tropical climate.

Material 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 of mountain and plain. 5 ml of blood was taken from 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:

Genomic DNA was extracted using an extraction kit (MBST Iran) according to Kit's instruction. 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 of which is found in all *Anaplasma* species (Table 1). The first product amplified 16S rRNA gene (1468bp) 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, the 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, and each step was conducted for 35cycles and then was 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 *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, chain lengthening step at 72 c for 45

seconds, and each step was conducted for 35-40 cycles and then was examined in 1.5% agarose gel of electrophoresis with ethidium bromide staining.

Table 1. PCR and n-PCR tested including primers, accession no. in GenBank and PCR product length

Name of primer	Accession No. in GenBank	Nucleotide sequences	PCR-product
Anaplasma all sense	AF414399	5` agagtttgatcctggctcag 3` 5'acagctaccttgttacgactt 3	1468bp
Anaplasma Phagocytophilum	M73220	5'gtcgaacggattattctttatagcttgc 3' 5'cccttccgttaagaaggatctaatctcc 3'	926 bp

Statistical analysis:

Chi-square ($\chi 2$) test was used to compare the variable factors included climate, altitude, season, farm type, hygiene, distance from other farms, farm density, and factor as vectors (Mosquito, Tick), use of acaricide, age, and Sex was performed for analyzing by using Statistical Package for Social Services (SPSS Inc, Chicago, USA) version 18.0 in the sheep infected with *A. phagocytophilum* ($P \leq 0.05$).

Results

After DNA extraction, 200 collected blood samples of sheep from Khuzestan province located in the southwest of Iran were amplified 16S rRNA gene (1468 bp) in the primary PCR with *Anaplasma sp.* 154 out of 200 sheep samples (77%) were positive for infection with *Anaplasma sp*. by PCR methods (Figure1).

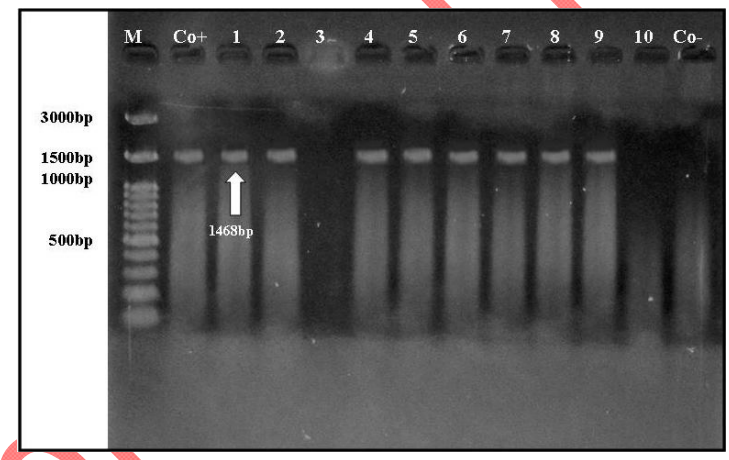

Fig 1- 1 to 10 amplified DNA samples with 1468bp Anaplasma primer (Co + Anaplasma positive control, Co- Anaplasma negative control, M: marker bp100)

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

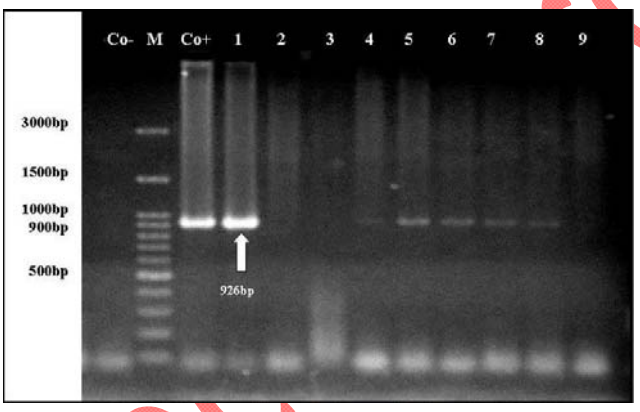

Fig2- Nested- PCR product amplified with a specific primer A. phagocytophilum (926 bp) (Co + A. phagocytophilum positive control, Co- A. phagocytophilum negative control, M: marker bp100)

Based on a statistical analysis of risk factors according to table 2 in the distribution of infection in different regions, it was determined that factors such as ages, hygiene, farm density, use of acaricide, and season were significantly different 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 (p=0.001) compared to good and normal hygienic farms. In the statistical study of the use of acaracid on the farm, all herds that did not use it were infected (p<0.0001). Farms with high density were significantly (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 altitude, farm type, vectors, distance from other farms, and sex with the occurrence of *A. phagocytophilum* infection Table 2.

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

Factors		*A. phagocytophilum*				
		Positive		Negative		
		Count	Row N %	Count	Row N %	p value
Climate	Mountain	4	12.5%	28	87.5%	0.460[a]
	Plain	30	17.9%	138	82.1%	
Altitude	1000-500	4	14.3%	24	85.7%	0.680[a]
	<500	30	17.4%	142	82.6%	
Longitude	48-50	34	17.0%	166	83.0%	0.000
Latitude	32-33	16	22.2%	56	77.8%	0.140[a]
	<31	18	14.1%	110	85.9%	
Season	Cold	2	5.3%	36	94.7%	0.032[a,*]

	Warm	32	19.8%	130	80.2%	
Hygiene	Good	4	66.7%	2	33.3%	0.001[a,*]
	Low	22	20.0%	88	80.0%	
	Normal	8	9.5%	76	90.5%	
Vectors	Tick	28	19.7%	114	80.3%	0.109[a]
	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	1Km>	30	16.1%	156	83.9%	0.232[a]
	1-5Km	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	1Year>	0	.0%	16	100.0%	0.027[*]
	1-3Years	10	12.8%	68	87.2%	
	3-5Years	24	22.6%	82	77.4%	
Sex	Female	28	17.7%	130	82.3%	0.598
	Male	6	14.3%	36	85.7%	

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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 the geographic distribution and emergence of this pathogen (Woldehiwet, *2010*). Epidemiological studies have been demonstrated that the changes in climate, temperature levels can lead to changes in the geographic distribution of ticks and as a result of the diseases transmitted by them *(*Atif, *2016;* Stuen *et al., 2013)*. The absence of clinical sign of ruminants infected with *A. phagocytophilum* highlights the need for molecular techniques to discriminate this species from other species. In the determination of the genus *Anaplasma sp*. it has been shown that the 16SrRNA gene has highly capable of detecting the genus (Atif, *2015)*. Various genes such as *gltA*, *msp4*, and *groESL* have been used to detect the *A. phagocytophilum in* sheep (Kang *et al., 2011*). The molecular results presented the high frequency (19.4%) of Candidatus *Anaplasma camelii* in camels, in 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 *A. phagocytophilum* and 34 of 154 (17%) positive samples were giving 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 ages, hygiene, farm density, use of acaricide, and season play an important role 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 prevalence of infection 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 wide 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 is usually the beginning of April until the middle of November. However, most cases are seen between the middle of May and the middle of June which is 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 cattle 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 is a good place to increase ticks and tick-borne diseases. In the study of anaplasmosis infection in Pakistan, the prevalence was highest (26.05%) in farms where cleaning

was done weekly than those farms which were cleaned one or two times daily *(*Shaukat *et al., 2019)*.

All age groups of lambs have been shown to be of epidemiological importance for the maintenance of *A. phagocytophilum* in tick populations. While 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* prevalence of *A. phagocytophilum* infections was 3% in dairy cattle in Southwest of Iran, in the evaluation of risk factors of the epidemiology of infection cattle <1 year age, with low milk yield and Low hygienic farms were significantly at lower risk, while cattle of mountain regions were significantly at higher risk.

In this study, the blood smears were taken from 160 cattle, 391 sheep and, 385 goats and examined for the presence of various *Anaplasma sp*. The results showed that 19.37% of cattle were infected with *Anaplasma marginale*, 80.3% of sheep, and 38.92% of goats were infected with *Anaplasma ovis*. In a study conducted by Jalali et al 2013, the contamination of Ahvaz sheep in Iran was reported to be 33%. Detected species in 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* showed that the most prevalent infection among *Anaplasma spp*. was related to *A. ovis* and *A. marginale* of infection rates and the lowest prevalence to *A. Phagocytophilum (Soosaraei et al, 2020)*. Noaman and Shayan (2009) in a study on *Anaplasma sp*. in cattle around Isfahan showed that of 150 extracted

DNA samples, 58 (38.67%) samples were positive for *Anaplasma marginale* in primary PCR, Semi- nested PCR, and RFLP– PCR. In a study performed by Noaman *et al. (2010)* on sheep in Isfahan province based on 16S rRNA genes, it was found that 33% of the samples were positive for PCR-RFLP. In the above research, no clinical signs were recorded in sheep that were positive, and in the analysis of blood smears of livestock, no inclusion was observed in neutrophils, which indicates that the measured livestock are vectors, and the results of this research are consistent with the research conducted. The report of genotyping and phylogenetic analysis of *A. 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 the epidemiological study of infection in different parts of Iran in cattle, showed that the various prevalence in different zones as 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 of *A. marginale*, *T. ovis* and *T. lestoquardi* in sheep in Pakistan was 07%, 06% and 1.2% respectively, phylogenetic analysis revealed that this isolates o were closely related to Iran, (Tanveer *et al., 2022*).

In order to control diseases such as *A. phagocytophilum*, due to its zoonotic potential and also ticks-born disease, it is necessary to study the important risk factors in the epidemiology of the infection. Based on the present study as well as other research temperature during the season, mountain climate, Farm health conditions, use acaricides long-term breeding and increasing herd

age, are risk factors that play a role in the prevalence of infection and should be considered in order to regulate controlling programs.

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Conflict of interest: All authors declare that there is no conflict of interest.

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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 می کند، بنابراین مطالعه اپیدمیولوژی و الگوی توزیع بیماری در مناطق مختلف حائز اهمیت است.

هدف: برای بررسی آلودگی به *آناپلاسما فاگوسیتوفیلوم* در جمعیت گوسفندان استان خوزستان در ایران، در مجموع 200 نمونه خون گوسفند به طور تصادفی جمع آوری شد.

روش کار: شیوع آلودگی پس از استخراج DNA نمونه های خون با استفاده از واکنش زنجیره ای پلیمراز (nested-PCR) بر اساس ژن 16S rRNA ارزیابی شد. همچنین عوامل موثر در پراکندگی بیماری در منطقه مورد آنالیز آماری قرار گرفت.

نتایج: شیوع آناپلاسما فاگوسیتوفیلوم 17٪بود و گوسفندان آلوده علائم بالینی نداشتند. بررسی آماری عوامل خطر موثر در شیوع عفونت در استان خوزستان شامل سن 5-3 سال، مزارع با بهداشت کم، تراکم بالا، استفاده از کنه کش ها در مزرعه و فصل گرما از عوامل تعیین کننده بود ((P ≤ 0/05) ارتباط معنی داری بین ارتفاع، نوع مزرعه، ناقلین، فاصله از مزارع دیگر و جنسیت با بروز عفونت *آناپلاسما فاگوسیتوفیلوم* وجود نداشت.

نتیجه گیری نهایی: از آنجایی که عفونت اغلب علائم بالینی ندارد، لذا شناسایی عوامل خطر موثر بر اپیدمیولوژی عفونت در توسعه برنامه ریزی کنترل و پیشگیری از بیماری مهم است.

کلمات کلیدی: *آنا پلاسما فاگوسیتوفیلوم، واکنش زنجیره ای پلیمراز، 16 srRNA، عوامل خطر، گوسفند.*