

## Investigation of *Anaplasma marginale* and *Anaplasma centrale* in Cattle in Adana Province by Serological and Molecular Methods

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Geliş Tarihi / Received: 10.10.2019, Kabul Tarihi / Accepted: 12.11.2019

**Abstract:** Anaplasmosis is a common disease in tropical and subtropical climate zone and is transmitted by vectors. Especially in large cattle management systems, it has started to be detected frequently in recent years. The aim of this study was to determine the prevalence of *Anaplasma* spp. in cattle in Adana province. For this aim, 187 blood samples were collected from cattle from fifteen districts of Adana that have different climatic zones and examined by Competitive ELISA (cELISA) and Nested-PCR methods. Seropositivity was determined as 38.5% (72/187) in cattle. The molecular prevalence was detected as 1.6% (3/187) for *Anaplasma centrale* and 3.2% (6/187) for *Anaplasma marginale* by Nested-Polymerase Chain Reaction (PCR) methods. In this study, epidemiological data related to bovine anaplasmosis in Adana province of Turkey were discussed in detail and it was thought that the obtained data would contribute to disease prevention and control programs.

**Key words:** *A. centrale*, *A. marginale*, cELISA, Nested-PCR, Adana

### Adana İlinde Sığırlarda *Anaplasma marginale* ve *Anaplasma centrale*'nin Serolojik ve Moleküler Yöntemler ile Araştırılması

**Özet:** Anaplazmozis tropikal ve subtropikal iklim bölgelerinde vektörler tarafından taşınan yaygın bir hastalıktır. Özellikle büyükbaş hayvancılık işletmelerinde son yıllarda sık tespit edilmeye başlanmıştır. Bu çalışmanın amacı, Adana ilindeki sığırlarda *Anaplasma* spp. prevalansını belirlemektir. Bu amaçla Adana'nın farklı iklim bölgelerine sahip on beş ilçesinde sığırlardan 187 kan örneği alınarak kompetitif ELISA (cELISA) ve Nested-PCR yöntemleriyle incelenmiştir. Sığırlarda seropozitiflik %38,5 (72/187) olarak belirlenmiştir. Ayrıca moleküler prevalans, Nested-PCR yöntemleriyle *Anaplasma centrale* için %1,6 (3/187), *Anaplasma marginale* için %3,2 (6/187) olarak tespit edilmiştir. Bu çalışma ile Türkiye'nin Adana ilinde sığır anaplazmozisi ile ilgili epidemiyolojik verileri ayrıntılı bir şekilde ele alınmış ve elde edilen verilerin hastalığın önlenmesine ve kontrol programlarına katkıda bulunacağı düşünülmüştür.

**Anahtar kelimeler:** *A. centrale*, *A. marginale*, cELISA, Nested-PCR, Adana

### Introduction

Anaplasmosis is a subclinical disease in animals and causes economic losses such as increase in cull rate, reduction in calf crop and mortality rate (Zabel et al. 2018). *Anaplasma* spp. have a wide range of hosts such as cattle, sheep, goats, wild ruminants, horses, mice, dogs, cats and transmitted by ticks or mechanical vectors (ear taggers, biting flies, surgical instruments and contaminated needles) and rarely transplacentally (Radostits et al. 2007; Kocan et al. 2010; Aubry et al. 2011). *Anaplasma* spp. can survive intracellularly within different cells as erythro-

cytes, monocyte, granulocyte and endothelial cells (Rar et al. 2011; Kocan et al. 2015)

The most common agents of anaplasmosis in cattle are *A. marginale*, followed by *A. centrale*, *A. bovis* and *A. phagocytophylum*. The hosts of *A. marginale* and *A. centrale* are cattle and wild ruminants and these agents infect erythrocytes. Competitive Enzim Linked Immuno Sorbent Assay (cELISA), polymerase chain reaction (PCR) is commonly used for infection detection. The advantage of PCR is early detection during the pre-patent period and identification of an "active" infection at a single time-point. The utility of PCR is widespread and can be

combined with the cELISA for thorough diagnosis of *Anaplasma* infection (Hairgrove et al. 2015). In this study, it was aimed to detect the prevalence of *A. marginale* and *A. centrale*, in cattle by molecular and serological methods in Adana.

## Materials and Methods

### Sampling

Blood samples were collected from 187 clinically healthy cattle (168 female and 19 male) in 15 districts (Çukurova, Seyhan, Ceyhan, Yumurtalık, Karaisalı, Kadirli, Kozan, İmamoğlu, Saimbeyli, Tufanbeyli, Pozantı, Feke, Karataş, Yüreğir and Aladağ) of Adana from June to September 2017. An information form which includes age, breed, gender of each cattle and altitudes of districts was prepared. Collected blood samples were stored at -20°C until used for serologic and molecular diagnosis.

### Sample Analysis

In this study, 187 sera samples were investigated by the c-ELISA method for the detection of anti-*Anaplasma* spp. antibodies. Sera samples were analyzed by *Anaplasma* antibody test kit (cELISA; VMRD Inc., Pullman, WA) for *A. marginale*, *A. ovis*, and *A. centrale* and cELISA kit was used according to the instructions of the manufacturer. The optical density (OD) of each well was measured at a wavelength of 620 nm.

Besides, a nested-PCR methods was used for the diagnosis of *A. marginale* and *A. centrale*. Blood samples was extracted with QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA). Extracted DNA samples were used as a template for *A. centrale* and *A. marginale* in species-specific nested-PCR reaction according to a previously described protocols (Kawahara et al. 2006; Molad et al. 2006) and Pearson's Chi-square tests were performed by SPSS 22.0 package software. P value of <0.05 was considered as statistically significant.

## Results

At the end of the study, *Anaplasma* spp. seropositivity was detected by c-ELISA method as 38.5% (72/187) in Adana. Sixty-nine (95.8%) of the seropositive animals were female and 3 (4.2%) were male and they were belonged to Holstein, Crossbred, Simmental, Local, Jersey, Brown Swiss and Aberdeen breeds. According to age ranges, se-

ropositivity (63.6% -21/33) was highest in the 7-13 over age group (Table 1).

**Table 1.** Seropositivity distribution according to breed, age and gender in cattle

	Seropositivity	Total
<b>Age</b>		
0-3	22 (29.3%)	75
4-6	29 (36.7%)	79
7-13	21 (63.6%)	33
<b>Gender</b>		
Female	69 (41.07%)	168
Male	3 (15.78%)	19
<b>Breed</b>		
Holstein	50 (35.46%)	141
Crossbred	14 (48.27%)	29
Simmental	3 (100%)	3
Local	2 (25%)	8
Jersey	1(100%)	1
Brown Swiss	1(50%)	2
Aberdeen	1(50%)	2
Anatolian Grey	-	1
<b>Total</b>	<b>72</b>	<b>187</b>

The highest seropositivity was observed in Çukurova (91.6% - 11/12) and Karataş (91.6% -11/12), followed by Seyhan (80% - 8/10) and Saimbeyli districts (60% - 6/10), respectively. *Anaplasma* spp. seropositivity was not found in Yumurtalık districts (Table 2).

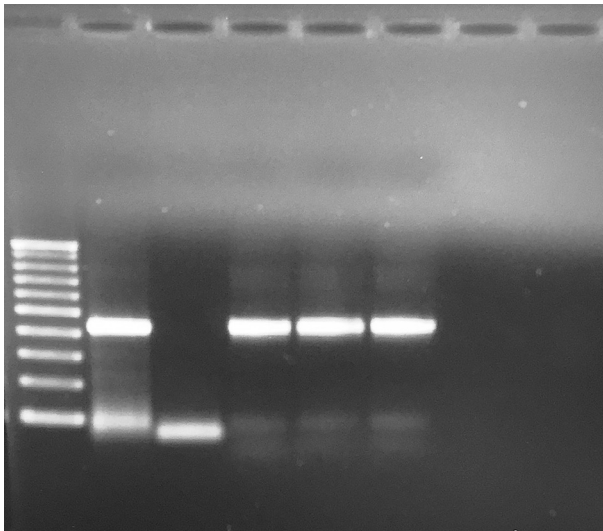
**Table 2.** Seropositivity distribution in districts of Adana.

Districts	<i>Anaplasma</i> spp. Seropositive Sample Size	Total Sample Size
Çukurova (150m)	11(%91.6)	12
Karataş (10m)	11(%91.6)	12
Seyhan (30m)	8(%80)	10
Saimbeyli (945m)	6(%60)	10
Yüreğir (20m)	5(%50)	10
Tufanbeyli (1472m)	11(%44)	25
Karaisalı (300m)	4(%40)	10
Pozantı (790m)	3(%30)	10
Sarıçam (100m)	3(%30)	10
Ceyhan (25m)	3(%25)	12
Kozan (150m)	2(%8.3)	24
Aladağ (860m)	2(%20)	10
Feke (560m)	2(%20)	10
İmamoğlu (84m)	1(%10)	10
Yumurtalık (20m)	-	12
<b>Total</b>	<b>72 (%38.5)</b>	<b>187</b>

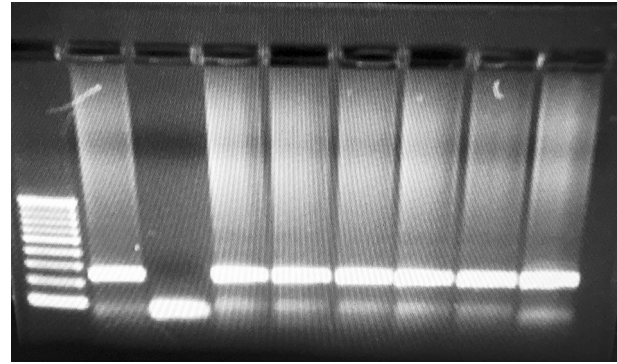
The molecular prevalence was detected as 1.6% (3/187) for *A. centrale* and 3.2% (6/187) for *A. marginale* by Nested-PCR methods. While *A. marginale* was determined in Tufanbeyli and Karataş, *A. centrale* was detected in Cukurova and Aladağ districts. All PCR-positive cattle were female. The cattle infected *A. centrale* were Holstein and were 3, 6 and 8 years old, respectively. Also, the cattle infected *A. marginale* were Crossbred, Simmental and Holstein and were 3, 4, 8, 10 and 13 years old (Table 3).

**Table 3.** Distribution of *Anaplasma* spp. in districts of Adana.

	Districts	Breed	Gender	Age	Total Cattle Number
<i>marginale</i>	Tufanbeyli	Crossbred	Female	8	6
		Simmental	Female	10	
		Crossbred	Female	13	
		Simmental	Female	3	
		Holstein	Female	3	
	Karataş	Holstein	Female	4	
<i>centrale</i>	Cukurova	Holstein	Female	8	3
		Holstein	Female	6	
		Aladağ	Holstein	Female	



**Figure 1.** *Anaplasma centrale* Nested-PCR (426 bp). Lane 1: 100bp DNA ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4-6: Positive samples.



**Figure 2.** *Anaplasma marginale* Nested-PCR (246 bp). Lane 1: 100bp DNA ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4-9: Positive samples.

### Statistical Analysis

**Table 4.** Statistically analysis between *Anaplasma* spp. seroprevalence and breed, gender, age and altitude

Parameters	<i>Anaplasma</i> spp.		Total	P value/X <sup>2</sup>	
	Negative	Positive			
Altitude	<100 m	45 59.2%	31 40.8%	76 100.0%	X <sup>2</sup> =0.283 p=0.595 p>0.05
	>100 m	70 63.1%	41 36.9%	111 100.0%	
	Total	115 61.5%	72 38.5%	187 100.0%	
Breeds	Holstein	91 64.5%	50 35.5%	141 100.0%	X <sup>2</sup> =2.240 p=0.135
	Others	24 52.2%	22 47.8%	46 100.0%	
	Total	115 61.5%	72 38.5%	187 100.0%	
Gender	Female	99 58.9%	69 41.1%	168 100.0%	X <sup>2</sup> =4.608 p=0.032
	Male	16 84.2%	3 15.8%	19 100.0%	
	Total	115 61.5%	72 38.5%	187 100.0%	
Age	0-3 age	53 70.7%	22 29.3%	75 100.0%	X <sup>2</sup> =11.574 p=0.003
	4-6 age	50 63.3%	29 36.7%	79 100.0%	
	7 over age	12 36.4%	21 63.6%	33 100.0%	
	Total	115 61.5%	72 38.5%	187 100.0%	

In this study, the relationship between *Anaplasma* spp. seroprevalence and gender, breed, age and altitude were statistically analyzed (Table 4). In the analysis, no significant difference was found between altitude, cattle breeds and *Anaplasma* spp. ( $p > 0.05$ ). While a statistically significant relationship was found between gender, age and the rate of *Anaplasma* spp. seroprevalence. The seroprevalence was higher than males ( $p < 0.05$ ) and in cattles over the age of seven ( $p < 0.01$ ).

## Discussion and Conclusion

For the detection of *Anaplasma* infections, conventional, serological and molecular methods are used alone or with in combination. Although microscopic examination is one of the most cost-effective and easiest way, it has low sensitivity and specificity. The serological methods as Indirect Immunofluorescence Assay (IFA), ELISA and Complement Fixation (CF) are generally used to detect the presence of specific antibodies. One of these methods, the cELISA test, has a very high sensitivity and specificity due to the presence of the ANAF16C1 monoclonal antibody, which recognizes the preserved antigen Major Surface Protein (MSP)-5. The gold standard for the diagnosis of anaplasmosis is combination of c-ELISA and microscopic examination. PCR, which is the most commonly used molecular method, is highly sensitive, specific and can easily distinguish the types and subspecies of *Anaplasma* species. Furthermore, it is also superior to other methods for detecting coinfections (Shabana et al. 2018). In this study, we used c-ELISA and PCR methods together.

In Turkey, many studies have been conducted to determine the anti-*A. marginale* antibodies with c-ELISA method and seroprevalence rates were determined as, 55.35% (357/645) in the Interior Aegean region (Birdane et al. 2006), 52.1% (98/188) in Kars (Gökçe et al. 2013), 45.9% (28/61) in Bursa (Selçuk et al. 2015), 37.8% (102/270) in the Black Sea region (Açııcı et al. 2016), 31.86% (223/700) in Konya (Işık et al. 2018). And also, anti-*Anaplasma* spp. antibodies were detected as 10.9% (5/46), 7% (3/43), 78.7% (37/47) and 15.2% (7/46) in Van, Muş, Siirt and Diyarbakır provinces, respectively (Oğuz et al. 2018).

Seroprevalence status was evaluated in various studies in neighboring countries and the seroprevalence rates were detected as 9.09% (4/44) in Erbil (Ameen et al. 2012), 13.04% (24/184) in Wassit (Jassem et al. 2015) regions of Iraq and 7.62% (8/105) in Iran (Khezri 2015).

In this study, *Anaplasma* spp. seroprevalence was found to be 38.5% (72/187). The highest seropositivity was determined in Çukurova and Karataş districts. In a study, cattle were found to be 1.18 times more positive in mountainous regions where altitude was 735-482 m above sea level and average temperature was between 26.9°C and 31.8°C. However, the altitude of the regions where the highest positivity was determined in our study was 10-150 m and the temperature was 35-41°C (Noaman et al. 2019). Geographical and climatic differences, reservoir host density and vector tick population that play a role in the spread of infection are the factors that directly affect the prevalence of *Anaplasma* infections. Depending on these reasons, the prevalence of the Anaplasmosis may vary districts to districts or from country to country (Ahmadi-Hamedani et al. 2009; Noaman et al. 2019).

We try to take samples from all districts of Adana with different altitudes and climatic characteristics and animals from different gender, breed and age for supplying reliable epidemiologic data. Seroprevalence was significantly higher in cattle older than 7 years. In a study examining risk factors, with multivariate logistic regression analysis, it was found that *Anaplasma* sp. positivity was 11.32 and 3.11 times higher in elderly (6-10 age) and adults (3-6 age) than in young animals (0-3 age), respectively, similar to this study (Abdela et al. 2018).

The seroprevalence of Anaplasmosis in females was statistically higher than males. In various studies, female hosts appeared to be more prone to tick-borne diseases than males. In this study, immunosuppression in cattle during pregnancy and high lactation may be the cause of high seroprevalence (Atif et al. 2012; RATHERA et al. 2016).

In Turkey, many molecular studies have been carried out to detect *Anaplasma* species. The molecular prevalence of *A. marginale* was detected as 31% in the Thrace region (Aktaş et al. 2017), and 29.1% (57/196) (Zhou et al. 2016), in six provinces

of Turkey. And also, the molecular prevalence of *A. marginale* and *A. centrale* was detected as 2.3% (9/389) and 0.8% (3/389) in Eastern Black Sea region (Aktaş et al. 2011), 6% (9/150) and 5.3% (8/150) in Karaman (Aydın et al. 2019), 7.21% (49/679) and 4.12% (28/679) in Aydın (Hoşgör et al. 2015), respectively.

There were also various molecular studies in countries with borders to Turkey. *Anaplasma marginale* positivity was reported as 44% (88/200) in Iran (Noaman et al. 2019) and 28.12% (18/64) in Al-Nasiriyah city in Iraq (Al-Kasar et al. 2018). Also, in northern Iran, the prevalence of *A. marginale* and *A. centrale* was detected as 9.33% (14/150) and 12% (18/150) (Salehi-Guilandeh et al. 2018), respectively. In this study, the overall molecular prevalence was detected as 4.81% (9/187) and seropositivity (38.5%) was higher than molecular prevalence (4.81%). We think that most of the animals survived after the infection but carrying the antibodies in their blood. It is known that anti-MSP5 antibodies remain approximately 15 to 72 months in cattle (Knowles et al. 1996).

Individual prevalence was detected as 1.6% (3/187) for *A. centrale* and 3.2% (6/187) for *A. marginale* with Nested-PCR. Zhou et al. (2016), studied on 196 cattle and find the overall prevalence as 29.1% from six different provinces (Konya, Karaman, Adana, Urfa, Diyarbakir and Kirklareli) of Turkey and 0% (0/13) in Adana provinces. In this study, there is no epidemiological data such as breed, age, gender and altitude of the sampling areas and also, sample size is so small for Adana region (Zhou et al. 2016).

In conclusion, this is the most comprehensive study for Anaplasmosis in cattle in Adana province. Serologic and molecular prevalence of *Anaplasma* spp., which are important for economic losses, were determined with this study. According to these results, vector control, vaccination and treatment protocols may be recommended in districts with high rates. And also, further studies are needed for determination of other *Anaplasma* and vector species in Çukurova region for clear understanding the epidemiology of Anaplasmosis.

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