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Research Article

Determination of Diagnostic Value of cELISA for the Diagnosis of Anaplasmosis in Clinically Suspected Ruminants [1]

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Abstract

The aim of this study was to determine diagnostic value of cELISA in anaplasmosis in clinically suspected animals and to compare the cELISA results with the clinical examination results. For this purpose a total of 720 ruminants (457 cattle, 146 sheep, 117 goat) were examined in terms of clinical signs. Eighty-eight ruminants consisting of 61 cattle, 11 sheep and 16 goat which had the symptoms of anemia, fever, icterus, weakness, depression and lack of appetite were selected for the study. Blood samples were collected from the jugular vein of all clinically suspected animals and serum samples were separated. A commercially available competitive enzyme-linked immunosorbent assay (C-ELISA) kit was used for determine antibodies to *Anaplasma* species. cELISA based diagnosis revealed that 47 of 88 serum samples (53.4%) were positive for anaplasmosis. In serological examination *Anaplasma* specific antibodies were determined in 45.9% of cattle, 63.6% of sheep and 56.2% of goats. Seropositivity rate was statistically differ among the age groups of cattle and the highest seropositvity rate was found in <12 month age ($P<0.005$). However no difference was found in the seropositivity rate of *Anaplasma* in sheep and goat in relation to age group. From the data obtained in this study it can be concluded that clinical findings are not sufficient criteria for the diagnosis of anaplasmosis and must be supported by serological examination.

Keywords: *Anaplasmosis, Ruminant, cELISA, Clinical diagnosis*

Klinik Olarak Anaplasmosis Şüphesi Olan Ruminantlarda cELISA'nın Tanısal Değerinin Belirlenmesi

Özet

Bu çalışmada cELISA'nın klinik olarak anaplasmosis şüphesi olan ruminantlardaki tanısal değerinin belirlenmesi ve klinik muayene sonuçları ile cELISA sonuçlarının karşılaştırılması amaçlanmıştır. Bunun için toplam 720 ruminant (457 sığır, 146 koyun, 117 keçi) klinik belirtiler yönünden muayene edilmiştir. Anemi, ateş, sarılık, güçsüzlük, durgunluk ve iştahsızlık belirtileri olan 61'i sığır, 11'i koyun ve 16'sı keçi olmak üzere toplam 88 ruminant çalışma için seçilmiştir. Klinik şüpheli bu hayvanların vena jugularisinden kan alınmış ve serumları çıkarılmıştır. *Anaplasma* antikorlarını belirlemek amacıyla ticari olarak temin edilen cELISA kiti kullanılmıştır. cELISA sonuçlarına göre 88 serum örneğinin 47'si (%53.4) anaplasmosis açısından pozitif bulunmuştur. Serolojik muayenede sığırların %45.9'unda, koyunların %63.6'sında ve keçilerin %56.2'sinde *Anaplasma* spesifik antikorlar saptanmıştır. İstatistiki olarak sığırlarda yaş gruplarına göre farklı seropozitivite oranları saptanmış olup, en yüksek oran 12 aydan daha küçük hayvanlarda saptanmıştır. Bununla birlikte koyun ve keçilerde yaş gruplarına göre seropozitivite oranlarında bir farklılık saptanmamıştır. Bu çalışmadan elde edilen verilere göre anaplasmosis tanısında klinik bulguların tek başına yeterli olmayacağı ve serolojik muayenelerle mutlaka desteklenmesi gerektiği sonucuna varılabilir.

Anahtar sözcükler: *Anaplasmosis, Ruminant, cELISA, Klinik tanı*

INTRODUCTION

Anaplasmosis is an important haemoricketsial disease caused by the tick-borne pathogen *Anaplasma* species [1,2]. Anaplasmosis is globaly the most prevalent tick-borne disease of ruminants and has a worldwide distribution with areas in endemicity on six continents. High prevalence rates are reported in tropical and subtropical regions of the world [2-6]. Prevalence of disease depends on distribution and density of reservoir host and tick vectors [2,5,6]. *Anaplasma* species is transmitted biologically by infected ticks, mechanically by biting flies (Tabanids) or blood contaminated fomites. Approximately 20 species of ticks have been determined as vectors worldwide

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including *Rhipicephalus spp., Ixodes spp., Hyalomma spp.,* and *Dermacentor spp.* Among these species *Rhipicephalus (Boophilus) spp.* is found to be major transmitting agent [2,7,8]. Although there are some prevalence reports of anaplasmosis in cattle with high rate (up to 80%) [1,3,9-11], information about prevalence rates in small ruminants is restricted in Turkey [12,13].

Acute phase of anaplasmosis is characterized by progressive haemolytic anemia associated with fever, jaundice, decreased milk production, weight loss, abortions, hyper excitability and in some cases sudden death [2,4,5,7]. After the first infection with *Anaplasma spp*. animals remain persistently infected carriers and serve as long term reservoirs for the maintenance of infection within herds [2,5,7]. These carrier animals are efficient sources of infection where they carry *Anaplasma* species in their bodies, but do not show any clinical signs and able to infect other animals.

Successful management of anaplasmosis in ruminants depends on accurate knowledge of prevalence and risk factors associated with transmission. For this reason diagnosis of acute infection and determination of carrier animals is vitally important. Diagnosis of disease usually based on microscopic examination of Giemsa stained blood smears in clinically suspected animals, during the acute infection [2,9,10]. However this method is not applicable for the detection of subclinical and/or latent infection in carrier animals, which serve as a reservoir for the spread of infection since the parasites are seldom detected microscopically in chronic infections [2,5]. Therefore, several serological tests have been established for the diagnosis of disease. However these tests cannot discriminate different *Anaplasma* species because of antigenic similarity. On the other hand detection of *Anaplasma* species using nucleic acid approach offers an alternative diagnostic tool [2,5,12,13].

Analyzing of diagnostic capacity of serological tests in anaplasmosis might provide further insight into the epidemiology, determination of carrier animals and may be helpful to management of disease. Although, there are few studies with respect to serologic diagnosis of this rickettsial disease in Turkey [3,9,10,12], there is no serological study conducted in clinically suspected ruminants.

Because of the scarcity of such data in the Turkish literature, the present study was undertaken to evaluate the ELISA in anaplasmosis clinically suspected ruminants from Bursa province, Turkey.

MATERIAL and METHODS

Ethical Committee Approval

The study protocols and experimental procedures were approved by the Uludag University Scientific Ethics Committee (No: 2010-05/04).

Study Area

This study was conducted at the South Marmara region of Turkey. Study area (Bursa) is located in southeast of the Marmara Sea (40°E, 28-30°N). This region is characterized by hot and dry summers with some rainfall. Winter conditions are changing mild to cool with more extended periods of light to moderate rainfall. The mean annual temperature in the area is 14-16°C with minimum and maximum averages of 5°C and 25°C. The area receives an average of 600-700 mm rain per year. There are generally in the form of chain of mountains running across the direction of east to west. Annual temperatures, rainfall distribution, are suitable for average humidity levels and forest covered areas are suitable for vector ticks [14].

Clinical Examination and Selection of Study Animals

The study was conducted during tick season between May to October, 2012 in four different districts (Alpagut, Koçuköy, Erenler, Gökçeören) of Bursa. In these districts a total of 720 ruminants (457 cattle, 146 sheep, 117 goat) were examined in terms of clinical symptoms and tick infestation. Collection of animal information such as age, breed, and origin were conducted with the help of animal owners. General physical examination was conducted on all animals in herds. Parotid, prescapular and prefemoral lymph nodes were palpated to assess whether they were enlarged. Mucous membranes of conjunctiva and mouth were examined for pallor or petechial haemorhages. All animals were examined for the presence of ocular and nasal discharges and diarrhea. The skin coat was examined any signs of roughness and ticks. According to clinic examination a total of 88 ruminants (61 cattle, 11 sheep and 16 goat) primarily having history of tick infestation, fever, jaundice and anemia were selected for the purpose of blood collection.

Blood Collection

A total of 88 ruminants were bled from the jugular vein into non-heparinised vacutainers tubes. About 5 ml of blood was taken from each animal into each tube and stored at 4°C until arrival at the laboratory. In the laboratory serum samples were separated by centrifugation at 3.000 rpm for 5 min and stored at -20°C until use.

Tick Collection and Identification

Whole bodies of animals were carefully checked for ticks and their specimens were placed into 70% ethanol in glass vials. In our laboratory ticks were identified according to the keys of Aydın [14] and recorded in data sheet.

Competitive ELISA

All sera collected from suspected animals were tested for the presence of antibodies against *Anaplasma* by competitive ELISA (cELISA). The cELISA was performed

using the *Anaplasma* antibody test kit (VMRD Inc., Pullman, WA- catalog number: 282-2) following the manufacturer's instructions. This kit can detect antibody specific to *Anaplasma marginale*, *A. ovis*, and *A. centrale* in serum samples [15]. All samples and controls were run in duplicate and the mean obligate density at 450 nm was determined.

Data Analyses

The associations of prevalence among three animal species were determined by Fischer exact test. The association of prevalence of the infection among the animals of different age groups was determined also by Fischer exact test. Results were considered to be significant at P<0.05. All results were analyzed statistically using Minitab (V-15) software package [16]. Clinical variables such as anemia, fever, weight loss, pallor of mucous membranes, diarrhea, lacrymation and lymph node enlargement were recorded as either present or absent.

RESULTS

During the study, 419 of 720 ruminants (58.16%) examined for tick infestation were carrying at least one of tick species. Tick infestation rate was found to be 62.29% in cattle, 72.27% in sheep and 68.75% in goats. As seen in the *Table 1* eight tick species belonging to five genus of Ixodidae family were identified. 26% of total ticks were *Ixodes ricinus, Rhipicephalus annulatus* 6%, *Rhipicephalus turanicus* %7.5, *Rhipicephalus bursa* 20%, *Dermacentor marginatus* 15%, *Haemaphysalis parva* 6.5%, *Hyalomma marginatum* 2%, *Hayalomma anatolicum* 3.7%, *Rhipicephalus spp nymph* 19%, *Ixodes nymph* 15.3%. *Rhipicephalus* species was the predominant tick vector followed by *Ixodes ricinus* and finally *Dermacentor marginatus*.

Clinical findings in all ruminants with anaplasmosis suspected as follows: anemia, pale mucous membrane, lack of appetite and decrease of milk production. Most animals demonstrated weakness, weight loss, depression, icterus and lethargy. Very few animals (5 cattle and 2 sheep) presented fever (>40°C) and dehydration. However there were no animal died within the study period.

cELISA based diagnosis revealed that overally 47 of 88 animals (53.4%) positive for *Anaplasma*. Serological examination of *Anaplasma* spesific antibodies proved that 45.9% of cattle, 63.6% of sheep and 56.2% of goats were positive. Detail of cELISA results according to age groups were presented in *Table 2*. According to statistical analyses results although there was a significant difference among the age groups in cattle, no difference was found in the seropositivity rate of *Anaplasma* in sheep and goat age group. In cattle the highest seropositvity rate was found in <12 age group (P<0.005). On the other hand concerning the seropositivity rate of anaplasmosis in different animal species, showed that sheep (data are not shown) had the highest infection rate. However the frequency of antibody existence were not statistically different between; cattle and sheep (P=0.279); cattle and goats (P=0.461); sheep and goats (P=1.000).

Table 1. *Tick species collected from cattle, sheep and goats in Bursa province*
Tablo 1. *Bursa yöresinde sığır, koyun ve keçilerden toplanan kene türleri*

Tick Species	Cattle	Sheep	Goat
Ixodes ricinus	+	+	+
Rhipicephalus annulatus	+	-	-
Rhipicephalus turanicus	+	+	+
Rhipicephalus bursa	+	+	-
Dermacentor marginatus	+	+	+
Haemaphysalis parva	+	+	+
Hyalomma marginatum	+	-	-
Hyalomma anatolicum	+	-	-
Ixodes spp. nymph	+	+	+
Rhipicephalus spp. nymph	+	+	+

Table 2. *Seropositivity rate obtained with cELISA related to age groups in ruminants*
Tablo 2. *Ruminantlarda yaş gruplarına göre cELISA ile elde edilen seropoziflik oranları*

Category	n	No of Positive Test Results
Cattle Age (month)		
<12	7	6[a]
13-24	18	6 [b]
25-48	21	13[ab]
>49	15	6 [ab]
P		P<0.005
Sheep Age (month)		
<12	2	1 [n.s.]
13-24	8	5 [n.s.]
>25	1	1[n.s.]
Goat Age (month)		
<12	5	3 [n.s.]
13-24	9	6 [n.s.]
>25	2	0 [n.s.]

[a, b] Values with different letters in each category are significantly different; ***n.s.*** *not significant*

DISCUSSION

Anaplasmosis is being recognized worldwide as a cause of extensive morbidity and mortality among farm animals. The disease is a major constraint to farm production in many countries and responsible for significant economic losses in endemic areas [2,4,5,9]. *Anaplasma* infections can be fatal in susceptible animals especially in cattle and partially responsible for the high rate of mortality observed in the

affected herd [17,18]. Infected animals may become carriers after recovery a long period and serving as source of infection. Naïve animals in non endemic areas may become infected with anaplasmosis following the introduction of a carrier animal from an endemic area. Therefore the reliable detection of acute infection and carriers is important issue in the epidemiology of anaplasmosis [11]. Scanning tests that are used in epidemiological studies must be reliable. In order to define whether the used test is a reliable sensitivity, specificity, false negative and false positive are calculated and it is required that these measurements have adequate level [9].

Diagnosis of anaplasmosis usually based on microscopic examination of stained blood smear. However this conventional method have some disadvantages and can only detect levels of 10^6 infected erythrosites per ml in acute infectons [19]. *A. marginale* and *A. centrale* multiplies within red blood cell of infected host, resulting in extravascular hemolysis and anemia. During this multiplying process ricketsiemia levels exceed 10^9 infected erythrocytes per ml. Recovery of acute anaplasmosis result in persistent infection characterized by $10^{2.5}$-10^7 infected erythrocytes [20]. In such cases the level of rickettsiemia is generally below the threshold level of microscopic examination. On the other hand lack of expertise among personnel performing smear examination and the occurrence of intracellular artifacts that difficult of differentiating the *Anaplasma* are other disadvantages of blood smear examination. Therefore microscopic examination of blood smears is not sufficiently sensitive and specific to detect chronic carriers. Sharma et al.[21] reported that the traditional Giemsa staining method is not applicable for determination of persistently infected cattle and buffalo. For these reasons mentioned above we have not examined blood smears of suspected animals. The present study is the first report providing serological evidence of anaplasma infections in clinical suspected animals in Turkey.

As an alternative to microscopic examination several serological tests and nucleic acid based assay can be used for detecting anaplasmosis in infected animals. Nevertheless, serological tests would be more practical for the diagnosis of large number of animals. Many authors stated that cELISA test has very high sensitivity and specificity in the diagnosis of antibodies against *Anaplasma* species [9,22]. In *Anaplasma marginale* infections cELISA can diagnose these antibodies 6 years after infection [9]. However, serodiagnostic assays did not distinguish between current infection and prior exposure. In the current study we evaluated the performance of c ELISA assay to detect infections with Anaplasma in clinically suspected animals. cELISA results indicated that overall 47 of 88 serum samples (53.4%) were positive for Anaplasma antibodies. Positivity rate was 45.9% of cattle, 63.6% of sheep and 56.2% of goats. Our finding showed that *Anaplasma* have been determined in nearly 50% of suspected animals with ELISA.

A few previous serological studies involving *A. marginale* reported that the sero-prevalence ranged from 14.86% to 59.3% in different regions of Turkey [3,9,10,12]. The differences between infection prevalence may be attributed to the changes in climatic condition, intensity of tick infestation, and also contaminated needles and instruments transmission is an efficient way of infection spreading in herds [23]. But the most important epidemiological factor for the establishment of high prevalence is the persistence of infection in the reservoirs. In this study concerning the seroprevalence of Anaplasmosis in different age groups, the results showed that adult animals (more than 12 months) of both sheep and goat had the highest seropositivity rate. This might be explained by the fact that; the age resistance which may lasts up to 12 months and as the animals get older, become more susceptible to infection. However statistical analyses revealed that seropositivity rates were not differ among age groups of sheep and goat. On the contrary; young cattle (0-12 months) showed also high infection rate, this may be due to maternal antibodies in the colostrum [24]. Our results cleared that Anaplasmosis is a disease of adults; a parallel findings were recorded by Chahan et al.[25] and Keleş et al.[26].

Meanwhile the frequency of antibody existence was not statistically different among animal species (cattle, sheep and goat). However, in our knowledge there was no any other report comparing the seroprevalence of Anaplasmosis in cattle, sheep and goat in Turkey. Therefore it was not possible for us discuss and compare our findings with others.

Farmers and veterinarians in endemic areas often suspect anaplasmosis based on a history of previous disease outbreaks and clinical signs in that locality. In this study, infected animals showed anemia, pale mucous membrane, lack of appetite, weakness, weight loss, depression, icterus, lethargy and few animals presented fever (>40°C) and dehydration. Similar findings had been reported previously by Sharma et al.[21], Birdane et al.[10] and Abao-Elnaga et al.[8]. Diagnosis of Anaplasmosis in Bursa province is based on clinical signs. However, nonspecific clinical signs (fever and anemia) could lead to misdiagnosis with other diseases such as theileriosis and babesiosis [29,30]. In addition, field veterinarians of the study region had not enough information about the symptoms of the disease. Hence, for the definitive diagnosis of the anaplasmosis the clinical findings should be supported by serological tests.

It is well known fact that ticks are biological vectors of *Anaplasma spp*. Worldwide approximately twenty species of ticks have been incriminated as biological vectors along with other mechanical means such as contaminated fomites, castration instruments and blood sucking diptera [2,5,7,10,31]. In the current study many potential tick vectors of *Anaplasma* infection were identified during the investigation in farms with seropositive animals. Overall eight tick species belonging to five genus were identified

and 58.16% of ruminants examined for tick infestation were carrying at least one of tick species. *Rhipicephalus* species was the predominant tick vector followed by *Ixodes ricinus* and *Dermacentor marginatus*. In northern part and black sea region of Turkey *I. ricinus* as the vector of Anaplasmosis was observed as the most common species [7,28]. Our findings is agreement with those of Aktas et al.[7] and Arslan et al.[27]. This results is also consistent with those of reported tick species by Aydin [28] in their comprehensive study in this region previously.

In conclusion the results obtained from the current study clearly indicated that anaplasmosis present in cattle, sheep and goat in Bursa province of Turkey. cELISA can detect 50% of anaplasma infection in clinically suspected ruminants and can serve as a valuable and practical tool under field conditions. Another important result of this study is that diagnosis of anaplasmosis only according to clinical symptoms may not be always right. Therefore clinical diagnosis of anaplasmosis must be supported by serological and molecular tests.

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