
MOLECULAR DETECTION OF ANAPLASMATACEAE AGENTS ON DOGS FROM THE DEPARTMENT OF PIURA, PERU

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ABSTRACT

This study aimed to study the prevalence of Anaplasmataceae organisms through the nested-PCR and phylogenetic analysis on domestic dogs in the Department of Piura, Peru. Two hundred and twelve canine blood samples were randomly collected on dogs from the central urban areas at the Piura Department in Peru. The extracted DNAs were tested, by nested-PCR based on 16SrRNA gene, to identify agents from Anaplasmataceae family. These results show that there was a prevalence of 18.5% (40/216) of positive dogs, 13.8% (30/216) for *Ehrlichia canis*, 7.4% (16/216) for *Anaplasma platys* and 0.1% (2/216) for *Ehrlichia* sp. confirmed by sequencing analysis. Co-positivity among Anaplasmataceae family species was present in 25% (10/40) of positive samples. There was a significant association among Anaplasmataceae family infection in dogs and the following variables: sex ($p=0.034$), presence of ticks ($p=0.0001$), and socio-economic status ($p=0.001$). There was no statistical association on the variables “living with other animals” and “age group” ($p=0.1074$). The partial sequences on the portion of the 16S rRNA gene, from positive samples for agents of Anaplasmataceae family demonstrated an identity of 97-100% with the isolated *E. canis* and *A. platys* obtained from the GenBank. This is the first study on infection by agents of Anaplasmataceae family in dogs in the Department of Piura, through molecular analysis.

KEY WORDS: Anaplasmosis; *Canis lupus*; ehrlichiosis; molecular analysis; South America.

INTRODUCTION

Dogs may be infected by several agents which belongs to Anaplasmataceae family. Among these, the most frequently described that

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causes canine diseases are *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum* and *Anaplasma platys*. These agents are mandatory Gram-negative intracellular microorganisms who are part of the Rickettsiales order, Anaplasmataceae family (Santos et al., 2009; Unver et al., 2003; Eiras et al., 2013; Vargas-Hernández et al., 2012).

Canine ehrlichiosis and anaplasmosis are considered two of the most significant diseases which affect dogs and that can be fatal for domestic dogs and other members of Canidae family (Borin et al., 2009). Clinical signs and laboratory findings are often nonspecific at different stages of this disease. In general, the most frequent laboratory abnormalities of ehrlichiosis in dogs include normochromic normocytic anemia, thrombocytopenia, pancytopenia (chronic disease phase), lymphocytosis, hyperglobulinemia, hypoalbuminemia and increased serum alanine aminotransferase activity (Macieira et al., 2005). In *A. platys* infections, in addition to cyclic episodes of thrombocytopenia, inconstant anemia, macro-platelets, hypoalbuminemia, and hypermagglobulinemia were observed (Macieira et al., 2005).

Due to the cosmopolitan distribution of the vector *Rhipicephalus sanguineus* the agents transmitted by this vector have a worldwide distribution, mainly in tropical and subtropical regions. This tick species which has a domestic habitat is important for public health due to the possibility of transmission of various pathogens to humans, despite of its low anthrophilia. Some studies have reported the presence of genera *Amblyomma*, *Dermacentor*, *Ixodes*, and *Rhipicephalus*, in dogs and in other hosts from different regions of Peru, such as Piura and Lima (Glenny et al., 2004; Vinasco et al., 2007).

Due to the clinical importance of these organisms which have a large population of vectors transmitting hemoparasites, and the fact that many of these parasites have zoonotic potential, it has been needed to characterize the agents present in the northern region of Peruvian territory. This will ease the tracing of these agents and it will help to set up the control strategies and methods for early diagnosis thus, it will help to have a greater knowledge of the epidemiological characteristics on dog infections caused by these agents. Furthermore, this study has aimed to obtain epidemiological information on the presence of agents from Anaplasmataceae Family which affect dogs on the Metropolitan Region of the Province of Piura, in the Department of Piura, Peru.

MATERIAL AND METHODS

This project was authorized by the Ethics Committee for Animal Research, Dean's Office for Research and Postgraduate Studies, Universidade Federal Fluminense, Niterói, RJ, Brazil (protocol 759).

A convenience sampling cross-sectional study was developed by analyzing the samples of domestic dogs from urban areas in Metropolitan Region of Piura, central area of the Piura department, Peru. The average altitude is 29 meters above sea level, latitude 4°59'24''S and longitude 80°24'36''W-GR, and the climate is characterized by the average maximum and minimum annual temperatures of 31.2°C and 17.7°C respectively. The average relative humidity is 83% in the morning and 51% in the evening. Two hundred and twelve canine blood samples (approximately 2 mL/sample) were randomly collected at the owner's house, by a puncture on the jugular or cephalic veins, using sterile disposables syringes and needles (21-gauge). Subsequently, EDTA blood samples were packaged in polypropylene bottles and kept at -20°C until the molecular analysis.

An epidemiologic questionnaire was administered to the animal owners, and it was individually tailored for each animal in order to obtain information about the dogs and its breeding routines, which included gender, presence of ticks, if they have been living with other animals, the socioeconomic level (High, Medium high, Medium, Medium low and Low) and age group categorized according to Hand et al. (2000) into young (< 1 year), adults (1 to 7 years) and advanced age (> 7 years). The selection criteria did not take clinical status into account.

The blood DNA extraction was performed using a commercial kit (Illustra blood genomic Prep Mini Spin Kit; GE Healthcare Life Sciences®, Buckinghamshire, England) following the manufacturer's instructions for usage.

To ensure the reliability of the negative results, all samples were tested for the presence of amplifiable DNA using the set of primers GAPDH-F and GAPDH-R following the protocol recommended by the original study (Birkenheuer et al., 2003), which provide amplification of a 399 bp fragment of the gene encoding the glyceraldehyde-3-phosphate dehydrogenase enzyme (GAPDH), present in all mammals. To identify agents from Anaplasmataceae Family, ECB and ECC primers (Murphy et al., 1998) were used, that amplify a fragment of approximately 478 bp from the 16S region of the ribosomal RNA (rRNA). Polymerase chain reaction (PCR) positive samples for ECB and ECC primers (first reaction) were tested by nested-PCR for ECAN-5/HE-3 primers for *E. canis*-specific amplification (Dawson et al., 1996, Murphy et al., 1998), which amplify a fragment of approximately 398 bp, and PLATYS-F/HE-3 for *A. platys*-specific amplification (Brown et al., 2001), amplifying 398 bp fragments. The reaction protocol and temperature conditions in the thermocycler were done according to the original articles. For each reaction, positive controls of a sample of *E. canis* culture and a blood sample known to contain *A. platys* were used. In addition, a negative control (ultrapure water) was included.

The products were applied on 1.5% agarose gel for electrophoresis and for identification, the gel was stained with GelRed™ (Uniscience, Miami, USA) visualized under ultra-violet light (Figure 1). The amplified PCR products from the positive samples were purified with the commercial kit (Illustra GFX PCR DNA and Gel Band Purification Kit; GE Healthcare Life Sciences) and they were sequenced using the Big Dye Terminator Cycle Sequencing Standart Version 3.1 protocol with POP7 polymer, following the specifications from the manufacturer, on the 3130/3130X / Genetic Analyzer Applied Biosystems sequencer Hitachi USA 850 Lincoln Drive Foster City CA94404USA using the commercial kit “Big Dye Terminator® v 3.1 Cycle Sequencing Kit” (Applied Biosystems®, CA, USA). The nucleotide sequences were compared with the corresponding counterparts available in the GenBank database, “National Center for Biotechnology Information” (www.ncbi.nlm.nih.gov), using the “Basic Local Alignment Search Tool” (BLAST; www.ncbi.nlm.nih.gov/blast.cgi).

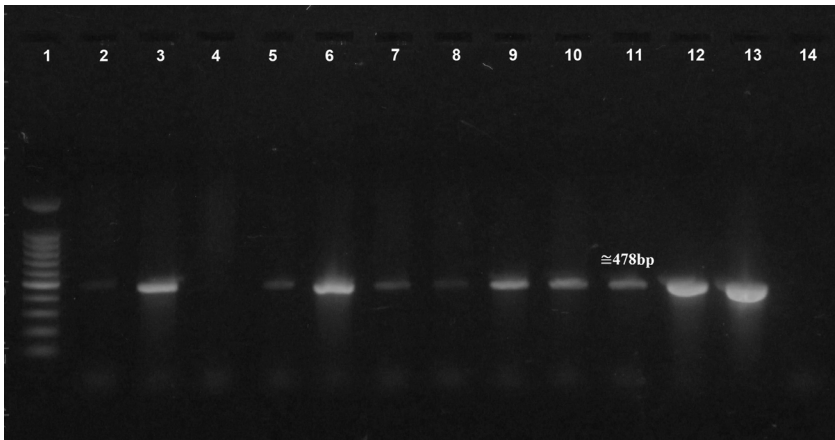


Figure 1. Agarose gel electrophoresis at 1% shows approximately 478 base pair products using ECC-ECB oligonucleotides. (Lanes 2-3, 5-11) Positive samples for Anaplasmataceae Family in dogs, (lane 4) negative sample, (lane 1) molecular marker, (lane 12-13) positive controls for *E. canis* and *A. platys*, and (lane 14) negative control. ≈ (Approximately equal).

Positive sequences were aligned using the Clustal W version 2.1 program, and the alignment gaps were removed. Subsequently, a phylogenetic analysis was performed by using the PHYLIP (Phylogeny Inference Package) (Felsenstein, 2005), using a maximum parsimony analysis with a bootstrap number of 1000, and a consensus tree was generated (the programs used were Seqboot, Dnapars and Consense). The graphic processing of the tree was performed using the Mega program version 6.06.

The data were subjected to statistical tests. Binary variables were analyzed by Fisher's test whereas age and socioeconomic stratum were analyzed by the binary logistic regression test, with a significance level of 5% with a 95% confidence interval to detect differences between frequencies found in the PCR results with the different variables which were mentioned above.

RESULTS

In this study, 216 blood samples were collected from domestic dogs. The absolute and relative frequency of positives for Anaplasmataceae Family and the risk factors associated with the infection are shown on the Table below. There was no statistical association in the variables "living with other animals" and "age group" ($p=0.1074$).

At the "gender" variable, a higher frequency of infection was observed in male animals. The relationship between this variable and the infection by agents of Anaplasmataceae Family showed statistical significance ($p=0.034$). By assessing the presence of ticks and the socioeconomic strata, an association was observed between these variables and the infection by agents of Anaplasmataceae Family ($p=0.001$). In addition, the socioeconomic stratum "Medium low" had a higher percentage of infected cases.

The positive samples for Anaplasmataceae Family were tested for the amplification of DNA fragments of *E. canis*-specific and *A. platys*-specific, which has revealed a positivity of 13.8% (30/216) and 7.4% (16/216) respectively. Among the positive results for Anaplasmataceae Family, co-positivity was confirmed in 25% (10/40). From these, eight were from *E. canis* and *A. platys* and two co-positivity's from *E. canis* and *Ehrlichia* sp. Thus, 32.5% (13/40) of the positive samples for primer ECC/ECB, were not positive, by Nested-PCR, nor for *E. canis*-specific primer and *A. platys*-specific primer. In two (2/40) samples, it was not possible to identify the infectious agents due to the non-amplification by the specific primers and a failure to sequence the first reaction.

The partial sequences of the 16S rRNA gene's portion from positive samples for agents of Anaplasmataceae Family demonstrated an identity of 99-100%, found by BLAST, with the isolate of *E. canis* obtained in Malaysia (KR920044) and an identity of 97-100 % with *A. platys* from Portugal, Brazil, Croatia, India and Trinidad and Tobago (accession numbers KX180944, KC109446, KY114935, KX818218, KY010670). Two sequenced fragments had similarities of 97.4% (310/318) and 98.4% (315/318) with sequences of *E. chaffeensis* from Mexico (MH487664) and the United States (MK611628) respectively.

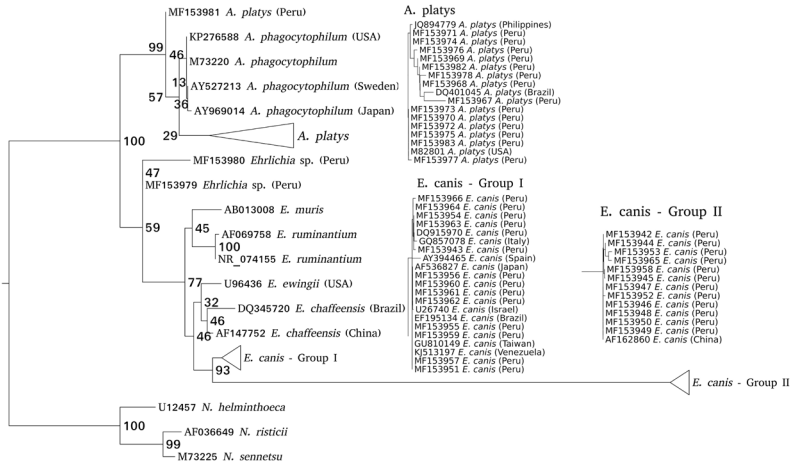
Table. Risk factors for infection by Anaplasmataceae Family in dogs from the Province of Piura, Peru metropolitan Region

Variables	Total	Positive	Positive (%)	% + CI	p-value
	216	40	18.5	18.5 +/- 6.5	
Gender					
Male	106	26	24.5	24.5 +/- 8.3	0.034
Female	110	14	14.0	14.0 +/- 6.4	
Age					
< 1 year	50	12	24.0	24.0 +/- 9.0	0.095
1 year-7 years	145	24	16.6	16.6 +/- 6.9	
> 7 years	21	4	19.0	19.0 +/- 8.3	
Cohabitation					
Yes	129	19	14.7	14.7 +/- 6.7	0.107
No	87	21	24.1	24.1 +/- 8.5	
Socioeconomic strata					
Upper	44	0	0.0	0.0 +/- 0.0	0.001
Upper-Middle	37	5	13.5	13.5 +/- 7.0	
Middle	41	8	19.5	19.5 +/- 8.3	
Low-Middle	74	22	29.7	29.7 +/- 9.4	
Low	20	5	25.0	25.0 +/- 9.5	
Ticks					
Yes	132	35	26.5	26.5 +/- 8.1	0.0001
No	84	5	6.0	6.0 +/- 4.6	

CI: confidence interval

Regarding phylogeny (Figure 2), the sequences used in the comparison were chosen because they are grouped in the same clade as *E. canis* and *A. platys* in the phylogeny proposed by Almazan et al. (2016), in addition to other sequences that have served as a comparison because they are other species of Anaplasmataceae Family. The sequences *Neorickettsia helminthoeca* (U12457), *N. risticii* (AF036649) and *N. sennetsu* (M73225) were chosen as an outgroup because they belong to a different clade from the species evaluated. The sequences of this study were deposited in Genbank and received the following access codes: MF153979, MF153980 (*Ehrlichia* sp.), MF153981, MF153971, MF153974, MF153976, MF153969, MF153982, MF153978,

MF153968, MF153967, MF153973, MF153970, MF153972, MF153983, MF153977 (*A. platys*), MF153966, MF153964, MF153954, MF153963, MF153943, MF153956, MF153960, MF153961, MF153962, MF153955, MF153959, MF153951, MF153957, MF153942, MF153944, MF153915, MF153944, MF153915, MF153946, MF153948, MF153950, MF153949 (*E. canis*).



PhyML ln(L)=-6482.2 1636 sites JC69 1000 replic. 4 rate classes 0.03

Figure 2. Phylogenetic tree based on the 16S rRNA partial sequence gene of positive samples for Anaplasmataceae Family, in the province of Piura-Peru metropolitan region, and similar sequences obtained through access to the GenBank. The phylogenetic analysis was done by the PhyML maximum likelihood algorithm, with the JC69 model, 1000 replicas and 1636 sites. The scale bar represents the difference (number of nucleotides) of each agent within each branch. The triangles showed represent the location of the sequences achieved in this work.

DISCUSSION

In this study, nested-PCR and sequencing techniques were used to verify the amplification of the DNA of *E. canis* and *A. platys* in the blood of dogs from urban areas. These results show that we might be facing with two endemic diseases in the province of Piura-Peru metropolitan region.

In South America, other studies have revealed varying positivity percentages in infection by agents of Anaplasmataceae Family. In Brazil, Argentina, Colombia and Venezuela the frequencies of 38.9% (86/221), 6.9% (6/86), 58.9% (23/39), and 35% (17/51) were reported, respectively (Santos et al., 2009; Unver et al., 2003; Eiras et al., 2013; Vargas-Hernández et al., 2012).

In Peru, at the Veterinary Teaching Hospital of the University of San Marcos in Lima Department, sera of 25 dogs were tested due to the existence of clinical signs compatible with *Ehrlichia* sp. for *E. canis*. The testing used the indirect immunofluorescence and all samples were positive. Besides, a real-time multiplex PCR (qPCR) protocol, amplifying a variable region of the 16S rRNA gene, was used to detect *E. canis*, *E. chaffeensis*, *E. ewingii*, *A. platys*, and *A. phagocytophilum*. Eleven of the 25 blood samples tested were positive for *E. canis* and none was positive for *E. chaffeensis*, *E. ewingii*, *A. platys*, or *A. phagocytophilum* (Vinasco et al., 2007).

In this study, the prevalence of agents of Anaplasmataceae Family in dogs were 18.5%. These differences in frequencies can be explained by the various factors in the design of the investigation, such as the methodologies used for diagnosis, geographic regions evaluated, sampling period and vector stage.

Although the samples were collected irrespective of the clinical condition of the animals, the owner or veterinarian ensured that none of these animals was receiving any medication by the owner nor by the veterinarian. This information was recorded at the time of the sample collection and it guarantees that the susceptibility of the animals studied about the infection is representative.

On the other hand, some studies have shown that the disease may be more severe with some strains of *E. canis* and when a co-positivity of *E. canis* with other hemoparasites is found (Santos et al., 2009). The co-positivity of *E. canis* and *A. platys* has been reported in the literature with a frequency of 30% in Brazil (Silva et al., 2010). Therefore, the cases of co-infection confirmed by sequencing analysis among agents of Anaplasmataceae Family were 25%.

This study has obtained a significant variation in relation to the “gender” variable, corroborating with Costa Jr et al. (2007) who have found a greater number on males rather than on females. However, other studies have not observed the differences among genders (Silva et al., 2010).

Environment of lower socioeconomic strata has favored the dogs’ contact with the vector, as they provide biotypes that are conducive to survival of *R. sanguineus* (Soares et al., 2006). The lack of recurring care in these environments, which associated with the failures in sanitary measures such as prophylactic treatments to control ectoparasites, may increase the risk of infection. By the reason that *R. sanguineus* usually hides in small holes, on cement and wood surfaces, this has allowed the transmission of different agents transmitted by this vector (Labruna & Pereira, 2001). This fact plus the environmental conditions, especially the climate from the studied region, have regain in favor of the proliferation of ticks and consequently the dissemination of the studied agents in dogs on this region.

A potential limitation of this study is that migratory history of the dogs was not assessed and some dogs could have been in contact with ticks from outside the studied area. However, based on our knowledge of the studied area and the studied population, we have considered that the probability of this event is low.

Using sequencing and phylogenetic analysis, we have been able to confirm for the first time the presence of *E. canis*, *A. platys* and the co-positivity by these two agents in the studied region. Moreover, after the phylogenetic analysis two of the products, identified as MF153979 and MF153980, could have not been grouped into the clades of either *E. canis* or *A. platys*, being classified as *Ehrlichia* sp. These findings are highly similar to that of the *E. chaffeensis* species, which increased the suspicion about the existence of other species of the genus *Ehrlichia* which have infected dogs in Latin America. *Ehrlichia chaffeensis* is an agent of Human Granulocytic Ehrlichiosis and it is primarily linked to the tick *Amblyomma americanum* (Padock & Childs, 2003). Three tick species, *R. sanguineus*, *Amblyomma triste* and *Amblyomma trigrinum* have been found during other studies in the same region investigated, with *R. sanguineus* being the most prevalent species (Cerro et al., 2018). Hence, more studies involving the understanding of pathogenicity and probable vectors for the agent found will be needed.

In conclusion, it has been possible to identify, for the first time in the studied region, through molecular evidence, the presence of isolated infections and co-infections caused by *E. canis* and *A. platys*. In addition, gender, the presence of ticks, and the socio-economic status demonstrated to be risk factors associated with the infection.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest to disclose.

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