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**Serological detection of antibodies to *Anaplasma* spp., *Borrelia burgdorferi* sensu lato and *Ehrlichia canis* and of *Dirofilaria immitis* antigen in dogs from Costa Rica**

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### **Highlights**

- *Ehrlichia canis* infection was detected most often and in all Costa Rican provinces
- About 8% of all tested dogs were seropositive for *Dirofilaria immitis*
- *Anaplasma* spp. was the third most frequent pathogen detected
- Puntarenas and Guanacaste were the provinces with highest numbers of seropositives
- Protection against vector-borne pathogens is essential for the whole of Costa Rica

### **Abstract**

In a study in Costa Rica 314 serum samples from dogs throughout all seven provinces were tested using a commercial kit for the detection of circulating antibodies against *Anaplasma* spp., *Borrelia burgdorferi* sensu lato and *Ehrlichia canis*, and of circulating antigen of *Dirofilaria immitis*.

A total of 6.4% (20/314) and 38.2% (120/314) were positive for *Anaplasma* spp. (An) and *E. canis* (Ec) antibodies. Overall, 8.0% (25/314) were positive for *D. immitis* (Di) antigen. One single dog reacted positive with *B. burgdorferi* s.l. (Bb) antigen (0.3%, 1/314). *E. canis* positive dogs were detected in all provinces (highest percentages in Guanacaste,

Puntarenas [both significantly different compared to the overall] and Limón). Guanacaste and Puntarenas also showed the highest prevalences of *Anaplasma* spp. (both significantly different compared to the overall). The highest prevalence of *D. immitis* was detected in Puntarenas (significantly different compared to the overall). Double pathogen exposure (Ec plus An; Ec plus Di; Ec plus Bb) were recorded in 8.9% (28/314). Two dogs showed a triple pathogen exposure (0.6%, 2/314; An, Ec and Di). There was a significant difference between male (11.5%, 18/156) and female (4.4%, 7/158) animals for *D. immitis* positive results. There was also a significant difference between breed and no breed dogs regarding the characteristics of a general positive test, as well as seropositivity to the single pathogens of *Anaplasma* spp., *E. canis* and *D. immitis*. Finally there was a significant difference in the presence of clinical signs again regarding the characteristics of a general positive test, as well as seropositivity to *Anaplasma* spp., *E. canis* and *D. immitis*. Practitioners in Costa Rica should be aware of the canine vector-borne diseases mentioned as dogs are at risk of becoming infected. Concerning the positive *B. burgdorferi* s.l. dog, an autochthonous occurrence cannot be confirmed due to a history of adoption and an unusual tattoo number. Veterinary advice to protect dogs and limit transmission of vector-borne pathogens, also to humans, by using prophylactic measures is strongly recommended.

**Keywords:** dog; canine vector-borne disease; CVBD; SNAP® 4Dx®; prevalence; distribution; Costa Rica

## 1. Introduction

Vector-borne diseases in dogs are widely distributed throughout the world in areas with climatic conditions that allow the development of transmitting arthropods (e.g. ticks, fleas, mosquitoes and sandflies). Canine vector-borne pathogens such as *Anaplasma* spp., *Borrelia* spp., *Ehrlichia canis* or *Dirofilaria immitis* can cause severe diseases in domestic dogs (Cohen et al., 1990; Levy and Magnarelli, 1992; Appel et al., 1993; Greig et al., 1996; Hoch and Strickland, 2008; Kohn et al., 2008; McCall et al., 2008; Harrus and Waner, 2011; Simón et al., 2012) and may also have a zoonotic character (Perez et al., 1996; Bakken and Dumler, 2008; Nicholson et al., 2010; Simón et al., 2012; Chomel, 2015). The role of these agents in animal and human health has become more and more evident in recent years. This trend can be illustrated also in the number of papers listed in PubMed searching with ‘vector-borne disease, dog’, which increased from 69 between approximately 1946 and 2009 to 221 within just the past 6.8 years (January 2010 – October 2016). For Mexico and South America diverse studies and data have been published (Labarthe et al., 2003; Eiras et al., 2013; Vieira et al., 2013; Movilla et al., 2016). In Central America data on vector-borne diseases of dogs are scarce. Two of the tested pathogens (i.e. *E. canis* and *D. immitis*) have both been reported in dogs both in Costa Rica (Scorza et al., 2011; Rojas et al., 2014, 2015a; Wei et al., 2015) and in some of the neighbouring countries, namely Honduras and Nicaragua (*E. canis*: Sosa-Ochoa et al., 2013; Wei et al., 2014) and Honduras, El Salvador, Cuba, the Dominican Republic, Puerto Rico, Curaçao and Bahamas (*D. immitis*: Sotolongo Guerra, 1977; Duménigo Ripoll et al., 1982, 1988; Grieve et al., 1986; Hesselink, 1988; Saleh et al., 1988; Manda, 1989; Kozek et al., 1995; Conde

Landaverde et al., 2005; Duran-Struuck et al., 2005; Soto Castro, 2007; McCown et al., 2013). In other countries of Central America the data on prevalence of infection is very limited or non-existent. Due to the fact that Costa Rica has similar conditions (environmental and pet managing) as an extensive area ranging from southern United States to South America, and is together with the Caribbean area also frequented as vacancy destination from abroad, data on the occurrence of canine vector-borne pathogens can be of interest for veterinary practitioners and pet owners beyond Costa Rica itself, including also recommendations for travel.

In Costa Rica, the first case of *E. canis* infection in a dog was reported in 1995 (Meneses, 1995). Later, the prevalence of infection ranged from 3.5% to 50%, but was either derived from restricted areas with smaller dog populations (Scorza et al., 2011; Rojas et al., 2014; Wei et al., 2015) or from a larger but preselected dog population with suspected clinical disease (Romero et al., 2011).

*D. immitis* has been reported in dogs in the country (2.3% [Scorza et al., 2011]; 15.1% [Rojas et al., 2015a]; 22.5% [Wei et al., 2015]), as well as in humans (Rodríguez et al., 2002, 2003a).

Regarding *Borrelia burgdorferi* sensu lato there are two reports for the Central American region. In Honduras, three out of 12 feral cats showed a positive Indirect Fluorescent Antibody (IFA) test for *Borrelia* species (McCown and Grzeszak, 2010). In Cuba antiborrelial antibodies and clinical signs resembling Lyme disease have been detected in humans (Rodríguez et al., 2004, 2012a), but the existence of *B. burgdorferi* s.l. is still much debated (Dessau, 2012; Rodríguez et al., 2012b). According to our search, no accessible

data have so far been published for Costa Rica demonstrating the occurrence of *B. burgdorferi* s.l., neither in humans nor animals.

There are two relevant canine species of *Anaplasma*: *Anaplasma phagocytophilum*, transmitted especially by ticks of the genus *Ixodes*, causing canine granulocytic anaplasmosis and mainly occurring in temperate zones of the world, and *Anaplasma platys*, the pathogenic agent of canine cyclic thrombocytopenia, occurring mainly in tropical and warm regions of the world and primarily transmitted by *Rhipicephalus sanguineus* sensu lato (s.l.). There have been several reports of *A. platys* in dogs in South America (e.g. Argentina [Eiras et al., 2013]; Brazil [da Silva et al., 2012]; Chile [Abarca et al., 2007]), and only very scarce information on the occurrence of *A. phagocytophilum* in dogs (by PCR [Santos et al., 2011, 2013] and by serology [Vieira et al., 2013]; all in Brazil). For Central America, apart from Costa Rica, we could not find any internationally published data on *A. platys* in dogs. For *A. phagocytophilum* occurrence was reported in dogs in Puerto Rico, detected by IDEXX SNAP® 4Dx® (McCown et al., 2013). Focusing on Costa Rica, one of the first descriptions of *A. phagocytophilum* and an *A. platys*-like pathogen in ticks as well as *A. platys* in a dog (all by PCR) was given by Campos (2011). Furthermore *A. platys* has been detected in 1% (Bonilla, 2014), 6.33% (Ábrego et al., 2009; Ábrego Sánchez et al., 2013), 7.5% (Wei et al., 2015) and 10% (Rojas et al., 2014) of dogs, in all cases by PCR. *A. phagocytophilum* has been detected only in 0.3% and 0.9% of dogs and 0.8% and 1.25% of ticks (*R. sanguineus* s.l.) by PCR (Bonilla, 2014; Campos et al., 2013) and in 2.7%, respectively 3.8% of dogs using serology (Bonilla, 2014; Barrantes-González et al., 2013). Most of this data, apart from Rojas et al. (2014), is mainly or exclusively

presented in Spanish. The general situation seems to be that there is a certain prevalence of *A. platys*, explainable also by the abundant occurrence of its transmitting tick vector *R. sanguineus* s.l. in Costa Rica, and a lower prevalence of *A. phagocytophilum*, where the diagnostic measures used were able to differentiate at all.

Here we investigate via a nationwide survey the occurrence and distribution of four major canine vector-borne pathogens in Costa Rica, as well as determine potential factors (sex, age, breed, tick history including current tick infestation, clinical signs) associated with the presence of seropositivity. A further aim was to characterise mixed exposures with the various pathogens and areas of high prevalence.

## **2. Material and methods**

### **2.1 Animals, sample collection, study area and study period**

Serum samples from 314 dogs, taken by 19 veterinarians in 17 randomly involved participating veterinary surgeries and two points of veterinary assistance of the Escuela de Medicina Veterinaria, were analysed in the study. The sample size of 314 was decided under statistical assumptions based on reported data elsewhere for Costa Rica or the region to receive valid prevalence data in general, but also to achieve a statement on presence or absence of seropositivity. Within the surgeries the dogs were randomly sampled and included in the study when inclusion criteria were fulfilled. The serum was collected from dogs seeking veterinary care for different reasons (check-ups, ectoparasitic, curative or surgical consultation). Dogs were clinically examined by participating veterinarians, and any clinical signs were recorded. Tested dogs had to fulfil the following criteria: consent of the owner, at least one year old, living for at least one



year in Costa Rica or born in Costa Rica in the case of dogs one year old, no treatment with ivermectin during the last six months, no treatment with doxycycline during the last 12 months. An identical questionnaire was completed by the participating veterinarians asking the dog owners. The sampling points were distributed throughout all seven Costa Rican provinces. The samples were either examined within the surgeries or transported to and analysed in the parasitological laboratory of the Escuela de Medicina Veterinaria. The origin of the dogs was determined by their owners' addresses within the different cantons, which were supplied with the sample. The serum samples were collected between January and August 2012.

## 2.2 Test analyses

The samples were tested for different canine vector-borne diseases. Serological testing was performed using a rapid assay test system (SNAP® 4Dx®, IDEXX Laboratories, Inc., Westbrook, ME, USA) following the manufacturer's instructions. SNAP® 4Dx® is a test system based on an enzyme immunoassay technique, checking for antibodies to *Anaplasma* spp., *B. burgdorferi* s.l. and *E. canis* using specific antigens, and checking for *D. immitis* antigen on the basis of specific antibodies. The sensitivity of the test ranges according to the manufacturer from 99.1% for *A. phagocytophilum* and 98.8% for *B. burgdorferi* s.l. to 96.2% for *E. canis* and 99.2% for *D. immitis*. The specificity for all four pathogens was around 100% according to Chandrashekar et al. (2010), but in experimentally infected dogs cross-reactions with the *A. phagocytophilum* analyte were observed for antibodies against *A. platys* in particular, so that in the present study *Anaplasma* spp. is stated. This was also reported for the *E. canis* analyte, possibly cross-

reacting with anti-*Ehrlichia chaffeensis* antibodies (O'Connor et al., 2006; Chandrashekar et al., 2010). Cross-reactivity of the *D. immitis* analyte in similar commercially available antigen tests with *Angiostrongylus vasorum*-positive dogs has also been described (Schnyder and Deplazes, 2012).

### **2.3 Statistical analyses**

The descriptive analysis was performed with the help of the validated statistical program TESTIMATE Version 6.5 from IDV Data Analysis and Study Planning. The presence of antibodies (of *Anaplasma* spp., *B. burgdorferi* s.l. and *E. canis*) or antigen (of *D. immitis*) for every variable was dichotomised into negative (= not present) and positive (= present) to calculate the prevalence and the 95% confidence interval (CI). Additionally, differences from the overall sum for each of the seven provinces were calculated using the Fligner-Wolfe test (many-to-one test,  $\alpha < 0.05$  two-sided) for all pathogens. Furthermore, the prevalence for the epidemiological parameters sex, age ( $\leq 2$  years and  $> 2$  years), breed (breed and no breed [mongrels]), tick history (including current tick infestation) and clinical signs was calculated, where applicable after dichotomising into negative (= not present) and positive (= present) (for tick history and clinical signs). The differences between the single categories were tested using the Chi-square test or the unconditional exact Röhmel-Mansmann test ( $\alpha < 0.05$  two-sided). Furthermore using backward elimination ( $\alpha$  to remove 0.100) factors with influence for the model were identified and subsequently analysed in a risk factor analysis using logistic regression.

### **2.4 Data visualisation**

The collected data were assigned to the different provinces and cantons by the dog owners' addresses in order to visualise the regional distribution of collected and analysed serum samples and antibody- and/or antigen-positive samples for the different pathogens on administrative maps. Cantons with positive serum samples were coloured, depending on the prevalence, on maps showing administrative boundaries.

### **3. Results**

The seroprevalences of all tested samples is summarised in Table 1. The overall prevalence of *E. canis* and *Anaplasma* spp. in dogs was 38.2% (n = 120; 95% CI: 32.8-43.4%) and 6.4% (n = 20; 95% CI: 3.9-9.7%), respectively. The overall prevalence based on the test results for *D. immitis* and *B. burgdorferi* s.l. in dogs was 8.0% (n = 25; 95% CI: 5.2-11.5%) and 0.3% (n = 1; 95%CI: 0.0-1.8%), respectively. The percentage of dogs serologically positive for at least one pathogen was 42.7% (n = 134; 95% CI: 37.1-48.4%).

The number of positive samples per province and canton is shown in Table 2. The epidemiological situation of the single pathogens differs greatly between the provinces. Generally, *E. canis* seropositivity was found across the whole country, but prevalences ranged from over 60% (62.2% in Guanacaste) to not much more than 10% (11.8% in Cartago). Even though the number of sampled dogs per province was sometimes quite low (e.g. 17 for Cartago), there was still a significant difference in the occurrence of *E. canis* seropositivity in the provinces of Cartago, Guanacaste and Puntarenas compared to the overall, with the latter two provinces showing the highest percentages. The highest prevalences of *E. canis* were detected (in descending order) in Guanacaste, Puntarenas and Limón. These provinces were also those with the highest prevalences of *Anaplasma*

spp. seropositivity, in the same descending order, but massively lower in number compared to *E. canis* prevalences, ranging from 16.2% (Guanacaste) down to 1.6% (San José) and 0% (Alajuela, Cartago, Heredia). Again, there was a significant difference between Alajuela, Guanacaste and Puntarenas and the overall, with Alajuela having no positive dogs in the examination and the latter two provinces showing the highest percentages. It was determined that living especially in the coastal provinces on the Pacific side of Costa Rica (Guanacaste and Puntarenas) was associated with seropositivity for *Anaplasma* spp. and *E. canis*.

Regarding the occurrence of *D. immitis* seropositivity, Puntarenas was the province with by far the highest prevalence rate (30.6%); all other provinces were either below 6% (5.4% in Guanacaste, 1.6% in San José) or even showed 0%. There was a significant difference between the provinces Alajuela and Puntarenas and the overall, again with Alajuela showing 0% in the examination and Puntarenas showing the highest percentage. Thus it can be determined that living in the province of Puntarenas was associated with *D. immitis* seropositivity.

Where the single dog seropositive for *B. burgdorferi* s.l. was concerned, the sample was taken in the province of Puntarenas. But the dog had a tattoo different to those done in Costa Rica and its owner confirmed that it had been adopted within Costa Rica, being of unknown origin. It can therefore not be excluded that the infection with *B. burgdorferi* s.l. occurred elsewhere and not in Costa Rica.

The cantons from which the positive dogs originated are coloured depending on the prevalence on administrative maps and are shown in Figures 1, 2 and 3 for *Anaplasma* spp., *E. canis* and *D. immitis*.

Co-seropositivity to *E. canis* and *Anaplasma* spp. were observed in 5.4% (n = 17; 95% CI: 3.2-8.5%), double seropositivity to *E. canis* and *D. immitis* in 3.2% (n = 10; 95% CI: 1.5-5.8%) and to *E. canis* and *B. burgdorferi* s.l. in 0.3% (n = 1; 95% CI: 0.0-1.8%) of the tested dogs. Two dogs proved to have a triple seropositivity with *Anaplasma* spp., *E. canis* and *D. immitis* (0.6%; n = 2; CI: 0.1-2.3%). The proportion of single, double and triple exposure in the sum of all tested samples is listed in Table 3.

Co-seropositivity, mostly to *E. canis* and *Anaplasma* spp. and *E. canis* and *D. immitis*, mainly occurred in the provinces of Guanacaste and Puntarenas, while in Alajuela, Heredia and Cartago no such cases were found (see also Table 2). There was a significant difference between Alajuela and Puntarenas and the overall regarding co-seropositivity, independent of the pathogen combination, with Alajuela having no double seropositive dogs in the examination and Puntarenas showing the highest percentage. Thus it can be determined that living in the province of Puntarenas was associated with pathogen co-seropositivity.

The following points were identified with respect to the additional epidemiological data (sex, age, breed, tick history including current tick infestation, clinical signs): The median age of the tested dogs was 4.0 years; 32.8% (n = 103) of these dogs were  $\leq 2$  and 67.2% (n = 211)  $> 2$  years old. Of the total dog population tested, 50.3% (n = 158) were females, while 49.7% (n = 156) were males. A categorisation of the tested dogs into breed

and no breed categories showed 53.2% (n = 167) of dogs with a breed and 46.8% (n = 147) with no breed (mongrels). Any clinical signs recorded during a comprehensive clinical examination, irrespective of whether they could be caused by one of the underlying pathogens, were noted and resulted in 113/314 dogs (36.0%; 95% CI: 30.7-41.6%) presenting with at least one clinical sign at the time of sampling. Furthermore, 67.2% (n = 211; 95% CI: 61.7-72.4%) of all examined dogs had a reported tick history with or without a current tick infestation.

The owners were asked about their animal's travel history and import status. Travel history abroad was negated in the tested dogs.

An analysis of seropositivity for one of the tested pathogens according to the above-mentioned epidemiological variables can be found in Table 4.

As far as the epidemiological variables are concerned, there was a significant difference for general positivity with the SNAP® test system and for positivity for all single pathogens (apart from *B. burgdorferi* s.l.) in the category 'breed', with 'no breed (mongrels)' showing the highest percentages in all four cases, as well as in the category 'clinical signs', with dogs presenting at least one clinical sign showing higher percentages. A significant difference was also detected for *D. immitis* seropositivity and sex, with more male dogs being positive ( $p = 0.0278$ ). All other variables showed no significant differences. Risk factor analysis with logistic regression calculated the risk for dogs with a breed to possess a positive SNAP® test result to be less than half as high as for mongrels (odds ratio [OR] = 0.41). Furthermore dogs showing at least one clinical sign during sampling possessed a 2.3-times higher risk for a positive SNAP® test result than their

counterparts. The same parameters were also of importance for *Anaplasma* spp. and *E. canis* seropositivity, with breed dogs possessing a risk less than 1/5 respectively less than half as high for a positive *Anaplasma* spp. (OR = 0.19) respectively *E. canis* (OR = 0.43) test result, whereas dogs showing at least one clinical sign possessed a 4.2- (for *Anaplasma* spp.) and 2.4- (for *E. canis*) times higher risk for a respective positive test result. Regarding a positive *D. immitis* test result, male dogs possessed a more than 2.5-times higher risk.

#### **4. Discussion**

Most previous studies in Costa Rica focused on geographically more restricted areas and/or smaller dog population sizes (Scorza et al., 2011; Rojas et al., 2014, 2015a; Wei et al., 2015). Reports with a comparable sample size either used clinically preselected dogs (Ábrego et al., 2009; Romero et al., 2011; Ábrego Sánchez et al., 2013) or information on the origin of the samples was not provided (Campos et al., 2013). Only Barrantes-González et al. (2013) also covered all seven Costa Rican provinces using a comparable sample size, but no data can be retrieved concerning the location of positive dogs as the last two studies are only accessible as congress abstracts (in Spanish). Thus, the present study offers a good overview of the whole country with an acceptable, not preselected dog population.

Within the population screened, by far the highest prevalence was detected for *E. canis* at 38.2%. This is comparable to data found by Rojas et al. (2014), yielding 34.2% in total. Also comparable to Rojas et al. (2014), seroprevalences were detected in all tested provinces, even though between the provinces percentages varied massively. The lower number of dogs surveyed in some of the provinces might preclude reliable estimates of

the real prevalence of this pathogen. However, the overall seropositivity confirms that a nationwide potential for canine infection with *E. canis* exists. This statement is supported by the widespread occurrence of the transmitting vector, *R. sanguineus* s.l., in Costa Rica (Troyo et al., 2012; Rojas et al., 2014) and by a significant association between *E. canis* infection and *R. sanguineus* s.l. infestation in Costa Rican dogs (Rojas et al., 2014). Potential cross-reactivities in the test system between *E. canis* antigen and *E. chaffeensis* antibodies have been reported (O'Connor et al., 2006; Chandrashekar et al., 2010). Both cross-reacting pathogens have been identified as source of human and canine disease in the United States (Anderson et al., 1992; Breitschwerdt et al., 1998; Buller et al., 1999). For *E. canis* infections in humans from Latin America see Perez et al. (1996, 2006). Regarding *E. chaffeensis* infection, only single positive cases in humans based on PCR detection have been reported for Costa Rica (Rojas et al., 2015b). Additionally, no *E. chaffeensis* or *E. ewingii* positive dogs could be detected in Costa Rica during a dog population screening using molecular and isolation techniques in cell culture (Romero et al., 2011).

Despite the good sensitivity and specificity of the used test to detect antibodies against *Anaplasma* spp. (99.1% and 100% respectively [Chandrashekar et al., 2010]), serological cross-reactivity between *A. phagocytophilum* analyte and *A. platys* antibodies has been described (Chandrashekar et al., 2010). A clear differentiation between the two species cannot be made without additional examination, e.g. via PCR. Whether the antibodies detected in this study were anti-*A. platys* antibodies is speculative, even though similar prevalences for *A. platys* have been detected elsewhere, all confirmed by



PCR (Rojas et al., 2014; Wei et al., 2015; Ábrego et al., 2009). Additionally, the data for *A. phagocytophilum* prevalence in Costa Rican dogs are scant and show very low numbers confirmed by PCR (Bonilla, 2014; Campos et al., 2013) or serology (Bonilla, 2014; Barrantes-González et al., 2013). In the two serological examinations moreover a test system was used in which potential cross-reaction with *A. platys* cannot be completely excluded (personal communication, L. Fuller). *A. phagocytophilum* thus seems to be much less frequent in comparison to the prevalence of *A. platys* in Costa Rica. Furthermore, ticks of the genus *Ixodes* are the expected vector of *A. phagocytophilum*. So far, however, the only reports of *Ixodes* spp. in Costa Rica we could find implicate *Ixodes venezuelensis* on didelphid marsupials and rodents (Durdén and Keirans, 1994), *Ixodes minor* and two unclassified *Ixodes* spp. on wild birds (Ogrzewalska et al., 2015), and *Ixodes boliviensis*, found on bovines (Alvarez et al., 2003) and also on Costa Rican dogs, but only containing an undescribed *Rickettsia* sp. (Troyo et al., 2014). Based on this scant information on *Ixodes* ticks in Costa Rica, it might be hypothesized that the vector for *A. phagocytophilum* and *B. burgdorferi* s.l. is either absent or at least very rare in the area. This could also explain the single *B. burgdorferi* s.l. seropositive dog with questionable autochthonous character detected in the present study. Nevertheless, the test specificity only allows 6.4% of *Anaplasma* spp. seropositive dogs to be stated in this study.

Where this single *B. burgdorferi* s.l. seropositive dog is concerned, as mentioned above, the expected transmitting vector seems to be absent or very rare in Costa Rica. The different tattoo number and the status of adoption with unknown origin argue for a possible contraction of the infection outside Costa Rica. The detection of *Borrelia*

antibodies against C6 peptide in the SNAP® 4Dx® test in dogs persists in untreated dogs for at least 12 months (Levy et al., 2008), so that even those dogs that had lived in the country for at least one year and had been infected before moving to Costa Rica could still produce a positive test result. A small question mark will remain, but as there are only two countries in the Central American region in which Lyme disease has been reported or debated (McCown and Grzeszak, 2010; Rodríguez et al., 2003b, 2004, 2012a), we claim it is more likely for the dog in question to have been confronted with the pathogen outside Costa Rica.

The overall prevalence of *D. immitis* in the present study with 8.0% is higher than in data generated in the province San José (2.3% [Scorza et al., 2011]), but lower than in data in studies by Rojas et al. (2015a) and Wei et al. (2015) (15.1% and 22.5%). The difference to Rojas et al. (2015a) might be explained by their use of molecular and serological detection techniques and the additional locations of sampling with low to zero prevalences in the present study, which lower the overall prevalence. If only serological data were compared between the two studies, the prevalence was quite similar at 8.0% and 10.96% in Rojas et al. (2015a). The higher percentages in Wei et al. (2015) might be explained by the fact that the tested dogs came from underprivileged areas, where heartworm preventatives are unaffordable (Wei et al., 2015), in contrast to the presumably better cared-for dogs in the present study. In the reported data and in the examinations carried out by Rojas et al. (2015a), Puntarenas was the province with by far the highest prevalence. Rojas et al. (2015a) had already shown the coastal region of Chomes in Puntarenas to be endemic for *D. immitis* (significant difference). This

phenomenon with higher prevalences of *D. immitis* infections on the coast has also been observed in the coastal states of the south-eastern United States (Bowman et al., 2009) and in Mexico as stated in Labarthe and Guerrero (2005). Regarding the cross-reactivity of the *D. immitis* analyte with *A. vasorum*-positive dogs (Schnyder and Deplazes, 2012), nowadays a revised version of the test system used in this study, SNAP® 4Dx® Plus (IDEXX Laboratories, Inc., Westbrook, ME, USA), is available which no longer shows that reaction (Schnyder and Deplazes, 2012). But this new version was not on the market when testing was performed. Nevertheless, this cross-reactivity can be ignored since, as far as we know, there have been no reports of *A. vasorum* in Costa Rica. The nematode was reported a long time ago in Brazil in dogs and wildlife (Goncalves, 1961; dos Santos Lima et al., 1985; Lima et al., 1994; Oliveira-Sequeira et al., 2002). However, the Brazilian specimen may represent a different species, or a distinct genetic population, to that observed in European dogs (Jefferies et al., 2009). For Costa Rica there have only been single reports of *Angiostrongylus costaricensis* with rodents as the main definitive host (Morera, 1973), detected in man (Morera and Céspedesu, 2002) and in one dog (Alfaro-Alarcón et al., 2015), causing abdominal angiostrongylosis. Cross-reactions with this nematode have not been tested. But the single canine case report in Costa Rica led us to presume that the *D. immitis* positive dogs detected in the present study represent true heartworm seropositive animals with the highest prevalence in Puntarenas.

Double exposure to *Anaplasma* spp. and *E. canis* are the most frequent combination at 5.4%, followed by *E. canis* and *D. immitis* at 3.2%. This order has also been reported in a comparable study in dogs in Mexico (Movilla et al., 2016). As *R. sanguineus*

s.l. is the main vector for *E. canis* and *A. platys*, which is possibly the more frequent pathogen rather than *A. phagocytophilum*, double seropositivity could result from a dually infected tick or from sequential or simultaneous infection by singly infected ticks. Synergistic effects and more clinically relevant immunosuppression in co-infected animals (Beall et al., 2008) as well as an altered clinical appearance potentially making diagnosis more difficult and probably leading to a more serious disease outcome (Krupka et al., 2007) should be borne in mind by veterinarians across the country, as exposure to several pathogens seems possible. The lower number of double or triple exposed dogs in the present study in contrast to data in Wei et al. (2015) could be influenced for example by keeping conditions and the state of care of the sampled dogs and by different, more sensitive detection methods in Wei et al. (2015).

With respect to the epidemiological parameters, no significant difference could be detected for any of the two age groups, reported also by Rojas et al. (2014), but in contrast for example to data from Cardoso et al. (2012), where older dogs showed a higher risk of seropositivity. The missing significance might have been influenced by the restricted sample size. The significant gender-related difference for *D. immitis* seropositivity, supported also by risk factor analysis, with male dogs showing higher percentages, was also reported by Rojas et al. (2015a), but without significance. This could possibly be caused by different keeping conditions or life style of male dogs, e.g. as guard dogs, which could imply a greater vector exposure. Furthermore significant differences and higher risks were detected for the variables 'breed' and 'clinical signs' for a general positive test result, as well as for positive *Anaplasma* spp. and *E. canis* test results. Dogs

with a breed showed a far lower risk, whereas dogs presenting clinical signs possessed a higher risk. Here, it can be assumed that mongrels often receive less care than pedigree dogs. Concerning the presence of clinical signs this information has to be regarded with caution as the clinical signs investigated here were not pathogen-specific but could also arise from different diseases. A significant difference was also detected for the same variables and a positive *D. immitis* test result, but this could not be confirmed by logistic regression.

The fact that a positive antibody test is not necessarily equivalent to the existence of the pathogen in the canine or vector population of a particular geographic region, but is only evidence of prior exposure at some point and some location in the dog's history is the limitation on the value of the serological survey published here.

## **5. Conclusions**

The number of dogs examined and the inclusion of all seven provinces provide a fairly comprehensive overview of important canine, but also zoonotic vector-borne pathogens in Costa Rica. Dogs seropositive for *E. canis* (38.2% prevalence) were detected in all seven Costa Rican provinces with highest prevalences and a significant difference to the overall in Guanacaste and Puntarenas. Dogs seropositive for *Anaplasma* spp. (6.4% prevalence) were detected in all coastal provinces (significant difference in Guanacaste and Puntarenas to the overall) plus San José, but absent from central provinces. Dogs seropositive for *D. immitis* (8.0%) were especially detected in the province of Puntarenas (significant difference to overall). Even though the Pacific side provinces seem to be especially affected, bearing in mind travel and relocation within the country, veterinarians

throughout the country should be aware that these three major canine vector-borne pathogens, affecting 42.7% of the tested population, may occur in their practice area, and that exposure of their canine clients is possible. Therefore, there is a strong advice for the use of repellents along with prophylactic measures, such as regular examination for ticks, removal of detected ticks and use of mosquito screens, countrywide in Costa Rica and as travel recommendation for dogs from abroad to prevent disease transmission by arthropod vectors and ideally to prevent pathogen expansion, especially also on the background of their zoonotic potential.

#### ***Conflict of interests***

The authors declare that they have no competing interests.

#### ***Compliance with ethical standards***

All investigations comply with the current laws of the country in which they were performed.

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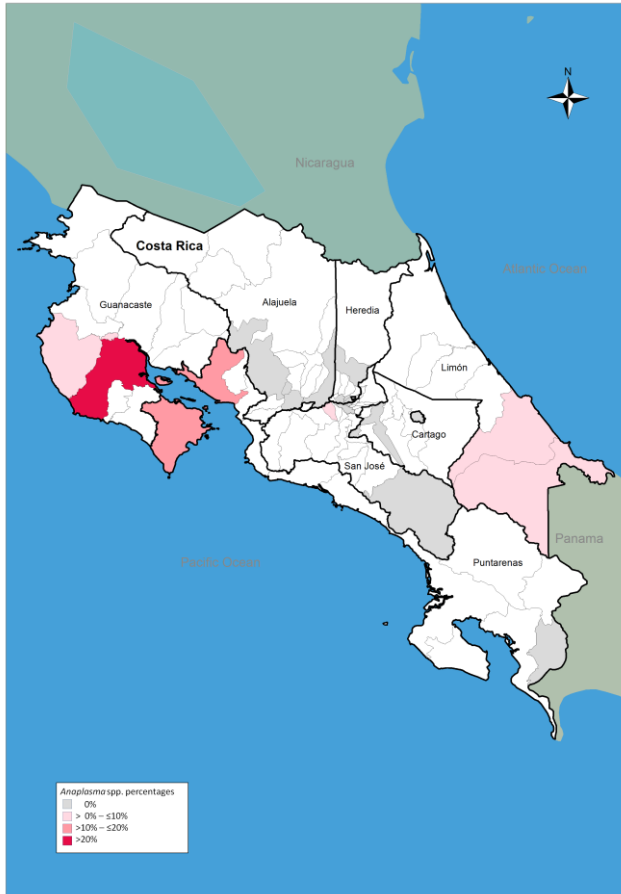
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**Fig. 1:** Occurrence of *Anaplasma* spp.-positive dogs detected by SNAP® 4Dx® in a population of 314 from Costa Rica. Grey areas represent the cantons in which dogs were sampled. Areas coloured in red shades represent the cantons in which positive dogs have been detected, depending on prevalence. In white areas no samples were collected.



**Fig. 2:** Occurrence of *Ehrlichia canis*-positive dogs detected by SNAP® 4Dx® in a population of 314 from Costa Rica. Areas coloured in green shades represent the cantons in which positive dogs have been detected, depending on prevalence. In all cantons sampled positive dogs were detected. In white areas no samples were collected.



**Fig. 3:** Occurrence of *Dirofilaria immitis*-positive dogs detected by SNAP® 4Dx® in a population of 314 from Costa Rica. Grey areas represent the cantons in which dogs were sampled. Areas coloured in blue shades represent the cantons in which positive dogs have been detected, depending on prevalence. In white areas no samples were collected.

**Table 1** Results of dog serum samples from Costa Rica (n = 314) tested for the presence of specific antibodies against *Anaplasma* spp. (An), *Borrelia burgdorferi* s.l. (Bb) and *Ehrlichia canis* (Ec) and of circulating antigen of *Dirofilaria immitis* (Di)

Pathogen	Antibody (An, Bb, Ec) or antigen (Di) positive dogs/all tested dogs	Prevalence	95% Confidence interval
<i>Anaplasma</i> spp.	20/314	6.4%	3.9-9.7%
<i>Borrelia burgdorferi</i> s.l.	1/314	0.3%	0.0-1.8%
<i>Ehrlichia canis</i>	120/314	38.2%	32.8-43.4%
<i>Dirofilaria immitis</i>	25/314	8.0%	5.2-11.5%

**Table 2** Distribution of *Anaplasma* spp. (An), *Borrelia burgdorferi* s.l. (Bb), *Ehrlichia canis* (Ec) and *Dirofilaria immitis* (Di) positive samples per province and canton (percentage and total numbers)

Province (number of sampling sites*)	Canton	Percentage An positive (x/y)	Percentage Bb positive (x/y)	Percentage Ec positive (x/y)	Percentage Di positive (x/y)	Percentage double positive (x/y)	Percentage triple positive (x/y)
San José (4 VS)	<b>Total</b>	1.6% (1/61)	0% (0/61)	26.2% (16/61)	1.6% (1/61)	1.6% (1/61)	0% (0/61)
	Tibás	0% (0/20)	0% (0/20)	25.0% (5/20)	0% (0/20)	0% (0/20)	0% (0/20)
	Santa Ana	9.1% (1/11)	0% (0/11)	27.3% (3/11)	0% (0/11)	9.1% (1/11; Ec+An)	0% (0/11)
	San José	0% (0/13)	0% (0/13)	23.1% (3/13)	7.7% (1/13)	0% (0/13)	0% (0/13)
	Pérez Zeledón	0% (0/17)	0% (0/17)	29.4% (5/17)	0% (0/17)	0% (0/17)	0% (0/17)
Alajuela (4 VS)	<b>Total</b>	<b>0% (0/68)<sup>a</sup></b>	0% (0/68)	29.4% (20/68)	<b>0% (0/68)<sup>e</sup></b>	<b>0% (0/68)<sup>i</sup></b>	0% (0/68)
	Alajuela	0% (0/5)	0% (0/5)	20.0% (1/5)	0% (0/5)	0% (0/5)	0% (0/5)
	San Ramón	0% (0/16)	0% (0/16)	6.3% (1/16)	0% (0/16)	0% (0/16)	0% (0/16)
	Palmares	0% (0/20)	0% (0/20)	30.0% (6/20)	0% (0/20)	0% (0/20)	0% (0/20)
	Atenas	0% (0/27)	0% (0/27)	44.4% (12/27)	0% (0/27)	0% (0/27)	0% (0/27)
Cartago (1 VS)	<b>Total</b>	0% (0/17)	0% (0/17)	<b>11.8% (2/17)<sup>d</sup></b>	0% (0/17)	0% (0/17)	0% (0/17)
	Cartago	0% (0/17)	0% (0/17)	11.8% (2/17)	0% (0/17)	0% (0/17)	0% (0/17)
Heredia (3 VS)	<b>Total</b>	0% (0/27)	0% (0/27)	22.2% (6/27)	0% (0/27)	0% (0/27)	0% (0/27)
	Heredia	0% (0/12)	0% (0/12)	16.7% (2/12)	0% (0/12)	0% (0/12)	0% (0/12)
	Santo Domingo	0% (0/9)	0% (0/9)	33.3% (3/9)	0% (0/9)	0% (0/9)	0% (0/9)
	San Pablo	0% (0/6)	0% (0/6)	16.7% (1/6)	0% (0/6)	0% (0/6)	0% (0/6)
Guanacaste (2 VS)	<b>Total</b>	<b>16.2% (6/37)<sup>b</sup></b>	2.7% (1/37)	<b>62.2% (23/37)<sup>e</sup></b>	5.4% (2/37)	18.9% (7/37)	0% (0/37)
	Santa Cruz	10.0% (2/20)	5.0% (1/20)	65.0% (13/20)	10.0% (2/20)	10.0% (2/20; Ec+An)	0% (0/20)



						5.00% (1/20; Ec+Di), 5.00% (1/20; Ec+Bb)	
	Nicoya	23.5% (4/17)	0% (0/17)	58.8% (10/17)	0% (0/17)	17.7% (3/17; Ec+An)	0% (0/17)
<b>Puntarenas</b> (2 VS, 1 PVA)	<b>Total</b>	<b>13.9%</b> <b>(10/72)<sup>c</sup></b>	0% (0/72)	<b>55.6%</b> <b>(40/72)<sup>f</sup></b>	<b>30.6%</b> <b>(22/72)<sup>h</sup></b>	<b>23.6%</b> <b>(17/72)<sup>j</sup></b>	2.8% (2/72)
	Puntarenas 1	15.6% (5/32)	0% (0/32)	59.4% (19/32)	31.3% (10/32)	15.6% (5/32; Ec+An), 9.38% (3/32; Ec+Di)	0% (0/32)
	Puntarenas 2	25.0% (5/20)	0% (0/20)	65.0% (13/20)	60.0% (12/20)	15.0% (3/20; Ec+An), 30.00% (6/20; Ec+Di)	10.0% (2/20; Ec+An+Di)
	Corredores	0% (0/20)	0% (0/20)	40.0% (8/20)	0% (0/20)	0% (0/20)	0% (0/20)
<b>Limón</b> (1 VS, 1 PVA)	<b>Total</b>	9.4% (3/32)	0% (0/32)	40.6% (13/32)	0% (0/32)	9.4% (3/32)	0% (0/32)
	Limón	8.3% (1/12)	0% (0/12)	8.3% (1/12)	0% (0/12)	8.3% (1/12; Ec+An)	0% (0/12)
	Talamanca	10.0% (2/20)	0% (0/20)	60.0% (12/20)	0% (0/20)	10.0% (2/20; Ec+An)	0% (0/20)
<b>Costa Rica</b>	<b>Total</b>	6.4% (20/314)	0.3% (1/314)	38.2% (120/314)	8.0% (25/314)	8.9% (28/314)	0.6% (2/314)

\*either veterinary surgery (VS) or point of veterinary assistance (PVA)

x: samples positive for a specific pathogen; y: total number of samples tested per province or canton

numbers in bold indicate a significant difference

<sup>a</sup> p = 0.0318; <sup>b</sup> p = 0.0430; <sup>c</sup> p = 0.0474; <sup>d</sup> p = 0.0364; <sup>e</sup> p = 0.0074; <sup>f</sup> p = 0.0081; <sup>g</sup> p = 0.0259;

<sup>h</sup> p < 0.0001; <sup>i</sup> p = 0.0180; <sup>j</sup> p = 0.0010

**Table 3** Proportion of single, double and triple exposure to *Anaplasma* spp. (An), *Borrelia burgdorferi* s.l. (Bb), *Ehrlichia canis* (Ec) and *Dirofilaria immitis* (Di) in all tested samples (n = 314)

	<b>An (alone)</b>	<b>Bb (alone)</b>	<b>Ec (alone)</b>	<b>Di (alone)</b>	<b>Ec+An</b>	<b>Ec+Di</b>	<b>Ec+Bb</b>	<b>An+Ec+Di</b>
<b>Positive samples (x/y)</b>	1/314	0/314	90/314	13/314	17/314	10/314	1/314	2/314
<b>Percentage/ [95% CI]</b>	0.3%/ [0.0- 1.8%]	0%/ [0- 1.2%]	28.7%/ [23.7- 34.0%]	4.1%/ [2.2- 7.0%]	5.4%/ [3.2- 8.5%]	3.2%/ [1.5- 5.8%]	0.3%/ [0.0- 1.8%]	0.6%/ [0.1- 2.3%]

CI: Confidence interval

x: samples positive for a specific single pathogen or pathogen combination; y: total number of samples tested

**Table 4** Seropositivity for *Anaplasma* spp. (An), *Ehrlichia canis* (Ec), *Dirofilaria immitis* (Di) and SNAP® 4Dx® in general according to the epidemiological variables analysed†

Epidemiological variable		Percentage positive (x/y)			
		An	Ec	Di	SNAP® 4Dx® in general
Sex	Male	7.1% (11/156)	39.1% (61/156)	11.5% (18/156)	46.2% (72/156)
	Female	5.7% (9/158)	37.3% (59/158)	4.4% (7/158)	39.2% (62/158)
	p value	0.7308	0.7855	<b>0.0278</b>	0.2316
	OR	*	*	2.63	*
Age (years)	≤2	5.8% (6/103)	41.7% (43/103)	8.7% (9/103)	47.6% (49/103)
	>2	6.6% (14/211)	36.5% (77/211)	7.6% (16/211)	40.3% (85/211)
	p value	0.9147	0.3905	0.7967	0.2323
	OR	*	*	*	*
Breed	Breed	1.8% (3/167)	26.9% (45/167)	4.8% (8/167)	30.5% (51/167)
	No breed (mongrels)	11.6% (17/147)	51.0% (75/147)	11.6% (17/147)	56.5% (83/147)
	p value	<b>0.0005</b>	<b>&lt;0.0001</b>	<b>0.0348</b>	<b>&lt;0.0001</b>
	OR	0.19	0.43	*	0.41
Tick history (including current tick infestation)	Yes	6.6% (14/211)	38.9% (82/211)	9.0% (19/211)	43.6% (92/211)
	No	5.8% (6/103)	36.9% (38/103)	5.8% (6/103)	40.8% (42/103)
	p value	0.9147	0.7576	0.3956	0.6588
	OR	*	*	*	*
Clinical signs	Not present	2.5% (5/201)	28.9% (58/201)	5.5% (11/201)	33.3% (67/201)
	Present	13.3% (15/113)	54.9% (62/113)	12.4% (14/113)	59.3% (67/113)
	p value	<b>0.0004</b>	<b>0.0001</b>	<b>0.0413</b>	<b>&lt;0.0001</b>

	<b>OR</b>	4.19	2.41	*	2.32
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x: samples positive for a specific pathogen or the SNAP® 4Dx® test in general; y: total number of dogs in the specific category

OR: odds ratio

\*parameter removed by backward elimination, thus no risk analysis

numbers in bold indicate a significant difference

†Due to only one single *B. burgdorferi* s.l. seropositive dog, this pathogen is not listed in the table.