UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Ciencias Biológicas y Ambientales

Detection of *Anaplasma, Babesia* and *Mycoplasma* in hunting dogs from the riverbank of Napo River.

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Ingeniería en Procesos Biotecnológicos

Trabajo de fin de carrera presentado como requisito
para la obtención del título de
Ingeniera en Procesos Biotecnológicos

Quito, 4 de mayo de 2020

UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

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HOJA DE CALIFICACIÓN
DE TRABAJO DE FIN DE CARRERA

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RESUMEN

La ribera del río Napo es una zona rica en vida silvestre, que además se comparte con las comunidades amazónicas y animales domésticos. En la Amazonía ecuatoriana, la caza sigue siendo un factor crítico en la subsistencia de las comunidades nativas y los perros domésticos se incorporan en esta actividad con fines de asistencia y apoyo. Se sabe que especies de hemoparásitos pueden ser alojadas en los eritrocitos de estos animales, sin embargo, la presencia de este grupo de microorganismos también se ha reportado en animales salvajes como ciervos, cánidos, felinos, suinos y roedores, lo que indica una posible zoonosis entre ambos grupos. Este estudio evalúa por primera vez la presencia de tres hemoparásitos con potencial zoonótico en 53 perros de caza (*Canis lupus familiaris*). Se utiliza a los perros de caza como animal "centinela" que nos permite comprender la circulación de enfermedades infecciosas en 5 comunidades locales de la ribera del río Napo, en la Amazonía ecuatoriana. La detección de los hemoparásitos se llevó a cabo amplificando el gen de ARN 16s por PCR convencional y PCR SYBR Green en tiempo real. Además, las especies de hemoparásitos detectadas fueron identificadas por secuenciación. En la detección de *Babesia spp.* y Anaplasma *spp.* se obtuvo resultados negativos para todas las muestras analizadas. Para *Mycoplasma* spp. se observó una positividad total de 30.18% (16/53) entre 4 de los 5 puntos de muestreo, pues una de las localidades no presento positivos. Se identificaron dos especies: *M. haemocanis* y *Candidatus Mycoplasma haematoparvum*. Los resultados indican que estos hemoparásitos están circulando en la zona y por lo tanto podrían estar infectando también la vida silvestre. Sin embargo, no es posible afirmar la direccionalidad de esta transmisión a partir de los resultados aquí presentados. Esta investigación corresponde a la primera etapa del análisis de los hemoparásitos que circulan entre animales domésticos y salvajes en la zona, y sus implicaciones y abre la puertas a nuevos estudios de investigación sobre los mecanismos de contagio y sus consecuencias sobre la biodiversidad. Así mismo, existe un gran potencial de tales estudios para ser utilizado con fines de conservación de vida silvestre.

Palabras clave: *Anaplasma*, *Babesia*, *Mycoplasma*, *Canis lupus familiaris*, zoonosis, vida silvestre, hemoparásitos, ribera del rio Napo.

ABSTRACT

The riverbank of the Napo River is a wildlife rich area, which is also shared with Amazonian communities and domestic animals. In the Ecuadorian Amazon, hunting is still a critical factor in native communities' subsistence and domestic dogs are incorporated as assistance and support. It is known that species of hemoparasites can be hosted in the erythrocytes of these animals, however, the presence of this group of microorganisms has also been reported in wild animals such as deer, canids, felids, swine, and rodents, indicating a possible zoonosis between both groups. This study evaluates for the first time the presence of three hemoparasites with zoonotic potential in 53 hunting dogs (*Canis lupus familiaris*). Hunting dogs are used as "sentinel" animals, that allows to understand the circulation of infectious diseases in 5 local communities on the banks of the Napo River, in the Ecuadorian Amazon. The detection was carried out by amplifying the 16s RNA gene by conventional PCR and PCR SYBR Green in real time. In addition, the species of the parasites detected were identified by sequencing. In the detection of *Babesia spp.* and Anaplasma *spp.* negative results were obtained for all the samples analyzed. For *Mycoplasma* spp. An overall positivity of 30.18% (16/53) was observed among 4 of the 5 sampling spots, as one of the localities sampled did not show any positives. Two species were identified: *M. haemocanis* and *Candidatus Mycoplasma haematoparvum*. The results indicate that these hemoparasites are circulating in the area and therefore could be infecting wildlife as well. However, it is not possible to affirm the directionality of this transmission from the results presented here. This research corresponds to the first stage of the analysis of the hemoparasites circulating between domestic and wild animals in the area, and its implications. It opens the doors to new research studies about mechanisms of contagion and its consequences on biodiversity. Likewise, there is a great potential of such studies to be used for wildlife conservation purposes.

Key words: *Anaplasma*, *Babesia*, *Mycoplasma*, *Canis lupus familiaris*, subsistence hunting, zoonoses, wild fauna, hemoparasites, riverbank of Napo River.

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INTRODUCTION

The bank of the Napo River is one of the richest biodiversity hotspots on the planet. It belongs to a tropical wet forest ecosystem that houses a very high number of species from all groups, especially unique terrestrial mammals such as the jaguar, the giant otter, the Yasuní round-eared bat, the bush dog and the giant armadillo, as well as more than 10 species of primates. This area is also known for its high cultural value, as it is home for several very representative Amazonian communities (WCS, 2018). Local communities base on natural resources to obtain daily goods, thus, they develop various subsistence activities in which hunting wild fauna is included. In the Ecuadorian Amazon, hunting is still a critical factor in native communities' subsistence (Zapata, Urgilés, & Suárez, 2009). Wild fauna´s meat is usually the only source of protein for communities living into neotropical forests. In this specific activity, domestic dogs are incorporated as assistance and support (Koster, 2009). These animals are therefore constantly exposed to arthropod bite and consequently are prone to acquire vector-borne diseases like hemoparasite infections (Akande, Adebowale, Idowu, & Sofela, 2018). Also, this sort of infections could be acquired by direct contact with infected blood, as a consequence of aggressive interaction with preys or ingestion of animal´s entrails. Previous studies assessing the relationship between hunting dogs and wildlife have reported several dog deaths and injuries, by wild fauna, which would also represent a vehicle for two-sided disease transmission (Fiorello, et al., 2017).

It is known that various species of hemoparasites can be hosted in erythrocytes of pets as dogs and cats. However, the presence of this groups of microorganisms in wild animals such as deer, canids, felids, swine, and rodents has also been reported, indicating a possible zoonosis between both groups (Ashford, 2001). The incidence of pathogens in free-ranging wildlife may

suppose a risk, other than to themselves, to the health of domestic animals and humans. Wild fauna represents both a target and a reservoir of pathogens. In general, wild animals are susceptible to infection by the same bacteria, viruses and parasites that affect pets. It is important to mention that infectious diseases can spread in any direction, so transmission between wild and domesticated animals should be considered in both directions. However, there are often differences in wild animal response to infection and a large variation in the potential role they could play in the epidemiology of these pathogens (Fischer & Gerhold, 2002) (OIE, 2015). This study focuses specifically on hemotropic pathogens belonging to *Mycoplasma*, *Anaplasma* and *Babesia* genus, which, are particularly common parasites of domestic, wild and farm animals.

Hemotropic mycoplasmas are gram positive, uncultivable, coccoidal-shaped, cell wall-less bacteria which belong to the Mycoplasmataceae family, Mycoplasmatales order and Mollicutes class (Neimark, Johansson, & Rikihisa, 2001). They parasite vertebrate hosts by adhering to their erythrocytes. Adherence mechanisms appear to be related to production of a dint in red blood cell membrane at the spot of attachment (Ashford, 2001). They have a wide range of hosts between mammals, which includes wild and domestic species. Infections with these agents are characterized by hemolytic anemia, which includes symptoms such as weight loss, lethargy, anorexia, acute hemolysis, fever, and thrombocytopenia for both wild and domestic animals (Willi, et al., 2007). In dogs, *Mycoplasma haemocanis* and *Candidatus Mycoplasma haematoparvum* have been described as their main parasites (Novacco, et al., 2010).

Anaplasma genus belongs to the Anaplasmataceae family and is comprised by seven species of gram negative obligate parasitic bacteria which colonize the interior of mammal`s erythrocytes. Some species of this genus, like *A. phagocytophilum*, have reported to be of great

zoonotic potential (Dantas-Torres & Otranto, 2016). The infection with these pathogens is named as Anaplasmosis. Disease is characterized by weight loss, muscular pain, weakness, nausea and in some cases even abortion and death. These symptoms are common between wild and domestic animals (Rymaszewska & Grenda, 2008).

Babesia is a protozoan hemoparasite characterized by their pear shape of merozoites. They infect erythrocytes from various hosts of the vertebrate group, including humans, and reproduce asexually when in the inside (Lempereur, et al., 2017). *Babesia* genus corresponds to Piroplasmida order and Babesiidae family. Infection caused by these microorganisms is named as Babesiosis and affects both wild and domestic animals similarly. It consists mainly of anemia caused by destruction of red blood cells, hyperthermia, hemoglobinemia, hemoglobinuria and common parasite-infection symptoms like fever, lethargy and anorexia (Dantas-Torres, Alves, & Uilenberg, 2016).

Anaplasma, *Mycoplasma* and *Babesia* prevalence data is not available in the study area. However, diseases caused by this hemoparasites are mainly relevant because of the high possibility of zoonosis between wild and domestic fauna, and even humans. They have been reported to be vector-borne and easily transmitted by contact with infected blood. (Bhattacharjee & Sarmah, 2013). Different detection techniques have been developed, including serology, microscopy and molecular tests. However, serology and microscopy tend to be unspecific techniques as bias is introduced by morphological and physiological similarities between hemoparasites. Most of them are coccoidal-shaped and under the microscope appear like dots inside or attached to erythrocytes. Additionally, their proteins are structurally similar, thus cross reaction could occur even with specific antibodies. Therefore, molecular tools such as polymerase chain reaction (PCR) represent the most accurate method to detect and distinguish

hemotropic bacteria. (Valenciano & Cowell, 2013) Preferred approach in PCR detection for bacterial pathogens is generally 16s rDNA targeting, as it is highly conserved and standardized protocols are available. 16s rDNA-mediated detection is commonly complemented by sequencing and sequence analysis. Sequencing can be done by a variety of methods; however, Sanger approach is preferred when the number of samples to be analyzed is low, and the research budget is reduced. Analysis comprises sequence aligning and phylogenetic tree construction and allows to make taxonomic assignations and differentiate pathogen species. This study evaluates for the first time the presence of *Mycoplasma*, *Anaplasma* and *Babesia* with zoonotic potential in *Canis lupus familiaris* in the Ecuadorian Amazon. The main purpose of the detection of these hemoparasites in hunting dogs is to comprehend if they are circulating in the area, since sampling of wild fauna would require more complex efforts. Thus, hunting dogs act as sentinel species, as they expose to blood-sucking arthropods and wild fauna´s blood.

MATERIALS AND METHODS

Sample collection

Blood samples were collected from 53 dogs from Pompeya, Sani Isla, San Roque, Indillama, and Nueva Providencia communities located in the riverbank of Napo River, in the Ecuadorian Amazon. Blood extraction was performed with previous verbal consent of the dog´s owners. Individuals tested develop subsistence-hunting assistance activities. Samples were preserved in EDTA and stored at -20ºC until processing. The molecular analysis on this study was performed under the Framework Contract of Access to Genetic Resources number MAE-DNB-CM-2018-0106.

DNA extraction

Total DNA was extracted from 50 µl of blood in EDTA using Chelex 100 (Singh, Kumari, & Iyengar, 2018). Briefly, 300 µl of 7% Chelex solution was heated at 100ºC for 10 minutes, blood sample was added to the solution and heated for 15 minutes. Tubes were centrifuged at 12000 rpm for 1.5 minutes and supernatants were transferred carefully to new tubes. Ammonium acetate was added (See table 1 for volume specifications) and the solution was left to rest for 5 minutes at 4ºC. Liquid was mixed by vortex and centrifuged at 12000 rpm and kept at 4ºC for 10 minutes. Afterwards, supernatant was recovered, 75% ethanol solution was added (See table 1 for volume specifications) and samples were stored at -20 ºC for 17-24 hours. Samples were centrifuged at 12 000 rpm for 1 minute and supernatant was carefully transferred to a new tube, pellets were discarded. For DNA precipitation, 3M sodium acetate solution (See table 1 for volume specifications) and 200 µl of 100% ethanol were added. Liquid was mixed by vortex and kept at 4ºC for 4 hours and centrifuged at 13000 rpm at 4ºC for 10

minutes. Supernatant was discarded and pellet was washed twice with ethanol. After, it was air dried and resuspended in 50 µl of ultra-pure water.

Hemoparasite detection

All samples were tested for the presence of *Mycoplasma* spp., *Anaplasma* spp. and *Babesia* spp. DNA. To test for PCR inhibitors and to discard the probability of false negatives, β-actin gene was amplified in 10% (n=53) of the samples (Du Breuil, Patel, & Mendelow, 2019). For *Mycoplasma* spp. detection 3 different assays were used (see Table 2 for primer and PCR details). Conventional PCR was performed using a Go Taq Green Master Mix (Promega, USA) in a total volume of 10 µl, using 0.8 µM of each primer and 2 µL of template DNA in a Bio-Rad T100 Thermal Cycler. Amplification conditions for the first two primer sets were as follows: 94^{o}C for 2 minutes, followed by 35 cycles at 94^{o}C for 45 seconds, 65^{o}C for 30 seconds and 70^{o}C for 1 minute, with a final extension at 70^{o}C for 5 minutes (Maggi, Colter, Kennedy-Stoskopf, & DePerno, 2013). SYBR Green PCR Master Mix (Invitrogen, USA) was used for the third *Mycoplasma* assay following the conditions reported by Willi et al., (Willi, et al., 2009) in a Bio-Rad CFX96 Touch Real-Time PCR Detection System

Anaplasma spp. detection was performed as previously described by Zobba et al. (Zobba, et al., 2014), PCR reactions were performed in a total volume of 10 µL using GoTaq Green PCR master mix, 0.8 µM of each primer and 1 µL of DNA template. Amplification was performed with an initial denaturation of 94 for 5 minutes, followed by 30 cycles at 94^{o}C for 1 minute, 55^{o}C for 1 minute and 72^{o}C for 3 minutes, with a final extension at 72^{o}C for 5 minutes.

Babesia spp. was tested using AccuPower *Babesia* PCR Kit (Bioneer, Daejeon, Republic of Korea) according to manufacturer`s instructions.

For all PCR runs a positive control and a no template control (ultra-pure water) were used. For *Mycoplasma spp*. detection, positive control was extracted from F VAX-MG® (MSD, United States) a freeze-dried live vaccine . For *Anaplasma spp*., a known positive sample previously detected and confirmed by sequencing was used as positive control. *Babesia spp.* positive control was provided with the PCR kit. Amplicons were run in a 1% agarose gel and visualized after electrophoresis using a Bio-Rad UV transilluminator.

Sequencing and taxonomic assignation

Amplicons were sequenced by Sanger sequencing at Macrogen Seoul, Korea. Sequences were aligned in MEGAX software (Kumar, Stecher, Lim, Knyaz, & Tamura, 2018) and compared to 16s rRNA gene sequences of hemotropic *Mycoplasma* species. Sequence identity and phylogenetic tree resolution allowed to make taxonomic assignation. The phylogenetic tree was inferred using the Neighbor-Joining method (Saitou & Nei, 1987), with a number of bootstrap replicates of 600. The analysis involved 31 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 454 positions in the final dataset (Kumar, Stecher, Lim, Knyaz, & Tamura, 2018). Sequence of 16s RNA from *Acholeplasma laidlawii* (GenBank U14905) was used as outgroup for tree construction.

RESULTS

No PCR inhibiting compounds were found in DNA extracts

For DNA validation, all samples tested for β-actin were positive, amplifying a fragment of 300bp, as shown in Figure No.1. Positive control amplified as expected and non-template control did not show any sign of amplification.

Hemoparasites found in dog blood samples

Testing for *Anaplasma* and *Babesia* showed all negative results. Positive controls amplified as expected and non-template controls showed no sign of amplification, confirming the correct functioning of the assays (Appendix A).

From 53 samples tested, 16 positives were detected, representing an overall positivity of 30.18%. Every sampled locality showed positivity over 16%, excepting Indillama, where no positives where found. Discrepancies were shown between the three detection assays, as presented in table No.3. A total of 16 positives were obtained, which are distributed among sampled localities as shown in Figure 2.

Sequencing allowed identification of two species: *Candidatus Mycoplasma haematoparvum* (CMh) and *Mycoplasma haemocanis* (Mhc), distributed as shown in Table No. 3. In one of the sampling sites, Indillama, no positives were found. In the present study samples identified as *M. haemocanis* showed melting temperatures of 74.50 ºC. Accordingly, samples identified as *Candidatus M. haematoparvum* showed melting temperatures from 73 ºC to 74 ºC (Appendix B). Phylogenetic tree (Figure 3) shows that sequences corresponding to *Mycoplasma spp.* isolates from dogs 2, 8 and 52 grouped with CMh sequence. Likewise, sequences of *Mycoplasma spp.* isolates from dogs 18, 19 and 23 grouped with Mhc sequence.

DISCUSSION

Dog hemoparasite infection has been widely reported in the last years. However, there are no previous data for this kind of infection in domestic dogs nor hunting dogs from the Ecuadorian Amazon.

Data indicates absence of *Anaplasma spp.* and *Babesia spp.*

From the three hemoparasites tested, *Anaplasma* and *Babesia* were not found in any of the dog samples analyzed. This finding indicates that whether species from this genus are not circulating in the area or their abundance is too low to be detected in the number of samples analyzed.

In contrast to the results presented in this study, a previous report stablished the presence of *Babesia conradae* in a kennel of coyote-hunting dogs from south-central California (Dear, et al., 2018). However, difference between both studies can be explained by the overall prevalence of *Babesia* spp. in their study areas. A review comparing articles that report prevalence of babesia worldwide show a wide difference in number of zoonotic *Babesia* studies, number of species, sporadic cases and seroprevalence between North America and South America (Young, et al., 2019). Similarly, a study conducted in Pastaza-Ecuador testing hemoparasites in cows showed total negativity for *Babesia*, evidencing a low prevalence of these genus in the Amazon region of the country. Data from different animal species results useful as information from dogs in the same geographic area is not available. Also, information about prevalence of these hemoparasites in other animal species in the area allow to understand their overall circulation rate, as vectors that carry them are shared.

Anaplasma spp. prevalence studies conducted in dogs in Ecuador report low positivity. In a study conducted in Azogues-Ecuador, where 150 canine individuals were tested, no positive samples were found (Ulloa, 2018). Another research conducted in dogs from Puerto Lopez, Manabí-Ecuador, showed a 12.4% (n=147) of positivity (Sarango & Alvarez, 2017). Also, a study from 2016 in Guayaquil-Ecuador showed 18.5% (n=65) positivity in samples taken from dogs in a veterinarian clinic. (Alcivar, 2018) All these previous results are relatively consistent with the results reported in the present study. However, the light difference in positivity showed between them can be explained by the difference of study areas, as no information is reported from Anaplasma tests conducted in dogs from the Amazon region.

A review from 2016 which analyzes data from *Anaplasma* spp. infection in dogs in the United states reveals a negative correlation between relative humidity, temperature and *Anaplasma* overall prevalence. (McMahan, et al., 2016) The sampling location is characterized by an annual average temperature of 26°C and humid to hyper-humid weather conditions (Gobierno Provincial de Napo, 2015). Therefore, according to the correlation mentioned before, *Anaplasma* spp. negativity turns out to be reasonable.

If compared to studies conducted in dogs in other countries, differences in positivity are found. However, this variation matches expectations, as research conditions and prevalence are also different. A similar study conducted in shepherd dogs, hunting dogs and stray dogs (n=126) in south Hungary showed a 11.1% of positivity for *Anaplasma phagocytophilum*, detected by nested PCR of 16s RNA gene (Hornok, et al., 2013). Another research of the presence of hemoparasites in dogs of Thailand reports a 3.70% of positive samples for *Anaplasma platys*, also tested by 16s RNA gene PCR (n=81). It is important to mention that the dogs sampled in the referred study were all affected with severe anemia (Kaewmongkol, y otros, 2017). Similarly, a

study conducted in Yogyakarta, Indonesia found an 11.7% (n=51) of *Anaplasma platys* positivity in domestic dogs tested by *GroEl* gene PCR (Disna, Haryanto, & Tjahajati, 2019). Presence of this hemoparasite has also been tested in dog`s ticks and fleas reporting 21.25% and 16% of positivity respectively (Pawełczyk, Asman, & Solarz, 2019) (Çetinkaya, et al., 2016).

On the other hand, *Anaplasma* spp. and *Babesia* spp. might not be present in the fauna that is being hunted by dogs tested. The study site, the riverbank of Napo river, is highly rich in bird, reptile, and mammal species. Most common mammals found in the area include *Tapirus terrestris, Tayassu pecari, Speothos venaticus, Atelocynus microtis, Panthera oca, Lophostoma Yasuni, Myrmecophaga tridactyla, Priodontes maximus Sphiggurus ichillus, Pteronura brasiliensis, Pithecia aequatoriali* and more than 10 primate species (Bass, et al., 2010) (WCS Ecuador, 2020). These mammals can be common targets of the hunting dogs tested in this study. However, it cannot be certainly said whether these preys are being colonized with *Anaplasma* and *Babesia* or not as no diagnosis test was done directly on them. Also, there are no reports from any of these hemoparasites in wildlife in the country, but plenty of information in other locations is available. A survey of hemoparasite infections in free-ranging mammals and reptiles in French Guiana reports positivity for *Anaplasma* spp. in 15 species, being *Myoprocta acouchi* (42%), *Dasyprocta agouti* (36%) and *Dasypu novemcinctus* (36%) the ones with higher percentages (de Thoisy, Michel, Vogel, & Vié, 2000). A study conducted in 625 wild mammals in Spain showed 64.2% of prevalence for *A. phagocytophilum* in cervids and total absence in wild boar and carnivores (Garcia-Perez, et al., 2015). Similarly, an article reporting zoonotic pathogens prevalence in White-Nosed Coatis (*Nasua narica*) in Monte Verde, Costa Rica presented a 100% (n=20) positivity for *Babesia* spp. (Mehrkens, et al., 2013).

Detection assays revealed occurrence of *Mycoplasma* spp.

Mycoplasma spp. was tested by 3 different PCR assays as described in methods, showing an overall positivity of 30.18%. Sequencing and posterior analysis allowed identification of *Mycoplasma haemocanis* and *Candidatus Mycoplasma haematoparvum*. Both species have been reported before as the most frequent canine parasites belonging to Mycoplasma genus (Compton, Maggi, & Breitschwerdt, 2012).

In this study, PCR protocols for *Mycoplasma* spp. detection exhibited differences in sensitivity. SYBR Green PCR revealed higher detection rates than both conventional assays, generating a greater number of positives. Conventional PCRs also showed sensitivity differences among them. The protocol with the smaller target size was more sensitive, allowing detection of new positive samples that larger amplicon-PCR could not recognize. Also, it is important to highlight that with SYBR Green PCR, melting curve analysis was performed. This technique usually allows to discriminate primer dimer and other unspecific products from specific target sequences (Nishizawa, Sato, Fujihara, Sato, & Harasawa, 2009). Temperatures of melting reported for *M. haemocanis* and *Candidatus M. haematoparvum* are 75 ^{o}C and 73-74 ^{o}C respectively (Willi, et al., 2009), thus, temperatures obtained in this study are consistent. Additionally , sample size is usually a crucial factor to consider in the design of research studies, as it determines significance of data obtained and therefore reliability on comparisons between sampling locations (Eng, 2003). In this study, this parameter (n=12) is even in most locations, however, one of them, Indillama, has a significant difference as only 5 individual could be sampled. Based on this statement, it could be inferred Indillama results might not reflect the actual scenario and *Mycoplasma* spp. could be preset in the area.

About taxonomic assignation, it is important to mention that 16s rRNA gene was selected as it is highly conserved in bacteria and also highly variable between smaller groups as family and genus (Clarridge, 2004). Also, tree was rooted to *Acholeplasma laidlawii* as this bacterium belongs to Mollicutes class, same as *Mycoplasma* spp., and to a different family: Acholeplasmatales. The bootstrap value obtained for the branches of main interest (marked blue and red in the phylogenetic tree: figure no. 3) is significant enough to assert that the inference is reliable, and the taxonomic assignation is correct. (Soltis & Soltis, 2003)

Several articles report the presence of these *Mycoplasma* species in dogs from different study areas. In the United states, a study reports prevalence data of 0.8% and 0.6% (n=506) for *Candidatus Mycoplasma haematoparvum* and *Mycoplasma haemocanis* respectively (Compton, Maggi, & Breitschwerdt, 2012). In south Hungary an 11.1% of positivity was obtained for *Candidatus M. haematoparvum* and 6.3% for *M. haemocanis* (Hornok, et al., 2013). In Curitiba, Brazil, results from 21 dogs sampled appear to be closer to the ones reported in the present study. Analyses showed that 33.3% were infected with *Mycoplasma haemocanis*, 42.8% with *Candidatus Mycoplasma haematoparvum* 19% were colonized by both (Constantino, et al., 2017). Similarity in results can be explained by both study areas location, which correspond to tropical territories, as prevalence of hemotropic mycoplasmas seem to be higher in tropical regions (Biondo, et al., 2009).

In Ecuador, no data is available about *Mycoplasma* prevalence in dogs from the Amazon regions, however, a few studies have been conducted in other locations inside the country. A hemoparasite survey conducted in Puerto Lopez showed a 24.4% of positivity for *Mycoplasma* spp. Nevertheless, methods in this case are different, as detection was determined by direct

observation of blood smears. Therefore, results are not fully comparable. (Sarango & Alvarez, 2017).

Presence of the two species of *Mycoplasma* found in dogs tested indicates that hemotropic bacteria of this genus are circulating in the area, therefore implying the potential infection of wildlife present in the surroundings. These and other species of *Mycoplasma* can colonize wild fauna and their presence has already been reported in previous studies. Screening of zoonotic pathogens in *Nasua narica* and *Vulpes vulpes* blood revealed 100% (n=20) and 1.1% (n=9) of positivity for *Mycoplasma* spp. respectively (Mehrkens, et al., 2013) (Koneval, Miterpáková, Hurníková, Blaňarová, & Víchová, 2017). Also, presence of *Candidatus M. haematoparvum* (25%) and a specie of *Mycoplasma* spp. closely related to *M. haemocanis* (99.4%) was reported in bears (n=17) from the east coast of North Carolina (Westmoreland, Stoskopf, & Maggi, 2017). Various *Mycoplasma* species were also identified in a molecular survey conducted in wild carnivores in Spain, including a wolf infected with *M. haemocanis* (Millan, y otros, 2018). *M. haemocanis* has also been reported in a racoon dog (*Nyctereutes procyonoides viverrinus*) from Morioka (Harasawa, Orusa, & Giangaspero, 2014). Data of prevalence of *Mycoplasma* in wildlife suggests high possibility of cross-species infection. However, in Ecuador, no reports assessing *Mycoplasma* spp. presence in non-domestic fauna are available.

Since hemoparasites as *Mycoplasma* spp. are difficult to be cultured, experimental studies assessing their mechanisms of infection are scarce, thus, ways of transmission are not entirely clear. However, evidence indicates they could be mainly transmitted by blood-sucking arthropods vectors such as ticks and fleas (Biondo, et al., 2009). *Rhipicephalus sanguineus* and *Ctenophalides* fleas are believed to be the main vectors of transmission of *M. haemocanis* and *M.*

haemofelis/Candidatus M. turiciensis respectively. Still, *M. haemofelis/Candidatus M. turiciensis* DNA has been found in *R. sanguineus* indicating both fleas and ticks can act as vectors for feline, canine and other mycoplasmas (André, Harumi, Marques, & Zacarias, 2011). Consequently, *Mycoplasma* present in dogs tested in the present study could easily be transmitted by arthropod vectors of the area, which could also be able to parasite wild fauna species such as rodents, felids, and swine, as they are constantly interacting (hunting) and transmission of hemoparasites is more likely to occur by strong interaction rather than slight contact. (André, Harumi, Marques, & Zacarias, 2011)

CONCLUSIONS

This study confirms the presence of *Mycoplasma haemocanis* and *Candidatus Mycoplasma haematoparvum* circulating in 4 locations on the banks of the Napo River. This finding represents a great possibility that these hemoparasites will be transmitted between dogs and wildlife as they are in constant direct contact. However, it is extremely important to do more research to generate data to determine the directionality of the transmission of these pathogens.

About the results obtained by the three protocols used, it is concluded that the most sensitive method of detection of *Mycoplasma* is SYBR Green PCR. In addition, it is essential to emphasize the suitability of *Canis lupus familiaris* as a sentinel species for the detection of infectious diseases circulating among wild animals. Still, this statement must be confirmed by testing ticks and other blood-sucking arthropods.

This research corresponds to the first stage of the analysis of the hemoparasites circulating between domestic and wild animals in the area, and its implications. It opens the doors to new research studies about mechanisms of contagion and its consequences on biodiversity. Likewise, there is a great potential of such studies to be used for wildlife conservation purposes. Finally, it would be interesting to investigate the presence of ticks in the area and thus know what types of vectors are present and which hemoparasites are transporting.

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TABLES

Table #1

Volume specifications for solutions used in DNA extraction protocol

Solution	Volume specifications
Ammonium acetate	$= \frac{Supernatant\ volume\ (\mu l)}{2}$
75% Ethanol solution	$= Supernatant\ volume\ (\mu l)$
3M Sodium acetate solution	$= \frac{Supernatant\ volume\ (\mu l)}{9}$

Table #2

Primers and PCR protocols details

Hemoparasite	Primer sequence	Target sequence and Amplicon size	Reference
Mycoplasma spp.	Myco16S-322s 5‘-GCCCATATTCCTACGGGAAGCAGCAGT-3’ Myco16S-938as 5‘-CTCCACCACTTGTTC AGGTCCCCGTC-3`	16s rRNA gene: 616 bp	(Maggi, Colter, Kennedy-Stoskopf, & DePerno, 2013)
	Myco16S-322s 5‘-GCCCATATTCCTACGGGAAGCAGCAGT-3’ HemMycop16S-1420as 5‘-GTT TGA CGG GCG GTG TGT ACA AGA CC-3’	16s rRNA gene: 1098 bp	Maggi, Colter, Kennedy-Stoskopf, & DePerno, 2013)
	SYBR_For 5‘-AGCAATRCCATGTGAACGATGAA-3’ SYBR_Rev1 5‘-TGGCACATAGTTTGCTGTCACTT-3’ SYBR_Rev2 5‘-GCTGGCACATAGTTAGCTGTCACT-3’	16s rRNA gene: 132 bp	(Willi, et al., 2009)
Anaplasma spp.	AnaplsppF 5’-AGAAGAAGTCCCGGCAAACT-3’ AnaplR3, 5’-GAGACGACTTTT ACGGATTAGCTC-3’	16s rRNA gene: 800 bp	(Zobba, et al., 2014)
Babesia spp.	AccuPower *Babesia* PCR Kit (Bioneer, Daejeon, Republic of Korea)		

Table #3

Mycoplasma spp. PCRs results by location

Locality	Total samples tested	Total positive samples	SYBR Green PCR positives	Conventional PCR positives (322s-938as)	Conventional PCR positives (322s-1420as)	No. samples sequenced	Species
Pompeya	12	6	6	5	2	4	*Candidatus Mycoplasma haematoparvum*
San Roque	12	5	5	3	1	2	*Mycoplasma haemocanis*
Sani	12	2	2	1	0	1	*Mycoplasma haemocanis*
Nueva Providencia	12	3	3	2	1	0	No identification
Indillama	5	0	0	0	0	0	No identification

FIGURES

Figure #1

Electrophoresis gel for β-actin PCR amplicons of samples P1 and P2

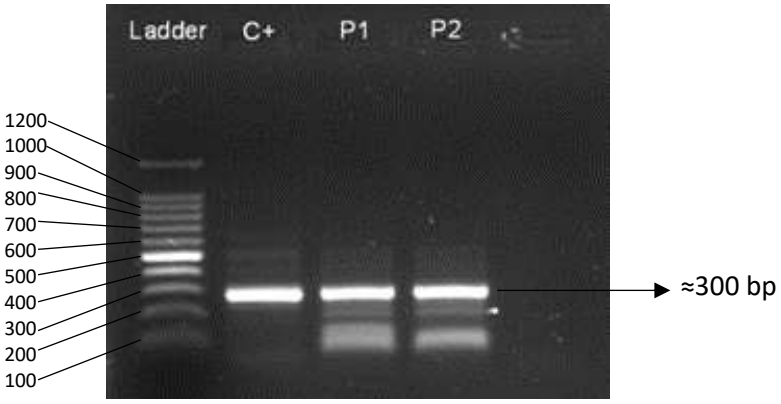


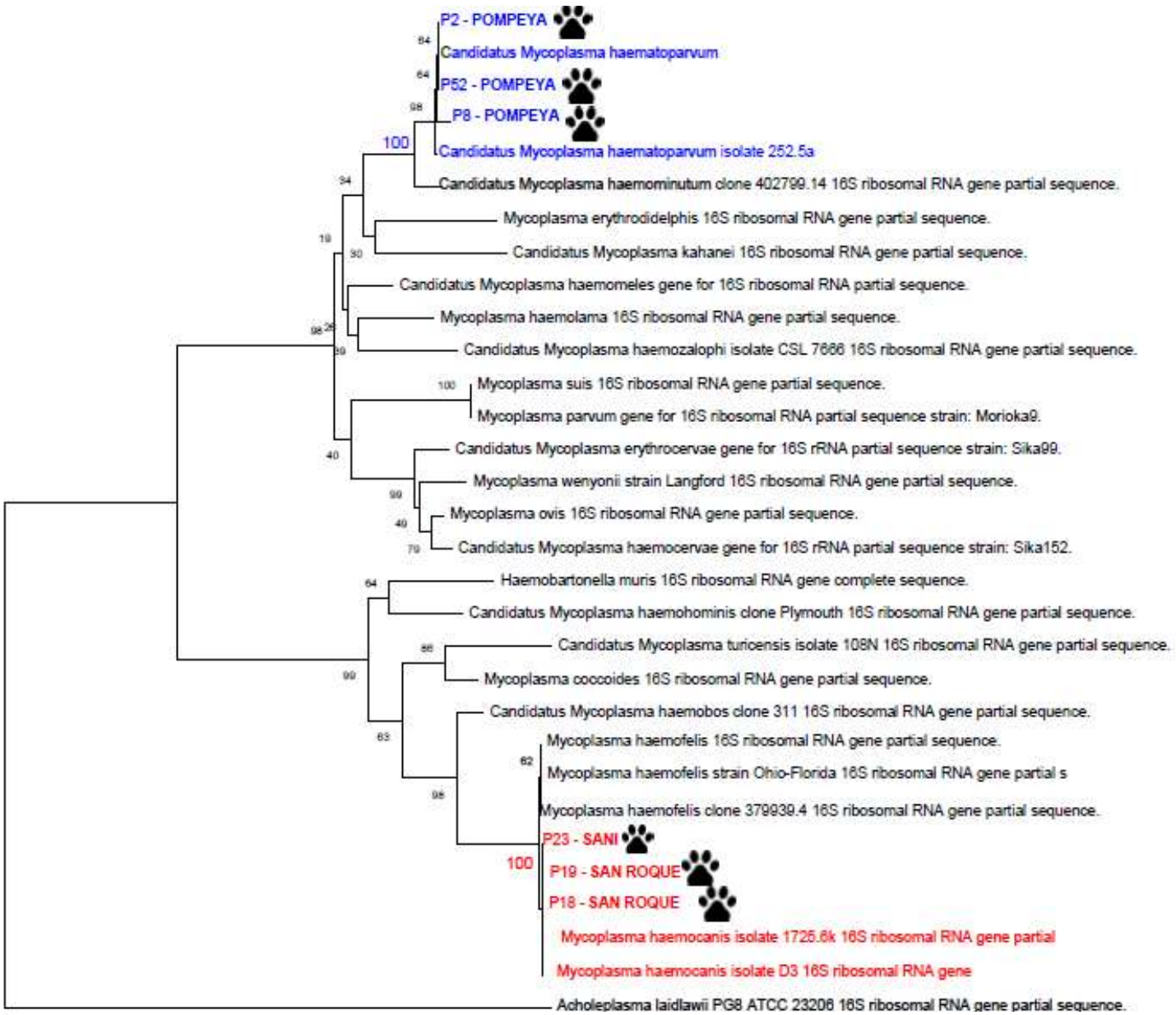
Figure #2

Positivity map

Map shows positivity results as percentage, obtained for each sampling site.

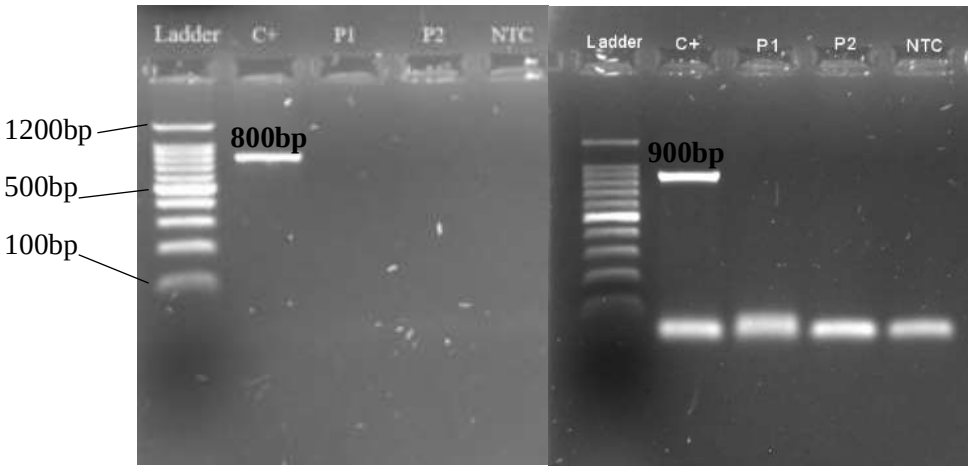
Figure #3

Maximum parsimony tree



The sum of branch length obtained for the optimal tree was 1.14153278. Bootstrap value for branches comprising sequences from isolates of dogs 2, 8 and 52 and CMh, and sequences from isolates of dogs 18, 19 and 23 and Mhc was 100 out of 600 replicates. This value corresponds to the number of times that a brunch appears in the same site, out of a total number of iterations that have been made to obtain a phylogenetic tree.

APENDIX A: ELECTROPHORESIS GEL OF PCR RESULTS FOR *Anaplasma* AND *Babesia*



Anaplasma (Left) and Babesia (Right) PCR results of samples P1 and P2

APENDIX B. Cq AND MELTING TEMPERATURES OF POSITIVE SAMPLES AMPLIFIED BY SYBR Green PCR FOR *Mycoplasma* spp.

Sample code	Cq	Melting temperature (^{o}C)
P2	38.65	73
P3	40.74	74.50
P7	35.33	73
P8	37.63	73.50
P10	34.78	73
P17	31.65	74.50
P18	35.87	74.50
P19	39.58	74.50
P21	39.66	74.50
P22	39.25	74.50
P23	40.03	74.50
P27	39.03	74.50
P38	39.11	74.50
P39	30.59	74
P43	39.74	74
P52	36.57	73.50