Demonstrating the presence of *Ehrlichia canis* DNA from different tissues of dogs with negative PCR in blood

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Abstract

Background: Nowadays, *Ehrlichia canis* receives more attention because of its great morbidity and mortality in animals. Dogs in the subclinical and chronic phases can be asymptomatic, and serologic tests show cross-reactivity and fail to differentiate between current and past infections. Moreover, there could be low parasitaemia, and *E. canis* might be found only in target organs, hence negative by PCR in blood. Methods: We evaluated by PCR the prevalence of *E. canis* in blood, liver, spleen, lymphatic nodules, and bone marrow in 59 recently euthanized dogs that had ticks but were clinically healthy. Results: In total, 52.55% of the blood PCRs for *E. canis* were negative, yet 61.30% yield positive results in tissue biopsies as follows: 63.15% from bone marrow, 52.63% from liver, 47.36% from spleen and 15.78% from lymphatic nodules. In addition, 33% had infection in three tissues (spleen, liver and bone marrow). Conclusions: Our results show prevalence of *E. canis* in tissue from dogs that were negative by PCR in blood. *E. canis* DNA in tissue was 30% lower in dogs that tested negative in blood samples by PCR, compared to those that were positive. However, it must be taken into account that some dogs with negative results were positive for *E. canis* in others tissues.

1. Introduction

Canine monocytic ehrlichiosis (CME) is caused by Ehrlichia canis, an intracellular parasitic bacteria and tick-borne pathogen. Recently, this pathogen has received further attention, because it leads to increasing morbidity and mortality in animals [1]. Transmission is mediated by the tick *Rhipicephalus sanguineus* sensu lato, and, before infection, the bacteria replicate in monocytes and macrophages [2]. Clinical presentation of CME results in acute, chronic or subclinical phases, with several clinical manifestations. The acute phase persists for 2 to 4 weeks [3] and is characterised
by signs in diverse systems, yet the most common are fever, weight loss, anorexia, depression, lymphadenomegaly, splenomegaly, and vasculitis [4]. In addition, dogs in this phase show thrombocytopenia as the most common laboratory abnormality [5]. In the subclinical phase, dogs have persistent thrombocytopenia and leukopenia in laboratory analysis; however, during this phase, in some dogs, the thrombocytopenia may be mild to non-existent [6] and they usually do not show clinical signs. Duration of this phase differs from months to years [7]. Additionally, in this phase is common that the microorganism is not circulating in blood and stays housed in some target organ, such as the spleen, bone marrow or liver [8–11]. Furthermore, previous research has shown that E. canis is widely distributed in different organs of infected dogs [8, 9, 12, 13]. Otherwise, in the chronic phase dogs have severe pancytopenia, haemorrhagic diathesis, and general debilitation [3]. Immune system deficiency, stress, co-infections, virulence strain, and geographical region are factors that influence the presentation of this phase in affected dogs [8].

In recent times, diagnosis of the disease has been challenging for practicing veterinarians [14, 15]. Identification of morulae in monocytes in a blood smear is diagnostic of the disease; however, a low frequency of morulae in buffy coat smears has been reported previously. It could be to the low parasitaemia observed in the natural infection [7, 11, 16–21]. Besides, other more specific methods are used as diagnostic, including the immunofluorescence antibody test (IFA) and ELISA (enzyme-linked immunosorbent assay), which are both able of detecting specific antibodies [21–27]. Also, other molecular techniques exist such as the polymerase chain reaction (PCR) [1, 19, 36, 28–35].

Presently, the infectious disease group of the American College of Veterinary Internal Medicine (ACVIM) requires that dogs diagnosed with this disease show suggestive clinical signs and have positive tests, either by serology and/or by PCR [37]. A complication in the diagnosis comes about in dogs in the subclinical phase of the disease, because normally
dogs do not have clinical signs. Furthermore, cross-reactivity and failure to differentiate between current and past infections with ELISA and IFA tests has been reported [25, 38, 39]. On the other hand, both in the subclinical and chronic phases, there is a possibility that parasitaemia is low in the dog [20, 26, 40, 41], as the bacteria are in the target organs [10]. Therefore, in these cases, the dogs will be negative in a PCR blood test [10]. Presently, the presence of DNA of E. canis in several organs, such as blood, bone marrow, spleen, liver, kidney, and lymph nodes has been demonstrated by PCR in infected dogs [7, 8, 11, 12, 40].

The goal of this study was to evaluate the occurrence of E. canis in different tissues, such as liver, spleen, lymphatic nodules and bone marrow, in dogs naturally infected with monocytic ehrlichiosis.

2. Materials And Methods

The present research has the approval of the Ethical Committee of the Universidad Autónoma de Ciudad Juárez. The handling of animals was carried out based on the regulation of laboratory animal handling and animal welfare (NORMA Oficial Mexicana NOM-062-ZOO-1999) [42] and in accordance with the International Guiding Principles for Biomedical Research Involving Animals.

For which an analysis of the variation in the infection by E. canis was carried out in four tissues, in two groups of dogs: positive and negative by PCR in blood.

2.1 Animals

Fifty-nine dogs were used for this research, which were obtained from the municipal Anti-Rabies Centre of Juarez. Based on the Centre’s internal regulations, animals that were not adopted 8 weeks after their arrival were euthanized. Euthanasia was performed by an overdose of sodium pentobarbital according to national and international animal welfare
regulations.

In order to increase the possibility that dogs will present with the subclinical phase of the disease, the inclusion criteria were that the dogs have ticks and were clinically healthy; therefore, dogs without ticks or with signs of any disease were excluded.

2.2 Sample collection

Whole blood samples were collected in tubes with EDTA (Vacutainer BD®, Mexico City, Mexico) by cephalic venepuncture previous to administration of sodium pentobarbital. The rest of the tissue samples were acquired by biopsies immediately after euthanasia, following the steps of surgical asepsis in order to prevent cross contamination. In addition, with the same purpose, a change of instruments was made to obtain the biopsy of each tissue; and particular attention was taken to avoid blood or other fluid from the dog having contact with the tissue samples.

Bone marrow aspirates were obtained with a Bone Marrow Aspiration Needles (Argon Medical Devices®, Dallas, USA) in the greater tubercle of the humerus, as described by Raskin and Messickin in 2013 [43]. Hepatic and splenic biopsies were obtained by celiotomy and with the ligature fracture technique [44]. Finally, prescapular lymph nodes were biopsied with a biopsy punch (Premier®, Plymouth Meeting, USA) as previously described [45]. Tissues samples were marked and frozen at -20 °C for future extraction of DNA and PCR analysis.

Biopsies obtained from spleen, liver and lymph node had an average weight 200 mg (range of 150 to 210 mg). The amount of whole blood obtained was 1.5 ml. and the bone marrow biopsy obtained 0.6 ml on average (range of 0.4 to 0.7 ml)

2.3 DNA extraction

In blood, the extraction of genomic DNA was performed from the cellular package of the
dogs samples with the UltraClean Blood DNA Isolation Kit (MoBio Lab®, Carlsbad, USA), according to the manufacturer's instructions.

The others tissues were handled in sterile fashion prior to the extraction of DNA. For the extraction of DNA from the biopsies, the protocol was modified with the previous addition of lysis reagents [46]. Then the tissues were macerated with the use of a low velocity drill (Jorvet Lab®, Loveland, USA) and a dental burn (JOTA Technical®, Rüthi Switzerland). Once each tissue was macerated, DNA extraction was performed in the same way as the blood.

2.4 PCR amplification and analysis

DNA detection of E. canis was achieved with the use of the nested PCR molecular test. Initially, to amplify the Ehrlichia genus 16SrRNA gene, 2 pmol of primer ECC (5´-AGA-ACGAACG-CTGGCGGCCAAGC-3´) and ECB (5´-CGTATTACC GCGGCTGCT-3´) were used [28]. In the second PCR, to amplify E. canis 16SrRNA gene, 2 pmol of primer HE-3(5´TATAGGTACCGTCATTATCTTCCCTAT-3´) combined with the reverse primer ECA (5´-CAATTATTTATAGCCTCTGGCTATAGGAA-3´) were used [28, 47].

Initially, PCR was performed in a thermocycler (BIO-RAD® C-1000 Touch, Hercules, USA) starting at 94 ºC for 1 minute and then for 35 cycles at 94 ºC for 1 minute (denaturation), 60 ºC for 1 minute (hybridisation), and 72 ºC for 3 minutes (extension). Afterwards, starting at 94 ºC for 5 minutes and then for 40 cycles at 94 ºC for 1 minute (denaturation), 60 ºC for 1 minute (hybridisation), and 72 ºC for 1 minute (extension), as described previously [28, 47, 48].

2.5 Statistical analyses

A multivariate logistic regression model was used for the response variable "infection" which was binary (variable dummy) with y = 1 if positive, and = 0 if negative, depending
on two explanatory variables: blood positivity (two levels) and positivity in 4 separate tissues (4 levels). Therefore, the model was: infection = blood + tissue + error.

The model analysed separately infection in both groups of dogs. In each group, the model compares infection among four tissues using statistical tests "z" between pairs of tissues, using a multiple comparison Scheffe Test. Comparison of the proportions of positive and negative results in blood, lymph node, liver and spleen samples were performed using Chi square and Fisher’s exact tests with the FREQ procedure of SAS (9.0). Significance was considered with a P value < 0.05.

3. Results

Of the 59 dogs analysed in this research, 28 (47.45%) had a positive result of *E. canis* by PCR in blood, and 31 (52.55%) were negative. When evaluating the 28 dogs that were positive by PCR in blood, it was observed that 16 (57.14%) were also positive by PCR in some of the tissues. Otherwise, when analysing dogs with negative PCR results in blood (n=31) and comparing them with the results of PCR in different tissues of the same dogs, it was observed that 19 dogs (61.30%) presented with positive results to *E. canis* in some of the tissues and 12 (38.70%) were negative in all tissues biopsied.

The tissue biopsy with the highest number of positive samples was the bone marrow with 26 (44.60%). This occurred both on positive and negative bloods. For example, 10 dogs (35.71%) that were positive in blood by PCR were also positive in the bone marrow.

Furthermore, it was found that were positive 12 of 19 cases (63.15%) with negative PCR blood samples. In half of the negative cases (n=6), the results of the PCR of other tissues were negative. Conversely, in two cases, the PCR was positive in all tissues analysed. The other combinations of positive and negative results of diverse tissues can be seen in Table 1.

The tissue with the second highest number of positive results was the spleen, with a
prevalence of 42.37% (n=25). When analysing PCR positive blood, 16 samples (57.14%) were also positive in spleen PCR. In PCR blood negative dogs, the splenic tissue had 9 (47.36%) positive PCR results, although there were spleen-only positive samples on two occasions. Also, on two occasions the PCR was positive in all the tissues analysed. The remaining of the combinations are presented in Table 2.

The liver had 22 PCR positive cases (37.28%) from the total of sample evaluated. Of the positive samples by PCR in blood, 12 (42.85%) were also positive in the liver tissue. Similarly with the spleen, of the 19 negative blood samples by PCR, 10 (52.63%) were positive in the liver tissue. In the negative blood samples, there was one liver-only positive result. In addition, the results were positive in all tissues twice. Other positive and negative mixtures of several tissues are presented in Table 3.

Finally, the tissue with the least positive results in the research was the lymph node, with five cases (8.47%). In the positive blood PCR samples, only two cases were positive (10.52%). On the other hand, the negative blood samples by PCR, were positive in lymphatic tissue in three occasions, that represented 15.78%. In none of these three cases was the lymph node the only tissue with positive results (Table 4).

Considering the infection in the four tissues, the infection rate was the same in both negative and positive dogs in blood (p > 0.05). The infection in tissues of negative dogs was an average rate of 0.23 ± 0.05 and for positive was 0.35 ± 0.04 (with 233 degrees of freedom and p <0.001).

4. Discussion

In this work, of the 59 clinically healthy dogs analyzed, 47.45% had a positive result with blood PCR to Ehrlichia canis. In addition, PCR recognised a higher prevalence of E. canis in different tissues of naturally infected dogs, both in dogs with positive and negative results by blood PCR.
At the present time, diagnosis by PCR is more useful than serology for the differentiation of concurrent infections and co-infections with diverse Ehrlichia spp. and is used for treatment monitoring [47]. However, in naturally-occurring CME, the diagnostic sensitivity and optimal tissue for PCR testing in the untreated dog or in the post-treatment setting has not yet been clarified [47]. Results obtained at this point demonstrate that, in dogs with naturally-occurring CME infection, it is feasible to detect E. canis in different tissues, even if they have had negative blood tests. Additionally, in the acute phase, E. canis is easily detected in blood, while in the subclinical and chronic phases, there is the possibility of false negatives. Therefore, some tissues are more appropriate for sampling, such as the spleen and the bone marrow [8, 9, 13, 49]. This research does not suggest performing PCR of tissues for routine diagnosis of CME in dogs, because perform biopsies in dogs with no clinical signs is unpractical. However, sampling tissues may be relevant to understand the distribution of CME in dogs.

Comparative information on the spread and presence of E. canis by PCR analysis in multiple organs is limited, especially in dogs with the natural form of the disease, although some research has been done in experimentally inoculated dogs. For example, it is proven that PCR is effective in detecting E. canis in diverse tissues in dogs with experimental disease [12]. In the same way, it has been described that the spleen is a tissue that can be useful to demonstrate the presence of E. canis DNA by PCR [8, 9]. In addition, the possibility of dogs in the subclinical phase being negative to the PCR in blood and positive to the PCR of splenic aspirates has also been established [8]. The difference between the last two studies and the present investigation is the spleen tissue analysed. In our study, the DNA was obtained through splenic biopsy, and DNA was obtained for blood through splenic aspirates in the others. In another investigation, it was found that out of 78 dogs with splenic disease, only one was positive for E. canis by PCR in a splenic
biopsy [50]. This results contrast with the ones obtained in the present study. Furthermore, another important difference in the results is the tissue with the highest number of positives was the bone marrow in our study, which was contrary to the others that obtained more positives in the aspirates of the spleen [8]. Nevertheless, other studies have demonstrated that other tissues besides the spleen are better to detect E. canis by PCR. For example, some authors describe results similar to those obtained in the present study, where it was shown that E. canis DNA was most often amplified from bone marrow [51, 52]. But in these cases there was experimental disease, and PCR was performed by aspirates. On the other hand, in one study performed on biopsies of dog cadavers, contrary to the results of the present study, none of the bone marrow biopsies were positive for E. canis by PCR [10].

An important limitation of the present study is the absence of blood analysis, especially blood counts. This would establish, in a more accurate way, the dogs presenting with the subclinical phase of monocytic ehrlichiosis [47]. However, it can be assumed that positive dogs are in this phase, since they are clinically healthy.

E. canis is widespread throughout the different body systems of infected dogs. In addition, the molecular detection of E. canis DNA has shown that it can be present in different target organs [13, 53, 54]. In the subclinical and chronic phases, E. canis could be “hiding” in splenic macrophages [8]; therefore, the spleen may be the principal reservoir of E. canis, probably because it has an abundance of macrophages. Moreover, some studies propose that it is the last organ to contain the microorganism before its elimination [8, 55]. Therefore, when containing a large number of bacteria, the spleen is considered by some authors as the organ of choice for molecular detection in different phases of the disease [4, 8, 49, 56]. Although, in our study E. canis DNA was detected in the spleen, the results differ slightly with this statement, since it was the third most
affected organ, surpassed by the bone marrow and liver. However, the results are similar with other studies, which suggested that the spleen was inferior to other tissues [10, 12, 51, 52].

5. Conclusion

In conclusion, results of this research could be applicable in some cases where the diagnostic sensitivity of PCR may be suboptimal [47]. In some special cases, it will be necessary to search for E. canis DNA by molecular methods in different organs. In this study we have demonstrated that although infection in organs was 30% lower in negative dogs by PCR in blood, a considerable number of dogs (n = 19 or 61.30%) that had negative results by blood PCR were positive for E. canis in some organs. Dogs with positive blood results were positive in three tissues (liver, bone marrow and spleen) in 48% of cases. At the same time, the three tissues were more positive than the lymph node, which was positive in only 8% of the samples evaluated, and was four times lower than in any of the other 3 tissues. Dogs with negative results in blood, showed 33% detection of E. canis DNA in the spleen, liver, and bone marrow; however, only the presence of DNA was higher in liver and bone marrow than in the lymph nodes. Because in some cases the DNA was detected only in one of these tissues, it is proposed to perform biopsies of at least these three structures. This assertion is stipulated with other rickettsial diseases, such as Anaplasma spp., where blood samples are routinely used for screening, but in persistently infected dogs with intermittent or low-level bacteraemia, other tissues might be useful [57]. The results open the possibility of performing similar research aimed to detect E. canis by PCR of different tissues in treated dogs that continue to show signs or alterations in blood tests as well as in dogs that have signs suggestive of the disease but have negative results in serologic and molecular blood analyses.
Declarations

Authors’ contributions
CARA, DMBR and JVFM conceived and designed the study. CARA, AOM, JTA, RRB and LSG collected samples and participated in data collection. DMBR, JAAM, JTA, JJLA OSCH and JVFM handle tissue samples, extracted in DNA and perform PCR in the laboratory. AQC, FPC and JAI analyzed the data. CARA, AOM and JAG wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data supporting the conclusions of this article are included within the article. Raw data used or analysed during the present study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate
This work was reviewed and approved by the Ethics and Animal Welfare Committee of the Universidad Autónoma de Ciudad Juárez, Mexico (CBE.ICB/001.01-25), and performed in compliance with the Mexican and American guidelines for animal research.

Consent for publication
Not applicable.

Competing interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Tables

Table 1

Comparison between negative blood-PCR of E. canis with positive bone marrow-PCR. In addition to the PCR results in the other tissues.
| Blood PCR | Bone marrow PCR | Liver PCR | Spleen PCR | Lymph node PCR |
|-----------|-----------------|-----------|------------|---------------|
| Negative  | Positive        | Positive  | Positive   | Positive      |
| Negative  | Positive        | Positive  | Negative   | Positive      |
| Negative  | Positive        | Positive  | Negative   | Negative      |
| Negative  | Positive        | Positive  | Negative   | Negative      |
| Negative  | Positive        | Positive  | Negative   | Negative      |
| Negative  | Positive        | Negative  | Positive   | Positive      |
| Negative  | Positive        | Negative  | Positive   | Positive      |
| Negative  | Positive        | Negative  | Positive   | Negative      |
| Negative  | Positive        | Negative  | Positive   | Positive      |

**Table 2**

Comparison between negative blood-PCR of *E. canis* with positive liver-PCR. In addition to the PCR results in the other tissues.

| Blood PCR | Liver PCR | Spleen PCR | Bone marrow PCR | Lymph node PCR |
|-----------|-----------|------------|-----------------|---------------|
| Negative  | Positive  | Positive   | Negative        | Negative      |
| Negative  | Positive  | Positive   | Negative        | Negative      |
| Negative  | Positive  | Positive   | Negative        | Negative      |
| Negative  | Positive  | Positive   | Negative        | Negative      |
| Negative  | Positive  | Positive   | Positive        | Positive      |
| Negative  | Positive  | Negative   | Positive        | Positive      |
| Negative  | Positive  | Positive   | Positive        | Positive      |
| Negative  | Positive  | Negative   | Positive        | Negative      |
| Negative  | Positive  | Negative   | Positive        | Positive      |

**Table 3**

Comparison between negative blood-PCR of *E. canis* with positive spleen-PCR. In addition to the PCR results in the other tissues.

| Blood PCR | Spleen PCR | Bone marrow PCR | Lymph node PCR | Liver PCR |
|-----------|------------|-----------------|---------------|----------|
| Negative  | Positive   | Negative        | Negative      | Positive |
| Negative  | Positive   | Negative        | Negative      | Positive |
| Negative  | Positive   | Negative        | Negative      | Positive |
| Negative  | Positive   | Positive        | Negative      | Positive |
| Negative  | Positive   | Positive        | Negative      | Negative |
| Negative  | Positive   | Positive        | Positive      | Positive |
| Negative  | Positive   | Positive        | Negative      | Positive |
| Negative  | Positive   | Negative        | Positive      | Positive |

**Table 4**

Comparison between negative blood-PCR of *E. canis* with positive lymph node-PCR. In addition to the PCR results in the other tissues.
