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Short communication

MOLECULAR DETECTION OF *EHRLICHIA CANIS* IN THE PET-DOG POPULATION IN R. N. MACEDONIA

Elena ATANASKOVA PETROV*, Irena CELESKA, Zagorka POPOVA, Kiril KRSTEVSKI, Igor DJADJOVSKI

University Ss. Cyril and Methodius - Skopje, Faculty of Veterinary Medicine, St. "Lazar Pop-Trajkov" 5-7, Skopje, R. of North Macedonia

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Canine monocytic ehrlichiosis (CME) is a widespread, tick-borne, canine disease, caused by an obligate intracellular bacterium, *Ehrlichia canis*. The main vector, a brown-dog tick, *Rhipicephalus sanguineus*, is widely distributed, especially in areas with tropic, subtropic, or Mediterranean climates (Central and South America, Eastern and Western Asia, Africa, Australia and Southern Europe). The study performed in 2012, by Stefanovska et al., determined a seroprevalence of 18.7% of *E. canis* among the Macedonian dog population. Up to date, the presence of *E. canis*, using molecular diagnostic methods, has not been investigated in Macedonia. Therefore, this study aimed to confirm the presence of *E. canis*, in the pet-dog population on the territory of the city of Skopje, North Macedonia, using a highly sensitive multiplex Real-Time PCR method (qPCR). Whole blood samples from 80 dogs of different breeds and ages, with clinical symptoms of CME and positive serology result for the presence of antibodies against *E.canis*, were collected for analyses. Out of 80 dogs, 36 (45%) were found as positive. The present work reports the first molecular detection of *E. canis* in pet dogs on the territory of the city of Skopje, Macedonia.

Key words: canine monocytic ehrlichiosis, dog, qPCR, 16S rRNA gene.

INTRODUCTION

Canine monocytic ehrlichiosis (CME) is a widespread, tick-borne, canine disease, caused by an obligate intracellular bacterium, *Ehrlichia canis*. The other two closely related species, *Ehrlichia ewingii*, and *Ehrlichia chaffeensis,* are known to infect dogs, as well, causing similar but milder clinical diseases [1].

The main vector of *E. canis*, a brown-dog tick, *Rhipicephalus sanguineus,* is widely distributed, especially in the areas with tropic, subtropic or Mediterranean climates (Central and South America, Eastern and Western Asia, Africa, Australia, and Southern Europe) [2-4]. Besides causing the disease in dogs, *E. canis* has a zoonotic potential

*Corresponding author: e-mail: eatanaskova@fvm.ukim.edu.mk

and can be a cause of human ehrlichiosis [5]. On the other hand, the epidemiological importance of *E. ewinvii* and *E. chaffeensis* seems to be limited only to North America, because the presence of their vectors, *Amblyomma americanum* and *Dermacentor variabilis* ticks, has not been reported outside this continent [6].

The infection with *E. canis* in dogs can be asymptomatic for months, or with the immediate development of clinical signs. The incubation period can vary from eight to twenty days. The disease is of multisystemic character and can be manifested as acute form with fever, depression, anorexia, splenomegaly, lymphadenopathy and hemorrhage predisposition, subclinical form with no apparent clinical signs of the disease, or chronic form with symptoms similar to those in the acute form, but more severe [2,7].

Different forms of the disease can hardly be distinguished in everyday clinical practice [8]. The main hematological alterations are moderate to severe thrombocytopenia, non-regenerative anemia, and depending on the disease stage, moderate to severe leucopenia [9].

Even though there is no perfect diagnostic test for *E. canis*, serological tests are most commonly used for the diagnosis of *E. canis* infections [10]. Antibodies against *E.canis* appear 7-35 days post infection and are not in correlation with the clinical manifestation and the duration of the disease [8]. Antibodies for *E.canis* can persist in the circulation for several months to years after treatment, and they cannot be used in the determination of active or past infection [11].

Therefore, the molecular diagnostic methods are considered as the most appropriate for the diagnosis of *E. canis* infection [12]. Polymerase chain reaction (PCR) is a very sensitive method for the detection of acute monocytic ehrlichiosis in dogs, and it is an irreplaceable diagnostic tool, especially in the first two weeks of infection, prior to antibody development and the onset of clinical signs [13]. However, the use of PCR for the detection of *E. canis* in the subclinical and chronic form may have limited value due to the low concentration of the microorganism in the circulation, which can result in false-negative results [14,15].

Molecular studies conducted around the world presented *E.canis* prevalence of 41.59% in India, 28.0% in Pakistan, 29.26% in Mexico, 37.0% in the Caribbean island of Saint Kitts, 34.50% in Northeastern Brazil, and 22.0% in Portugal [16-21].

Serological evidence for the presence of CME in dogs has been reported in almost all Balkan countries (Greece, Albania, Serbia, and Bulgaria) [22-25]. The study performed in 2012, by Stefanovska et al, determined the seroprevalence of 18.7% of *E. canis* among the Macedonian dog population [26]. However, published data on studies for molecular detection of *E. Canis* are reported only from Greece [27].

Up to date, the presence of *E. canis*, using molecular diagnostic methods, has not been investigated in Macedonia. Therefore, this study aimed to confirm the presence and determine the prevalence of *E. canis*, in the pet-dog population on the territory of the city of Skopje, using a highly sensitive multiplex Real-Time PCR method (qPCR).

MATERIAL AND METHOD

Sample selection

The targeted group was pet dogs brought to the University Veterinary Clinic (UVC) at the Faculty of Veterinary Medicine – Skopje (FVMS), and several other private veterinary clinics on the territory of the city of Skopje, exhibiting clinical symptoms characteristic for tick-borne diseases (fever, anorexia, lethargy, and pale mucosa), and hematological alterations with the presence of thrombocytopenia and/or anemia. All dogs were screened for the presence of antibodies against *E. canis* using a commercially available immunochromatography based point-of-care (POC) test (SNAP® 4Dx®; IDEXX Laboratories, Inc. U.S.A). Finally, only the seropositive animals were selected for further investigation and detection of *E. canis*.

From May 2018 until September 2019, 80 whole blood samples from naturally infected pet dogs (47 males and 33 females), of different breeds and ages, were collected and analyzed using the qPCR method. Whole blood samples were collected in 3ml vacuum tubes sprayed with Ethylenediaminetetraacetic Acid (Vacuette, K3E K3EDTA, Greiner Bio-One), and stored at -20°C until DNA extraction.

DNA extraction

Total DNA was obtained from 200μL of whole blood, using an automated extraction system (SaMag-24, Sacace, Italy), and an appropriate DNA extraction kit (*SaMag Blood DNA Extraction kit*, REF SM001), according to the manufacturer's protocol. For optimization of the DNA input in the qPCR reaction, the quantity and purity of the extracted DNA was evaluated for each sample, using the UV-Vis spectrophotometer (NanoDrop 2000C, Thermo Scientific), and determination of the A 260/280 ratio.

Detection of *Ehrlichia canis* by qPCR

For detection of *E. canis* DNA, a highly sensitive, multiplex, Taqman-based qPCR protocol, previously described by *Peleg et al., 2010*, was used. [28] The protocol is designed to amplify a short sequence within the 16S rRNA of *E. canis*, and additionally, the canine beta actin gene (ACTB) as an internal control (IC). Even though this protocol detects *E. ewingii* and *E. chaffeensis*, as well, as neither the presence of competent vectors nor the pathogens themselves have been confirmed in Europe so far, we considered the positive amplification of the 16S rRNA fragment of Erlichia genom to be specific for *E. canis*.

Shortly, the primers used for amplification of the 16S rRNA sequence of *E. canis* were: forward *E.c 16S fwd – 5'-TCGCTATTAGATGAGCCTACGT-3'*, reverse *E.c 16S rev – 5'-GAGTCTGGACCGTATCTCAGT-3'*, and the probe *E. canis 16S rRNA - 5'-Fam-GTCTGAGAGGACGATCAGCCACACT-3'-BHQ1*. The primers for amplification of the ACTB were: forward Canine actin fwd- 5'- GCGCAAGTACTCTGTGTGGAT-3',

revers Canine actin rev – 5'- GTCGTACTCCTGCTTGCTGAT-3', and Canine beta actin pro - 5'-JUN-TCCTGGCCTCACTGTCCACCTTCCAGCA-3'-QSY. The primers and probes were synthesized by Applied Biosystems. Amplification reaction was performed on *QuantStudio® 5 Real-Time PCR System* (Applied Biosystems™, Cat.no. A28138), using the *Path-ID™ Multiplex One-Step RT-PCR Kit* (Applied Biosystems™, Cat.no. 4442136). The total volume of the reaction was 25 μl, containing: Multiplex RT-PCR Buffer 12.5 μl, *E. canis* primer-mix 2 μl (final concentration of primers and the probe in the mix 0.8 μM and 0.2 μM, respectively), ACTB primer-mix 2 μl (final concentration of primers and the probe in the mix 0.25 μM and 0.12 μM, respectively), Multiplex Enzyme Mix 2.5 μl, DNase/RNase free water 3 μl, and 3 μl of template DNA. The thermal cycling conditions were as follows: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 45 seconds. The intensity of the fluorescence signal was measured at the end of each cycle. DNase/RNase free water, as a non-template control (NTC), and *E. canis* extracted DNA optimized at Ct 32 (±2), as a positive control, was used in each run.

Based on the internal verification and determination of the limit of detection (LOD) of the qPCR protocol, the cut-off value for the positive result was set at < Ct 39 (data not presented).

RESULTS AND DISCUSSION

Eighty blood samples from pet dogs, living on the territory of the city of Skopje, were analyzed for the presence of *E. canis* using the qPCR method, of which 36 were found as positive, giving an overall prevalence of 45%. The prevalence of *E. canis* was calculated as 55.6% (20 of 47) among the male dogs, and 44.4% (16 of 33) among the female dogs, included in this study. A chi-square test of independence showed that there was no significant association between gender and the prevalence of *E. canis*, $X2$ (2, N = 80) = 0.275, p = .59. The obtained Cycle threshold (Ct) values, as an indicator of the quantity of the extracted *E. canis* DNA, ranged from 19.39 to 37.89, with the average value of 31.25. Of this, 36.11% (13 of 36) of the positive results were with Ct values lower than 30, and 68.89% (23 of 36) with Ct values higher than 30. The main hematological alterations characteristics of CME, thrombocytopenia and anemia, were present in 95% (76 of 80) and 66.25% (53 of 80) of the dogs included in this study, respectively.

Unspecific clinical manifestation, together with the lack of sensitive diagnostic procedures impairs the overall diagnosis of CME [12]. Thus, the combination of clinical, serological and molecular diagnostic procedures is required for early detection of circulating *E. canis*. Through personal communication with the major veterinary clinics/ambulances in Macedonia, the commercial POC tests for detection of IgG antibodies against *E. canis*, was found to be the most commonly used tests for diagnosis of CME. Even though, the presence of *E. canis* has been indirectly confirmed with previous serological studies [26], direct detection of the pathogen, using molecular

diagnostic methods, has never been performed in Macedonia. This study presents the results of the first molecular survey designed for confirmation of the presence and determination of the prevalence of *E. canis* among pet-dogs, exhibiting clinical signs of a tick-borne disease on the territory of the city of Skopje. In addition to clinical signs, the presence of hematological alterations (thrombocytopenia and/ or anemia), and confirmation of the presence of antibodies against *E. canis*, were used as a diagnostic approach to maximize the probability of detecting circulating *E. canis* using the qPCR method. From the 80 seropositive pet dogs included in this study, in 36, successful amplification of the specific fragment within the 16S rRNA of *E. canis*, was obtained. The established prevalence of 45.0%, was similar to the prevalence observed in molecular studies conducted in several countries from different continents [16-21]. However, some studies report a rather low prevalence of *E. canis* (Malaysia – 2%, Myanmar – 0.75%, etc.), or even fail to detect the presence of *E. canis* in the studied dog population (Croatia) [10,29,30]. The established differences in the prevalence of *E. canis* in different studies are mainly influenced by the wide variety of approaches used for the study design. In general, studies reporting a low prevalence of *E. canis* mostly used a simple random sampling method of apparently healthy dogs, as a method for selection, while the majority of studies reporting higher prevalence used a more targeted approach selecting only dogs exhibiting clinical symptoms of tick-borne disease or only seropositive dogs, such was the case with our study. The established high prevalence of *E. canis* among pet dogs living on the territory of the city of Skopje, indicates a frequent exposure, as well as a low level or poor protection against ectoparasites of this population. No significant difference ($p > 0.05$) was observed when comparing the prevalence on a gender level. However, the slightly higher prevalence observed in the male in comparison to female dogs (55.6%, and 44.4%, respectively), goes in line with the data from similar studies conducted in Portugal and Malaysia [21,10].

In 55% (44 of 80) of the seropositive dogs, we failed to detect circulating *E. canis* in the analyzed whole blood samples. The whole blood samples were chosen because of the noninvasive nature of this sampling technique and the established suitability of these samples for molecular detection of *E. canis* [31,32]. The reason for this is the ability of the bacteria to “hide” in the spleen and other organs, in the subclinical phase of the disease, while the level of antibodies is high and the level of circulating bacteria is very low (severe pancytopenia in the chronic phase) [31,33,34].

Based on the results from the study conducted by Waner et al. (2014), the sensitivity of the qPCR method dramatically decreases 17 days post infection (DPI) [35]. Thus, only 36.11% (13 of 36) of the confirmed positive dogs in this study, with Ct values lower than 30, could be considered as being detected within 15 DPI (acute phase). The use of spleen aspirates for the confirmation of *E. canis*, in seropositive/qPCR negative dogs, will contribute to increasing the level of sensitivity of the overall diagnostic approach, and identification of the carrier status for CME in dogs with a subclinical or chronic form of the disease [15].

Besides the presence of clinical symptoms (fever, depression, anorexia, etc.), and serological confirmation of the antibodies against E. canis (100%), thrombocytopenia was the most prevalent finding, present in 95% (76 of 80) of the dogs included in this study. This highlights the diagnostic value and significance of this parameter for the general approach in CME diagnostic.

The study should be further continued with the determination of the genetic characteristics of Macedonian E. canis isolates, and the establishment of epidemiological relations with the closely related isolates deposited in the accessible, open-source, genetic databases.

Authors' contributions

EAP participated in designing and coordination of the study, as well as sample selection and immunochromatography. IE participated in laboratory analyses (haematology and biochemistry), and helped to draft the manuscript. ZP carried out the molecular protocol. KK participated in molecular and the statistical analysis. IDj participated in the design of the study, molecular analyses, performed the statistical analysis, helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting 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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MOLEKULARNA DETEKCIJA *EHRLICHIA CANIS* U POPULACIJI PASA -KUĆNIH LJUBIMACA U SEVERNOJ MAKEDONIJI

Elena ATANASKOVA PETROV, Irena CELESKA, Zagorka POPOVA,
Kiril KRSTEVSKI, Igor DJADJOVSKI

Monocitna erlihioza pasa (CME) je široko rasprostranjena bolest pasa, koja se prenosi krpeljima, a uzrokuje je obligatno-intracelularna bakterija *Ehrlichia canis*. Glavni vektor, smeđi krpelj pasa, *Rhipicephalus sanguineus*, široko je rasprostranjen, posebno u oblastima sa tropskom, subtropskom ili mediteranskom klimom (Centralna i Južna Amerika,

Istočna i Zapadna Azija, Afrika, Australija i Južna Evropa). U Studiji izvedenoj 2012. godine, Stefanovska i saradnici su utvrdili da među severnomakedonskom populacijom pasa, seroprevalencija *E. canis* iznosi 18,7%. Do danas, prisustvo *E. canis*, koristeći molekularne dijagnostičke metode, nije istraženo u Severnoj Makedoniji.

Stoga je ova studija imala za cilj da potvrdi prisustvo *E. canis* u populaciji kućnih ljubimaca na teritoriji grada Skoplja u Severnoj Makedoniji, koristeći visoko osetljivu *multiplex Real-Time PCR* (qPCR). Za analize su prikupljeni uzorci pune krvi od 80 pasa različitih rasa i uzrasta, sa kliničkim simptomima CME i pozitivnim serološkim rezultatom na prisustvo antitela protiv *E.canis*. Od 80 pasa, 36 (45%) je ocenjeno kao pozitivno. Ovaj rad izveštava o prvom molekularnom otkrivanju *E. canis* kod pasa kućnih ljubimaca na teritoriji grada Skoplja, Severna Makedonija.