Biotechnological Communication

Biosci. Biotech. Res. Comm. 10(2): 187-191 (2017)

BBRC Bioscience Biotechnology Research Communications

PCR based analysis of Haemobartonellosis (*Candidatus mycoplasma haematoparvum* and *Mycoplacma haemocanis*) and its prevalence in dogs in Isfahan, Iran

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ABSTRACT

The present study was conducted to determine prevalence of *Candidatus mycoplasma haematoparvum* and *Mycoplacma haemocanis* in dogs in Isfahan (Central province of Iran). Total 294 dogs were the materials of the study. To determine molecular and haematological parameters, 2 ml blood with and without anticoagulant were taken according to technique from vena cephalica antebrachii. *Candidatus mycoplasma haematoparvum* and *Mycoplacma haemocanis* was detected in blood smears preparations of 26 (8.82%) by Geimsa staining and (20.06%) by PCR. Based on the 16S rDNA sequence, a specific PCR assay was developed.

KEY WORDS: HAEMOBARTONELLOSIS, DOGS, IRAN, PCR

INTRODUCTION

The organisms formerly known as *Haemobartonella* and *Eperythrozoon* spp. are small, pleomorphic bacteria that parasitize red blood cells of a wide range of vertebrate animals. Haemobartonellosis, infectious anaemia of dogs is caused by *Candidatus mycoplasma haematoparvum* and *Mycoplacma haemocanis*, an anaplasma species belonging to the rickettsia family. The infection is characterized by extreme fatigue, depression, anorexia, weight loss and anaemia and may cause death (Lumb, 2001; Neimark et al., 2001; Torkan et al., 2014).

The pathogen can be identified as small coccoids, rings or strings on erythrocyte membrane or free in

ARTICLE INFORMATION:

**Corresponding Author: borji_milad@yahoo.com*
Received 21st March, 2017
Accepted after revision 21st June, 2017
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA

Thomson Reuters ISI ESC and Crossref Indexed Journal
NAAS Journal Score 2017: 4.31 Cosmos IF : 4.006
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Online Contents Available at: http//www.bbrc.in/

plasma in Giemsa staining of blood smears (Murray et al., 1995; Lumb, 2001).Mode of transmission has not been clearly identified but bloodsucking arthropods like ticks were the suspected vectors. Another possible mode of transmission is close fighting among dogs. Intrauterine and lactation-related transmission was also reported (Neimark et al., 2002; Jane et al., 2005).Acute disease presents with fever, anorexia, weight loss, jaundice, apathy, adenopathy, motor incoordination and splenomegaly (North, 1978; Sykes et al., 2004). Chronic disease has atypical symptoms like anaemia, weight loss, paraplegia, dehydration, hyperesthesia and depression (Saykes et al., 2008). Latent form of the infection has also been described (Pitulle et al., 1999).Diagnosis of haemobartonellosis depends on clinical and hematological findings together with microscopic examination of blood smears and specific serological and PCR testing for the pathogen (Lappin et al., 2006). Various antibiotics were reported to be effective in the treatment of haemobartonellosis. Haemobartonellosis was first described in 1953 in the United States (Splitter et al., 1956) but the number of studies about incidence and prevalence of the disease and the risk factors in transmission remains limited after 50 years (Kemming et al., 2004). In addition, studies examining *Candidatus mycoplasma haematoparvum* and *Mycoplacma haemocanis* infection have not been came across in this country except for one study (Foley et al., 2001).Therefore, this study was planned to investigate prevalence of *Candidatus mycoplasma haematoparvum* and *Mycoplacma haemocanis* infection in dogs.

MATERIAL AND METHODS

Study population consisted of 294 dogs (140 females, 154 males). To determine molecular and haematological parameters, 2 ml blood with and without anticoagulant were taken according to technique from V. cephalica. The dogs were clinically examined and blood samples with and without anticoagulant were drawn into tubes for haematological and molecular analysis. Prepared blood smears were stained with Geimsa method and examined under light microscope according to the literature (Foley et al., 2001).

Blood was processed for DNA extraction as described by d'Oliveira *et al.* (1995). Briefly, 200: L of thawed blood was washed 3-5 times by mixing with 0.5 mL PBS (137mM NaCl, 2.6 mM KCl, 8.1mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4), each time followed by centrifugation at maximum speed (13,000 rpm) for 5 min. After the final wash, the cell pellet was resuspended in 100: L of lysis mixture (10mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5% Tween 20, and 100: g/mL of proteinase K). This mixture was incubated overnight at 56°C, followed by 10 min of boiling to inactivate the Proteinase K and kept at -20°C until needed for PCR for amplification of the 16S rRNA gene (Dean et al., 2008).

The DNA samples from cattle blood were used in PCR reactions (Reverse line blot-PCR) to amplify any *Anaplasma* (or even any *Ehrlichia*) 16S rRNA gene present. One primer set was used to amplify a 309-328 bp fragment being part of the V1 region of the16S rRNA gene. The forward primer was (EF416566) (5'-GAAACTAAGGCCATAAATGACGC -3') for *Mycoplacma haemocanis* and (HQ918288) (5'-ACGAAAGTCTGATGGAGCAATAC-3') for *Candidatus mycoplasma haematoparvum* whereas the reverse primer was (5'- ACCTGTCACCTCGATAACCTCTAC-3') for *Mycoplacma haemocanis* and (5'-TATCTACGCATTCCACCGCTAC-3') for *Candidatus mycoplasma haematoparvum*. The 1xPCR reaction constituents in a final volume of 25: L were as follows: 1xPCR buffer (Invitrogen), 3.0 mM MgCl2 (Invitrogen), 200: M each dATP, dCTP, dGTP, 100: M dTTP (ABgene) and 100: M dUTP (Amersham), 1.25 U of Taq polymerase (Invitrogen), 0.1U of UDG (Amersham), 25 pmol of each primer and 2.5: L of template DN A. This was over laid with about 12.5: L of mineral oil. Positive control DNA (*E. canis*) from Molecular Biology Laboratory, Makerere University and negative control (reaction constituents without DNA) tests were included. The reactions were performed using a three phase touchdown program as previously described (Barker et al., 2009; Brinson et al., 2001). The possible presence of ectoparasites on the dogs was also looked for carefully. Statistical analyses were done using SPSS for Windows.

RESULTS AND DISCUSSION

Clinical signs including temperature, pulsation and respiration rates were in normal ranges. Some of the infected cats had anorexia and weight loss. Microscopic examination revealed *Candidatus mycoplasma haematoparvum* and *Mycoplacma haemocanis* in 26 (8.82%) cats. The animals had no ectoparasites on them. Baseline haematological and biochemical findings did not differ after the treatment. Appearance of *Candidatus mycoplasma haematoparvum* and *Mycoplacma haemocanis* in Geimsa staining of blood smears is presented. According to the results sex had no effect on the infection of the dogs ($P > 0.05$) (Tables1, 2 and 3).

In this study, we used histological methods and novel molecular techniques to determine the regional prevalence and identity of *Candidatus mycoplasma haematoparvum* and *Mycoplacma haemocanis*. Of 294 samples, 66 supported amplification of parasite rRNA by PCR. 25 samples were then examined in parallel by microscopic

Table 1. Distributions of the findings according to the sex of the cats *Candidatus mycoplasma haematoparvum* by pcr

Sex	Number	Positive	%
Female	140	12	8.57
Male	154	17	11.03
Total	294	29	9.86

Table 2. Distributions of the findings according to the sex of the cats *Mycoplacma haemocanis* by pcr

Sex	Number	Positive	%
Female	140	15	10.71
Male	154	18	11.68
Total	294	33	11.22

Table 3. Distributions of the findings according to the sex of the cats *Mycoplacma haemocanis* and *Candidatus mycoplasma haematoparvum* by pcr

Sex	Number	Positive	%
Female	140	26	18.57
Male	154	33	21.42
Total	294	59	20.06

examination and PCR. One samples positive by initial microscopic examination were not amplified by PCR. Thirty-nine PCR-positive samples did not contain sarcocysts on initial microscopic examination, but additional sections from these samples revealed sarcocysts in an additional 12 samples. The partial sequence analysis of the16S rRNA gene (both sequences had 150 bp) indicated a homology of 100% between the PCR amplicons of the positive samples and *Candidatus Mycoplasma haematoparvum*.

The infection was most probably transmitted by ectoparasites (Wood et al., 2006). In conclusion, a rate of haemobartonellosis of 22.45% was determined in dogs and it is believed that haemobartonellosis should always be suspected in dogs presented to veterinary clinics with non-specific symptoms. In addition, serological investigations should also be done in future studies to document the prevalence of the disease in this country.

Polymerase chain reaction (PCR) has been increasingly applied to detect these pathogens in both blood and tick vectors instead of microscopy. Although a number of publications report the use of PCR, most publications are based on 6 original methods for all pathogens (Roura et al.,2005; Sykes et al., 2005; Bauer et al., 2008; Sasaki et al., 2008;). Many reports summarised compare PCR detection with serology to demonstrate assay specificity. However, the most suitable detection method depends upon whether antigen or antibody detection is relevant for the particular investigation, detection of parasites, or current infection, prevalence studies or evidence of exposure to parasites.

Although PCR is more sensitive than light microscopy (Kemming et al., 2004), this method complicated post-PCR detection methods to further enhance the sensitivity and confirm the specificity of the PCR technique such as PCR-ELISA and PCR-probe hybridisation. A quantifiable PCR technique referred to as real time PCR (also known as 5' *Taq* nuclease assays, fluorogenic probe assays, or TaqMan_ assays), are increasingly applied for the detection and identification of animal pathogens and do not require post PCR electrophoresis or processing steps (Gentilini et al.,2009).

Real time assays exploit the 5' nuclease activity of *Taq* DNA polymerase cleaving a dual labelled fluorescent probe which has annealed to a specific sequence between two primers (Francino et al., 2006; Peters et al., 2008; Wengi et al., 2008). To date, the applications of real time PCR for the detection of tick borne disease pathogens have been described for *Theileria spp*. and *Anaplasma spp*. (Taber et al.,2009; Lako et al., 2010). It may not be feasible for certain laboratories to use PCR-ELISA, PCR-probe hybridisation or real time PCR assays as the application of each of these methods requires specific and expensive reagents and equipment. Microscopy remains the most economic and sustainable method of parasite detection for all laboratories.

This is the first case of canine infection with *Candidatus Mycoplasma haematoparvum* in Iran. This organism, named *Candidatus Mycoplasma haematoparvum* is smaller (0.3 _m in diameter) and does not form chains on the erythrocyte surface of dogs (Sykes et al., 2005). The infection has been confirmed by methods of molecular biology (Jensen *et al*., 2001). In France, a 15.4% prevalence of haemoplasma infection in dogs, as well as the presence of coinfection of both haemoplasmas, has been registered using the PCR test (Kenny *et al*., 2004).

Further researches on haemotropic mycoplasmas are indispensable, regarding that: haemoplasmas might act as cofactors in the progression of retroviral, neoplastic and immunologically mediated diseases; the factors of the virulence and pathogenic mechanisms in the development of these infections, as well as the functions of the immunologic system, which is in this case again responsible for the occurrence of new opportunistic infections, are not known (Messick, 2004). Until now, there is no evidence that canine haemoplasmas cause human diseases, but regarding the fact that feline and swine haemotropic mycoplasmas have zoonotic potential, future researches should be conducted in this direction (Xavier Roura et al., 2010; Wu et al., 2006).

The hemoplasma sample prevalence was significantly higher in Switzerland (8.7%) than in Spain (2.5%) (Willi

et al.,2006; Lako et al., 2010; Xavier et al., 2010). Risk factors for infection included living in kennels, young age, crossbreeding, and mange infection. Among the PCR-positive dogs, 40% were infested with blood-sucking arthropods. No association was found with anemia.

ACKNOWLEDGEMENTS

We thank Hamid Reza Arshad Riahi and Manochehr Momeni for their enthusiastic support of this work.

FINANCIAL SUPPORT AND SPONSORSHIP

Nil.

CONFLICT OF INTEREST

There are no conflicts of interest.

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