Evaluation of PCR Assay Using Specific Primers in Diagnosis of Canine Ehrlichiosis and Babesiosis:A Study on Herd and Stray Dogs in Shiraz

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Abstract

Canine tick-borne diseases are regarded as emerging problems within Iran and throughout the world. The present article aims at investigating *Ehrlichia canis and Babesia canis* infections in herd and stray dogs in Iran. The present study is therefore an attempt to compare PCR assay with specific primers in order to detect the organisms at the species level. This study is based on PCR amplification of the 16S rRNA, 18S rRNA and p28 genes. Concerning *Babesia canis*, species-specific primers BAB3 and BAB1 are compared with genus-specific primers B-GA1B and 16S8FE. Moreover, for *Ehrlichia canis,* species-specific primers ECp28-F and ECp28-R are compared with genus-specific primers RLB - F2 and RLB - R2. For the purpose of this study, 280 herd and stray dogs from seven different regions in south of Iran were monitored. As the results revealed, molecular surveillance of tick-borne diseases was based on PCR amplification of the 16S rRNA, 18S rRNA and p28 genes. For *Ehrlichia canis*, PCR was positive with genus-specific primers (B-GA1B and 16S8FE) and species-specific primers (ECp28-F and ECp28-R),1.4% (4 of 280) of blood samples. For *Babesia canis*, PCR was positive with genus-specific primers (RLB - F2 and RLB - R2), 1% (3 of 280) and species-specific primers for *Babesia canis* (BAB1 and BAB3) 0.4% (1 of 280) of blood samples, respectively. Sequencing results showed that one herd dog was infected with *Babesia canis*, four herd dogs were infected with *Ehrlichia canis*, and one herd dog showed co-infection with *Ehrlichia canis and Babesia canis*. This has been the first report of the co-infections of both *Ehrlichia canis and Babesia canis* in naturally infected dogs in South of Iran.

Keywords: *Ehrlichia canis, Babesia canis, Shiraz, Iran*

Introduction

Piroplasmosis and Ehrlichiosis are tick-borne diseases in dogs, caused by *B. canis* and *E. canis*. They are common worldwide zoonosis, concentrated in tropical and subtropical regions due to the geographical distribution of its vector ticks *Rhipicephalus sanguineus* and *Dermacentor reticulatus* (Caeiro 1992; Perez, 2006; Dantas-Torres, 2008). *R. sanguineus* is widely distributed throughout the world. This tick is reported as a common tick in Iran (Rahbari et al., 2007), but there has been inadequate epidemiological data regarding the prevalence of ehrlichiosis in different geographical parts of the country. *E. canis* is recognized in dogs in Iran (Avizeh et al., 2010). Ehrlichiosis is characterized by fever, anorexia, depression, lymphadenopathy, emaciation, bleeding, epistaxis and in some cases death (Neer, and Harrus 2006, Cardenas et al., 2007). Canine babesiosis is an infectious disease caused by protozoans of the genera Babesia that is characterized by fever, anemia and hemoglobinuria. The main hosts, *B. canis* and *E. canis*, are dogs. The parasites are transmitted to the host by

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the bite of ticks and infect the spleen, the liver and lymph nodes. Definitive diagnosis of canine ehrlichiosis and babesiosis are based on hematological, biochemical, and serologic findings. Direct detection of the intracytoplasmatic *E. canis* morulae is a rapid and low-cost diagnosis method in blood smears. The proportion of infected cells in a serological positive animal may be less than 1% and the absence of the *E. canis* morulae do not exclude positive diagnostic. The immunofluorescence assay test (IFAT) and Dot-ELISA are the serology tests usually used to detect antibodies anti- *E. canis* in sera of suspected dogs. Though serology is a sensitive test, it cannot detect current infection from either exposure without establishment of infection or previous infection (Iqbal, Chaichanasiriwithaya, and Rikihisa, 1994), and titers might remain high after infection for an additional period of more than 11 months (Harrus et al., 1998). Because of the disadvantages of these methods, PCR has been used as a complement in the diagnosis of canine ehrlichiosis and babesiosis, due to its high sensitivity and specificity for detection of low levels of *E. canis* DNA (Iqbal, and Rikihisa, 1994). This technique can be used to confirm active infection, and establish failure or success of treatment of Ehrlichia infections (Iqbal, Chaichanasiriwithaya, and Rikihisa, 1994). Many PCR methods for the detection of infections by Ehrlichia species have been described (Chang, and Pan, 1996; Engvall, 1996; Stich, 2002). Primers used to detect Ehrlichia organisms may be generic or species-specific. Generic primers detect many organisms in related genus groups, while specific primers designed to amplify highly variable portions of the genome can be chosen to identify only a particular species of organism (Breitschwerdt et al.,1998).

The PCR protocol, widely employed to detect *E. canis*, is the 16S rRNA PCR, but limited sequence variation of this gene between related bacteria results in unspecific amplification (Sumner, 1997; Whitlock, 2000). Although ehrlichiosis pathogenesis is poorly understood, some studies show that multigene families described in members of the genus Ehrlichia may be involved in the evasion of host immune system by the variation of major surface antigen expression (Mcbride, Yu, and Walker, 2000). The 28 kDa immunodominant outer membrane protein of *E. canis* encoded by a multigene family (p28 gene) has been recently reported, and this gene may be conserved in North America. It is likely that the p28 protein of *E. canis* has a similar location and function to that of the p28 of *E. chaffeensis*. There is evidence that the *E. canis* p28 protein may be a reliable serodiagnostic antigen (Mcbride, Yu, and Walker, 1999). The microscopically direct examination of blood smear is the conventional method used for the differentiation *B. canis*, which is based on the morphometric characteristics. This is a rapid and low-cost method with high specificity, but lacks sensitivity to detect low-level parasitemia (Carret 1999; Dantas-Torres, and Figueredo, 2006). Capillary parasitemias are usually higher than venous parasitemias as frequently found in dogs with *B. canis* infections. Thus capillary samples are the most appropriate diagnostic samples (Bohm et al., 2006).

Recently, technologies based on molecular biology have provided a wide variety of methods that are currently being adopted as alternative tools for the genetic characterization of all sort of pathogens (Gasser, 2006). Among these, the phylogenetic analysis of fragments of the SSU rRNA gene has been frequently applied for molecular studies of *B. canis* isolates (Caccio 2002; Inokuma, and Jefferies, 2003; Oyamada, and Passos, 2005; Gu¨ lanber, and Trapp, 2006; Criado- Fornelio, and Yamasaki, 2007). PCR assays have been validated as alternative methods to achieve reliable species-specific DNA detection of *B. canis* (Martin et al., 2006) with high level of sensitivity and relatively less time-consuming. A study has reported a semi-nested PCR assay to amplify specific DNA fragments varying from 185 bp to 200 bp of either *B. gibsoni*, *B. c. canis*, *B. c. vogeli* or

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B. c. rossi which has been demonstrated to be effective for the detection of these organisms. (Birkenheuer et al., 2003).

The present study, therefore, has been an attempt to evaluate a PCR assay, based on amplification of the *E. canis* p28 gene with species-specific primers, comparing that with PCR method used to amplify the 16S rRNA genes with genus-specific primers. It also aims at comparing PCR assay used to genus-specific primers with species-specific primers for the diagnosis of *B. canis*, based on amplification 18S rRNA genes. It will therefore lead to detect molecular prevalence rate *E. canis* and *B. canis*.

Materials and Methods

Blood samples and DNA extraction

A total of 280 blood samples were monitored. Samples were collected in seven regions of the Southern Iran. Shiraz as the capital of Fars province is located in the South of Iran. Blood samples were removed and stored at −20°C until PCR analysis. Blood smears were prepared and allowed air drying. The smears were fixed in methanol for 5 min and stained with 10% Giemsa to detect morulae of E. canis in monocytes and B. canis in red blood cells.

Selection of PCR primers *B.canis and E.canis*

The primers chosen for this purpose were as follows: partial 18S rDNA sequences of B. canis. A genus forward (BAB1) was then selected from a Babesia conserved region in the 3′ end of the 18S rRNA gene and species-specific reverse primers from variable regions of the large subunit rRNA genes of B. canis (BAB3) could amplify the 714-732 bp open reading frame.(Duarte et al., 2008). Genus-specific Primers used in PCR to amplify a portion of the 18S rRNA gene for Babesia were RLB - F2 and RLB - R2 could amplify the 460- 520 bp open reading frame. (Gubbels et al.,1999). (Table I).

Table 1. Primers used in PCR assays to amplify *Babesia canis*

Gene	Primers	Sequence 5′- 3′
18S rRNA	*BAB1 (f)	5′-GTGAACCTTATCACTTAAAGG-3′
	†BAB3 (r)	5′-CTACACAGAGCACACAGCC-3′
18S rRNA	‡RLB- F2 (f)	5′-ACACAGGGAGGTAGTGACAAG-3′
	§RLB-R2 (r)	5′-CTAAGAATTTCACCTCTGACAGT-3′

* forward genus-specific primers; †reverse species-specific primers; ‡forward genus-specific primers;§genus-specific primers

Specific PCR primers were chosen based on a previously sequenced conserved region to amplify a partial locus of the p28 gene (Mcbride, Yu, and Walker, 1999). Forward primer (ECp28-F) and reverse primer (ECp28-R) could amplify the 843-bp open reading frame. (Nakaghi et al., 2010). Genus-specific Primers used in PCR to amplify a portion of the 16S rRNA gene for Ehrlichia were B-GA1B and 16S8FE could amplify the 492-498 bp open reading frame. (Bekker et al., 2002). (Table II)

Table 2. Primers used in PCR assays to amplify *Ehrlichia canis*

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Gene	Primers	Sequence 5′- 3′
P28	*ECP28-F (f)	5′-ATGAATTGCAAAAAAATTCTTATA-3′
	†ECP28-R (r)	5′-TTAGAAGTTAAATCTTCCTCC-3′
16S rRNA	‡B-GA1B (f)	5′-CGAGTTTGCCGGGACTTYTTCT-3′
	§16S8FE (r)	5′-AGAGTTGGATCMTGGYTCAG-3′

*forward genus-specific primers; †reverse species-specific primers; ‡forward genus-specific primers; §genus-specific primers

DNA isolation, PCR amplification, Sequencing

For PCR amplification of the 16S rRNA gene spanning the V1 region primers 16S8FE and B-GA1B were used as described by Bekker (2002). For p28 gene spanning the specific primers ECp28-F and ECp28-R were used as described by Nakaghi (2010) and of the 18S rRNA gene genus-specific primers RLB F2 and RLB R2 covering the hypervariable region 4 described by Gubbels (1999). BAB1 and BAB3 were used as described by Duarte (2008). The reaction mixture contained 25 µl of REDExract–N-Amp PCR Ready Mix, 0.25 µl of forwarding primer, 0.25 µl of reverse primer, 5 µl of DNA extract and water to a final volume of 50 µl. PCR amplification was performed in an automatic DNA thermocycler (Eppendorf). The reaction was incubated at 94°C for 10 min to denature genomic DNA. The thermal cycle reaction program was as follows: 94°C for 20 sec, 67°C for 30 sec and 72 °C for 30 sec for two cycles. During the subsequent two-cycle sets the annealing temperature was lowered by 2°C until it reached 59°C following a traditional touch-down program. Then, for the next 30 cycles annealing, temperature was 57 °C. The PCR reaction was ended by a final extension at 72°C for 5 min. Samples were held at 4°C until analysis. Samples containing DNA of the various species were used as positive controls and those containing water as negative controls were part of each PCR amplification. PCR products were visualized on 1.2% agarose gels stained with ethidium bromide and under UV light using a Bioimaging system SYNGENE. After amplification generated DNA, fragments were sequenced by Lark Technologies, Inc., Essex. Sequencing reaction contained 200 ng of the template with 1.8 pmol of primer, using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit version 1.1. to final volume 10 µl. Reaction condition's modifications of the companies recommended conditions. The completed sequence reactions are then run on an ABI 3730 xl capillary machine. Data analyzed using ABI sequence analysis software version 5.0.

Statistical analysis

Dogs were grouped based on age, sex, breed, and season to determine whether these factors were associated with the prevalence of E. canis and B. canis. Statistical evaluations were carried out using SPSS 15.0. Chi-square test and Fisher's exact test were computed. Differences were considered significant when $P< 0.05$.

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Results

PCR assays to amplify *E. canis* and *B. canis*

A total of 280 DNA samples were tested by 16S rRNA and p28-based PCR. Amplification of the 16S rRNA gene of Ehrlichia with genus-specific primers was demonstrated by a 492-498 bp fragment (Figure 1), and it was visualized in four samples (1.4%). The Other 276 samples (98.6%) were negative in this PCR. Positive samples were demonstrated by the amplification of an 843-bp fragment of the p28-based PCR gene of E. canis with species-specific primers (Figure 2). Results detected in this PCR system was similar to 16S rRNA -based PCR. All positive samples by p28-based PCR were also detected in 16S rRNA gene. Furthermore, samples were tested by the amplification of the 18S rRNA gene of Babesia with genus-specific primers demonstrated by a 460-520 bp fragment (Figure 3), and it was visualized in three samples (1%). The Other 297 samples (99%) were negative in this PCR. Positive samples were demonstrated by the amplification of a 714-732 bp (BAB1/BAB3-B. canis fragment of the 18S rRNA gene of B. canis (Figure 4). Amplification of the 18S rRNA gene with B. canis-specific primers was visualized in one sample (0.4%). The Other 279 samples (99.6%) were negative in this PCR. 1 out of 6 (16.7%) were Co-infection E. canis and B. canis. Co-infection E. canis and B. canis were found 1 of 280 samples (0.4%), in one adult male dog ≥ 3 years-old.

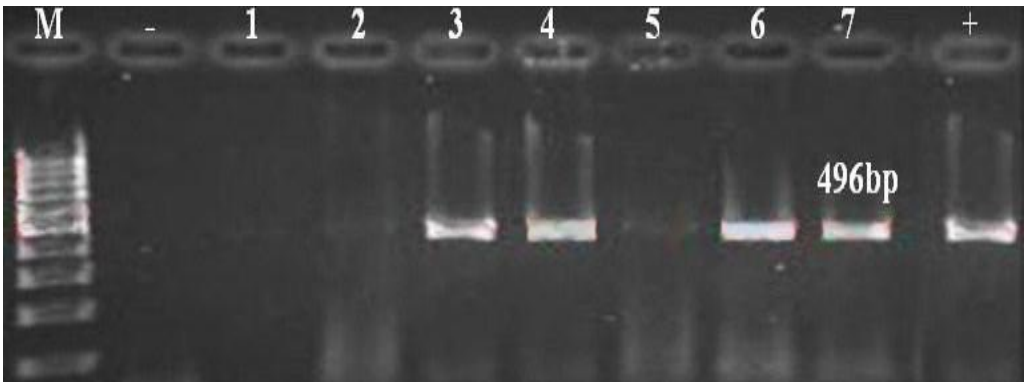

Figure 1. *Ehrlichia* PCR with genus-specific primers B-GA1B/ 16S8FE by 1.2% agarose gel electrophoresis. M, 100 bp DNA ladder; lane 3,4,6 and 7 positive; +, positive control; -, negative control.

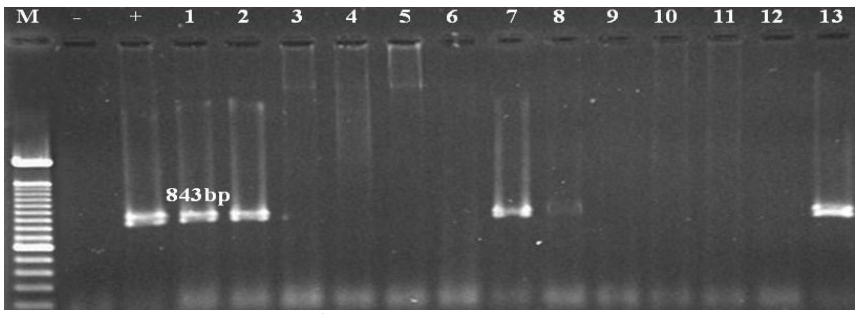

Figure 2. *Ehrlichia canis* PCR with species-specific primers ECp28-F/ ECp28-R by 1.2% agarose gel electrophoresis. M, 100 bp DNA ladder; lane 1,2,7 and 13 *E.canis*; +, positive control; -, negative control.

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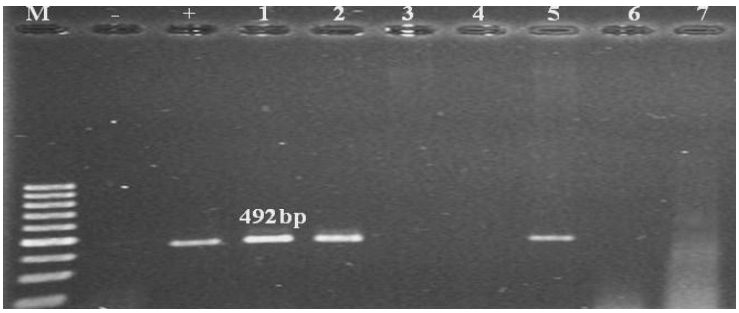

Figure 3. *Babesia* PCR with genus-specific primers RLB-F2/RLB-R2 by 1.2% agarose gel electrophoresis. M, 100 bp DNA ladder; lane 1,2 and 5 positive; +, positive control; -, negative control.

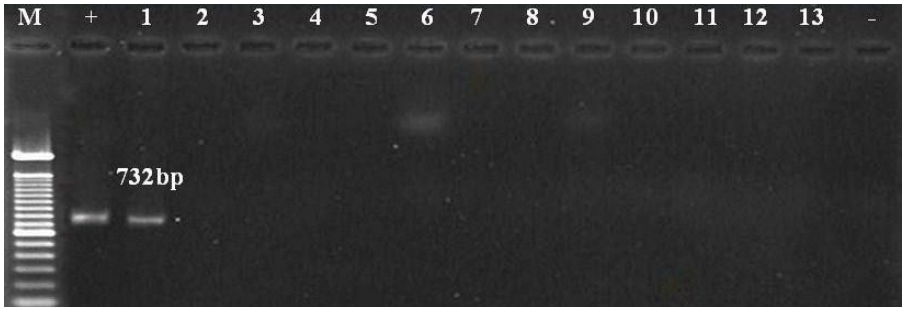

Figure 4. *Babesia canis* PCR with species-specific primers BAB1/BAB3 by 1.2% agarose gel electrophoresis. M, 100 bp DNA ladder; lane 1, *B.canis*; +, positive control; -, negative control.

Molecular prevalence rate *E. canis* and *B. canis*

Molecular prevalence rate E. canis was in herd dogs 2.9% (4 of 140) and positive sample was not observed in stray dogs. There were significant differences between maintenance environment in the herd and stray dogs and prevalence of infection ($P<0.05$). Molecular prevalence rate B. canis was in herd dogs 0.7% (1 of 140). There were not any significant differences between the molecular prevalence rate of infection and maintenance environment. ($P>0.05$). The positive sample was not visualized in stray dogs. Molecular prevalence rate E. canis was higher in male dogs (2.1%) than female dogs (0.7%), and B. canis was higher in male dogs (0.7%). There were not significant differences between the molecular prevalence rate of infection and host gender, season and region ($P>0.05$). Molecular prevalence rate E. canis was significantly higher in adult dogs, 3 years old (4.1%) compared with 1-3 years old dogs ($P= 0.05$) and young dogs of 1 year old ($P<0.05$). B. canis was found in adult ≥ 3 years old ($P>0.05$).

Sequencing

Sequencing results from four samples of blood showed that four samples from dogs were infected with *E. canis* (97-98%, identities with *E. canis*, GeneBank accession numbers AF082744.2 and EF014897.1), one sample with *B. canis* (98-99%, identities with *E. canis* GenBank accession numbers HQ148664.1 and AY371197.1). *E. canis* was identified in Bidzard, Dariun and Dinakan, *B. canis* in Bidzard. Co-infection *E. canis* and *B. canis* were found in one region-Bidzard.

Discussion

In this study, canine babesiosis and ehrlichiosis were diagnosed when climate conditions favor the activity of Dermacentor and Rhipicephalus sp. ticks (Caeiro1992; Perez, 2006; Dantas-Torres, 2008). History of travel outside this area, where canine ehrlichiosis and babesiosis were endemic, was not obtained for any of the dogs. This situation supports the assumption that infections with B. canis and E. canis and the other vector-borne agents were acquired locally. In addition, to our best knowledge, this is the first report of molecular identification of B. canis and E. canis in dogs from south of Iran. B. canis was detected as (0.4%) and E. canis as (1.4%) in 280 herd and stray dogs. This is in agreement with other studies conducted in Iran (Ashrafi et al., 2001). In fact, only 0.3% of the dogs studied by Ashrafi (2001) were clinically suspected of B. canis, and infection with Babesia sp. was not assessed molecularly by the researcher. This could be due to a lower prevalence of infected dogs or tick vectors in the study area, in comparison to Babesia sp. in other animals in Iran. Prevalence of ehrlichiosis was reported from other regions of Iran. Akhtardanesh (2009) noted that the seroprevalence of canine monocytic ehrlichiosis using IFA and ICA was 13.8% and 10.6%, respectively.

In West Azerbaijan, 67% of wild dogs and 38% of domestic dogs were serologically positive for E. canis. These rates of infection reported 58% and 39%, respectively, in east Azerbaijan (Asri and Mahmoudian, 2001). Studies indicate that the incidence of ehrlichiosis can vary greatly between countries and regions. The high seroprevalence of E. canis infection was detected as 21% in Turkey (Batmaz et al., 2001), whereas low prevalence (2.9- 9.7%) was obtained in Italy (Solano-Gallego et., al 2006). In the present study, the differences between these low prevalence rates for E. canis (1.4%) as detected by molecular methods, and those observed in the present study may be explained by a different sample population, and the methods used. In this study, infection with E. canis was more prevalent than with B. canis. Due to relatively lower parasite loads of B. canis and E. canis in the blood, compared with other tissues, the use of blood to assess infection with B. canis and E. canis may demonstrate limited sensitivity of detection. In this study, However, the use of highly sensitive quantitative PCR for B. canis and E. canis with specific primers probably improved the prospects of detection, when compared to other conventional methods. In the present study, B. canis and E. canis were not detected in blood smears. Parasites were not detected in the smears of four dogs which were found infected with E. canis. Moreover, 1 of four dogs was detected with co-infection with B. canis and diagnosed by PCR and sequencing. Microscopy may lack sensitivity in dogs clinically suspected of babesiosis and ehrlichiosis, possibly due to low parasitaemia. We used PCR assays based on the p28, 16S rRNA, and 18S rRNA genes of E. canis and B. canis species infecting dogs, mainly in the tropics and subtropics. The detection was carried out by species-specific primers, and no cross-reactions were observed. The catchall E. canis and B. canis control is of importance in case a PCR product is amplified. Dog blood sample infected by B. canis and E. canis as a representative was used, but no specific reaction was seen. Additional species or strains can be identified by the PCR with species-specific, and subspecies-specific primers should be subsequently sequenced to identify these organisms, and if necessary, they can be included in the assay. The previously described molecular methods applied to discriminate among the species of B. canis were either based on RFLP-PCR technique (Zahler, 1998; De Sa´, 2006) or on phylogenetic analysis of PCR products (Caccio 2002; Inokuma, 2004; Oyamada, 2005; Passos, 2005; Gu¨ lanber, 2006; Criado-Fornelio, 2007; Yamasaki, 2007). These methods are recommended for definite genetic characterization of the organism. The results obtained from this study give support to PCR

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assay with the specific-primers pair to be used as reliable tools for species detection and discrimination of B. canis and E. canis. These methods account for less time-consuming and cheaper procedures for the molecular diagnosis of canine babesiosis and ehrlichiosis at the species level of B. canis and E. canis that could be steadily applied for further research on different aspects of the disease such as etiology, geographic distribution, molecular epidemiology, etc. The assays used in this paper were PCR products of wide and clear differences between the species (B. canis = 732 bp and E. canis= 843 bp) to reinforce the specificity of the results while testing large numbers of samples. This feature may also facilitate the simultaneous use of these primer pairs in a multiplex PCR from where the results could be instantly read and concluded.

The PCR results in the specificity evaluation for each primer's pair (BAB1/BAB3 and ECp28-F/ ECp28-R) strongly demonstrated the lack of B. canis and E. canis species PCR cross-amplification as well as with other common dog haemoparasites, i.e., H. canis, T. evansi, M. haemocanis and B. gibsoni. Ehrlichia infections can mimic several other diseases, and nonspecific clinical signs make the diagnosis difficult (Cohn, 2003). The diagnosis of ehrlichiosis can be achieved by a combination of clinical and hematological presentation, serology and molecular biology tests (Nakaghi et al., 2008). DNA amplification by PCR has improved the sensitivity and specificity of the diagnosis of ehrlichiosis (Iqbal, Chaichanasiriwithaya, and Rikihisa, 1994). There have been many reports about PCR techniques for the detection of E. canis DNA, but only a few studies compared their sensitivity and specificity. To determine the sensitivity and specificity of the PCR to detect gene p28, a comparison with 16S rRNA-based PCR was performed. In this study, the p28 PCR system exhibited sensitivity like the 16S rRNA-based PCR assay. PCR assays with species-specific sequences, rather than highly conserved bacterial sequences, are likely to provide sensitive and specific tests (Wagner et al., 2004). However, this method can result in unspecific amplification due to the highly conserved 16S rRNA gene among strains of species of Ehrlichia, and it is at higher risk of DNA cross-contamination (Labruna et al.,2007). Therefore, a PCR assay that amplifies the p28 gene seems to be the most specific method to detect E. canis DNA. The only dog found infected with B. canis in our study also had the co-infection with E. canis. Dogs in our study had not clinical signs for B. canis and E. canis. It is possible that chronic subclinical infection with B. canis and E. canis are not clinically apparent by these co-infections. The co-infected dog may have been exposed to arthropods infected with single pathogen species at different points in time or to vector(s) concurrently infected with multiple agents (Otranto et al., 2009). Co-infections with tick-borne organisms may affect the severity of canine vector-borne disease and the variety of associated clinical signs (Tabar et al., 2009). In a study, it was reported that there was a significant difference between animals with dual infection and those with single infection (Mekuzas et al., 2009). However, the clinical signs of co-infections with two or more vector-borne organisms are often difficult to be specifically assigned to each one of the infecting agents (Kordick et al., 1999).

In the present study, although a complete clinical-pathological evaluation was not performed, especially blood cell counts, no significant differences among HCT values were found between the co-infected dogs, and those with one single infection detected. In the recent investigation, prevalence was significantly differed among age groups. This is in line with other studies (Watanabe 2004; Rodriguez-Vivas, 2005; Costa, 2007). Possible explanations can be attributed to the increased probability of a dog being exposed to E. canis. In some other studies, age was not associated with E. canis (Matthewman 1993; Inokuma, 1999; Waner, 2000).

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Seroprevalence of infection in male dogs (2.1%) was higher than females (0.7%), though, it was not significantly differed. It can be explained by the territorial habits associated with males, as they have a wider area of operation than females, and the result was in line with some other studies (Waner 2000; Watanabe, 2004; Rodriguez-Vivas, 2005; Solano- Gallego, 2006). However, findings were not in agreement with Batmaz (2001). Sainz (1996) reported no difference between breeds, though, he found a correlation between the utility of dogs and seropositivity to E. canis. In the present study, as the findings demonstrated, there was a low prevalence (1.4%) of E. canis infection. However, the results were in agreement with the low prevalence of infection (3.5%) in dogs in Rio de Janeiro, Brazil (Macieira et al., 2005).

A study in urban and rural dogs (n = 651) from Hungary revealed a 6% seropositivity to B. canis (Hornok et al.,2006). In the present study, B. canis was found in one old male herd dog, only. A herd dog littermates, aged 3 years, was found co-infected with B. canis and E. canis. This finding could suggest the possibility of keeping environment and having contact with the domestic animal transmission. However, it should be regarded as the most likely source of transmitting Babesia and Ehrlichia to them. E. canis was found in male and female old herd dogs. There was no clear distinction of age and sex between single infected and co-infected dogs. Rather than a genetic or breed predisposition, this situation probably reflects the fact that these dog breeds and crosses are popular and over-represented in the southern part of Iran. Also, a considerable percentage of these dogs lives outdoors and are used for herding activities in the field, where they face a higher risk of contact with infected arthropod vectors. Nevertheless, it is due to the increasing mobility of dogs and the existence of competent or presumptive vectors (Otranto et al., 2009).

1.4% and 0.4% of the blood from Iranian herd and stray dogs were infected by tick-borne bacteria and protozoa, respectively. Sequencing analysis showed infection by E. canis and B. canis occurred in blood samples of dogs in monitored regions of southern Iran. Veterinarians should be aware that babesiosis and ehrlichiosis seem to be endemic in Iran. Since babesiosis and ehrlichiosis are a zoonotic disease that is mostly transmitted by ticks Dermacentor reticulatus and Rhipicephalus sanguineus, a complex study has to be done, where both dogs and humans are included with an appropriate sample size and the evaluation of risk factors. Furthermore, preventive and control measures to control ticks should be established in order to minimize the risk of infection.

Conclusion

It is concluded that further investigation and additional molecular studies are necessary to identify the strains of the organism. Development of PCR assays can provide sensitive and/or specific tests to be used in the diagnosis of experimental or natural infections. The p28-based and 18S rRNA-based PCR seem to be useful tests for the molecular detection of E. canis, and B. canis. However, studies with primers targeting a specific region inside gene p28, and 18S rRNA may improve the sensitivity of this method that in return it can be an important alternative in the diagnosis of canine ehrlichiosis and babesiosis. This study confirmed the presence of B. canis and E. canis as agents of babesiosis and ehrlichiosis in dogs in south of Iran. A higher sensitivity of Babesia and Ehrlichia sp. detection was obtained by the use of PCR assays, compared to microscopy of blood smears. Co-infections with some other vector-borne agents were also detected and molecularly characterized.

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Detection and identification of species and subspecies of pathogens, either in single or in co-infection are necessary for the treatment, clinical management, and prevention of canine vector-borne disease.

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References

Akhtardanesh B., Ghanbarpour R., and Blourizadeh H. Serological evidence of canine monocytic in ehrlichiosis Iran. Comparative Clinical Pathology, 2009; 24: 889-895.

Ashrafi H. J., H. R. Haddadzadeh, D. Shirani, P. Khazraiinia, and S. Mostofi. Histopathologic, hematologic and clinical study on canine Babesiosis. Journal of the faculty of Veterinary Medicine University of Tehran, 2001; 56: 93-96.

Asri S., and A. Mahmoudian. Serological study of Canine Ehrlichiosis in western and eastern Azerbaijan's of Iran. 2001; 26th WSAVA Congress. proceedings, Vancouver, British, Columbia, Canada.

Avizeh R., B. Mosallanejad, M.H. Razi Jalali, and A.R. Alborzi. Seroprevalence of Ehrlichia canis in dogs referred toVeterinary Hospital of Shahid Chamran University of Ahvaz, Iran. Archives of Razi Institute, 2010; 65: 21-26.

Batmaz H., E. Nevo, T. Waner, S. Senturk, Z. Yilmaz, and S. Harrus. Seroprevalence of Ehrlichia canis antibodies among dogs in Turkey. The Veterinary Record, 2001; 148: 665-666.

Bekker C.P.J., S. de Vos, A. Taoufik, O.A.E. Sparagano, and F. Jongejan. Simultaneous detection of Anaplasma and Ehrlichia species in ruminants and detection of Ehrlichia ruminantium in Amblyomma variegatum ticks by reverse line blot hybridization. Vet. Microbiol, 2002; 89: 223-238.

Birkenheuer AJ., MT. Correa, MG. Levy, and EB. Breitschwerdt. Geographic distribution of babesiosis among dogs in the United States and association with dog bites. J Am Vet Med Assoc, 2003; 227: 942-947.

Bohm M., A.L. Leisewitz, P.N. Thompson, and J. Schoeman. Capillary and venous Babesia canis rossi parasitaemias and their association with outcome of infection and circulatory compromise. Vet. Parasitol, 2006; 141: 18–29.

Breitschwerdt E., B. B. C. Hegarty, and S. I. Hancock. Doxycycline hyclate treatment of experimental canine ehrlichiosis followed by challenge inoculation with two Ehrlichia canis strains. Antimicrobial Agents Chemother, 1998; 42: 362-368.

[DOR: 20.1001.1.2717378.2017.1.1.1.7]

Caccio S.M., B. Antunovic, A. Moretti, A. Mangili, A. Marinculic, R.R. Baric, S.B. Slemenda, and N.J. Pieniazek. Molecular characterization of Babesia canis canis and Babesia canis vogeli from naturally infected European dogs. Vet. Parasitol, 2002; 106:285–292.

Caeiro V.M.P. As carraças em Portugal: seus hospedeiros domésticos e silvestres. Ciclos vitais, preferências de vegetação e clima. Med Vet, 1992; 28:7-25.

Cardenas A.M., C.K. Doyle, X. Zhang, K. Nethery, R.E. Corstvet, D.H. Walker, and J.W. McBride. Enzyme-linked immunosorbent assay with conserved immunoreactive glycoproteins gp36 and gp19 has enhanced sensitivity and provides species-specific immunodiagnosis of Ehrlichia canis infection. Clin Vaccine Immunol, 2007; 14:123-128.

Carret C., F. Walas, B. Carey, N. Grande, E. Precigout, K. Moubri, T.P. Schetters, and A. Gorenflot. Babesia canis canis, Babesia canis vogeli, Babesia canis rossi: differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small subunit ribosomal RNA genes. J. Euc. Microbiol, 1999; 46:298–303.

Chang W. L., and M. J. Pan. Specific amplification of Ehrlichia platys DNA from blood specimens by two-step PCR. Journal Clinical Microbiology, 1996; 34: 3142-3146.

Cohn L. A. .Ehrlichiosis and related infections. Veterinary Clinics Small Animals, 2003; 33:863-884.

Costa L.M., K. Rembeck, M.F. Ribeiro, P. Beelitz, K. Pfister, and L.M. Passos. Seroprevalence and risk indicators for canine ehrlichiosis in three rural areas of Brazil. The Veterinary Journal, 2007; 174: 673-676.

Criado-Fornelio C. Rey-Valeiron, A. Buling, J.C. Barba-Carretero, R. Jefferies, and P. Irwin. New advances in molecular epizootiology of canine hematic protozoa from Venezuela, Thailand and Spain. Vet. Parasitol, 2007; 144: 261–269.

Dantas-Torres F., and L.A. Figueredo. Canine babesiosis: a Brazilian perspective. Vet. Parasitol, 2006; 141: 197–203.

Dantas-Torres F. The brown dog tick, Rhipicephalus sanguineus (Acari: Ixodidae): from taxonomy to control. Veterinary Parasitology, 2008; 152:173-185.

De Sa´ A.G., A.M.F. Cerqueira, L.H. O'dwyer, D.B. Macieira, F.S. Abreu, R.F. Ferreira, A.M. Pereira, P.B. Velho, and N.R.P. Almosny. Detection and molecular characterization of Babesia canis vogeli from naturally infected Brazilian dogs. Int. J. Appl.Res. Vet. Med, 2006; 4: 163–168.

Duarte S.C., G.F.C. Linhares, T. N. Romanowsky, O. J. S. Neto, and L. M. F. Borges. Assessment of primers designed for the subspecies-specific discrimination among Babesia canis canis, Babesia canis vogeli and Babesia canis rossi by PCR assay. Veterinary Parasitology, 2008; 152:16–20.

Engvall E. O. et al. A 16S rRNA-based PCR assay for detection and identification of granulocytic Ehrlichia species in dogs, horses, and cattle. Journal Clinical Microbiology, 1996; 34: 2170-2174.

Gasser R.B. Molecular tools advances, opportunities and prospects. Vet. Parasitol, 2006; 136: 69–89.

Gubbels M.J., A.P. de Vos, M. vander Weide, J. Viseras, L.M. Schouls, E.de Vries, and F. Jongejan. Simultaneous detection of bovine Theileria and Babesia species by reverse line blot hybridization. J. Clin. Microbiol, 1999; 37:1782-1789.

Gulanber A., A. Gorenflot, T.P.M. Schetters, and B. Carcy. First molecular diagnosis of Babesia vogeli in domestic dogs from Turkey. Vet. Parasitol, 2006; 139:224–230.

Harrus S. et al. Amplification of ehrlichial DNA from dogs 34 months after infection with Ehrlichia canis. Journal Clinical Microbiology, 1998; 36:73-76.

Hornok S., R. Edelhofer, and R. Farkas. Seroprevalence of canine babesiosis in Hungary suggesting breed predisposition. Parasitol Res, 2006; 99:638-642.

Iqbal Z., W. Chaichanasiriwithaya, and Y. Rikihisa. Comparison of PCR with other tests for early diagnosis of canine ehrlichiosis. Journal Clinical Microbiology, 1994; 32:1658-1662.

Inokuma H., K. Ohno, and S. Yamamoto. Serosurvey of Ehrlichia canis and Hepatozoon canis infection in dogs in Yamaguchi Prefecture, Japan. The Journal of Veterinary Medical Science, 1999; 61: 1153-1155.

Inokuma H., T. Beppu, M.Okuda, Y. Shimada, and Y. Sakata. Epidemiological survey of Anaplasma platys and Ehrlichia canis using ticks collected from dogs in Japan. Vet. Parasitol, 2003; 115:343–348.

Inokuma H., Y. Yoshizaki, K. Matsumoto, M. Okuda, T. Onishi, K. Nakagome, R. Kosugi, and M. Hirakawa. Molecular survey of Babesia infection in dogs in Okinawa, Japan. Vet. Parasitol, 2004; 121: 341–346.

Jefferies R., U.M. Ryan, C.J. Muhinickel, and P.J. Irwint. Two Species of Canine Babesia in Australia: detection and characterization by PCR. J. Parasitol, 2003; 89: 409–412.

Kordick SK., EB. Breitschwerdt, BC. Hegarty, KL. Southwick, CM. Colitz, SI. Hancock, JM. Bradley, R. Rumbough, JT. McPherson, and JN. MacCormack. Coinfection with multiple tick-borne pathogen in a Walker Hound kennel in North Carolina. J Clin Microbiol, 1999; 37:2631-2638.

Labruna M. B. et al. A preliminary investigation of Ehrlichia species in ticks, humans, dogs, and capybaras from Brazil. Veterinary Parasitology, 2007; 143:189-195.

Linhares G.F.C., A.P. Santana, L.H. Lauerman, and C.R. Madruga. Assessment of primers designed from the small ribosomal subunit RNA for specific discrimination between Babesia bigemina and Babesia bovis by PCR. Cieˆn. An. Bras, 2002; 3:27–32.

Macieira D.B., J.B. Messick, A.M. Cerqueira, I.M. Freire, G.F. Linhares, N.K. Almeida, and N.R. Almosny. Prevalence of Ehrlichia canis infection in thrombocytopenic dogs from Rio de Janeiro, Brazil. Veterinary Clinical Pathology, 2005; 34:44-48.

Martin A.R., R.H. Dunstan, T.K. Roberts, G.K. Brown. Babesia canis vogeli: a novel PCR for its detection in dogs in Australia. Exp. Parasitol, 2006; 112:63–65.

Matthewman L.A., P.J. Kelly, P.A. Bobade, M. Tagwira, P.R. Mason, A. Majok, P. Brouqui, and D. Raoult. Infections with Babesia canis and Ehrlichia canis in dogs in Zimbabwe. The Veterinary Record, 1993; 133: 344-346.

McBride J. W., X. J. Yu, and D. H. Walker. Molecular cloning of the gene for a conserved major immunoreactive 28-kilodalton of Ehrlichia canis: a potential serodiagnostic antigen. Clinical and Diagnostic Laboratory Immunology, 1999; 6:392-399.

McBride J. W., X. J. Yu, and D. H. Walker. A conserved, transcriptionally active p28 multigene locus of Ehrlichia canis. Gene, 2000; 254:254-252.

Mekuzas Y., L. Gradoni, G. Oliva, V. F. Manzillo, and G. Baneth. Ehrlichia canis and Leishmania infantum co-infection: a 3-year longitudinal study in naturally exposed dogs. Clin Microbiol Infect, 2009; 2:30-31.

Nakaghi A. C. H. et al. Canine ehrlichiosis: clinical, hematological, serological and molecular aspects. Ciência Rural, 2008; 38: 766‑770.

Nakaghi A. C. H., R. Z. Machado, J. A. Ferro, M. B. Labruna, A. L. Chryssafidis, M. R. André, and C. D. Baldani. Sensitivity evaluation of a single-step PCR assay using Ehrlichia canis p28 gene as a target and its application in diagnosis of canine ehrlichiosis. Vet. Jaboticabal, 2010; 19: 75-79.

Neer T.M., and S.H. Harrus. Canine monocytotropic Ehrlichiosis and Neorickettsiosis. In Infectious diseases of the dog and cat, C.E. Greene (3rd eds.). W.B. Saunders Co, St. Louis, Philadelphia, 2006; p. 203-219.

Ogliari J.B., R.L. Boscariol, and L.E.A. Camargo. Optimization of PCR amplification of maize microsatellite loci. Gen. Mol. Biol, 2000; 23:395–398.

Oyamada M., B. Davoust, M. Boni, J. Dereure, B. Bucheton, A. Hammad, K. Itamoto, M. Okuda, and H. Inokuma. Detection of Babesia canis rossi, B. canis vogeli, and Hepatozoon canis in Dogs in a Village of Eastern Sudan by using a screening PCR and sequencing methodologies. Clin. Diag. Lab. Immunol, 2005; 12:1343–1346.

[DOR: 20.1001.1.2717378.2017.1.1.1.7]

Otranto D., F. Dantas-Torres, and EB. Breitschwerdt. Managing canine vector- borne diseases of zoonotic concern: part one. Trends Parasitol, 2009; 25:157-163.

Otranto D., F. Dantas-Torres, and EB. Breitschwerdt. Managing canine vector- borne diseases of zoonotic concern: part two. Trends Parasitol, 2009; 25:228-235.

Passos L.M.F., S.M. Geiger, M.F.B. Ribeiro, K. Pfister, and M. Zahler-Rinder. First molecular detection of Babesia vogeli in dogs from Brazil. Vet. Parasitol, 2005; 127: 81–85.

Perez M., M. Bodor, C. Zhang, Q. Xiong, and Y. Rikihisa. Human infection with Ehrlichia canis accompanied by clinical signs in Venezuela. Annals of the New York Academy of Sciences, 2006; 1078: 110-117.

Persing D. Target selection and optimization of amplification reactions. In Diagnostic Molecular Microbiology Principles and Applications, D.H. Persing, T.F. Smith, F.C.Tenover, and T.J. White (eds.). Library of Congress Cataloging-in-Publication Data, Washington, 1993; p. 88–104.

Rahbari S., S. Nabian, and P. Shayan. Primary report on the distribution of tick fauna in Iran. Parasitology Research, 2007; 101: 175-177.

Rodriguez-Vivas R.I., R.E.F. Albornoz, and G.M.E. Bolio. Ehrlichia canis in dogs in Yucatan, Mexico: seroprevalence, prevalence of infection and associated factors. Veterinary Parasitology, 2005; 127: 75-79.

Sainz A., S. Delgado, I. Amusategui, M. Tesouro, and P. Cármenes. Seroprevalence of canine ehrlichiosis in Castilla-León (north-west Spain). Preventive Veterinary Medicine, 1996; 29: 1-7.

Solano-Gallego L., M. Trotta, L. Razia, T. Furlanello, and M. Caldin. Molecular survey of Ehrlichia canis and Anaplasma phagocytophilum from blood of dogs in Italy. Annals of the New York Academy of Sciences, 2006; 1078: 515-518.

Stich R. W. et al. Detection of Ehrlichia canis in canine carrier blood and in individual experimentally infected ticks with a p30-based PCR assay. Journal Clinical Microbiology, 2002; 40: 540-546.

Sumner J. W., W. L. Nicholson, and R. F. Massung. PCR amplification and comparison of nucleotide sequences from groESL shock operon of Ehrlichia species. Journal Clinical Microbiology, 1997; 35:2087-2092.

Tabar MD., O .Francino, L. Altet, A. Sánchez, L. Ferrer, X. Roura. PCR survey of vectorborne pathogens in dogs living in and around Barcelona, an area endemic for leishmaniosis. Vet Rec, 2009; 164:112-116.

Trapp S.M., J.B. Messick, O.Vidotto, F.S. Jojima, and H.S.A. Morais. Babesia gibsoni genotype A´ sia in dogs from Brazil. Vet. Parasitol, 2006; 141: 177–180.

[DOR: 20.1001.1.2717378.2017.1.1.1.7]

Wagner E. T. Development of a p28-based PCR assay for Ehrlichia chaffeensis. Molecular and Cellular Probes, 2004; 18:111-116.

Waner T., I. Leykin, M. Shinitsky, E. Sharabani, H. Buch, A. Keysary, H. Bark, and S. Harrus. Detection of platelet-bound antibodies in beagle dogs after artificial infection with Ehrlichia canis. Comparative Clinical Pathology and Immunology, 2000; 77:145-150.

Watanabe M., M. Okuda, M. Tsuji, and H. Inokuma. Seroepidemiological study of canine ehrlichial infections in Yamaguchi prefecture and surrounding areas of Japan. Veterinary Parasitology, 2004; 124: 101-107.

Whitlock J. E. Prevalence of Ehrlichia chaffeensis (Rickettsiales: Rickettsiaceae) in Amblyomma americanum (Acari: Ixodidae) from the Georgia coast and barrier Islands. Journal Medicine Entomology, 20003; 7: 276-280.

Yamasaki M., H. Inokuma, C. Sugimoto, S.E. Shaw, M. Aktas, M. J. Yabsley, O. Yamato, and Y.C. Maede. Comparison and phylogenetic analysis of the heat shock protein 70 gene of Babesia parasites from dogs. Vet. Parasitol, 2007; 145: 217–227.

Zahler M., E. Schein, H. Rinder, and R. Gothe. Characteristic genotypes discriminate between Babesia canis isolates of differing vector specificity and pathogenicity to dogs. Parasitol. Res, 1998; 84: 544–548.

[DOR: 20.1001.1.2717378.2017.1.1.1.7]