

# Sensitivity evaluation of a single-step PCR assay using *Ehrlichia canis* p28 gene as a target and its application in diagnosis of canine ehrlichiosis

Avaliação da sensibilidade da PCR em uma etapa com base no gene p28 de *Ehrlichia canis* e sua aplicação no diagnóstico da erliquiose canina.

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Recebido em 19 de Outubro de 2009

Aceito em 26 de Abril de 2010

## Abstract

The aim of this study was to optimize a PCR assay that amplifies an 843 pb fragment from the p28 gene of *Ehrlichia canis* and compare it with two other PCR methods used to amplify portions of the 16S rRNA and dsb genes of *Ehrlichia*. Blood samples were collected from dogs suspected of having a positive diagnosis for canine ehrlichiosis. Amplification of the p28 gene by PCR produced an 843-bp fragment and this assay could detect DNA from one gene copy among 1 billion cells. All positive samples detected by the p28-based PCR were also positive by the 16S rRNA nested-PCR and also by the dsb-based PCR. Among the p28-based PCR negative samples, 55.3% were co-negatives, but 27.6% were positive in 16S rRNA and dsb based PCR assays. The p28-based PCR seems to be a useful test for the molecular detection of *E. canis*, however improvements in this PCR sensitivity are desired, so that it can become an important alternative in the diagnosis of canine ehrlichiosis.

**Keywords:** *Ehrlichia canis*, PCR, p28, dsb gene, 16S rRNA gene.

## Resumo

O objetivo deste estudo foi aperfeiçoar um ensaio de PCR que amplificasse um fragmento de 843 pares de bases do gene p28 da *Ehrlichia canis* e compará-lo com outros dois métodos de PCR utilizados para amplificar partes do gene 16S rRNA e dsb do gênero *Ehrlichia*. Amostras sanguíneas foram colhidas de cães com diagnóstico clínico de erliquiose. A amplificação do gene p28 pela PCR produziu um fragmento de 843pb e esse ensaio permitiu a detecção do DNA de um parasita dentre 1 bilhão de células. Todas as amostras positivas detectadas pela PCR baseada no gene p28 foram também positivas pela nested PCR para detecção do gene 16S rRNA e também pela PCR dsb. Dentre as amostras negativas para a PCR p28, 55,3% foram co-negativas, mas 27,6% foram positivas pela PCR baseada nos genes 16S rRNA e dsb. A PCR p28 parece ser um teste útil para detecção molecular de *E. canis*, entretanto otimizações na sensibilidade nesta PCR são necessárias, para que esta técnica se torne uma importante alternativa no diagnóstico da erliquiose canina.

**Palavras-chave:** *Ehrlichia canis*, PCR, p28, gene dsb, gene 16S rRNA.

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## Introduction

Canine monocytic ehrlichiosis is an important tick-borne disease caused by the obligate intracellular bacterium *Ehrlichia canis*, which has worldwide distribution especially in tropical and subtropical areas. In Brazil, this parasite was first described in 1973 (COSTA, 1973), and is the most common species among the *Ehrlichia* genus (DAGNONE et al., 2003; MACHADO, 2004; LABRUNA et al., 2007; NAKAGHI et al., 2008).

Definitive diagnosis of canine ehrlichiosis is based on hematological, biochemical, and serologic findings. Direct detection of the intracytoplasmatic *E. canis* morulae in blood smears is a rapid and low cost diagnosis method, however, according to Cowell et al. (1988), 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 (EWING, 1969). 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. Despite of serology is a sensitive test, it cannot distinguish current infection from either exposure without establishment of infection or previous infection (IQBAL; CHAICHANASIRIWITHAYA; 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, due to its high sensitivity and specificity for detection of low levels of *E. canis* DNA (Iqbal; Rikihisa, 1994). This technique can be used to confirm active infection, and establish failure or success of treatment of ehrlichial infections (IQBAL; CHAICHANASIRIWITHAYA; RIKIHISA, 1994).

A single-step PCR targeting *E. canis* 16S rRNA gene in blood and tissues of experimentally infected dogs was often not sensitive enough to detect a few organisms, therefore, nested-PCR test was performed to increase sensitivity and specificity in the detection of *E. canis* in blood specimens (WEN et al., 1997; HARRUS et al., 1998; NAKAGHI et al., 2008; SEAMAN et al., 2004). However, nested-PCR has the disadvantage of a high risk for sample cross-contamination and detection of false positives (KWOK; HIGUCHI, 1989; LABRUNA et al., 2007). Single-step PCR assays offer several advantages over nested PCR, including reduced labor, reduced cost and cross-contamination, and adaptability to quantitative technique such as competitive or real-time PCR (WAGNER et al., 2004).

Many PCR methods for the detection of infections by *Ehrlichia* species have been described (CHANG; PAN, 1996; ENGVALL et al., 1996; STICH et al., 2002; DUMLER, 2003). Primers used to detect ehrlichial organisms may be generic or species-specific. Generic primers detect many of the 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 nested-PCR, but limited sequence variation of this gene between related bacteria results in unspecific amplification (SUMNER et al., 1997; WHITLOCK et al., 2000). A highly

sensitive and specific real-time PCR was used, targeting the *Ehrlichia* disulfide bond formation protein (*dsb*) gene, which was identical to the corresponding sequence of the North American isolate (LABRUNA et al., 2007). This *dsb* PCR protocol has been successfully employed in conventional PCR analyzed in agarose gel (AGUIAR et al., 2007).

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; 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 are evidences that the *E. canis* P28 protein may be a reliable serodiagnostic antigen (McBRIDE; YU; WALKER, 1999).

The purpose of this study was to develop a PCR assay based on amplification of the *E. canis p28* gene and to compare this technique with two PCR methods used to amplify the 16S rRNA and *dsb* genes of *Ehrlichia*.

## Material and Methods

### 1. Blood samples and DNA extraction

Ninety-six blood samples were collected from dogs suspected of having a positive diagnosis for canine ehrlichiosis by the attending clinician at the Veterinary Teaching Hospital, UNESP, Jaboticabal, SP. These samples were previously tested by nested-PCR to detect *E. canis* 16S rRNA gene. Positive control blood was collected from an *E. canis* experimentally-infected dog used in previous studies in the Immunoparasitology Laboratory – UNESP/ Jaboticabal (CASTRO et al., 2004) and negative control was collected from the same dog before the infection. DNA was extracted by using the QIAamp Blood Kit according to the manufacturer's recommendations (QIAGEN, Inc.).

### 2. Selection of PCR primers

PCR primers were designed based on a previously sequenced conserved region to amplify a partial locus of the *p28* gene (McBRIDE; YU; WALKER, 1999) (GenBank accession number EF014897). Forward primer (ECp28-F) and reverse primer (ECp28-R) could amplify the 843-bp open reading frame. Primers used in nested-PCR to amplify a portion of the 16S rRNA *Ehrlichia* genus were ECC and ECB, and HE3 and ECAN primers were used to amplify the *E. canis* 16S rRNA gene (MURPHY et al., 1998; MACIEIRA et al, 2005; NAKAGHI et al., 2008). Primers used in another PCR protocol to amplify a 409-bp fragment of the disulfide bond formation protein gene (*dsb*) of *Ehrlichia* spp. were designated as Dsb-330 and Dsb-728 (LABRUNA et al., 2007) (Table 1).

**Table 1.** Primers used in PCR assays to amplify *Ehrlichia* genes.

| Gene       | Primers     |             | Sequence<br>5' - 3'                   |
|------------|-------------|-------------|---------------------------------------|
|            | (f) forward | (r) reverse |                                       |
| <i>p28</i> | ECp28-F (f) |             | 5' ATG AAT TGC AAA AAA ATT CTT ATA 3' |
|            | ECp28-R (r) |             | 5' TTA GAA GTT AAA TCT TCC TCC 3'     |
| 16S rRNA   |             | ECC (f)     | 5'- GAACGAACGCTGGCGGCAAGC -3'         |
|            |             | ECB (r)     | 5'- CGTATTACCGCGGCTGCTGGCA -3'        |
|            |             | ECAN (f)    | 5'- GAACGAACGCTGGCGGCAAGC -3'         |
|            |             | HE3 (r)     | 5'- TATAGGTACCGTCATTATCTTCCCTAT -3'   |
| <i>dsb</i> | Dsb-330 (f) |             | 5'- GATGATGTCTGAAGATATGAAACAAAT -3'   |
|            | Dsb-728 (r) |             | 5'-CTGCTCGTCTATTTTACTTCTTAAAGT -3'    |

### 3. PCR assays

Amplification of a portion of the gene *p28* consisted of a 50  $\mu$ L reaction containing 5  $\mu$ L of template DNA in 5  $\mu$ L PCR buffer 10 $\times$  (100 mM Tris-HCl, pH 9.0, 500 mM KCl), 0.2 mM each dNTP, 2.5 mM MgCl<sub>2</sub>, 0.5 pmol each primer, 1.25 U of *Taq* DNA polymerase. The amplification profile consisted of 95 °C for 5 minutes, 30 cycles at 95 °C for 30 seconds, annealing temperature gradient ranging from 40 to 60 °C for 1 minute and 72 °C for 2 minutes, followed by a final extension at 72 °C for 5 minutes. The PCR results were visualized in a 1.3% agarose gel containing ethidium bromide. The 16S rRNA gene fragment amplification was performed as previously described (NAKAGHI et al., 2008). The *dsb* gene fragment was amplified as previously described (AGUIAR et al. 2007). The sensitivity of the *p28*-based PCR was analyzed from an *E. canis*-infected DH82 monolayer 100% infected diluted 10-fold in distilled water.

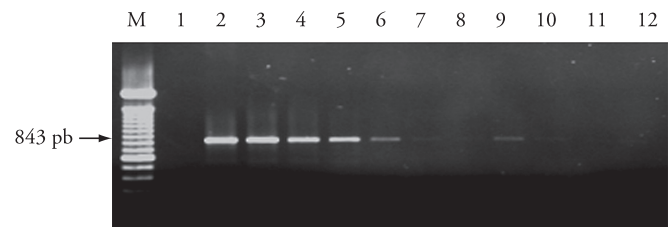
## Results

### 1. Optimization of the *p28*-based PCR.

Detection of *E. canis p28* gene by PCR was optimized with genomic DNA extracted from the blood sample of an infected dog. Reactions were submitted to an annealing temperature gradient ranging from 40 to 60 °C. Amplification could be observed up to 55 °C and the best amplification was observed at 52 °C, then this was the annealing temperature chosen for the *p28*-based PCR. Sensitivity was analyzed from an *E. canis* culture with 100% rickettsemia. This PCR assay was able to amplify target template diluted as much as 1 $\times$ 10<sup>-7</sup>% (Figure 1), or 1 parasite among 10<sup>9</sup> cells.

### 2. PCR assays to amplify *Ehrlichia* genes.

A total of 96 DNA samples were tested by *p28*-based PCR, nested PCR to amplify the 16S rRNA gene, and by PCR to detect *dsb* gene. Amplification of the *p28* gene was demonstrated by an 843-bp fragment and it was visualized in 49 samples (51%). The



**Figure 1.** Sensitivity of the *p28*-based PCR assay analyzed from an *E. canis*-infected DH82 monolayer 100% infected diluted 10-fold in distilled water. M, molecular standard size; lane 1, negative control; lanes 2 to 12, represent assays of the same template dilutions of 1 $\times$ 10<sup>0</sup>, 1 $\times$ 10<sup>-1</sup>, 1 $\times$ 10<sup>-2</sup>, 1 $\times$ 10<sup>-3</sup>, 1 $\times$ 10<sup>-4</sup>, 1 $\times$ 10<sup>-5</sup>, 1 $\times$ 10<sup>-6</sup>, 1 $\times$ 10<sup>-7</sup>, 1 $\times$ 10<sup>-8</sup>, 1 $\times$ 10<sup>-9</sup>, 1 $\times$ 10<sup>-10</sup>, respectively.

other 47 samples (49%) were negative in this PCR. Nested-PCR positive samples were demonstrated by the amplification of a 398 bp fragment of the 16S rRNA gene of *E. canis*. This PCR system was able to detect 66 positive samples (68.7%), and 30 samples (31.3%) were negative. *Ehrlichia dsb* gene was detected by PCR in 66 samples (68.7%) and it was not observed in 30 samples (31.3%). All positive samples by *p28*-based PCR were also detected in 16S rRNA and *dsb* gene. On the other hand, among the PCR negative samples (47), only 26 (55.3%) were simultaneously negative by nested PCR and the *dsb*-based PCR, and 13 negative samples (27.6%) were positive for these later two tests (Table 2).

## Discussion and Conclusions

Canine monocytic ehrlichiosis is an important widely spread disease in Brazil, caused by *Ehrlichia canis* (DAGNONE et al, 2003; MACHADO, 2004; NAKAGHI et al, 2008). Ehrlichial 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).

**Table 2.** Comparison between results obtained by *p28*-based PCR, nested PCR for the amplification of 16S rRNA gene, and PCR for the detection of *dsb* gene in 96 canine DNA samples.

| 16S rRNA | <i>p28</i> positives<br>n = 49 |          | 16S rRNA | <i>p28</i> negatives<br>n = 47 |          | Total<br>n = 96 |
|----------|--------------------------------|----------|----------|--------------------------------|----------|-----------------|
|          | <i>dsb</i>                     |          |          | <i>dsb</i>                     |          |                 |
|          | Positive                       | Negative |          | Positive                       | Negative |                 |
| Positive | 49                             | 0        | Positive | 13                             | 4        | 17              |
| Negative | 0                              | 0        | Negative | 4                              | 26       | 30              |
| Total    | 49                             | 0        | Total    | 17                             | 30       | 47              |

DNA amplification by PCR has improved the sensitivity and specificity of the diagnosis of ehrlichiosis (IQBAL; CHAICHANASIRIWITHAYA; RIKIHISA, 1994). There are many reports about PCR techniques for the detection of *E. canis* DNA, but only a few studies compared their sensitivity and specificity. In this study the optimization of PCR to detect a partial locus of the *E. canis p28* gene was developed and this assay could amplify an 843bp fragment, in agreement with the sequence found in GenBank (EF014897).

To determine the sensitivity and specificity of the PCR to detect gene *p28*, a comparison with 16S rRNA-based nested PCR and *dsb*-based PCR was performed. The *p28* PCR system exhibited less sensitivity than the other PCR assays. The nested PCR sensitivity was previously evaluated and it could detect *E. canis* DNA to an equivalent rickettsemia of one infected monocyte in  $10^{36}$  cells (NAKAGHI et al., 2008). The *dsb* primers used to amplify the *Ehrlichia* spp *dsb* gene was previously evaluated in a real-time assay, in which it was able to detect as few as 10 copies of *E. canis* (LABRUNA et al., 2007). The *p28*-based PCR was able to detect 1 parasite among  $10^9$  cells. Probably, the lower sensitivity found in this study could be associated to the large size of the *p28* amplicon. Single-step *p28* based PCR assay used to detect *E. chaffeensis* was 1000-fold more sensitive than nested 16S rDNA-based PCR assay, when PCR amplified a 277 bp fragment. PCR assays with species-specific sequences, rather than highly conserved bacterial sequences are likely to provide sensitive and specific tests (WAGNER et al., 2004). Development of a *p30*-based PCR assay with primer sequences located within a specific region of 135 bp or less increased sensitivity and specificity (STICH et al., 2002).

The nested PCR assay to detect 16S rRNA gene is the most common molecular method used to diagnose ehrlichiosis (BULLA et al., 2004; MACIEIRA et al., 2005; SANTOS et al., 2007; NAKAGHI et al., 2008). However, this method can result in unspecific amplification, due to the highly conserved 16S rRNA gene among strains of species of *Ehrlichia*, and is at higher risk of DNA cross-contamination (LABRUNA et al., 2007). Besides that, single-step PCR offers some advantages over nested PCR, including reduced labor, cost and timing, and lower risk of DNA cross-contamination (KWOK; HIGUCHI, 1989; WAGNER et al., 2004). Real-time PCR is usually a very sensitive PCR system because primers are designed under highly developed softwares that indicate the most efficient combination of oligos. Once these oligos are chosen and tested their efficiency, they can be applied with the same success to routine PCR assays

that are interpreted in agarose gel runs (AGUIAR et al., 2007). In addition, the *dsb* gene is a conserved gene among *Ehrlichia* species. Nevertheless, this system detects the presence of the *Ehrlichia* genus but digestion with restriction enzymes or DNA sequencing is necessary to differentiate the ehrlichial species (LABRUNA et al., 2007). Therefore, a single-step PCR assay that amplifies the *p28* gene seems to be the most specific method to detect *Ehrlichia canis* DNA.

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 PCR seems to be a useful test for the molecular detection of *E. canis*, but studies with primers targeting a specific region inside gene *p28* may improve the sensitivity of this method, so it can be an important alternative in the diagnosis of canine ehrlichiosis.

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