

Molecular detection of *Ehrlichia canis* from blood of naturally infected dogs in India

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ABSTRACT

The nested PCR assay for the detection of 16S rRNA gene fragment of *Ehrlichia canis* from the blood of dogs was standardized for the first time in India. The genus-specific primers amplified a 477 bp band of *Ehrlichia* sp. in the first PCR. The nested PCR assay using species-specific primers produced a 387 bp band of *E. canis*. The nested PCR detected *E. canis* organisms in 50% of samples as against the routine blood smear examination, which revealed morulae in only 19.38% of samples. This protocol could detect the presence of *E. canis*, even one month after specific antibiotic therapy.

Key words: *Ehrlichia canis*, nested PCR, 16S rRNA gene, dogs

Introduction

Canine Monocytic Ehrlichiosis (CME) is a fatal tick-borne disease caused by the obligate intracellular pathogen, *Ehrlichia canis*. Affected dogs manifest a wide spectrum of clinical signs varying from mild weight loss to a severe and often fatal haemorrhagic syndrome. Dogs in the acute phase of the disease show dramatic improvement in haematologic and clinical responses within 24-48 hr. after therapy. It is when the disease is not treated in the initial stages that it goes on to damage the systems of the host. However, clinically healthy dogs are carriers of the organism in the sub-clinical phase of

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the disease, which increases the possibility of transmission of the disease to other dogs. Dogs in the chronic phase of the disease may not improve on treatment, thus leading to poor prognosis. Hence, diagnosis of canine ehrlichiosis in the early stage of the infection is important to ensure successful treatment.

Currently definite diagnosis of canine ehrlichiosis in India is based on haematological, biochemical and serological results. Microscopic evaluation of stained blood smears is not sensitive as the organisms are not readily demonstrable in smears. Serological tests fail to distinguish a current infection from either previous infection or exposure without establishment of infection. Furthermore, serologic cross-reactivity between ehrlichial species poses a serious problem in differentiating the infecting *Ehrlichia* species. Cell culture isolation, although sensitive and specific, takes a long time to produce results, thus limiting its use as a rapid diagnostic tool (WANER et al., 2001).

Sensitivity, specificity, ease of use, rapidity, and the ability to analyse a large number of samples at the same time make nested PCR a superior option for detection of early as well as persistent canine ehrlichiosis. Only limited attempts have been made in India to diagnose canine ehrlichiosis using PCR. In this paper we report a nested PCR protocol for diagnosis of canine ehrlichiosis.

Materials and methods

Collection of blood samples. Ninety-eight whole blood samples collected in EDTA from the dogs brought to the Small Animal Clinics of Madras Veterinary College were selected for PCR analysis. Peripheral blood smears were made from these animals and subjected to direct microscopic examination using Giemsa stain.

Genomic DNA extraction. DNA extraction was carried out using Genomic DNA Purification Kit (MBI Fermentas, Lithuania) following the manufacturer's protocol. Two hundred μL of the blood sample were mixed with 400 μL of the lysis solution and incubated at 65 °C for 5 min. Six hundred μL of chloroform was immediately added, mixed and centrifuged at 10,000 rpm for 3 min. The supernatant was discarded and the DNA pellet was dissolved in 100 μL of 1.2 M NaCl. Then, 200 μL of cold ethanol were added, kept at -20 °C for 10 min. and spun at 10,000 g for 4 min. The supernatant was discarded. The DNA pellet was washed once with 70% cold ethanol and then dissolved in 50 μL of sterile TDW. DNA concentration and purity was measured spectrophotometrically as per SAMBROOK et al. (2001).

DNA amplification. The PCR for the detection of *E. canis* in the blood samples was carried out based on the method of MURPHY et al. (1998) with some modifications, using DyNAzyme™ II DNA Polymerase kit (Finnzymes, Finland). Primers were selected based on MURPHY et al. (1998). Genus specific primers (Alpha DNA Canada.) used for

the amplification of Ehrlichial DNA were ECC (5' -AGA ACG AAC GCT GGC GGC AAG C- 3') and ECB (5' - CGT ATT ACC GCG GCT GCT GGC A -3').

Extracted DNA (10 µL) was used as a template to amplify a fragment of the 16S rRNA gene in 50 µL of reaction mixture containing 5 µL of Mg - free DyNAzyme™ buffer (1X), 2 µL of Mg Cl₂ (2 mM), 1.5 µL of dNTP mix (10 mM each), 3 µL each of primers ECC and ECB (10 p mol/ µL), 1.5 µL of DyNAzyme™ II DNA Polymerase (2 U/µL) and 24.0 µL of sterile triple distilled water.

PCR was carried out in a thermal cycler (MJ Research Inc, USA). The thermocycle profile consisted of initial denaturation at 94 °C for one min, followed by 30 cycles of denaturation at 94 °C for one min., annealing at 65 °C for 2 min. and extension at 72 °C for 2 min. This was followed by a final extension at 72 °C for 5 min. The amplicons obtained were subjected to nested PCR for confirmation of the species of *Ehrlichia*. Nested reactions were performed using 1 µL of this amplicon as a template with species specific primers of *E. canis*, namely, ECAN5 (5'- CAA TAA TTT ATA GCC TCT GGC TAT AGG A- 3') and HE3 (5'- TAT AGG TAC CGT CAT TAT CTT CCC TAT - 3') under the reaction conditions described above. The DNA retrieved from the 100% infected DH 82 cell lines, provided by Prof. I Kakoma, Illinois State University, USA, served as positive control. Sterile deionised water served as negative control.

PCR amplicons, known positive and negative controls and a molecular weight marker (100 bp ladder) were electrophoresed in a 2% agarose gel stained with ethidium bromide using submarine gel electrophoresis. The products were visualized using the UV transilluminater (Fotodyne, USA) and photographed using a video documentation system (Pharmacia Biotech., USA).

Results

The nested PCR assay for the detection of *Ehrlichia canis* 16S rRNA gene fragment was standardized. Of the total 98 samples subjected to PCR, 49 (50%) were found to be positive for *Ehrlichia* infection using primers ECC and ECB. These primers produced a 477 bp band in accordance with the known positive control. All of the PCR-positive samples subjected to nested PCR produced a 387 bp band using *E. canis* species-specific primers, ECAN5 and HE3 (Fig. 1). Of those 49 dogs, 2 were found to be positive for *E. canis* by nested PCR one month after after a combined parenteral oxytetracycline and oral doxycycline treatment for 2 weeks. Of the total 98 samples only 19 (19.38%) were positive by blood smear examination (Table 1).

Table 1. Comparison of the results of PCR and blood smear examination

Technique	Staining positive	Staining negative	Total
PCR positive	15	34	49
PCR negative	4	45	49
Total	19	79	98

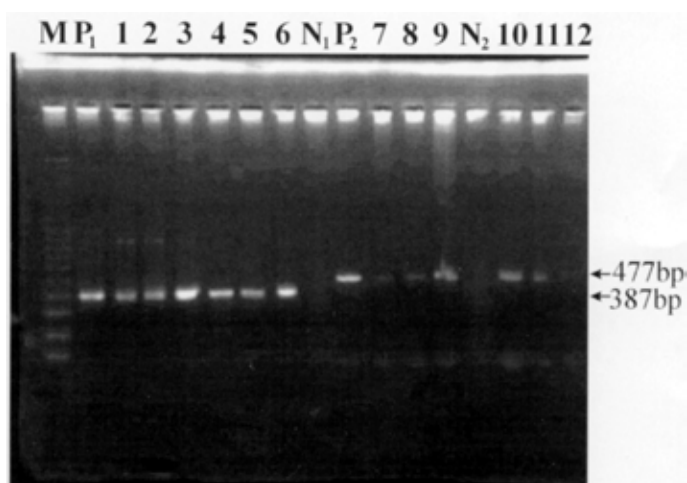


Fig. 1. Agarose gel electrophoresis of nested PCR products. M - 100 bp ladder; P1- known positive control of *E. canis*; P2 - known positive control of *Ehrlichia* sp.; N1- known negative control of *E. canis*; N2 - known negative control of *Ehrlichia* sp.; 1-6 - positive samples of *E. canis*; 7-12 - positive samples of *Ehrlichia* sp.

Discussion

Comparison of PCR and microscopic findings reveals the extremely low sensitivity of blood smear examination for diagnosis of the disease. This might be due to the fact that the percentage of *E. canis*-infected cells within the peripheral blood is low. Similar findings have been presented by a number of authors (WOLDEHIWET and RISTIC, 1993; IQBAL et al., 1994; HARRUS et al., 1998). To date, the epidemiological studies of canine ehrlichiosis in India has been based on blood smear results, as well as on serological evidence of circulating antibodies. Nested PCR would provide more accurate epidemiological data of the disease

Amplification of the genomic DNA by species-specific primers in a single step PCR failed to yield positive results. Hence, nested PCR was adopted and found to be a reliable

and sensitive technique for the diagnosis of low level parasitaemia, as reported by IQBAL and RIKIHISA (1994) and WEN et al. (1997). The enhanced sensitivity of nested PCR for the detection of canine ehrlichiosis was earlier reported by WARNER and DAWSON (1996) and EGENVALL et al. (2000).

In the present study, 2 dogs were found to be positive for *E. canis* by nested PCR one month after specific antibiotic treatment, indicating the possibility of reinfection or persistence of infection. It may also be an indicator of the short duration of treatment, especially in immunocompromised dogs. Further research is required to explore the carrier status in dogs, if any, even after specific antibiotic therapy. All 49 samples that were positive by PCR, were also positive by nested PCR. This indicated that these dogs were infected with *E. canis* only.

In conclusion, nested PCR is a reliable and rapid test for the diagnosis of early stages of canine ehrlichiosis, which is imperative to ensure successful treatment of this disease. Nested PCR could detect the presence of *Ehrlichia* even after specific antibiotic therapy. This is a highly effective tool for assessing the clearance of organisms after antibiotic therapy. It can be used as a tool to evaluate the efficiency of the antibiotic therapy by testing various tissue aspirates.

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SAŽETAK

Ugniježđena lančana reakcija polimerazom za dokaz odsječka gena 16S rRNA vrste *Ehrlichia canis* iz uzoraka krvi pasa standardizirana je prvi put u Indiji. U prvom krugu lančane reakcije polimerazom umnožene su bile početnice od 477 baznih parova specifične za rod *Ehrlichia*. Postupkom ugniježdene PCR upotrebom vrstno specifičnih početnica umnožen je odsječak gena *E. canis* od 387 baznih parova. Ugniježđenom lančanom reakcijom polimerazom vrsta *E. canis* mogla se dokazati u 50% uzoraka u odnosu na rutinske pretrage razmazaka krvi pomoću kojih su se morule mogle dokazati samo u 19,38% uzoraka. Ugniježđenom lančanom reakcijom polimerazom mogla se dokazati prisutnost *E. canis* čak do mjesec dana nakon specifičnoga liječenja antibioticima.

Ključne riječi: *Ehrlichia canis*, ugniježđena lančana reakcija polimerazom, gen 16S rRNA, pas
