

Anaplasma platys: an improved PCR for its detection in dogs

Anthony R. Martin*, Graeme K. Brown, R. Hugh Dunstan, Timothy K. Roberts

The Molecular Structure and Detection Group, The University of Newcastle, Callaghan, NSW 2308, Australia

Received 16 September 2004; received in revised form 23 November 2004; accepted 24 November 2004

Available online 6 January 2005

Abstract

This study compares two PCR assays for the detection of *Anaplasma platys* in dog blood using primers based on the *A. platys* 16S rRNA gene. The first approach utilized a “standard” PCR protocol composed of a “single-step” direct amplification using an *Ehrlichia* genus-specific primer set. The second assay being a “nested” PCR screen that first involved a universal bacterial primer set that amplified the majority of the 16S rRNA gene, followed by the nested round of PCR using an *A. platys*-specific primer set. Of the 22 dogs sampled, 10 were found to contain *A. platys* DNA using both protocols, and an additional two dogs were found positive using the nested technique. An extract of *A. platys* positive genomic DNA was serially diluted and comparison of sensitivities determined between the nested PCR, and a direct assay using *A. platys*-specific primers. The nested protocol demonstrated an increased sensitivity by at least 2 orders of magnitude when compared to the direct assay alone. Our results indicated that the nested PCR assay with its increased sensitivity would be useful for experimental research investigations as well as offer the potential for use as a routine test in diagnostic pathology.

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Keywords: *Anaplasma platys*; PCR; Polymerase chain reaction; Nested PCR; *Ehrlichia*

1. Introduction

Anaplasma platys, which causes canine infectious cyclic thrombocytopenia (CICT), was first reported in the USA in 1978 (Harvey et al., 1978). *A. platys* is an obligate intracellular rickettsial organism that appears to only parasitize platelets of dogs. It is thought to be transmitted by the Brown Dog Tick *Rhipicephalus sanguineus* (Harrus et al., 1997; Woody and Hoskins, 1991).

Diagnosis of *A. platys* infection has usually been made using microscopy, by demonstrating the organisms in platelets on blood films or buffy-coat smears that have been stained using Romanowsky-type stains such as Giemsa or Diff-Quik. However, due to the cyclic parasitemia, this is not a reliable method and,

additionally, in many regions of the world, the organisms are quite often absent or only present in very low numbers (Bradford et al., 1996; Harrus et al., 1997).

A previous study examined free-roaming dogs in Central Australia to determine whether they harbored any blood-borne infectious agents of zoonotic significance. Blood samples were initially tested by polymerase chain reaction (PCR) using primers to amplify a fragment of the 16S rDNA. DNA analysis of amplicons provided evidence of *A. platys*, and further analyses using a specific primer set for *A. platys* led to the first confirmation and report of the carriage of *A. platys* by dogs in Australia (Brown et al., 2001).

The original PCR method employed a “single-step” direct amplification using an *Ehrlichia* genus-specific primer set. To increase the sensitivity of the PCR, a nested PCR approach was developed in the present study.

* Corresponding author. Fax: +61 2 4921 7281.

E-mail address: Anthony.Martin@newcastle.edu.au (A.R. Martin).

2. Materials and methods

For this study, blood samples were collected from another group of 22 free-roaming dogs in Central Australia. A total volume of 4 mL of blood was collected in labeled EDTA vacutainers from the cephalic vein of the dogs after aseptic preparation of the skin. At the time of sampling, thin blood films were prepared from each of the dogs. After blood sampling was completed, a small amount of EDTA blood from each dog was placed in individual plain micro-hematocrit tubes and then sealed. The samples were kept cool (4–8 °C) during transport to the University of Newcastle laboratory, and stored at 4 °C until extraction of the DNA within 5 days. The QIA-amp DNA Mini-Kit (Qiagen, Vic., Australia) was used for the isolation and extraction of DNA as per manufacturer's protocol with the following modifications; the starting blood volume was increased to 360 µL, overnight incubation at 56 °C, and a final elution volume of 40 µL. Extracted DNA was stored at 4 °C until use in the PCR.

2.1. Standard PCR

The 22 dog samples were first subjected to a “standard” PCR using an *Ehrlichia* genus set of primers that amplify a 345 bp fragment in the 5' half of the 16S rRNA gene. This primer set, EHR16SD (5'-GGT ACC YAC AGA AGA AGT CC-3') and EHR16SR (5'-TAG CAC TCA TCG TTT ACA GC-3'), can amplify various species including *E. canis*, *E. chaffeensis*, *E. muris*, *E. ruminantium*, *Anaplasma phagocytophilum*, *A. platys*, *A. marginale*, *A. centrale*, *Wolbachia pipientis*, *Neorickettsia sennetsu*, *N. risticii*, and *N. helminthoeca* (Inokuma et al., 2000). The thermal-cycling conditions for the standard PCR were as previously described by Brown et al. (2001). Amplicons produced using this primer set were purified using the QIAquick DNA Purification Kit (Qiagen, Vic., Australia) for sequence analysis. Big Dye Terminator Cycle Sequencing technology and an ABI 377 automated sequencer (Applied Biosystems Division) were used. The BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) was used for the comparison and analysis of sequence data obtained.

2.2. Nested PCR

A nested PCR procedure was established employing primers 8F (5'-AGT TTG ATC ATG GCT CAG-3') and 1448R (5'-CCA TGG CGT GAC GGG CAG TGT G-3') which amplify the majority of the 16S rRNA gene (Warner and Dawson, 1996) in a first round of PCR, followed by a second round of PCR using an *A. platys*-specific primer, PLATYS (5'-GAT TTT TGT CGT AGC TTG CTA TG-3'), and an *Ehrlichia* genus-specific primer EHR16SR (Motoi et al., 2001) to produce a 678 bp amplicon.

Amplification was performed in a Peltier model Dyad DNA Engine thermal-cycler (MJ Research, Watertown, MA, USA). Template DNA (5 µL) was amplified within a 50 µL reaction containing 12.5 pmol of each primer, 1.25 U *Taq* DNA polymerase, 0.2 mM deoxynucleoside triphosphates, 20 mM Tris-HCl, 100 mM KCl, 3 mM MgCl₂, and a final volume of diethyl-pyrocabonate (DEPC)-treated water. First round amplification was performed using 40 cycles of 1 min at 94 °C, 1 min at 45 °C, and 1 min 40 s at 72 °C (with an additional 1 s extension on each 72 °C cycle). The second round amplification involved 1 µL of first round PCR product used as template DNA within another 50 µL reaction. The cycle profile is as follows: initial denaturation of 94 °C for 1 min then 40 cycles of 55 °C for 30 s, 72 °C for 30 s, and 94 °C for 30 s, with a final 5 min extension at 72 °C. In each test, DEPC-treated water was used as the negative control, and positive *A. platys* sequenced genomic DNA for use as a positive control. The amplicons were detected after electrophoresis in a 1.5% agarose gel stained with ethidium bromide. To minimize the chances of contamination, DNA was extracted under a biosafety hood in a separate room with equipment exclusively used for DNA extraction. Nested PCR was performed in separate pre- and post-PCR areas.

3. Results

Ten of 22 dogs were shown to be *Ehrlichia* genus “positive” using the standard PCR. The 10 amplicons were purified and sequenced. The sequences obtained were closely related to *A. platys* with an homology of between 99.7 and 100% (AF 286699 strain).

The 22 genomic DNA samples were then subjected to the new nested PCR-based assay. The same 10 dogs testing positive for the standard assay were confirmed positive by the nested PCR assay (Figs. 1C and D). The amplicons obtained were substantially greater in intensity than the 345 base pair products obtained using the standard assay. In addition, two other dogs tested positive for *A. platys* (lanes 6 and 12). This result was reproducible in ensuing experiments and these additional two positives were confirmed by sequence analysis as *A. platys* [AF 286699 strain]. Subsequently, *A. platys* DNA was serially diluted and subjected to PCR using both a direct and the nested protocol with the primer combination of PLATYS and EHR16SR. The results of the comparative serial dilutions of the same sample measured by the two assays were reproducible (Fig. 2). It is evident from these gels that the nested PCR was able to detect approximately 100-fold less *A. platys* DNA than the standard PCR.

By examining Diff-Quik stained buffy-coat smears prepared from the *A. platys* positive dogs, we were able to determine that no more than 1% of platelets were

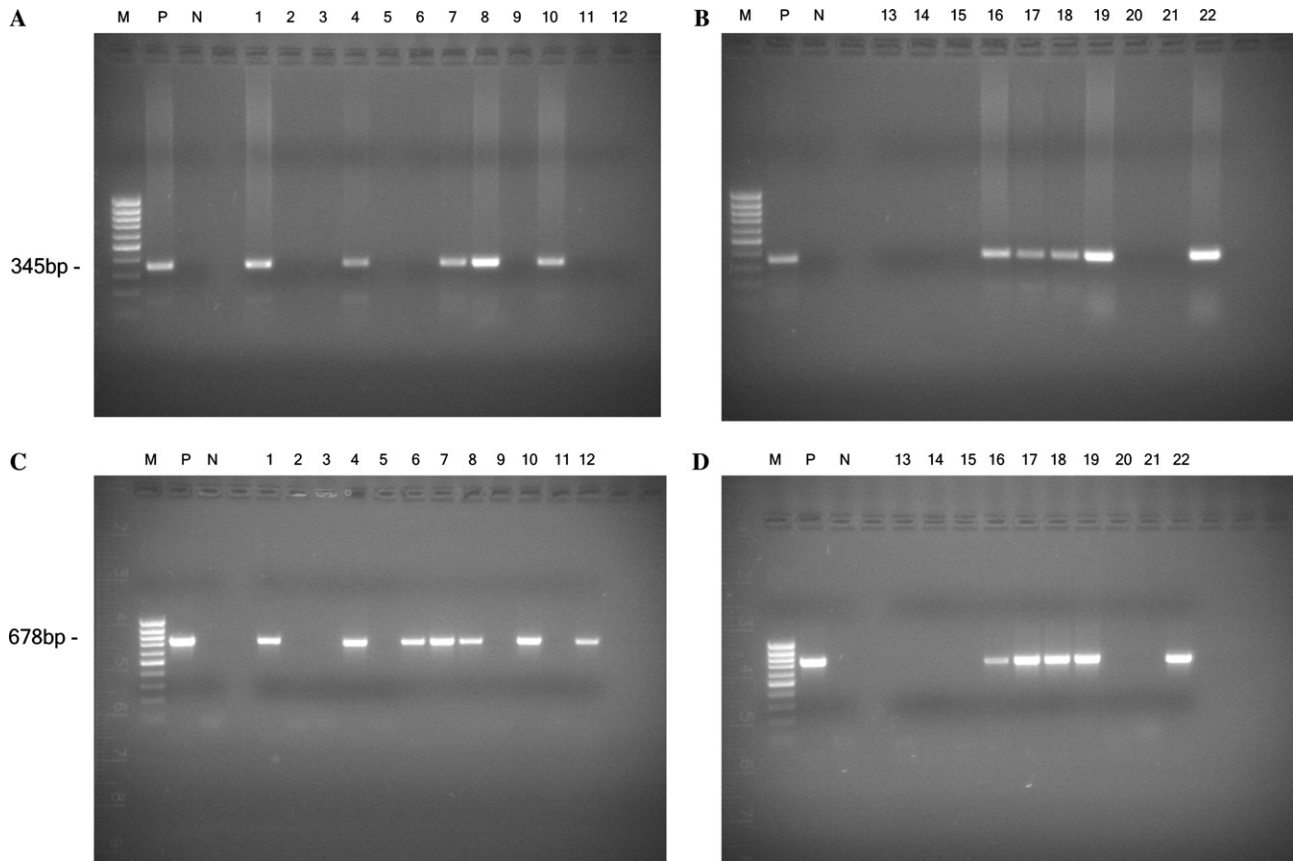


Fig. 1. Frames A and B represent PCR amplicons obtained from dogs 1 to 12 and 13 to 22, respectively, after screening with the *Ehrlichia* genus primer set. Lane M, molecular size markers; lane P, positive control (*A. platys*); and lane N, negative control. Dogs 1, 4, 7, 8, 10, 16, 17, 18, 19, and 22 are positive for *A. platys*. Frames C and D represent PCR amplicons obtained from dogs 1 to 12 and 13 to 22, respectively, after screening with the nested PCR protocol. Lane M, molecular size markers; lane P, positive control (*A. platys*); and lane N, negative control. Dogs 1, 4, 6, 7, 8, 10, 12, 16, 17, 18, 19, and 22, are positive for *A. platys*. Dogs # 6 and # 12 were previously negative using the *Ehrlichia* genus primer set (see A).

parasitized. These results were later confirmed using an indirect immunofluorescence assay (data not shown).

4. Discussion

Intracellular pathogens are notoriously difficult to detect by clinical pathological investigations. Examining stained blood films for the intra-platelet inclusions of *A. platys* is usually time-consuming and not very rewarding as the morulae are often absent or present in very low numbers (Bradford et al., 1996; Harrus et al., 1997). On the other hand, Arraga-Alvarado et al. (2003) reported that in Venezuela, where up to 97% of platelets are parasitized, diagnosis of *A. platys* infection is made routinely by examining stained blood smears. These authors also used electron microscopy and found structural differences between experimentally infected and naturally infected dogs suggesting different strains. To confirm this, a comparative serologic and molecular characterization of the organism still had to be performed. Serological methods using an indirect immunofluorescent antibody (IFA) technique have been used using *A.*

platys-infected platelets as the antigen (Baker et al., 1987; French and Harvey, 1983). The diagnosis is made retrospectively but at times it is difficult to access antigen for use in the IFA testing because, as yet, *A. platys* has not been cultured. In Venezuela, Arraga-Alvarado et al. (2003) also used the IFA test for diagnosis when the parasitemia was absent or low and used platelet-rich plasma (PRP) as the source of antigen.

Chang and Pan (1996) were the first to demonstrate the increased sensitivity obtained by using a two-step (nested) PCR on an experimentally infected dog. They were able to demonstrate that the nested PCR was 10 times more sensitive than the single PCR for the diagnosis of *A. platys*. They confirmed the authenticity of the amplified products by Southern blot hybridization. The authors concluded that the sensitivity of the nested PCR in detecting *A. platys* from blood samples was equal to that obtained from a single (one round) PCR and Southern blot hybridization. However, a nested PCR can be performed in less than 2 h whereas Southern blot hybridization procedures are time-consuming and require a radio-labeled probe that must be prepared frequently.

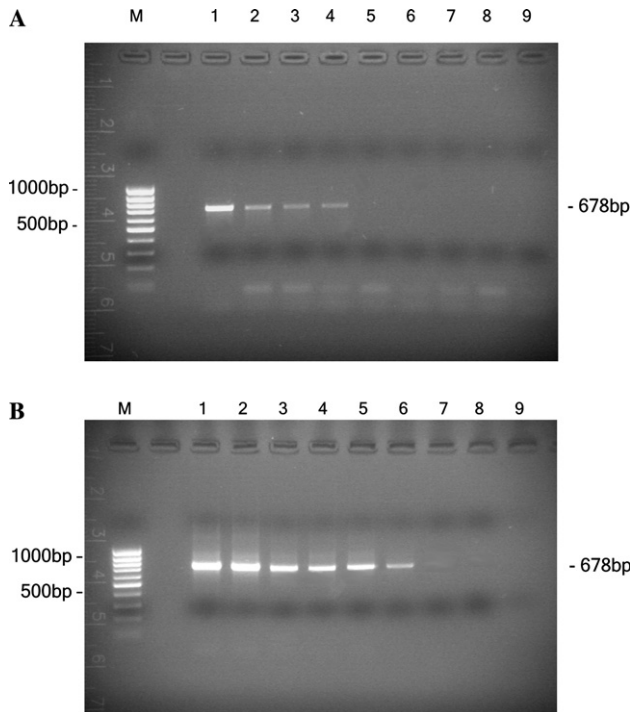


Fig. 2. Sensitivity of the direct assay (A) in comparison to the nested reaction (B) using primers PLATYS/EHR16SD. DNA was isolated from an infected canine and subjected to a dilution series for simultaneous amplification with the two assays. Lanes 1–9, respectively, represent assays of the same template dilutions of 1×10^0 , 1×10^{-1} , 1×10^{-2} , 1×10^{-3} , 1×10^{-4} , 1×10^{-5} , 1×10^{-6} , 1×10^{-7} , and 1×10^{-8} for each panel.

Chang et al. (1997) also demonstrated the usefulness of a nested PCR in assessing the duration of antibiotic treatment required for dogs infected with *A. platys*. The duration of treatment of ehrlichial diseases is more important than the dosage or frequency, particularly in chronic cases (Woody and Hoskins, 1991) and nested PCR is helpful in assessing the efficacy of antibiotic treatment (Chang et al., 1997).

Sparagano et al. (2003) used several PCRs and a reverse line blot hybridization (RLB) method to identify *A. platys* in dogs in Italy. The RLB approach was able to rule out some species known for dogs (such as *E. canis* and *A. phagocytophilum*) that are endemic in Europe. An advantage of the RLB method is the ability to specifically identify mixed infections based on genera and species-specific probes. It is useful for the diagnosis of several pathogens at the same time, but the hybridization and incubation steps are time-consuming and costly compared to the nested PCR for the specific detection of *A. platys*.

Nested PCR is more “sensitive” as it has allowed us to diagnose active cases of CICT which otherwise would have gone undetected by the nature of the low sensitivity of microscopy, i.e., the low numbers of platelets actually found to be infected with *A. platys* ($\leq 1\%$). Serological assays fail to detect acute cases of infection and are

non-specific due to potential cross-reactivity with organisms from the same genogroup (Beaufils et al., 2002; French and Harvey, 1983). The results of this study demonstrate the value of PCR testing for ehrlichial organisms, particularly *A. platys* where the procedures were able to reproduce results of high sensitivity. The standard PCR method was able to detect *Anaplasma*-infected dogs with a lower specificity. The sequence analyses of the 345bp amplicons subsequently revealed the samples to be closely related to *A. platys* (99.7–100%) [AF 286699 strain]. This finding further supports the work of Unver et al. (2003) indicating that there is little diversity between the 16S rRNA gene sequences of *A. platys* obtained from different continents of the world.

The nested PCR assay confirmed the *Anaplasma* infections detected by the standard assay, and also detected a further two infected animals with the increase in sensitivity. This nested assay was facilitated via the use of two pairs of primers based on the known 16S rRNA gene sequences of ehrlichial species (Warner and Dawson, 1996). The first pair of primers allowed amplification of most of the 16S rRNA, and the second pair of primers used were specific to the internal section of the initial PCR amplicons. The nested reaction demonstrated a reproducible improvement in sensitivity by at least 2 orders of magnitude compared with the standard PCR assay.

These results indicate that the nested PCR was specific for *A. platys* and displayed enhanced sensitivity to reduce the incidence of reporting false negative results. The sequence analyses confirmed a higher integrity of specificity. Further testing could now be performed in a cost-efficient manner for specific detection of *A. platys* without the need for additional sequence analysis.

Acknowledgment

We thank the Judith Mason Chronic Fatigue Research Fund for the financial support of this work.

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