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Specific Amplification of *Ehrlichia platys* DNA from Blood Specimens by Two-Step PCR

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A two-step PCR method for diagnosis of canine infectious cyclic thrombocytopenia was established. Three primers derived from the 16S rRNA gene sequence were used to amplify genomic DNA specifically from *Ehrlichia platys*. Two-round amplification with DNA templates prepared from *E. platys*-infected blood specimens produced 742- and 385-bp fragments, but these products were not found when an *Ehrlichia canis*-infected blood sample and *Escherichia coli* were used. This method, for which the minimum detectable copy number in the blood specimen was estimated to be five ehrlichial inclusions within platelets, is more sensitive than single PCR amplification. These results demonstrate that this two-step PCR is highly sensitive and efficient for detecting the etiologic agent of canine infectious cyclic thrombocytopenia in blood. The same technique was applied to blood specimens collected from a dog inoculated with *E. platys*. Amplification of the target DNA fragments was observed with blood collected on the fifth day after inoculation, which indicates that this method is also feasible for early diagnosis of *E. platys* infection.

Canine infectious cyclic thrombocytopenia (CICT), a disease caused by *Ehrlichia platys*, was first described in the United States in 1978 (6). *E. platys* is an obligate intracellular rickettsial organism that parasitizes only thrombocytes of the host (6) and is suspected to be transmitted between dogs by the Brown Dog tick (18). Dogs with CICT are not clinically ill and rarely show signs of significant hemorrhage, even with severe thrombocytopenia (3, 6, 18). However, more virulent strains of *E. platys* may exist in nature. One report from Europe suggests that a different, more pathogenic form of *E. platys* exists in Greece (11). Another report indicates that there was a case of *E. platys* associated with overt signs of hemorrhage in the United States (16). Acute CICT is characterized by cyclic parasitemia of platelets followed by thrombocytopenia and generalized lymphadenomegaly (18). In the chronic stage of the disease, the cyclic nature of parasitized platelets sometimes diminishes, resulting in cyclic thrombocytopenias associated with sporadically occurring parasitemias (3, 6).

Since the clinical signs associated with CICT are not specific, *E. platys* infection often presents diagnostic dilemmas. Currently, diagnosis of CICT is carried out either by demonstrating basophilic inclusions within platelets in Giemsa-stained blood smears (Fig. 1) (6, 18) or by immunofluorescence assay (IFA) (5). Detection of the morulae in platelets is time-consuming and usually not rewarding because of the cyclic nature of the appearance of parasitized platelets (3, 6). IFA appears to be a relatively specific means of identifying dogs that have been exposed to *E. platys*, since there is minimal serologic cross-reaction among different species of ehrlichiae that commonly infect dogs (5). Nevertheless, IFA recognizes the infected host's response rather than indicating active infection of the pathogen. Consequently, a simple and more sensitive laboratory diagnostic method for identifying active *E. platys* infection seems to be needed.

PCR is a method which amplifies target nucleotide sequences specifically (13) and has been widely used for the diagnosis of infectious diseases. In this research, we developed a rapid and sensitive method for detection of *E. platys* DNA by two-step (nested) PCR. By constructing PCR primers derived from the 16S rRNA gene sequence, we were able to amplify *E. platys* DNA specifically. Our results demonstrate that this two-step PCR has high sensitivity and specificity for diagnosis of *E. platys* infection. The etiologic agent of CICT was detected in the blood specimen collected from a dog as early as the fifth day postinoculation (p.i.), which indicates that this technique is also useful for diagnosis at the early phase of CICT.

MATERIALS AND METHODS

Ehrlichial strains. *E. platys* organisms used in this study for experimental inoculation and PCR amplification were isolated from dogs with CICT (3). A blood specimen collected with EDTA as an anticoagulant and mixed with an equal volume of 10% dimethyl sulfoxide in phosphate-buffered saline (PBS) (0.01 M potassium phosphate, 0.15 M NaCl, pH 7.2) was divided into 1-ml aliquots, which were then stored in liquid nitrogen. *Ehrlichia canis* was isolated from a canine ehrlichiosis patient in Taipei, and the buffy coat of this blood sample was extracted and stored at −20°C.

Experimental infection. One 6-month-old female crossbred dog in healthy condition was used for experimental infection. This dog was dewormed and dipped for both internal and external parasites, and clinical pathologic exams (including hematogram, plasma biochemistry, and Giemsa-stained blood smear observation) were taken 2 weeks before inoculation. Experimental infection was carried out by intravenous inoculation. Rectal temperature, appetite, attitude, and other indicators were monitored daily. For hematologic evaluation and detection of *E. platys* by PCR, venous blood samples were collected from the cephalic vein every day with EDTA as an anticoagulant except days 6 and 13 p.i.

Selection of PCR primers and hybridization probe. The 16S rRNA gene sequences of four species of *Ehrlichia* that are currently known to cause clinical and subclinical infections in dogs have been published in GenBank under the following accession numbers: M82801 (*E. platys*), M73221 (*E. canis*), M73223 (*Ehrlichia equi*), and M21290 (*Ehrlichia risticii*). These sequences, together with the 16S ribosomal DNA (rDNA) sequences of three other ehrlichial species, *Ehrlichia ewingii*, *Ehrlichia sennetsu*, and *Ehrlichia chaffeensis* (GenBank accession numbers M73227, M 73225, and M73222, respectively), were aligned for the maximal homology by using MegAlign sequence alignment software (DNASTAR Inc.). Oligonucleotide primers and probes were obtained from a commercial source (DNAFax Co., Ltd., Taipei, Taiwan) and used without further purification. Primer EP1 was constructed to correspond to nucleotides 53 to 69 on the sense strand of *E. platys*, a region of the 16S rDNA near the 5′ end that has been reported to be highly variable in other ehrlichiae (1). Table 1 shows the sequences of three primers and the corresponding sequences for all ehrlichiae that are currently known to infect dogs and some other ehrlichial species. Primer

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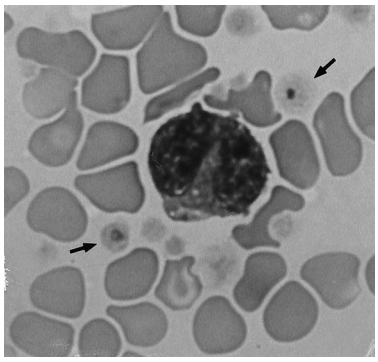

FIG. 1. Giemsa-stained blood smear of a dog experimentally inoculated with *E. platys*. Basophilic inclusions within platelets (arrows) were observed. Magnification, ca. ×1,500.

EP2, corresponding to nucleotides 410 to 428 within the conserved region of the 16S rRNA gene, was constructed (Table 1). The reverse primer EP3, which is complementary to nucleotides 777 to 794, defines a 742-bp product with EP1 and a 385-bp product with EP2 upon PCR amplification (Table 1). A 30-nucleotide hybridization probe was also constructed, one which consists of the sequence 5′-CAGTGGGGAAGATAATGACGGTACCCACAG-3′, corresponding to nucleotides 403 to 432, and locates within the 742-bp PCR product obtained upon amplification with primers EP1 and EP3.

Hematologic evaluation and sample processing for PCR amplification. Complete blood cell counts and platelet counts were performed with an electronic cell counter (Sysmex K-1000; Toa Medical Electronics Co., Ltd., Kobe, Japan). Giemsa-stained blood smears were examined, and the proportion of infected platelets was estimated by observing 200 platelets in one blood smear.

DNA of *E. platys* and *E. canis* was extracted from 50-μl blood specimens by the method described by Higuchi (7). Blood cells and platelets were lysed by adding an equal volume of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, and 1% Triton X-100), and the lysate was centrifuged at 13,000 × *g* for 20 s. The supernatant was removed, and the pellet was again subjected to lysis and centrifugation. The pellet was dissolved and incubated in 50 μl of PCR lysis buffer (0.45% Nonidet P-40, 0.45% Tween 20, 50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.1 mg of gelatin per ml, and 60 μg of proteinase K per ml) at 56°C for 1 h. Proteinase K was inactivated by heating at 95°C for 10 min. DNA of *Escherichia coli* was extracted directly by boiling. Each nucleic acid sample was amplified with 16S rDNA universal primer pairs described by Weisburg et al. (15) with TaKaRa Ex Taq PCR kit (TaKaRa Shuzo Co., Ltd., Shiga, Japan) to demonstrate the efficacy of these as PCR templates.

Two-step PCR amplification of ehrlichial DNA. The first PCR amplification was performed in a 15-μl reaction mixture containing 1 μl of DNA template; 250 μM (each) dATP, dCTP, dGTP, and dTTP; 1 μM (each) primers EP1 and EP3; 250 μg of bovine serum albumin per ml; and 0.4 U of Replitherm thermostable DNA polymerase (Epicentre Technologies Co.) in reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.005% Tween 20, 0.005% Nonidet P-40, and 1.5 mM $MgCl_2$). The second round of PCR was carried out under the same conditions, except that the DNA template was replaced with 1 μl of the first PCR product and the primer EP1 was replaced with primer EP2. All reactions were performed in capillary tubes with an air thermal cycler (Idaho Technology Inc.), and the cycling program was denaturation at 94°C for 1 s, annealing at 55°C for 1 s, and then chain extension at 74°C for 20 s. The PCR cycle was repeated 40 times for the first amplification and 35 times for the second step of PCR.

Amplified DNA analysis and hybridization. The PCR products from each step were electrophoresed through 2% agarose gels (SeaKem LE; FMC BioProducts) in Tris-acetate–EDTA (TAE) buffer. The DNA fragments were visualized by ethidium bromide staining under UV fluorescence and were transferred to nylon membranes (MagnaGraph; Micron Separations Inc.) by a vacuum transfer apparatus (VacuGene XL; Pharmacia-LKB Biotech, Uppsala, Sweden); then the membranes were exposed to 0.15 J/square cm of UV irradiation (Fluo-Link; Vilber Lourmat, Marne la vallée France).

A synthetic oligonucleotide probe (10 pmol) was labeled in 20 μl of reaction buffer (70 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, and 5 mM dithiothreitol) containing 50 μCi of [γ-^{32}P]ATP (specific activity, >5,000 Ci/mmol; Amersham, Little Chalfont, England) and 10 U of T4 polynucleotide kinase (Promega, Madison, Wis.) by incubating at 37°C for 45 min. The kinase activity was inactivated by adding 2 μl of 0.5 M EDTA (pH 8.0). The labeled probe was then purified with the MicroSpin column (S-300 HR; Pharmacia-LKB Biotech).

Hybridization was performed by the standard techniques. The membranes were rinsed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then were soaked in prehybridization solution (6× SSC, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate [SDS], 100 μg of denatured DNA per ml, 50% formamide) at 42°C for 4 h. Hybridization was done in 10 ml of solution containing 6× SSC, 0.5% SDS, 100 μg of denatured DNA per ml, 50% formamide, and 5 pmol of labeled oligonucleotide probe at 42°C for 16 h. The membranes were washed by high-stringency procedures: 15 min at room temperature in 2× SSC–0.5% SDS twice, 15 min at 37°C in 1× SSC–0.5% SDS twice, and 15 min at 65°C in 0.1× SSC–1% SDS three times. Autoradiography was carried out for 36 h at −70°C with XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) in a cassette containing an intensifying screen.

RESULTS

According to the published sequence of the 16S rDNA of *E. platys*, the predicted PCR products with the primer pairs EP1-EP3 and EP2-EP3 would be 742 and 385 bp in length, respectively. The amplified DNA resulting from using primer pair EP1-EP3 was shown by electrophoresis in 2% agarose gels (Fig. 2a) to be approximately 742 bp, in accordance with the prediction. The specificity of the product was also confirmed from the signals obtained by Southern blot hybridization using an internal oligonucleotide probe end labeled with ^{32}P (Fig. 3b). The approximately 385-bp size of the amplified DNA produced by using primer set EP2-EP3 again confirmed the specificity of amplification (Fig. 2b). The negative control with no DNA template and with DNA from *E. canis* and *E. coli* as templates failed to produce any bands of product with both sets of primers (Fig. 2). Since DNA samples extracted from *E. canis* and *E. coli* could serve as templates for PCR amplification with 16S rDNA universal primers (data not shown), we conclude that both primer sets are specific for *E. platys* in our experiment.

The combination of one internal primer (EP2) with one of the external primers (EP3) yielded a specific 385-bp DNA fragment in our investigation. In this method, two bands amplified by two primer sets (EP1-EP3 and EP2-EP3) were expected to appear after the second amplification (Fig. 2b). Obviously, continued amplification of the 742-bp fragment did not visibly influence the results, and the use of one new primer is sufficient to prevent preferential amplification of aberrant PCR products.

TABLE 1. Nucleotide sequence alignment for the regions of the ehrlichial 16S rDNA corresponding to primers[a]

Ehrlichia sp.	EP1	EP2	EP3 (rc)[b]
E. platys	TGTCG-TAGCTT---GCTA-T--G	GAAGATAATGACGGTACCC	CGTTTTGTCTCTGTGTTG
E. canis	AT.TA.....C.CTG........A	..................T	GA....A...T...A...
E. equi	CT.TA..................A	..................T	TT....A..........
E. risticii	ATCA.G-...GCTT..AGCCTT.	....	GT.A..T.A....CT...
E. ewingii	CC.AAA...TC.CTGA.....TTA	..................T	GA....A...T...A...
E. sennetsu	ATCAAA-...GCTT..C.GCTT.	.......G..........	GT.A..T.A....CT...
E. chaffeensis	TC.TATA.C...TTG.T...-.TA	..................T	GA....A...T...A...

[a] The sequences were aligned by the Clustal method. The source for sequences used in the alignment was GenBank. All sequences are shown from 5′ to 3′ with respect to the 16S rDNA. Conserved positions are indicated with periods; gaps are indicated with hyphens.

[b] The reverse complementary sequence of the primer EP3 is shown as EP3 (rc).

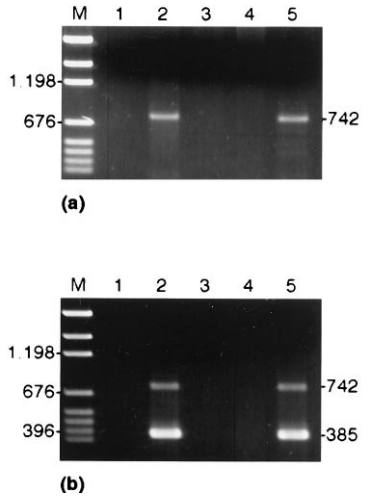

FIG. 2. Specificity of PCR primers. (a) Agarose gel electrophoresis of PCR products amplified with primers EP1 and EP3. (b) PCR products reamplified with primers EP2 and EP3. DNA templates were extracted from blood specimens of *E. platys* (lanes 2)- or *E. canis* (lanes 3)-infected patients and from *E. coli* (lanes 4). Lanes 1, no DNA control; lanes 5, a blood sample of the dog experimentally inoculated with *E. platys*; lanes M, molecular size standards (base pairs) (pGEM DNA marker; Promega). The sizes of molecular size standards are marked on the left of the gel, and the approximate sizes of PCR products are marked on the right of the gel.

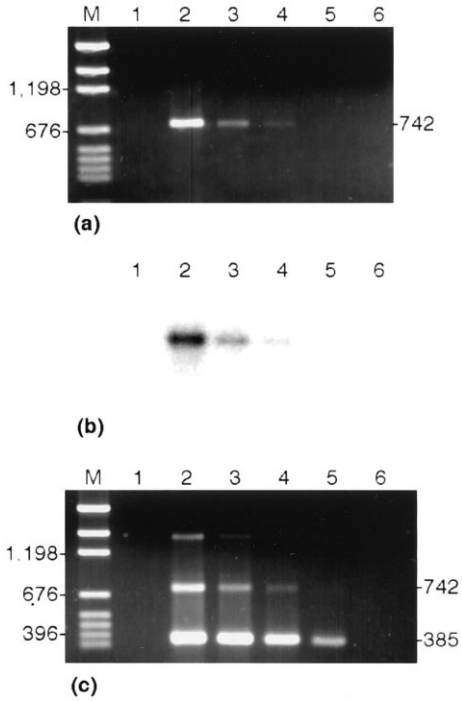

FIG. 3. Sensitivity test of two-step PCR for detecting *E. platys* DNA. (a) Agarose gel electrophoresis of first PCR products. (b) Corresponding Southern blot hybridized with a ^{32}P-labeled oligonucleotide internal probe. (c) Second PCR products amplified with primers EP2 and EP3. Lanes M, molecular size standards (base pairs); lanes 1, no DNA control; lanes 2 to 6, DNA extracted from 10-fold serial dilutions (10^0 to 10^{-4}) of a blood specimen containing about 5,000 n/μl of parasitized platelets.

A blood specimen (platelet count, 26,000 n/μl with 21% parasitized) collected from the experimentally inoculated dog on day 11 p.i. was used for testing sensitivity of the method. Blood was 10-fold diluted with PBS, and then DNA was extracted as templates for amplification. Amplified DNA with primer set EP1-EP3 either was detected by agarose gel electrophoresis and ethidium bromide staining or served as templates for the second round of amplification with primer set EP2-EP3. While DNA from 50 ehrlichial inclusions probably represents an absolute limit for detection after single PCR amplification, a faintly staining 742-bp DNA fragment was observed after agarose gel electrophoresis (Fig. 3a). In contrast, even with only five infected platelets in the specimen, two rounds of PCR amplification were able to produce a strong 385-bp DNA fragment (Fig. 3c), which indicates that a higher sensitivity of detection can be achieved.

For some unknown reason, a large faintly stained DNA fragment was produced. We noted these 1,500- to 1,600-bp products present in the lanes with high concentrations of the first PCR products as templates after amplification (Fig. 3c, lanes 2 and 3; see also Fig. 5b, lanes 8 and 11). Since this unexpected DNA neither influences the PCR amplification nor disturbs our judgment for positive results, we do not think the specificity of this PCR method would be reduced.

The typical hematologic manifestation of *E. platys* infection of parasitemia and concomitant thrombocytopenia was observed in this experimental dog (Fig. 4). Platelet count number dropped below the normal range on day 5 p.i. From day 9 to 12 p.i., the platelet counts of this dog were even less than 30,000 n/μl. Basophilic inclusions within platelets (Fig. 1) began to appear on day 7 p.i., and the peak of this parasitemic episode reached 21% on day 11 p.i. The parasitized platelets soon disappeared on day 14 p.i. in this episode (Fig. 4).

Blood specimens collected from the experimental dog served as templates for PCR amplification. The 16S rDNA of *E. platys* was detected in the single PCR product of DNA extracted from blood specimens collected on days 8 and 11 p.i. (Fig. 5a). Faint bands stained by ethidium bromide to be approximately 742 bp were also observed on days 7, 9, 10, and 12 p.i. (Fig. 5a). Evidently, two-step PCR is the most sensitive method, as a clear and strong 385-bp PCR product became detectable on day 5 p.i. However, probably because of that blood specimen containing a very small number of ehrlichial inclusions, the sample collected on day 14 p.i. was negative by two-step PCR.

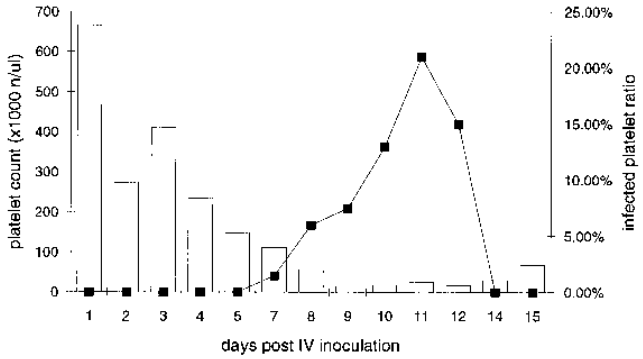

FIG. 4. Proportion of infected platelets and the platelet counts of an experimental dog intravenously inoculated with *E. platys*. The number of platelets (bar area) is on the left axis. The infected platelet ratio over a 15-day period p.i. (solid line) is on the right axis. Blood specimens were collected on the days p.i. as the numbers indicate.

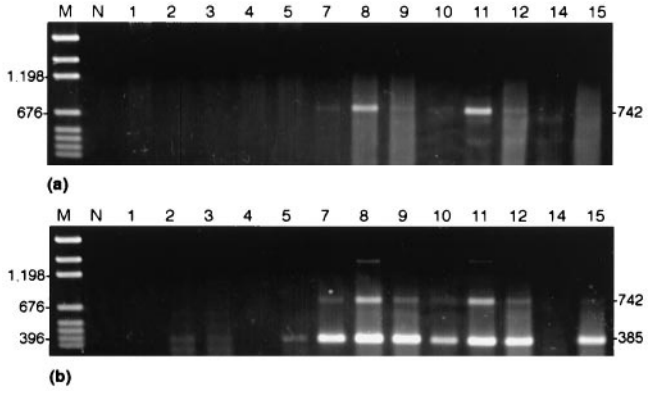

FIG. 5. Two-step PCR for detection of *E. platys* in blood samples of an experimental dog after inoculation. (a) Products of the first-step PCR amplification. (b) The second-step PCR-amplified DNA fragments. DNA templates were blood specimens collected on the days p.i. as the numbers indicate. Lanes M, molecular size standards (base pairs); lanes N, no DNA control.

DISCUSSION

According to the serologic studies reported by French and Harvey, *E. platys* infection is distributed throughout the United States (5). In our previous study, we confirmed that the etiologic agent of CICT and its vector are well established in Taiwan (3). Therefore, the development of a specific, sensitive, and efficient diagnostic method that indicates active infection of *E. platys* is of great interest.

Originally, laboratory diagnosis of CICT was done by direct observation of *E. platys* morulae within platelets in stained peripheral blood smears (6). Since morulae are most prevalent in the first episode of thrombocytopenia and may be present only intermittently thereafter, and since the appearance of parasitized platelets is cyclic in nature (3, 6), diagnosing CICT by demonstration of *E. platys* within platelets is unreliable, especially in the chronic stage of the disease. The IFA, which evaluates titer of specific antibodies against *E. platys* in serum, has been used widely; however, this method suffers from a lack of reliability in terms of assessing current status (8). Furthermore, serologic diagnosis is limited by the fact that successful cultivation of *E. platys* has never been reported, and the antigens for IFA are not readily available.

The recently introduced technique of PCR has great potential for improving the ability to diagnose infectious diseases caused by obligate intracellular bacteria. It has been employed to detect various kinds of rickettsial agents of humans and animals (1, 2, 8, 14). A disadvantage of PCR-based DNA detection is the need to confirm the authenticity of the amplified products either by Southern blot hybridization, by DNA sequencing, or by restriction endonuclease cleavage patterns. Kaneko et al. demonstrated that two-step PCR (nested PCR) amplifying with two successive primer pairs is equivalent to one round of PCR and Southern blot hybridization analysis (10). The amplification of the first PCR product with another set of primers not only increases the sensitivity and confirms the authenticity but also greatly reduces the manipulation time. This technique has been applied to detect some obligate intracellular bacteria like *Chlamydia psittaci* (9), *Rickettsia tsutsugamushi* (12), and the etiologic agent of human granulocytic ehrlichiosis (4) in the blood specimens.

A primer set (EP1-EP3) derived from the 16S rRNA gene of *E. platys* was constructed for use in PCR amplification. As clinical samples (patients' blood) contain a very low concentration of ehrlichiae, even in the ehrlichiaemia stage, but contain a large number of the host's blood cells, detecting *E. platys* DNA by single PCR seems not to be sensitive enough for clinical use. To increase the sensitivity, another upstream primer (EP2) was selected within the sequence between EP1 and EP3 for two-step PCR. The results obtained from Giemsa stain observation, one round of PCR amplification, and two-step PCR indicate that this two-step method provides the highest sensitivity for detecting *E. platys* in blood specimens. Sensitivity testing shows that two-step PCR is 10 times more sensitive than single PCR, as it can detect as few as five ehrlichial inclusions in the blood sample, whereas single PCR is only good enough to amplify the DNA templates from a sample containing at least 50 infected platelets (Fig. 3a and c).

As most strains of *E. platys* currently isolated rarely cause obvious clinical signs (3, 6, 18), when we inspect a blood specimen with severe thrombocytopenia, it is very difficult to give strong evidence of *E. platys* infection. Showing the *E. platys* parasitizing platelets in Giemsa-stained blood smears is a direct indication but is usually not compensatory, because of the cyclic nature of the appearance of parasitized platelets. To present the *E. platys* DNA within the blood specimen provides a better chance to give the definitive diagnosis of CICT directly. We applied this nested PCR to several thrombocytopenic blood samples, which were Giemsa stain negative. Two of them were positive after two rounds of PCR amplification (data not shown), which indicated that this PCR-based method is applicable for clinical use.

We conclude that the two-step PCR is very sensitive in detecting *E. platys* in blood specimens. Though one round of PCR and Southern blot hybridization is equivalent to nested PCR (10), Southern blot hybridization procedure involves several critical and time-consuming steps and utilizes a radiolabeled probe that must be prepared frequently. In contrast, the two-step PCR we developed is much more efficient, all the amplifications were performed in capillary tubes by an air thermal cycler (17), and it took less than 2 h to complete the total 75 cycles of the extension program. This report shows that the application of this technique is useful in detecting *E. platys* in the early phase or the chronic stage of CICT and is helpful in differential diagnoses of thrombocytopenia in dogs.

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