JOURNAL OF CLINICAL MICROBIOLOGY, July 1997, p. 1852–1855 Vol. 35, No. 7
0095-1137/97/$04.00+0

Comparison of Nested PCR with Immunofluorescent-Antibody Assay for Detection of *Ehrlichia canis* Infection in Dogs Treated with Doxycycline

BOHAI WEN,[1] YASUKO RIKIHISA,[1]* JASON M. MOTT,[1] RUSSELL GREENE,[2] HYUNG-YONG KIM,[1] NING ZHI,[1] GUILLERMO C. COUTO,[3] AHMET UNVER,[1] AND ROBERT BARTSCH[2]

Department of Veterinary Biosciences[1] and Department of Veterinary Clinical Sciences,[3] Ohio State University, Columbus, Ohio, and Southwest Veterinary Diagnostic Center, Phoenix, Arizona[2]

Received 13 May 1996/Returned for modification 1 November 1996/Accepted 11 April 1997

A partial 16S rRNA gene was amplified in *Ehrlichia canis*-infected cells by nested PCR. The assay was specific and did not amplify the closely related *Ehrlichia chaffeensis*, *Ehrlichia muris*, *Neorickettsia helminthoeca*, and SF agent 16S rRNA genes. The assay was as sensitive as Southern hybridization, detecting as little as 0.2 pg of *E. canis* DNA. By this method, all blood samples from four dogs experimentally infected with *E. canis* were positive as early as day 4 postinoculation, which was before or at the time of seroconversion. One hundred five blood samples from dogs from Arizona and Texas (areas of *E. canis* endemicity) and 30 blood samples from dogs from Ohio (area of *E. canis* nonendemicity) were examined by nested PCR and immunofluorescent-antibody (IFA) test. Approximately 84% of dogs from Arizona and Texas had been treated with doxycycline before submission of blood specimens. Among Arizona and Texas specimens, 46 samples were PCR positive (44%) and 80 were IFA positive (76%). Forty-three of 80 IFA-positive samples (54%) were PCR positive, and 22 of 25 IFA-negative samples (88%) were negative in the nested PCR. None of the Ohio specimens were IFA positive, but 5 specimens were PCR positive (17%). Our results indicate that the nested PCR is highly sensitive and specific for detection of *E. canis* and may be more useful in assessing the clearance of the organisms after antibiotic therapy than IFA, especially in areas in which *E. canis* is endemic.

Ehrlichia canis is an obligatory intracellular pathogen which causes canine ehrlichiosis (15). Canine ehrlichiosis was first described in Algeria in 1935 (5); now, it is recognized worldwide, particularly in tropical and subtropical areas (7, 13). In the United States, canine ehrlichiosis is primarily found in southern states (6, 13, 17). Brown dog tick, *Rhipicephalus sanguineus*, is the primary vector for *E. canis* transmission (9). Oral doxycycline is commonly used for treating canine ehrlichiosis (8).

The immunofluorescent-antibody (IFA) test with *E. canis* antigen has been the most widely used test for the diagnosis of *E. canis* infection of dogs since it was developed in 1972 (17). Although the IFA test is very sensitive in detecting the prevalence of exposure to *E. canis*, it is not useful for determining current infection status or assessing clearance of *E. canis* after antibiotic treatment, since dogs remain IFA positive for a long period of time after clearance of the organism (12). Especially in the geographic regions where *E. canis* is endemic, the serologic test alone is often unreliable, since dogs may be IFA positive due to multiple exposure to *E. canis*. Serologic cross-reactivities among *E. canis*, *E. chaffeensis*, *E. ewingii*, and other *Ehrlichia* spp. (14, 16, 20) pose another potential problem for the specificity of the IFA test. Our previous study found that cell culture isolation is the most sensitive and reliable diagnostic test for canine ehrlichiosis, since it detects the presence of live *E. canis* (10). However, isolation is laborious and impractical in clinical microbiology laboratories, because it takes too long (14 to 34 days) and requires an appropriate cell culture technique and facility, in addition to its high cost. Therefore, for the diagnosis of canine ehrlichiosis, a more sensitive, specific, and simple method to directly detect the organism is desirable. Along this line, we previously developed a one-step PCR test to detect *E. canis* DNA in blood and tissues from dogs experimentally infected with *E. canis* (10, 11), but it was often not sensitive enough to detect a few organisms in blood specimens from asymptomatic, naturally infected dogs, especially those which had been treated with antibiotics. In this study, we describe a more sensitive and specific nested PCR test to detect *E. canis* in blood specimens from experimentally and naturally infected dogs.

MATERIALS AND METHODS

Culturing *Ehrlichia* spp. *E. canis*, *E. muris*, *E. chaffeensis*, *Neorickettsia helminthoeca*, and SF agent were cultured as previously described (16, 19–21).

Blood specimens. Blood specimens were collected in a series from eight dogs at 4- to 7-day intervals after inoculation with 10^7 *E. canis*-infected DH82 cells as previously described (10). All dogs were seronegative for *E. canis* prior to inoculation with *E. canis*. Mononuclear cells were separated and kept at −80°C as described previously (10). Dog blood specimens (2 to 5 ml), in EDTA anticoagulant tubes, were obtained through the Southwest Veterinary Diagnostic Center in Phoenix, Ariz. (90 specimens were from Arizona and 15 specimens were from Texas), and from the Ohio State University Veterinary Teaching Hospital (30 specimens). Each blood sample was centrifuged at 2,500 × *g* for 5 min. Plasma was saved for the IFA test, and 200 μl of the buffy coat layer was harvested for PCR.

DNA extraction. DH82 cells (approximately 50% infected; 10^6 cells per 200 μl) or 200 μl of buffy coat were used for DNA extraction with a QIAamp Tissue Kit (QIAGEN Inc., Chatsworth, Calif.). At the final step, DNA was eluted from the column with 50 μl of H_2O, and the DNA concentration and purity were determined by measuring optical density at both 260 and 280 nm with a DNA calculator (GeneQuant II; Pharmacia Biotech, Piscataway, N.J.) and kept at −20°C.

PCR amplification of *E. canis* 16S rRNA gene. Extracted DNA (0.5 to 1 μg) was used as a template to amplify a fragment of the 16S rRNA gene in a 50-μl reaction mixture containing 5 μl of 10× PCR buffer, 5 μl of 50 mM $MgCl_2$, 1 μl of 10 mM deoxynucleoside triphosphate mixture, 1.25 U of *Taq* polymerase (GIBCO BRL, Gaithersburg, Md.), 2 pmol of primer ECC (5′-AGAACGAAC GCTGGCGGCAAGCC-3′) (4), and 2 pmol of primer ECB (5′-CGTATTACC

* Corresponding author. Mailing address: Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Rd., Columbus, OH 43210-1096. Phone: (614) 292-9677. Fax: (614) 292-6473.

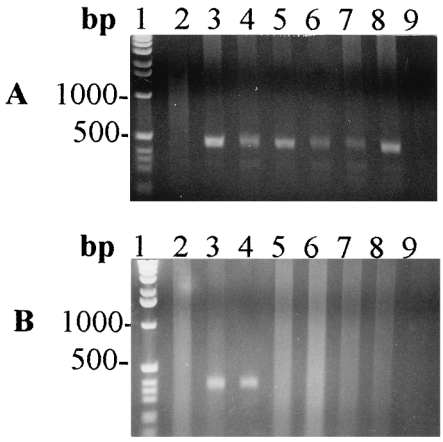

FIG. 1. Specificity of the nested PCR. The first (A) and second (B) PCR products are shown. Lanes 1 contain a 1-kb DNA ladder (GIBCO BRL) and lanes 2 to 9 contain DNA purified from various cells as follows: lanes 2, uninfected DH82 cells; lanes 3, *E. canis*-infected DH82 cells; lanes 4, purified *E. canis*; lanes 5, *E. chaffeensis*-infected DH82 cells; lanes 6, *E. muris*-infected DH82 cells; lanes 7, *N. helminthoeca*-infected DH82 cells; lanes 8, SF agent-infected DH82 cells; lanes 9, blood sample from an *E. canis*-seronegative dog. One microgram of DNA from each sample was used as the template in the first PCR. The numbers on the left indicate molecular sizes in base pairs.

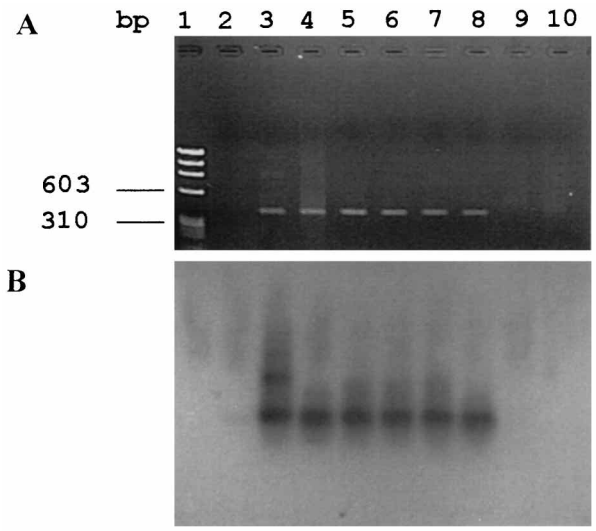

FIG. 2. (A) Sensitivity of the nested PCR to detect *E. canis* DNA. Lane 1, DNA standards (*Hae*III-digested ϕX174 replicative-form DNA fragments); lane 2, negative control; lanes 3 to 10, serially diluted *E. canis* DNA (100, 10, 1, 0.8, 0.5, 0.2, 0.1, or 0.01 pg, respectively). The numbers on the left indicate molecular sizes in base pairs. (B) Southern hybridization with a ^{32}P-labeled *E. canis* probe of the nested PCR products shown in panel A.

GCGGCTGCTGGC-3′) (4). PCR was performed at 94°C for 5 min and then for 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min in a Minicycler (MJ Research, Inc., Watertown, Mass.).

In the second PCR, the reaction mixture and conditions were the same as those in the first amplification, except for the primers and DNA templates. Two picomoles of primer HE-3 (5′-TATAGGTACCGTCATTATCTTCCCTAT-3′) (4) and 2 pmol of primer ECA (5′-CAATTATTTATAGCCTCTGGCTATAG GAA-3′), which is the sequence of the *E. canis* gene corresponding to *E. chaffeensis* primer HE-1 (2), were used, and 1 μl of the product of the first PCR was used as the DNA template. A positive control with 1 pg of *E. canis* DNA as the template and a negative control without DNA template were included in each PCR run. The resulting PCR products were electrophoresed through a 1.4% agarose gel, stained with ethidium bromide, and photographed with UV illumination.

Southern hybridization. The PCR products electrophoresed in an agarose gel were transferred to a Hybond-N$^+$ nylon membrane (Amersham Co., Arlington Heights, Ill.) by a standard method (18). The 389-bp *E. canis* nested PCR product was purified by using a Wizard PCR preps DNA purification system (Promega, Madison, Wis.), labeled with [α-^{32}P]dATP by the random primer method with a kit (Boehringer Mannheim, Co., Indianapolis, Ind.), and used as a DNA probe. Hybridization was performed at 60°C in rapid hybridization buffer (Amersham) for 20 h. The nylon sheet was washed in a solution containing 0.1× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate, and the hybridized bands were exposed to Hyper film (Amersham) at −80°C.

IFA test. The IFA test was performed as previously described (16). Briefly, *E. canis*-infected DH82 cell suspensions were placed onto 12-well Teflon-coated slides (Cel-Line Associates, Newfield, N.J.) at a concentration of 10^3 cells per well. The cells were air dried and fixed with cold acetone. The cells were incubated with serially diluted dog plasma at 37°C for 1 h. After three rinses in phosphate-buffered saline, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-dog immunoglobulin G (Organon Teknika Co., Durham, N.C.) at a dilution of 1:200 at 37°C for 1 h. The slides were observed with an epifluorescence microscope.

RESULTS

Specificity of the nested PCR. In the first PCR, a single 478-bp DNA fragment of a 16S rRNA gene was amplified from *E. canis*, and similar-sized DNA fragments were amplified from the rest of the *Ehrlichia* spp. used in this study (Fig. 1A). However, a DNA fragment (389 bp) of 16S rRNA gene was amplified only from the first PCR product of *E. canis* with the second pair of primers (Fig. 1B).

Sensitivity of the nested PCR. By using serially diluted DNA from purified *E. canis*, as little as 0.2 pg of *E. canis* DNA could be detected by the nested primer PCR (Fig. 2A). The Southern hybridization sensitivity was the same as that of the nested PCR (Fig. 2B).

PCR and IFA tests of experimentally infected dogs. A single 389-bp DNA fragment was amplified from all specimens of dogs 11250, 12925, 12931, and 13034 at day 4 postinoculation with *E. canis*, which was the earliest time when the blood specimens were collected from these dogs (Fig. 3). IFA titers at day 4 postinoculation were 1:20 for dog 11250 and negative for dogs 12925, 12931, and 13034. These four dogs remained PCR positive up to 2 months postinoculation when the experiment was terminated.

Dogs 011, 307, 320, and 340 were PCR positive at day 6 postinoculation, which was the earliest time when the specimens were available from these four dogs. At day 6 postinoculation IFA titers were 1:160 (dogs 011, 307, and 340) and 1:320 (dog 320). By one-step PCR, dogs 340 and 320 became positive at day 6 postinoculation (10). Dogs 011 and 307 were negative at day 6 postinoculation and became positive at day 8 and 10 postinfection, respectively, by one-step PCR (10). Dogs 011,

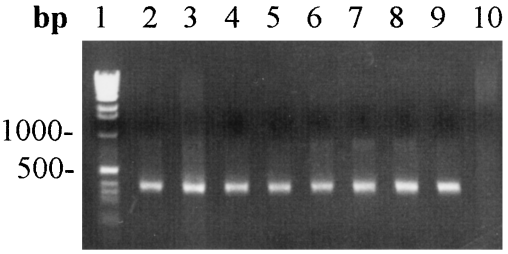

FIG. 3. Detection by nested PCR of *E. canis* DNA in the blood specimens of dogs experimentally infected with *E. canis*. Lane 1, DNA ladder. Lanes 2 to 5 contain day 4 specimens from experimentally infected dogs as follows: lane 2, dog 11250; lane 3, dog 13034; lane 4, dog 12931; lane 5, dog 12925. Lanes 6 to 9 contain day 6 specimens as follows: lane 6, dog 011; lane 7, dog 320; lane 8, dog 340; lane 9, dog 307. Lane 10, dog 011 day 0 specimen (negative control). The numbers on the left indicate molecular sizes in base pairs.

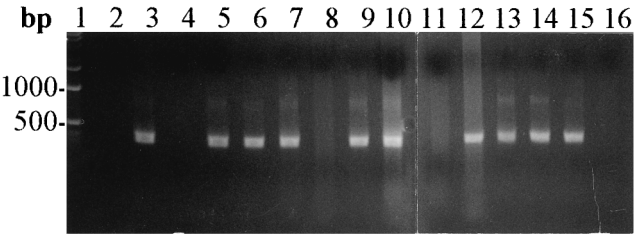

FIG. 4. Detection of *E. canis* DNA in blood specimens of dogs from Arizona and Texas by nested PCR. Lane 1, DNA ladder; lanes 2 to 14, blood samples from selected individual dogs (blood samples in lanes 2, 4, 8, and 11 were negative the remaining 9 dogs were positive); lane 15, positive control (0.2 pg of purified *E. canis* DNA); lane 16, negative control (no DNA template). The numbers on the left indicate molecular sizes in base pairs.

307, and 340 were both one-step PCR and culture isolation positive up to 4 months postinoculation despite 1 week of doxycycline therapy at 2 months postinoculation, as reported previously (11). Dog 320 was one-step PCR positive up to 2 months postinoculation and became PCR negative after 1 week of daily doxycycline treatment at 2 months postinoculation. The blood of dog 320 remained PCR and culture isolation negative throughout the 2-month period after withdrawal of the antibiotic, and its liver, spleen, kidney, mesenteric lymph node, and poplitial lymph node were also one-step PCR as well as culture isolation negative at necropsy at 2 months posttreatment (i.e., at 4 months postinoculation) (11, 12). All these four dogs developed mild clinical signs of transient fever, weight loss, thrombocytopenia, and increased gamma globulins before treatment with antibiotic, as previously reported (10).

PCR and IFA tests of naturally infected dogs. Genomic DNAs were extracted from 105 blood samples of dogs from Arizona and Texas and from 30 blood samples of dogs from Ohio, and the nested PCR was performed. The 389-bp DNA fragment was amplified in 46 samples (44%) among the Arizona-Texas specimens and in 5 samples (17%) among the Ohio specimens. One set of results of Arizona-Texas specimens is shown in Fig. 4. Eighty-six dogs in Arizona and Texas had been treated with doxycycline for periods ranging from 1 to 2 months (24 dogs), 3 to 6 months (28 dogs), 7 to 12 months (16 dogs), 13 to 24 months (7 dogs), and more than 24 months (11 dogs) at the time of the blood specimen collection for the PCR and IFA tests. Sixteen dogs had not been treated with doxycycline, and the treatment status of the remaining three dogs was unknown. Three (19%) of 16 non-antibiotic-treated dogs were PCR positive. None of the Ohio dogs had been treated with doxycycline at the time of blood specimen collection.

By the IFA test, 80 of 105 plasma specimens of dogs from Arizona and Texas were positive (titer $\geq$ 1:20). Forty-three of 80 IFA-positive samples were PCR positive, and 22 of 25 IFA-negative specimens were negative in the PCR (Table 1). The relation between PCR results and IFA titers is shown in Fig. 5. At higher IFA titers (>1:2,560) more specimens were PCR positive than negative. Thirty-seven samples (35%) were IFA positive but PCR negative. The median IFA titer of PCR-positive samples was 1:2,560 and that of the PCR-negative samples was 1:40.

DISCUSSION

In this study, we evaluated the nested PCR with two pairs of primers based on the known 16S rRNA gene sequences of *Ehrlichia* spp. (1, 2, 4) for detection of *E. canis* DNA in both experimentally and naturally infected dogs. The first pair of primers was common to the segment of the 16S rRNA genes of all *Ehrlichia* spp. The second pair of primers was specific to the internal segment of the first PCR product. The second PCR product was detected only in *E. canis*-infected cells but not in the cells infected with other ehrlichial organisms, including *E. chaffeensis* and *E. muris*, which are the species most closely related to *E. canis* (the sequence similarity of 16S rRNA genes among them is >98%) (1, 20). These results demonstrated that the nested PCR is specific to *E. canis*. The nested PCR was highly sensitive. It could detect as little as 0.2 pg of purified *E. canis* DNA, which was as sensitive as Southern hybridization and 100 times more sensitive than the previous one-step PCR, which could detect up to 20 pg of *E. canis* DNA (10). At day 4 postinoculation when three dogs were still seronegative and one dog had an IFA titer of 1:20, these four dogs were positive by the nested PCR, indicating that the nested PCR is more sensitive than IFA in detecting early stage *E. canis* infection. In a previous report, blood samples of dogs 011 and 307 were negative at day 6 postinoculation by one-step PCR (10); however, by nested PCR, they were positive.

Previously we reported that after 1 week of oral doxycycline treatment at 10 mg/kg of body weight once a day, only two of five experimentally infected dogs got rid of infection (11, 12). The remaining three dogs were continuously infected until the termination of the experiment (11, 12). Both the dogs which got rid of *E. canis* and those which remained infected were IFA positive at 2 months after antibiotic treatment (12). Our current study showed that doxycycline resistance of *E. canis* and persistence of IFA titer after doxycycline treatment also occur in natural infection. Of 105 blood specimens from dogs in Arizona and Texas, 84% of dogs had been treated with doxycycline, 46 samples (44%) were positive by the nested PCR, and 80 samples (76%) were positive by IFA. There were 37 dogs that were IFA positive and PCR negative, giving a false impression that IFA is more sensitive than the nested PCR. But based on the nested PCR sensitivity study using experimentally infected dogs and our previous study with doxycycline-treated dogs (12), it is most likely that positive IFA titers of PCR-negative dogs from Arizona and Texas were residual, and doxycycline treatment eliminated most *E. canis* organisms in approximately 50% of the dogs treated. Another possibility is that since *E. canis* is endemic in Arizona and Texas, some of these dogs were IFA positive due to multiple exposure to the antigen but were not currently infected; thus, they were PCR negative. An additional possibility is that the PCR result was negative due to infection of the dogs with *E. chaffeensis*, *E. ewingii*, or variant *E. canis* strains, which are IFA cross-reactive by using *E. canis* Oklahoma strain as antigen. 16S rRNA base sequences among new isolates of *E. risticii* were different at several bases (21), and they are serologically cross-reactive. Recently Dawson et al. reported a high prevalence of *E. chaffeensis* PCR-positive dogs in southeast Virginia (3). Their result also showed that some dogs with relatively high antibody titers against *E. chaffeensis* or *E. canis* were PCR negative. In the present study, 3 of 46 IFA-negative samples in Arizona and Texas and 5 of 30 IFA-negative samples in Ohio were PCR positive, which may have resulted from immunosuppression in the dogs, low levels of immunoglobulin G antibodies in the

TABLE 1. Results of examination of 105 dog blood samples by nested PCR and IFA test

Status by IFA test	Status by PCR (no. [%])		
	Positive	Negative	Total
Positive	43 [41.0]	37 [35.2]	80 [76.2]
Negative	3 [2.8]	22 [21.0]	25 [23.8]
Total	46 [43.8]	59 [56.2]	105 [100]

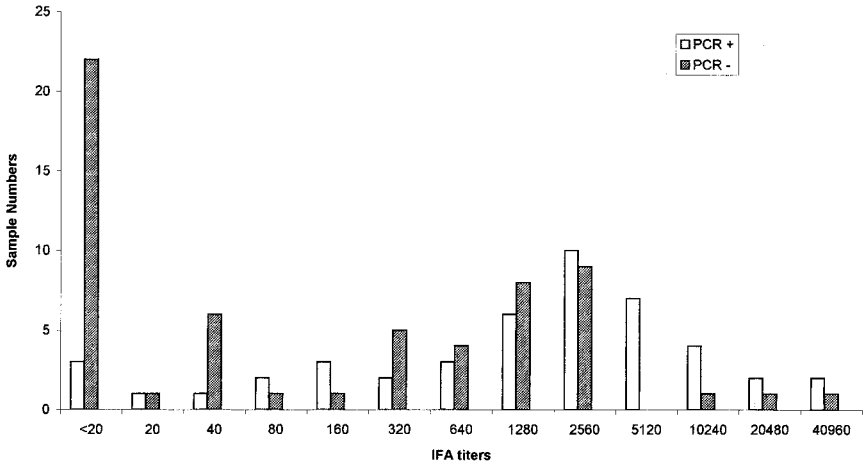

FIG. 5. Correlation between the nested PCR results and IFA titers for 105 blood specimens from Arizona and Texas.

early phase of *E. canis* infection, or a new undefined *Ehrlichia* sp. which may cross-react with our test. Dawson et al.'s study also showed four of eight (50%) *E. chaffeensis* PCR-positive dogs were seronegative to either *E. canis* or *E. chaffeensis* (3).

Our results in this study suggest that the nested PCR is highly specific and sensitive for the detection of *E. canis*, and it may be useful for laboratory diagnosis and assessment of the efficacy of antibiotic therapy for *E. canis* infection. Especially in combination with the IFA test, it can provide a more reliable yet quick diagnosis of canine ehrlichiosis. If the results of PCR and IFA tests are both positive or negative (62% of cases), dogs are either infected or not infected, respectively. If the IFA test result is positive but the PCR results are negative and the dogs have no clinical signs, we recommend withdrawing the antibiotic treatment and retesting the dogs 2 months later by IFA and nested PCR. If the dogs remain without clinical signs and IFA titers drop more than 16-fold and the dogs remain PCR negative, the infection is most likely cleared. If dogs are IFA negative but PCR positive (in this study, this was true of 2.8% of cases in areas in which *E. canis* was endemic and 17% of cases in areas of nonendemic *E. canis*), we recommend treatment, even without clinical signs of infection, until they become PCR negative.

ACKNOWLEDGMENT

This work was supported in part by the Ohio State University Canine Research Fund.

REFERENCES

1. **Anderson, B. E., J. E. Dawson, D. C. Jones, and K. K. Wilson.** 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J. Clin. Microbiol. **29:**2838–2842.
2. **Anderson, B. E., J. W. Sumner, J. E. Dawson, T. Tzianabos, C. R. Greene, J. G. Olson, D. B. Fishbein, M. Olsen-Rasmussen, B. P. Holloway, E. H. George, and A. F. Azad.** 1992. Detection of the etiologic agent of human ehrlichiosis by polymerase chain reaction. J. Clin. Microbiol. **30:**775–780.
3. **Dawson, J. E., K. L. Biggie, C. K. Warner, K. Cookson, S. Jenkins, J. F. Levine, and J. G. Olson.** 1996. Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from southeast Virginia. Am. J. Vet. Res. **57:**1175–1179.
4. **Dawson, J. E., D. E. Stallknecht, E. W. Howerth, C. Warner, K. Biggie, W. R. Davidson, J. M. Lockhart, V. F. Nettles, J. G. Olson, and J. E. Childs.** 1994. Susceptibility of white-tailed deer (*Odocoileus virginianus*) to infection with *Ehrlichia chaffeensis*, the etiologic agent of human ehrlichiosis. J. Clin. Microbiol. **32:**2725–2728.
5. **Donatien, A., and F. Lestoquard.** 1935. Existence en Algerie d'une Rickettsia du chien. Bull. Pathol. Exot. **28:**418–419.
6. **Ewing, S. A.** 1963. Observations on leukocytic inclusion bodies from dogs infected with *Babesia canis*. J. Am. Vet. Med. Assoc. **143:**503–506.
7. **Ewing, S. A.** 1969. Canine ehrlichiosis. Adv. Vet. Sci. Comp. Med. **13:**331–354.
8. **Greene, C. E., and J. W. Harvey.** 1990. Canine ehrlichiosis, p. 404–414. *In* C. E. Greene (ed.), Clinical microbiology and infectious diseases of the dog and cat. The W. B. Saunders Co., Philadelphia, Pa.
9. **Groves, M. G., G. L. Dennis, H. L. Amyx, and D. L. Huxsoll.** 1975. Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). Science **227:**522–524.
10. **Iqbal, Z., W. Chaichanasiriwithaya, and Y. Rikihisa.** 1994. Comparison of PCR with other tests for early diagnosis of canine ehrlichiosis. J. Clin. Microbiol. **32:**1658–1662.
11. **Iqbal, Z., and Y. Rikihisa.** 1994. Application of the polymerase chain reaction for the detection of *Ehrlichia canis* in tissues of dogs. Vet. Microbiol. **42:**281–287.
12. **Iqbal, Z., and Y. Rikihisa.** 1994. Reisolation of *Ehrlichia canis* from blood and tissues of dogs after doxycycline treatment. J. Clin. Microbiol. **32:**1644–1649.
13. **Keefe, T. J., C. J. Holland, P. E. Sayler, and M. Ristic.** 1982. Distribution of ehrlichia canis among military working dogs in the world and selected civilian dogs in the United States. J. Am. Vet. Med. Assoc. **181:**236–238.
14. **Perez, M., Y. Rikihisa, and B. Wen.** 1996. Antigenic and genetic characterization of an *Ehrlichia canis*-like agent isolated from a human in Venezuela. J. Clin. Microbiol. **34:**2133–2139.
15. **Rikihisa, Y.** 1991. The tribe *Ehrlichieae* and ehrlichial diseases. Clin. Microbiol. Rev. **4:**286–308.
16. **Rikihisa, Y., S. A. Ewing, J. C. Fox, A. G. Siregar, F. H. Pasaribu, and M. B. Malole.** 1992. Analysis of *Ehrlichia canis* and a canine granulocyte *Ehrlichia* infection. J. Clin. Microbiol. **30:**143–148.
17. **Ristic, M., D. L. Huxsoll, R. M. Weisiger, P. K. Hildebrandt, and M. B. A. Nyindo.** 1972. Serological diagnosis of tropical canine pancytopenia by indirect immunofluorescence. Infect. Immun. **6:**226–231.
18. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 9.31–9.58. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Wen, B., Y. Rikihisa, P. A. Fuerst, and W. Chaichanasiriwithaya.** 1995. Diversity of 16S rRNA genes of new *Ehrlichia* strains isolated from horses with clinical signs of Potomac horse fever. Int. J. Syst. Bacteriol. **45:**315–318.
20. **Wen, B., Y. Rikihisa, J. Mott, P. A. Fuerst, M. Kawahara, and C. Suto.** 1995. *Ehrlichia muris* sp. nov., identified on the basis of 16S rRNA base sequences and serological, morphological, and biological characteristics. Int. J. Syst. Bacteriol. **45:**250–254.
21. **Wen, B., Y. Rikihisa, S. Yamamoto, N. Kawabata, and P. A. Fuerst.** 1996. Characterization of the SF agent, an *Ehrlichia* sp. isolated from the fluke *Stellantchasmus falcatus*, by 16S rRNA base sequence, serological, and morphological analyses. Int. J. Syst. Bacteriol. **46:**149–154.