Tissue Diagnosis of Ehrlichia Chaffeensis in Patients with Fatal Ehrlichiosis by Use of Immunohistochemistry, In Situ Hybridization, and Polymerase Chain Reaction


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Abstract. In the United States, human ehrlichiosis is a complex of emerging tick-borne diseases caused by 3 distinct Ehrlichia species: Ehrlichia chaffeensis, Ehrlichia ewingii, and the human granulocytotropic ehrlichiosis agent. Ehrlichioses are characterized by a mild to severe illness, and ~4% of cases are fatal. Because these obligate intracellular bacteria are difficult to resolve with routine histologic techniques, their distribution in tissues has not been well described. To facilitate the visualization and detection of ehrlichiae, immunohistochemistry (IHC), in situ hybridization (ISH), and polymerase chain reaction (PCR) assays were developed by use of tissues from 4 fatal cases of E. chaffeensis infection. Evidence of E. chaffeensis via IHC, ISH, and PCR was documented in all 4 cases. Abundant immunostaining and in situ nucleic acid hybridization were observed in spleen and lymph node from all 4 patients. Significantly, in 2 of these patients, serologic evidence of infection was absent. Use of IHC, ISH, and PCR to visualize and detect Ehrlichia in tissues can facilitate diagnosis of ehrlichial infections.

INTRODUCTION

In 1986, a man from Michigan developed a severe febrile illness characterized by anemia, thrombocytopenia, encephalopathy, mild hepatitis, and acute renal failure 14 days after sustaining multiple tick bites in Arkansas. Careful examination of a Wright-Giemsa–stained buffy coat smear revealed rare inclusion bodies (morulae) in lymphocytes, atypical lymphocytes, neutrophils, and monocytes. This was the first reported evidence in humans in the United States of a disease caused by a member of the genus Ehrlichia. The agent was subsequently isolated and named Ehrlichia chaffeensis. Since the initial report, ~750 cases of disease attributable to this agent have been reported to state health departments. Case fatality ratios are as high as 5%.

In 1992, a Wisconsin man was hospitalized with an acute febrile illness similar to the index patient with E. chaffeensis infection. Once again, intracellular inclusions were observed in the patient’s peripheral blood smear. However, organisms were observed exclusively in neutrophils. The agent of this disease, human granulocytotropic ehrlichiosis (HGE), has also been isolated and shown to be very similar to E. phagocytophila and E. equi, agents of disease in domestic ruminants and horses, respectively.

In 1999, Buller and others described 4 febrile patients from Missouri, an area where E. chaffeensis (but not HGE) is endemic. Although inclusions were seen in granulocytes from 2 of the patients, anti-ehrlichial antibodies detected in these patients reacted with E. chaffeensis and the closely related canine pathogen, E. canis, but not with the agent of HGE. The polymerase chain reaction (PCR) products from the patients revealed sequences that matched the etiologic agent of canine granulocytic ehrlichiosis, E. ewingii.

These obligate intracellular bacteria are extremely difficult to detect upon routine examination of blood smears or hematoxylin and eosin (HE)-stained tissues. Morulae have proved to be difficult to find in immunocompromised patients in whom large numbers of ehrlichiae would be expected. Currently, most laboratory diagnoses are accomplished by indirect immunofluorescence assay (IFA). Few reports use immunohistochemistry (IHC) to detect E. chaffeensis infection have been published, and with the exception of one study that was limited to bone marrow, all reports have been single cases. Furthermore, PCR diagnosis based on testing of formalin-fixed, paraffin-embedded tissues (PET) has been reported for only one case. In the present study, we developed probes specific for ehrlichiae and used these probes to detect E. chaffeensis in various tissues from 4 patients with fatal disease.

MATERIALS AND METHODS

Patient 1. A 41-year-old woman with advanced human immunodeficiency virus (HIV) disease developed a febrile illness in June 1992, ~10 days after receiving a tick bite in northern Arkansas. She died of pulmonary hemorrhage after a 10-day hospital stay. Infection with E. chaffeensis was diagnosed postmortem by demonstration of morulae in the peripheral blood, PCR, electron microscopy, and IHC. Patient serum samples collected 1 day before death and at autopsy were negative for antibodies reactive with E. chaffeensis as determined by IFA.

Patient 2. A 52-year-old man with advanced HIV disease developed high fever and pancytopenia ~2 weeks after being bitten by a tick in May 1996 in central Georgia. The patient died of acute renal failure and pneumonia 10 days after the onset of illness. Infection with E. chaffeensis was confirmed by visualization of morulae in the peripheral blood, PCR, electron microscopy, and isolation of the organism in cell culture. Serum samples collected 4 and 7 days after onset of disease were negative for antibodies reactive with E. chaffeensis by IFA.

Patient 3. A 66-year-old man from northeast Texas was hospitalized in December 1997 with a 10-day history of fever, malaise, and cough. Laboratory abnormalities on admission included a white blood cell count of 3.7 cells/L, a platelet count of 62 × 10^9/L, an aspartate aminotransferase level of 156 U/L, and an alanine aminotransferase level of 156 U/L.
level of 222 U/L. His condition deteriorated over the next several days as he developed seizures, systemic hemorrhages, adult respiratory distress syndrome, hemolysis, and acute renal failure. The patient died on the eighth day of hospitalization. *Ehrlichia chaffeensis* DNA was detected in fresh frozen lung tissue by PCR. 15 A single serum sample collected 16 days after onset of disease was positive for antibodies reactive with *E. chaffeensis* (IFA titer = 2.048).

**Patient 4.** An 80-year-old woman from Delaware with a low-grade monoclonal gammapathy of unknown significance became ill with fever, chills, sore throat, weakness, and severe epigastric pain in May 1999, 24 hr after an embedded tick was noted on her back. She presented to an emergency room 6 days after the onset of symptoms, at which time she was found to be hypotensive (blood pressure 100/50 mm Hg) and pancytopenic (white blood cell count 1.6 × 10⁹/L, hemoglobin 12.5 g/dL, and platelets 26 × 10⁹/L). Additional abnormal laboratory values included elevated levels of aspartate aminotransferase (350 U/L), alanine aminotransferase (174 U/L), lactate dehydrogenase (1218 U/L), and creatinine (4.9 mg/dL). Doxycycline therapy was begun immediately, but she soon developed jaundice and diffuse ecchymoses. Respiratory failure requiring mechanical ventilation ensued, and the patient became bradycardic.

She died ∼24 hr after admission to the hospital. Polymerase chain reaction testing of acute-phase whole blood detected DNA of *E. chaffeensis*. A single serum sample collected 7 days after onset of disease was positive for antibodies reactive with *E. chaffeensis* (IFA titer = 512).

**Patient tissues.** Patient tissues examined included bone marrow (n = 4), liver (n = 3), lung (n = 3), spleen (n = 3), lymph node (n = 3), kidney (n = 1), and adrenal gland (n = 1). For all cases, routine H&E-stained sections were examined, and clinical and laboratory reports were reviewed.

**Cell and tissue controls.** We developed IHC and *in situ* hybridization (ISH) assays by using controls of 24-hr, formalin-fixed, paraffin-embedded pellets of minced normal human tissue mixed with uninfected DH82 cells,5 ISE6, 6 or IDE8 tick cells,18 P388D1 or Hep-2 cells, and cells infected with *E. chaffeensis* (Arkansas isolate), *E. canis* (Ebony isolate), HGE agent (Minnesota isolate), *E. sennetsu* (Miyayama isolate), *E. risticii* (Illinois isolate), or *Chlamydia pneumoniae* (CWLO-029 isolate) (Table 1). Normal human tissues provided a matrix to which the infected and uninfected cells could bind and assisted in determining background staining levels. Tissues from patients infected with pathogens other than *Ehrlichia* spp., including *Rickettsia rickettsii*, *R. prowazekii*, *Coxiella burnetii*, or hantavirus, were used to determine if nonspecific antibody binding or probe hybridization occurred.

**Antibodies.** Hyperimmune anti-serum from a dog experimentally infected with *E. canis*-infected ticks was used to detect *E. chaffeensis* in formalin-fixed tissues by IHC assay. Antigens of *E. canis*, *E. chaffeensis*, and *E. ewingii* may cross-react in serologic assays. 19 Human anti-*E. chaffeensis* sera resulted in much higher background on human tissues than canine anti-*E. canis* sera. The sensitivity of the 2 high-titer sera were the same; therefore, the canine antisera was utilized. Serum collected from a naive dog served as a negative control.

**Immunohistochemistry.** Formalin-fixed PET were cut at 3 μm, and sections were placed on Fisher Plus slides (Fisher Scientific, Pittsburgh, PA) and incubated for 30 min at 60°C to melt the paraffin. Paraaffin was removed from the slides with xylene, and the samples were rehydrated by sequential immersions in 100, 95, and 70% ethanol and deionized water. The tissue sections were digested in 0.1 mg/mL proteinase K (Roche, Indianapolis, IN) in 0.6 M Tris (pH 7.5)/0.1% calcium chloride (PK buffer) for 15 min. All of the subsequent steps were performed at room temperature in a Dako autostainer (Dako, Carpinteria, CA). A blocking step was performed with 20% normal sheep serum. The optimal dilution and specificity of the primary antibody was determined in pilot experiments by use of a series of titrations applied to control slides consisting of *E. chaffeensis*-infected and uninfected DH82 cells. The primary antibody was applied to the tissue section and incubated for 90 min. This step was followed with a 60-min incubation of alkaline phosphatase conjugated goat anti-dog immunoglobulins (1:20; Kirkegaard and Perry Laboratories, Gaithersburg, MD) and a subsequent 15-min incubation in naphthol/fast red substrate (Dako). Sections were counterstained in Mayer hematoxylin (Fisher Scientific).

**The RNA probe design and labeling.** The 16S rRNA probes used in this study were generated from nested PCR products amplified from *E. chaffeensis* (genus- and *E. chaffeensis*-specific probes) or the HGE agent (HGE-specific probe) by use of primers with RNA polymerase promoter sequences. The nucleotide sequences of the primers and their positions relative to *Escherichia coli* or *E. chaffeensis* are shown in Table 2. The forward primers, designated T3-270F and T3-15F were complementary to the 16S ribosomal RNA gene and contained the T3 promoter sequence at the 5' end. The reverse primers, designated T7-398R and T7-151R, were complementary to 16S ribosomal RNA and had T7 promoter sequence at the 5' end. Template DNA was extracted with phenol and precipitated with ethanol before transcription to optimize incorporation of the digoxigenin-

| Table 1 | Cells and tissues used in the development of the IHC, ISH, and polymerase chain reaction assays* |  |
|---|---|---|---|---|---|
| *E. chaffeensis/DH82† | E. chaffeensis DH82† | E. chaffeensis DH82† | E. chaffeensis DH82† | E. chaffeensis DH82† | E. chaffeensis DH82† |
| Ehrlichia canis/IDE8‡ | + | + | + | + | + |
| HGE/ISE6§ | - | - | - | - | - |
| Ehrlichia sennetsu/P388D1¶ | - | - | - | - | - |
| Ehrlichia risticii/P388D1# | + (small subset) | + | + | + | + |
| Chlamydia pneumoniae/Hep-2 | - | - | - | - | - |
| Rickettsia rickettsii (lung) | - | - | - | - | - |
| Rickettsia typhi (skin) | - | - | - | - | - |
| Cosxiella burnetii (heart) | - | - | - | - | - |
| Hantavirus (liver, lung, spleen) | - | - | - | - | - |

* Uninfected cells lines tested in parallel and found to be negative. HGE = human granulocytic ehrlichiosis; IHC = immunohistochemistry; ISH = *in situ* hybridization.
† Miyayama isolate of *E. chaffeensis*. 1
‡ Ebony isolate of *E. cans* (Ewing SA and others, unpublished data).
§ Canine isolate of HGE. 19
¶ Miyayama isolate of *E. sennetsu* (ATCC VR-367).
§ Maryland isolate of *E. risticii* (ATCC VR-3996).
labeled nucleotides. Digoxigenin-labeled RNA probes were generated by in vitro transcription of the PCR products with either T3 or T7 RNA polymerase by use of a digoxigenin RNA labeling kit (Roche). Adequate labeling of the probes was confirmed by dot-blot analysis.21

**In situ hybridization.** In situ hybridization assays were performed by a biotin-streptavidin method essentially as previously described.22 Briefly, 3-μm sections of formalin-fixed PET were placed on Fisher Plus slides (Fisher Scientific) and incubated, and paraffin was removed as described above for the IHC slides. The tissue sections were digested in PK buffer for 30 min, then postfixed in 4% paraformaldehyde (Polysciences, Warrington, PA) and acetylated with 0.025% acetic anhydride in 0.1 M triethanolamine (Sigma Chemical, St. Louis, MO). Subsequently, the tissues were denatured in 70% ethanol followed by room-temperature 95 and 100% ethanol. The slides were air dried and prehybridized for 30 min at room temperature in hybridization buffer containing 50% deionized formamide and 50% hybridization buffer (Roche). The slides were rinsed in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and air dried. The RNA probes were diluted (1:20) in hybridization buffer, denatured at 95°C for 5 min, and applied to the sections. The tissues were covered with Parafilm (American National Can, Chicago, IL) and hybridized overnight at 37°C in a humidified chamber. The slides were then washed in 6× SSC at room temperature, followed by 25 min in 50% formamide/2× SSC at 44°C. Sections were placed in the Dako autosetter and blocked with 20% normal sheep serum. Mouse anti-digoxigenin antibodies (Roche) diluted 1:500 were applied to the sections for 2 hr and detected by serial application of anti-mouse biotinylated immunoglobulins and streptavidin alkaline phosphatase and naphthol fast red substrate (Dako). Sections were counterstained in Mayer hematoxylin (Fisher Scientific) and mounted with aqueous mounting medium (Signet Laboratories, Dedham, MA). The specificity of the probes was confirmed by means of a digoxigenin-labeled *Chlamydia pneumoniae* RNA probe as a negative control probe, as well as incubation of each *Ehrlichia* probe with all cell and tissue controls.

**Polymerase chain reaction.** We extracted DNA from 10-μm sections of the formalin-fixed PET by the QIAamp Tissue Kit (Qiagen, Valencia, CA), following the manufacturer’s protocol for PET. Each DNA template was examined by heminested PCR. The region of the 16S gene selected for *Ehrlichia* identification and the reaction conditions used for PCR amplification of that region have been described previously.23 In this study, however, Roche PCR buffer and Taq polymerase were used. The primary amplification reaction used primers 15F23 and HE3 in a 100-μL reaction containing 20 μL of template DNA. These products were amplified in a thermocycler (Perkin-Elmer/Applied Biosystems, Foster City, CA) for 5 min at 94°C followed by 40 cycles of 1 min at 94°C, 2 min at 45°C, and 2 min at 72°C. A final 7-min extension was performed at 72°C. The conditions for the heminested amplification reactions were identical to those used for the primary reactions except for the primers and target DNA concentrations. Each heminested reaction contained 1 μL of the product from the outside reaction as DNA template and primers HE1 and HE3 for the specific amplification of *E. chaffeensis.* The temperature profile was the same as that described above, except that the annealing temperature was increased to 55°C and the annealing and extension times were each shortened to 1 min. The 389-bp amplification products were analyzed by electrophoresis in 1.3% agarose gels and visualized with ethidium bromide.

**Sequencing of DNA.** Sequencing of DNA was performed on amplified products from 2 different tissues from each patient, except one for whom only bone marrow was available. A total of 7 PCR products were prepared for sequencing with the Wizard PCR Prep DNA Purification System following the protocol for direct purification of DNA from PCR amplifications (Promega, Madison, WI). The products were labeled for sequencing with the PRISM BigDye Terminator Cycle Sequencing Kit (Perkin Elmer/Applied Biosystems). The unincorporated fluorescent nucleotides were separated from labeled products with Centri-Sep columns (Princeton Diagnostics, Princeton, NJ). The sequence was collected with an Applied Biosystems 373A automated sequencer and edited with Sequence Navigator (Perkin Elmer/Applied Biosystems). The sequence obtained from the amplified PCR products was compared with pertinent *Ehrlichia* sequences by GCG software (Genetics Computer Group, Madison, WI).

**RESULTS**

**Visualization of morulae by routine stains.** A total of 18 blocks representing 7 tissues from 4 patients were ex-
of PCR are presented in Table 3. Seventy-two percent of staining was present by ISH than by IHC. This was especially evident in sections of lung evaluated by both methods. The greatest amount of nucleic acid was observed in the spleen and lymph node.

**Immunohistochemistry.** The specificity of the *E. canis* antibody to the various antigens is presented in Table 1. As expected, staining was observed with *E. canis* - and *E. chaffeensis*-infected cells. Unexpectedly, 10% of the *E. risticii*-infected cells were IHC positive. This finding was repeated several times.

Immunohistochemical staining of ehrlichial antigens was observed in all tissues examined (Table 3). Antigen-positive cells were widely distributed in various tissues (Figure 1). Immunostaining was observed in cells of the mononuclear phagocyte system as typical morulae and as less defined antigen aggregates. Macrophages in the spleen, hilar lymph node, and bone marrow revealed the greatest number of inclusions. Morulae were less frequent in the lung and liver (Kupffer cells) and rare in the kidney and adrenal gland.

**In situ hybridization.** Three different digoxigenin-labeled probes were developed for this study. The specificity of the probes are detailed in Table 3. The T7-labeled 130-nucleotide genus-wide probe was more sensitive (16 of 18 positive tissues; 89%) than the T7-labeled 80-nucleotide *E. chaffeensis*-specific probe (12 of 18 positive tissues; 67%) in detecting *E. chaffeensis* rRNA in patient tissues.

The ISH signal was primarily in the form of typical morulae, which were easily visualized in cells of the mononuclear phagocyte system (Figure 1). Although the tissue distribution of nucleic acid was similar to the distribution of antigen as visualized by IHC, in general, quantitatively less staining was present by ISH than by IHC. This was especially evident in sections of lung evaluated by both methods. The greatest amount of nucleic acid was observed in the spleen and lymph node.

**Polymerase chain reaction and sequencing.** The results of PCR are presented in Table 3. Seventy-two percent of tissues were positive by PCR for *E. chaffeensis* 16S rDNA. Approximately 300 nucleotides of sequence were determined from PCR-amplified material from Patient 1 (lymph node and bone marrow), Patient 2 (bone marrow only), Patient 3 (lymph node and spleen), and Patient 4 (lymph node and bone marrow). The 7 sequences were identical with the *E. chaffeensis* 16S rDNA sequences in GenBank (accession numbers M73222, U23503, U60476, U86664, and U86665).

**DISCUSSION**

This study demonstrates the diagnostic utility of IHC, ISH, and PCR and provides insight into the distribution of *E. chaffeensis* in human tissues. The epidemiologic and clinical characteristics of the 4 fatal cases described in this report were strongly compatible with ehrlichiosis caused by *E. chaffeensis*. Three of the 4 patients had a history of tick bite preceding the illness, all resided in areas where *E. chaffeensis* was endemic, and all presented with an acute syndrome characterized by fever, headache, malaise, and myalgia. Two HIV-positive patients failed to develop a diagnostic antibody response before death; the other 2 patients had antibodies to *E. chaffeensis*, but only single serum samples were available. Although highly suggestive of acute infection, paired sera with a 4-fold rise in titer are required for confirmed infection. Childs and others concluded that many, if not most, *E. chaffeensis*-infected patients who present early in the course of the disease may be missed by serologic methods due to lack of detectable antibody. If IFA had been the only test attempted, none of these 4 fatalities would have met the Council of State and Territorial Epidemiologists’ criteria for confirmed ehrlichiosis.

In addition to the evaluation of serologic response, current diagnostic laboratory testing includes microscopic examination of Wright-Giemsa-stained blood smears for intracytoplasmic inclusions. However, inclusions in *E. chaffeensis* infection are rare and are often missed during initial examination of blood smears. In routine HE-stained tissue sections, it is also extremely difficult to identify organisms. We often failed to identify morulae unequivocally in HE-stained sections where antigen, as determined by IHC, was abundant. Therefore, routine HE staining should not be used to exclude a diagnosis. Specific diagnostic tests, such as IHC, ISH, and PCR, are essential in this regard and are useful to the accurate identification of these organisms.

All tissue blocks from all cases were positive by IHC, establishing the IHC technique as a sensitive diagnostic tool. Similarly, a recently published IHC study demonstrated that the granulocytotropic ehrlichiosis agent infects many tissues. The greatest number of ehrlichiae were seen in the spleen, lung, and liver. Infected neutrophils occur infrequently in bone marrow, and rare morulae were detected in lymphatic mononuclear cells in one patient. In contrast, in our study, *E. chaffeensis* antigens were consistently seen in mononuclear phagocytic cells in the spleen, lymph nodes, bone marrow, lung, and liver. In this context, spleen, lymph nodes, or bone marrow appear to be excellent tissues for confirmation of *E. chaffeensis* infection by IHC.
Immunohistochemical and in situ hybridization localization of ehrlichiae in 4 patients with fatal *Ehrlichia chaffeensis* infection. Immunohistochemical staining results are presented on the left side of the panel (A [Patient 1], C [Patient 2], E [Patient 4], G [Patient 1], and I [Patient 4]) and identical tissues tested by in situ hybridization are presented on the right (B [Patient 1], D [Patient 2], F [Patient 4], H [Patient 1], and J [Patient 1]). *Ehrlichia chaffeensis* organisms appear as red inclusions by both techniques (immunoalkaline phosphatase stain with naphthol phosphate–fast red substrate and hematoxylin counterstain). Staining in lung (A and B, bar = 10 μm), bone marrow (C and D, bar = 15.9 μm), spleen (E and F, bar = 10 μm), lymph node (G and H, bar = 15.9 μm), and liver (I and J, bar = 10 μm) revealed a larger bacterial load than previously recognized by routine histologic methods.
This is to our knowledge the first report on the detection of *E. chaffeensis* nucleic acids in histologic samples. Testing with ISH demonstrated nucleic acids in 89% of the tissue blocks when the genus-wide probe was used and 67% of the blocks when the *E. chaffeensis* probe was used. However, the latter probe had the advantage of being more specific. Digoxigenin-uridine triphosphate (DIG-UTP) is incorporated into the transcript approximately every 25 nucleotides. Therefore, the 130-nucleotide genus-wide probe contains 5–6 DIG-UTP whereas the 80-nucleotide species-specific probe contains only 3–4 DIG-UTP. The reduced number of DIG-UTP could account for the decreased sensitivity of the *E. chaffeensis*-specific probe. Although ISH testing identified *E. chaffeensis* nucleic acids in each of the locations where antigens were stained by IHC, staining of the former was generally less pronounced. Future refinement of the *Ehrlichia* ISH procedure and testing of other probes will be necessary to enhance the sensitivity of this technique.

Seventy-two percent of the tissue blocks examined were PCR positive and sequence confirmed for *E. chaffeensis* 16S rDNA. The 5 PCR-negative tissues were also negative when the *E. chaffeensis*-specific probe was used in the ISH procedure. Several variables known to cause nucleic acid degradation affect the yield of amplifiable DNA in an unpredictable manner. The RNA obtained from formalin-fixed PET is often degraded, not only by ribonuclease during tissue handling and processing, but also by routine formalin fixation. Advantages of PCR over classic techniques are that rapid detection can accelerate diagnosis in comparison to isolation of the agent in cell culture. Polymerase chain reaction also allows detection in specimens that are unsuitable for cultivation attempts. With the addition of xylene and ethanol extraction steps, PCR on formalin-fixed tissues can be routinely performed in classical microbiology laboratories. Polymerase chain reaction amplification from formalin-fixed PET should facilitate a wide range of studies on archival tissue, as well as improve the utility of diagnostic PCR assays when fresh tissue cannot be obtained.

Routine histopathologic examination of tissues does not totally explain the mechanism of tissue injury or cause of mortality associated with *Ehrlichia* infection. The present study, which may be biased because all were fatal infections and some were in immunocompromised patients, revealed a larger bacterial load than previously recognized by routine histologic methods. Characterization of tropism of *E. chaffeensis* in liver, lymph node, lung, spleen, kidney, adrenal gland, and bone marrow by IHC and ISH provides useful insights into the pathogenesis of this disease. Infection of mononuclear phagocytes may play a critical role in the pathogenesis of *E. chaffeensis* infection through the secretion of physiologically active substances, including cytokines and other inflammatory mediators.

In conclusion, we were able to visualize *E. chaffeensis* nucleic acids and antigens in formalin-fixed PET from 4 fatal cases of ehrlichiosis by IHC and ISH techniques; and PCR was used to amplify 16S rDNA, thereby providing a thorough characterization of the infection in autopsy tissues. Use of IHC and ISH to visualize *Ehrlichia* can facilitate the diagnosis of the ehrlichioses and should assist in elucidating the pathogenesis of these diseases.

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