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Comparison of PCR and Culture to the Indirect Fluorescent-Antibody Test for Diagnosis of Potomac Horse Fever

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Potomac horse fever is an acute systemic equine disease caused by *Ehrlichia risticii*. Currently, serologic methods are widely used to diagnose this disease. However, serologic methods cannot determine whether the horse is presently infected or has been exposed to ehrlichial antigens in the past. The purpose of the present study was to compare the sensitivities of the nested PCR and cell culture with that of the indirect fluorescent-antibody (IFA) test for the diagnosis of Potomac horse fever. Blood and fecal specimens serially collected from a pony experimentally infected with *E. risticii* Maryland, blood specimens serially collected from mice inoculated with *E. risticii* Ohio 380, and blood and/or fecal specimens collected from 27 horses which had clinical signs compatible with Potomac horse fever were examined. These horses resided in Kentucky, Indiana, Pennsylvania, and Vermont. The IFA test titer became positive after 6 days postinoculation (p.i.) for the pony. A culture of the blood of the pony was positive for *E. risticii* starting on day 1 and was positive through day 28 p.i. By the nested PCR, *E. risticii* was detectable in the blood and feces of the pony starting on day 1 and was detectable through day 32 p.i. *E. risticii* was detected in the blood of subclinically infected mice by the nested PCR. Twenty-two clinical specimens were seropositive for *E. risticii* by the IFA test, with titers ranging from 1:20 to 1:1,280. *E. risticii* was cultured from 95% (20 of 21) of seropositive clinical blood specimens. *E. risticii* was detected in the blood by PCR in 81% (17 of 20) of the culture-positive clinical specimens. The study indicated that the nested PCR is as sensitive as culture for detecting infection with *E. risticii*.

Ehrlichia risticii is an obligatory intracellular bacterium in the family *Rickettsiaceae* (9, 21). *E. risticii* is the causative agent of Potomac horse fever (PHF), characterized by anorexia, fever, depression, diarrhea, leukopenia, dehydration, and laminitis (9, 18, 21). Serologic evidence of infection is found throughout the United States, Canada, and Europe (7, 23, 27). However, culture for *E. risticii* was only done with specimens from horses residing in Maryland (5, 8, 21), Ohio (4), and Kentucky (4, 24). Experimentally infected horses develop immunoglobulin M and immunoglobulin G antibodies against *E. risticii* (15, 16, 18), and therefore, an indirect fluorescent-antibody (IFA) test has been widely used for diagnosis (23). The IFA test titer, however, does not distinguish between present and past infection or vaccination. Bacterins made of the type strain of *E. risticii* are available from three commercial sources. The efficacies of vaccines in the field are perceived to be marginal (13). In agreement with this perception, *E. risticii* has been isolated from sick horses which had been vaccinated (4, 26). Although, rising IFA test titers with accompanying clinical signs suggest active infection, an IFA test at a single time point is useless in diagnosing PHF in vaccinated horses. Seroepidemiologic studies reported high incidences of seropositive horses without clinical signs (1, 6, 10, 17, 19). A recent comparative serologic study of Californian horses for PHF suggests that this might be partially due to a high incidence of false-positive IFA test results in some laboratories (12). Therefore, a test which directly detects the presence of ehrlichial organisms in clinical specimens is desirable. A one-step PCR which detects unknown target DNA of *E. risticii* was previously used to detect *E. risticii* in the blood and feces of two experimentally infected horses (3), and a nested PCR which detects a partial 16S rRNA gene of *E. risticii* (2) was developed. These PCR methods were not compared with the culture isolation method. In this study we compared the sensitivities of culture and the nested PCR, which detects an *E. risticii*-specific sequence of the 16S rRNA gene, with the IFA test using blood and fecal specimens collected from both experimentally and naturally infected horses.

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MATERIALS AND METHODS

E. risticii. *E. risticii* Maryland (31) and Ohio 380 (4) were cultured in P388D$_1$ murine macrophage cells (American Type Culture Collection, Rockville, Md.) in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, Ga.) and 2 mM L-glutamine (GIBCO) at 37°C in 5% CO_2–95% air.

Experimental infection of a pony. One pony negative for an *E. risticii* antibody titer was intravenously inoculated with 5×10^7 *E. risticii* Maryland-infected P388D$_1$ cells. Blood samples were taken from the jugular vein of the pony from 1 to 32 days postinoculation (p.i.) at 2- to 3-day intervals to be used for determination of the antibody titer by the IFA test and buffy coat collection. Naturally voided fecal samples were collected from days 1 to 32 p.i. for use in a PCR amplification. The rectal temperature, depression, anorexia, diarrhea, leukopenia, and other clinical signs were monitored daily for 34 days. Blood and fecal specimens were also collected from two healthy horses which were seronegative for PHF; these served as negative controls.

Experimental infection of mice. A group of five female CF1 mice (age, 6 weeks; Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) were inoculated intraperitoneally with 10^4 P388D$_1$ cells infected (80%) with *E. risticii* Ohio 380 (4). The control group of 5 CF1 mice received 10^4 uninfected P388D$_1$ cells intraperitoneally. Blood (~0.2 ml) was collected every 3 to 4 days from the retro-orbital plexus of all mice in both experimental groups until day 17 p.i.

Peripheral blood mononuclear cell separation. Heparinized blood samples were aseptically collected from the experimentally infected pony (~30 ml), experimentally infected mice (pooled from five mice each ~1 ml), several healthy horses (~30 ml), and 27 horses (~10 to 30 ml) with clinical signs of PHF in Indiana, Kentucky, Michigan, Pennsylvania, and Vermont during 1994 and 1996. Heparinized blood was added to a sterile 50-ml centrifuge tube, and the tube was centrifuged at 1,600 × *g* for 5 min. The plasma was removed and was used to determine antibody titers by the IFA test. The buffy coat containing the mononuclear cell fraction was overlaid on 10 ml of Histopaque 1077 (Sigma, St. Louis, Mo.) in another 15-ml centrifuge tube, and the tube was centrifuged at 800 × *g*

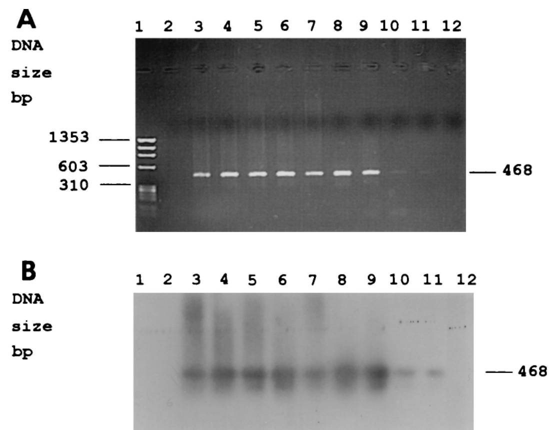

FIG. 1. Agarose gel electrophoresis of nested PCR-amplified products (A) and Southern hybridization (B) of purified nested PCR products showing test sensitivity. Lane 1, *Hae*III-digested ϕX174RF DNA; lane 2, negative control (no template); lanes 3 to 12, 100, 10, 1, 0.8, 0.5, 0.2, 0.08, 0.05, 0.02, and 0.01 pg of *E. risticii* DNA template, respectively.

for 15 min. The interface containing mononuclear cells between the Histopaque 1077 and the remaining plasma was collected and resuspended in RPMI 1640 medium containing 1% 100× antibiotic-antimycotic mixture (10,000 U of penicillin G per ml, 10,000 μg of streptomycin per ml, and 25 μg of amphotericin B per ml) (GIBCO). The tube was centrifuged at 1,000 × *g* for 7 min. The pellet was used as the mononuclear cell fraction.

***E. risticii* isolation from mononuclear cell fraction.** One milliliter of mononuclear cell suspension was added to a $P388D_1$ cell monolayer in a 25-cm^2 flask, and 4 ml of RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum and 2 mM L-glutamine was added. The floating lymphocytes were removed after 2 days of culture. Cultured cell samples were taken and examined every other day after centrifugation in a Cytospin V centrifuge (Shandon Inc., Pittsburgh, Pa.) and Diff-Quik staining (Baxter Scientific Products, Obetz, Ohio). For positive cultures, the number of days required for infection of more than 3% of the $P388D_1$ cells with more than 5 to 10 clearly identifiable organisms or a morula (inclusion) was recorded. The culture was considered negative when *E. risticii* was not found after 35 days of culture.

Titration by the IFA test. The antibody titer was determined by the IFA test with *E. risticii* Maryland as the antigen, as described previously (15, 16). The positive fluorescein isothiocyanate staining of intracellular *E. risticii* at serum dilutions greater than 1:20 is considered a positive result (15, 16).

DNA extraction from mouse blood, horse blood monocytes, and horse feces. DNA was extracted from peripheral blood mononuclear cell fractions by the phenol-chloroform method as described previously (28) or by using a Qiagen tissue DNA extraction kit (Qiagen, Inc., Chatsworth, Calif.). DNA from 1 g of feces was extracted with a Genomix DNA extraction kit (Genomix; Washington Biotechnology, Bethesda, Md.).

PCR amplification of monocyte and fecal DNA. The 16S rRNA genes of several *E. risticii* isolates have been sequenced and were used to prepare primers (4, 28). ER5-3 (5′-ATTTGAGAGTTTGATCCTGG-3′; forward primer), which is specific for all *E. risticii* strains, and ER3-2 (5′-GTTTTAAATGCAGTTCTTGG-3′; reverse primer) were chosen as the external primer pair. ER5-3 and ER3-2 (2 pmol each) were used to prime the amplification of *E. risticii* DNA. DNA (approximately 1 μg) extracted from mononuclear cells of the buffy coat and fecal samples was used as the template in 50 μl of a reaction mixture containing 5 μl of 10× PCR buffer, 2 μl of 50 mM $MgCl_2$, 1 μl of 10 mM deoxynucleoside triphosphate mixture, and 1.25 U of *Taq* polymerase (Gibco BRL, Gaithersburg, Md.). DNAs (10 pg) extracted from purified *E. risticii* Maryland cultured in $P388D_1$ cells and in H_2O were used as positive and negative control templates, respectively, for each reaction. To determine the sensitivity of the PCR, serially diluted *E. risticii* DNA template (100 to 0.01 pg) was used. Samples were amplified by a hot-start method at 92°C for 5 min, followed by denaturation (92°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min) for 40 cycles in a DNA Thermal Cycler 480 (Perkin-Elmer, Foster City, Calif.). Final extension followed at 72°C for 10 min. In a second PCR, 1 μl of the PCR product from the first reaction was added as template to a 50-μl reaction mixture containing 5 μl of 10× PCR buffer, 1 μl of 50 mM $MgCl_2$, 1 μl of 10 mM deoxynucleoside triphosphate mixture, 1.25 U of *Taq* polymerase, and 2 pmol of primer ECC (5′-AGAACGAACGCTGGCGGCAAGCC-3′; forward primer) and ECB (5′-CGTATTACCGCGGCTGCTGCG-3′; reverse primer), which are specific for all ehrlichial species (29). PCR products were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and photographed by using a still video photodocumentation system, Gel Print 2000I (Biophotonics Corporation, Ann Arbor, Mich.) A 1-kb DNA ladder (Gibco BRL) and the amplified product of the *E. risticii* Maryland DNA template (positive control) were included in each agarose gel electrophoresis run to identify accurately the sizes of the amplified bands. Reagent mixing, addition, DNA purification, etc., were done in a Biosafety II laminar flow hood designated for the PCR. Filtered tips were used for all PCR reagents and templates.

Southern hybridization. The PCR products electrophoresed in the agarose gels were transferred to a Hybond-N^+ nylon membrane (Amersham Life Sci. Inc., Arlington Heights, Ill.) by a standard method (25). The 468-bp *E. risticii* nested PCR product was purified by using the Wizard PCR preps DNA purification system (Promega, Madison, Wis.), labeled with [α-^{32}P]dATP by the random primer method by using 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 0.1× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) with 0.1% sodium dodecyl sulfate, and the hybridized bands were exposed to Hyperfilm (Amersham) at −80°C.

RESULTS

Sensitivity of the nested PCR and Southern blot analysis. By using the nested PCR, a partial sequence (468 bp) of the 16S rRNA gene was detectable at a level as low as 0.02 pg of purified *E. risticii* DNA (Fig. 1A). An additional Southern hybridization did not improve the sensitivity of the nested PCR (Fig. 1B).

Experimentally infected pony. The experimentally infected pony developed moderate clinical signs consisting of a fever (rectal temperature, >38.3°C), depression, anorexia, nonbloody (watery) diarrhea, leukopenia on days 8 to 15 p.i., and leukocytosis on day 17 p.i. (16). Culture for *E. risticii* Maryland from peripheral blood occurred from day 1 p.i. to day 28 p.i., with culture times ranging from 3 to 35 days. *E. risticii* antibody titers steadily increased over the course of the experiment before reaching a peak of 1:2,560 at 32 days p.i. (17). The *E. risticii* 16S rRNA gene PCR product was detected by agarose gel electrophoresis by a nested PCR amplification of DNA extracted from blood mononuclear cell fractions and feces of the pony on days 1, 6, 8, 11, 13, 15, 18, 20, 22, 25, 28, and 32 p.i. (Table 1; Fig. 2 and 3).

Experimentally inoculated mice. Mice inoculated with 10^4 *E. risticii* Ohio 380-infected $P388D_1$ cells did not develop any clinical signs, unlike mice inoculated with the same number of a 1984 Virginia isolate of *E. risticii* (20). The 16S rRNA gene of *E. risticii* was detected by nested PCR amplification of the blood of inoculated CF1 mice on days 1, 3, 7, 10, 13, and 17 p.i., indicating subclinical infection (Fig. 4). Control mice inoculated with uninfected $P388D_1$ cells were PCR negative throughout the experimental period.

Specimens from naturally infected horses. In 1994 and 1996, blood specimens were obtained from 27 horses in Indiana, Kentucky, Michigan, Pennsylvania, and Vermont; these horses

TABLE 1. Culture and PCR detection of *E. risticii* in an experimentally infected pony

Day p.i.	IFA test titer	No. of days required for cultivation	Nested PCR result	
			Buffy coat	Feces
1	<1:20	31	+	+
6	1:40	9	+	+
8	1:20	3	+	+
11	1:20	4	+	+
13	1:80	5	+	+
15	1:160	3	+	+
18	1:640	14	+	+
20	1:640	12	+	+
22	1:320	17	+	+
25	1:640	12	+	+
28	1:640	35	+	+
32	1:2,560	Negative (>35)	+	+

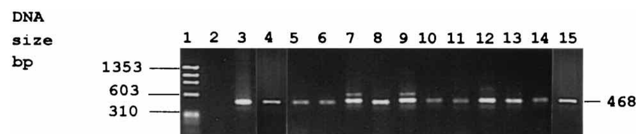

FIG. 2. Agarose gel electrophoresis of nested PCR-amplified products of the *E. risticii* 16S rRNA gene from mononuclear cell fractions of experimentally inoculated pony. Lane 1, *Hae*III-digested ϕX174RF DNA; lane 2, negative control (no template); lane 3, positive control (DNA from purified *E. risticii*); lanes 4 to 15, DNA purified from mononuclear cell fraction at days 1, 6, 8, 11, 13, 15, 18, 20, 22, 25, 28, and 32 p.i.

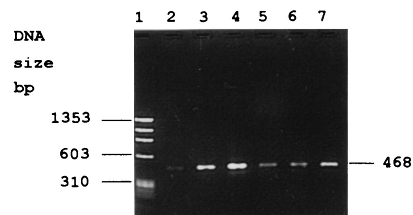

FIG. 4. Agarose gel electrophoresis of nested PCR-amplified products of the *E. risticii* 16S rRNA gene from the blood of mice inoculated with *E. risticii* Ohio 380. Lane 1, *Hae*III-digested ϕX174RF DNA; lanes 2 to 7, DNA purified from mouse blood mononuclear cell fractions from days 1, 3, 7, 10, 13, and 17 p.i.

were suspected of having PHF on the basis of clinical signs ranging from leukopenia, lethargy, fever, anorexia, diarrhea, thumps, and/or laminitis. Eight horses had been vaccinated against PHF with one of three commercially available formalin-killed bacterins during the spring of 1994 (five Pennsylvania horses and one Kentucky horse) or a few days (2 Indiana horses) prior to the time that clinical signs appeared (the results for 23 seropositive and/or culture-positive horses are presented in Table 2). The vaccination statuses of five horses whose blood was obtained in 1996 were unknown. Twenty-two horses were seropositive and four horses were seronegative (<1:20) for *E. risticii* by the IFA test with *E. risticii* Maryland as the antigen. The titers of seropositive horses ranged from 1:20 to 1:1,280 at the time of blood sample collection (Table 2). A blood specimen was not obtained from one horse (horse Ar), but this horse was positive by an IFA test performed in another laboratory. *E. risticii* was cultured from the blood mononuclear cell fraction from 95% of the horses (20 of 21 seropositive horses, including 6 of 7 vaccinated horses), with the culture time for isolation ranging from 5 to 21 days (Table 2). One seropositive but culture-negative horse had an IFA test titer of 1:20 (data not shown in Table 2). Four seronegative horses were all found to be culture negative (data not shown in Table 2). We previously reported that several recent *E. risticii* isolates grow as morulae (4). On light microscopy all isolates were small cocci that stained dark purple by Diff-Quik staining. The isolate from horse Ro from Indiana and the isolate from horse Gr from Kentucky were primarily seen as individual organisms in the cytoplasm, while all other isolates were seen as morulae of various sizes. The *E. risticii* 16S rRNA gene was detectable by nested PCR amplification of DNA from the mononuclear cell fractions in 81% (17 of 21) of culture-positive clinical specimens (Table 2; Fig. 5). PCR amplification of DNA from fecal specimens was positive for 60% (three of five) of the clinical specimens tested (Table 2; Fig. 5).

DISCUSSION

In this study, culture and PCR were compared to IFA test detection of *E. risticii* infection. Nested PCR appeared to be more sensitive than culture for an experimentally infected pony, since at day 32 p.i. the *E. risticii* PCR was positive, while culture was negative. However, for naturally infected horses, 81% of culture-positive specimens were positive by the nested PCR and 95% of seropositive specimens were positive by culture. This does not seem to be due to strain variations of *E. risticii*, since our previous study showed that the same pair of primers can amplify the 16S rRNA genes of more than 11 strains of *E. risticii* in Maryland, Ohio, Kentucky, and California (2, 4, 11). Besides, PCR-negative but culture-positive horses were not found in any particular geographic regions. Rather, since the same amount of DNA template was used, the negative PCR result for culture-positive specimens is most likely due to the presence of *E. risticii* DNA below the threshold level in the 1-μg DNA template (most is host cell DNA). This may be caused by the poor recovery of *E. risticii* DNA from clinical specimens compared to that from experimental specimens, perhaps due to suboptimal storage of and shipment conditions for the clinical specimens. The PCR, however, is the most convenient test for the quick and accurate diagnosis of active infection. The PCR was also useful in detecting subclinical infection with *E. risticii* Ohio 380 in mice. Mice have been used as experimental infection or disease models for PHF (16, 20). Since it is very difficult to monitor subclinical infection in mice by culture isolation due to the small amount of blood that is available, PCR is useful for experimentation with the mouse model.

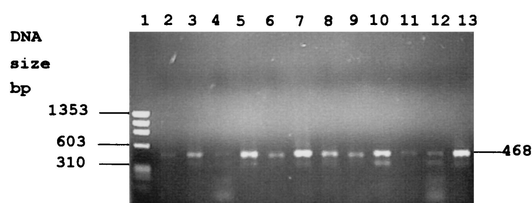

FIG. 3. Agarose gel electrophoresis of nested PCR-amplified products of the *E. risticii* 16S rRNA gene from fecal samples of an experimentally inoculated pony. Lane 1, *Hae*III-digested ϕX174RF DNA; lanes 2 to 13, DNA purified from fecal samples from days 1, 6, 8, 11, 13, 15, 18, 20, 22, 25, 28, and 32 p.i.

Additional bands other than bands of 468 bp were amplified by using DNAs extracted from some of the blood and fecal specimens. These are unlikely derived from *E. risticii* DNA, since the *E. risticii* DNA control included in each PCR run did not produce these bands. These extra bands may have been derived from the DNA of equine cells or organisms in the fecal specimens which happened to have base sequences with which the primers could hybridize. These nonspecific amplifications may be eliminated by optimizing the PCR conditions. Generally, one of the disadvantages of PCR is possible contamination. To prevent false-positive PCR results, in addition to the use of filtered tips and a maximally clean environment, the inclusion of negative controls (reaction mixtures without template and a template of monocyte DNA from a healthy horse) in each PCR run, as described for the current study, is recommended. The addition of positive control *E. risticii* DNA prevents a false-negative PCR test result due to reagent or equipment failure. A DNA size marker and an amplified product should be included as positive controls in each agarose gel run for accurate product size determination. The addition of Southern hybridization did not improve the sensitivity of the nested PCR.

The number of days required for cultivation varied greatly. In general, the less the volume of blood used to separate the mononuclear cell fractions or the fewer number of organisms

TABLE 2. *E. risticii* IFA test titers, culture, and PCR of clinical specimens[a]

Horse	State	Date that blood was collected (mo/day/yr)	No. of days required for cultivation	IFA test titer (mo/day)		Nested PCR result	
				First	Second	Mononuclear cells	Feces
Wa	Vt.	3/25/1994	15	1:160 (3/25)	N[a]	N	N
Bi[b]	Pa.	7/21/1994	7	1:640 (7/21)	1:640 (8/10)	+	N
Al[b]	Pa.	7/21/1994	7	1:320 (7/21)	1:640 (8/10)	+	+
Jo[b]	Pa.	7/26/1994	14	1:320 (7/26)	1:640 (8/9)	+	N
Sa[b]	Pa.	7/26/1994	7	1:160 (7/26)	1:640 (8/9)	+	+
Ar[b]	Pa.	7/21/1994	N	N	N	N	+
Ro[b]	Ind.	7/26/1994	21	1:20 (7/26)[c]	N	+	N
Je[b]	Ind.	8/3/1994	9	1:640 (8/3)	N	−	−
Gr	Ky.	8/4/1994	7	1:160 (8/4)	N	−	N
Ma[b]	Ky.	8/16/1994	21	1:80 (8/16)	N	−	N
Bl	Ky.	8/24/1994	13	1:320 (8/24)	N	+	N
Mg	Ky.	8/30/1994	13	1:80 (8/30)	N	+	N
So	Ky.	8/30/1994	7	1:40 (8/30)	N	+	N
Ta	Ky.	9/6/1994	5	1:320 (9/7)	N	+	N
Pa	Ky.	9/7/1994	5	1:320 (9/7)	N	+	N
Sp	Ky.	9/8/1994	6	1:160 (9/9)	N	−	−
Cy	Ky.	9/9/1994	6	1:20 (9/9)	N	+	N
Su	Ky.	12/1/1994	14	1:320 (12/1)	N	+	N
Hi	Ind.	9/12/1996	N	1:1,280 (9/17)	N	+	N
Cl	Mich.	9/12/1996	13	1:640 (9/17)	N	+	N
Io	Mich.	9/12/1996	—[d]	1:80 (9/17)	N	+	N
Se	Mich.	9/16/1996	21	1:640 (9/17)	N	+	N
Li	Ind.	9/17/1996	15	1:640 (9/17)	N	+	N

[a] N, not done.
[b] Vaccinated.
[c] Low titer may be due to severely hemolyzed plasma.
[d] —, negative.

present per milliliter of blood, the longer the number of days required for cultivation. Other factors, however, such as the growth rates and survivabilities of *E. risticii* strains in cell culture, the presence of activated monocytes and lymphocytes, the viability of monocytes, and the number of monocytes per milliliter of blood, may have influenced the number of days required for cultivation. The culture isolation of *E. risticii*, however, is impractical for most clinical situations because of the time required before a positive diagnosis is made (up to 3 weeks in this study). Other requirements, such as a sterile culture facility, trained personnel, and the availability of fresh sterile specimens, make culture cost prohibitive to the average horse owner. Even with these drawbacks, culture isolation may be acceptable in extremely important cases or for determining the efficacy of treatment.

It was previously suggested that a titer of less than 1:80 does not indicate that *E. risticii* is the cause of the current disease, since experimentally infected horses have IFA test titers of greater than 1:80 by the time that they develop clinical signs (17). However, in the current study, *E. risticii* was cultured from the blood of five horses which had IFA test titers of less than 1:80 (titers, 1:20 and 1:40). The low titers for these infected horses may be due to poor sample collection methods and poor storage conditions or may be due to infection with a strain antigenically different from that used for IFA testing. One culture-negative, seropositive (titer, 1:20) horse probably indicates that the current disease in this horse is not caused by *E. risticii*. Because low titers are epidemiologically more difficult to correlate with disease (17), culture isolation or PCR is helpful when the IFA test titer is low.

The current study revealed that *E. risticii* DNA can be detected in the feces of both experimentally and naturally infected horses. Others have also reported the detection of *E. risticii* in the feces of two experimentally infected horses by immunomagnetic separation and PCR of unknown target DNA of *E. risticii* (3). To the contrary to our concern that PCR based on the 16S rRNA sequence may produce many false-positive results with fecal specimens due to fecal bacteria, all fecal specimens from seronegative horses were PCR negative. *E. risticii* was detectable in DNA from the peripheral blood mononuclear cell fraction and feces of the experimentally infected pony as early as day 1 p.i. Since the pony did not have diarrhea at day 1 p.i. and *E. risticii* does not cause bloody diarrhea (18, 19), *E. risticii* DNA in the feces is unlikely derived from the blood but is derived from infected cells shed into the gut lumen. We have shown previously by electron microscopy that ehrlichial organisms are present in the intestinal epithelial cells, macrophages, and mast cells in the intestinal wall of

FIG. 5. Agarose gel electrophoresis of PCR-amplified products of the *E. risticii* 16S rRNA gene from the blood and feces of naturally infected horses. Lane 1, *Hae*III-digested ϕX174RF DNA; lane 2, negative control (no template); lane 3, positive control (DNA from purified *E. risticii*); lanes 4 to 20, DNA purified from the blood of horses Bi, Al, Jo, Sa, Ro, Bl, Mg, So, Ta, Pa, Cy, Su, Hi, Cl, Io, Se, and Li, respectively; lanes 21 to 23, DNA purified from feces of horses Al, Sa, and Ar, respectively.

affected horses and that infected cells containing ehrlichiae are shed into the gut lumen (22). Our preliminary study also showed that *E. risticii* DNA can be detected in the intestinal tissue of a naturally infected horse by PCR (30). Our results further support the possibility of oral transmission of *E. risticii*, which was experimentally demonstrated previously (14). In naturally infected horses, detection of *E. risticii* DNA by the PCR method appears to be more effective with peripheral blood specimens than with fecal specimens, although there was no difference between specimens from an experimentally infected horse. This may probably reflect the concentration of *E. risticii* DNA present in these specimens. Therefore, for the diagnosis of PHF, blood monocytes rather than fecal specimens should be examined.

As in our previous study (4), live *E. risticii* was isolated from seven vaccinated horses. Two of the Indiana horses may not have had sufficient time to develop immunity before infection took place. However, the remaining horses had received repeated vaccinations during the spring, yet they had active infection during the following summer, indicating vaccine failure. Thus, PCR and culture are also useful for diagnosing PHF in previously vaccinated horses.

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