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Diagnostic Application of Polymerase Chain Reaction for Detection of *Ehrlichia risticii* in Equine Monocytic Ehrlichiosis (Potomac Horse Fever)†

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Genomic amplification by the polymerase chain reaction (PCR) was used to identify a unique genomic sequence of *Ehrlichia risticii* directly in DNA isolated from peripheral-blood buffy coat cells of *E. risticii*-infected horses (Potomac horse fever) and from infected cell cultures. A specific primer pair, selected from a cloned, species-specific, 1-kb DNA fragment of the *E. risticii* genome as a template, was used for the amplification of the target DNA of 247 bp. The optimal number of 40 PCR cycles, determined by analyzing an amplification profile obtained with a constant *Taq* polymerase concentration, was used to achieve maximum amplification of the *E. risticii* DNA segment. Efficient amplification of target DNA was achieved with specimens processed by either the phenol extraction or rapid lysis method. The specificity of the amplified DNA product was confirmed by the proper size (247 bp) and appropriate restriction enzyme cleavage pattern of the amplified target DNA, as well as by the specific hybridization signal obtained by using a PCR-amplified 185-bp internal DNA probe. A 10^5- to 10^6-fold amplification of target DNA, which allowed detection of *E. risticii* from as few as two to three infected cells in culture and from a very small volume of buffy coat cells from infected horses, was achieved. This PCR amplification procedure was found to be highly specific and sensitive for the detection of *E. risticii* for the study of Potomac horse fever.

Equine monocytic ehrlichiosis, or Potomac horse fever (PHF), is a recently recognized disease of pathogenic importance which is caused by *Ehrlichia risticii* (3, 8, 14). The main clinical signs of the disease include depression, biphasic rise in body temperature, decreased leukocyte count, diarrhea, and occasional laminitis (4). Mortality ranges from 8 to 12%. Since the first recognition of the disease in Maryland and Virginia along the Potomac River in 1979, it has been reported in most of the states of the United States and other parts of the world (9).

Because of the pathogenic nature of the organism, the disease is an alarming concern to the equine industry. Early diagnosis of PHF is of prime importance for initiating specific therapeutic measures, because the clinical signs of PHF can mimic other horse diseases that cause abdominal distress and diarrhea. Confirmation of diagnosis of PHF is presently done by detecting seroconversion of serum samples collected at 2 to 3 weeks postinfection (5, 13, 15, 20) and by isolating *E. risticii* in cell cultures (3), which is a time-consuming and impractical procedure for field studies. Methods for direct detection of antigens of the organism are not available at present.

In addition to the need for an early and rapid diagnosis of acute PHF, there is also a need to substantiate the seroepidemiological evidence for the prevalence of subclinical disease (11) by the detection of *E. risticii*. Cats (2), foxes, and wild rabbits (19) have been found to carry antibodies to *E. risticii*; cats are also susceptible to experimental infection (2). This suggests that domestic and wild animals may be involved as a carrier or reservoir of the organism. The mode of transmission of *E. risticii* remains unknown, but the etiological and epidemiological factors (12, 18) suggest the involvement of blood-feeding arthropods as potential vectors in the transmission of PHF. To study the above-mentioned cases, there was an unquestionable need for an efficient method of detecting *E. risticii*. Recently, a cloned *E. risticii* DNA was used as a probe for the detection of DNA of the organism in blood mononuclear cells of experimentally infected horses (21). In this procedure, a large number of mononuclear cells was required for the detection of *E. risticii* DNA. Thus, in order to study the cases in which an extremely low number of organisms is expected to be present, a highly sensitive and specific method for the detection of the organisms is necessary.

This work, a continuation of earlier study (21), presents the development of genomic amplification of *E. risticii* DNA by the polymerase chain reaction (PCR) and the detection of the organism in infected cell cultures and from buffy coat cells of infected horses to aid in the diagnosis of PHF.

MATERIALS AND METHODS

Extraction of *E. risticii* DNA. Purification of *E. risticii* and extraction of *E. risticii* DNA was performed as previously described (6).

***E. risticii* recombinant clone and extraction of DNA.** An *E. risticii* recombinant from the genomic library constructed in a pUC13 plasmid vector system (21) containing *Hin*dIII-digested insert DNA of 1 kb was employed. The insert DNA had exhibited species specificity by failing to cross-hybridize with the genomes of *Ehrlichia sennetsu*, *Ehrlichia canis*, and *Ehrlichia equi* (21). The extraction of DNA from this clone

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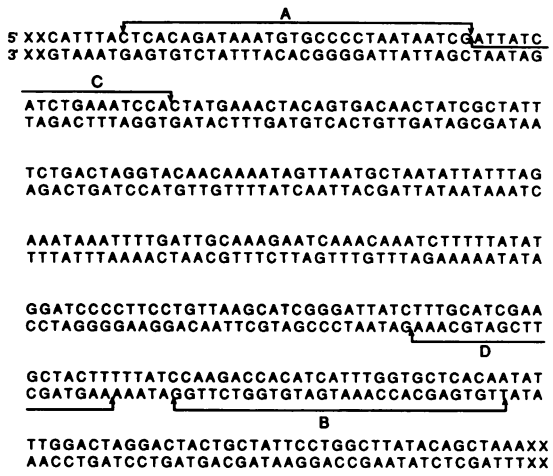

FIG. 1. Sequences (297 bp) of the *E. risticii* template DNA (C5) and selection of primers. The two external primers, primers A (29 bp) and B (28 bp), were used for the amplification of specific target DNA of 247 bases. The two internal primers, primers C (18 bp) and D (18 bp), were used to amplify a DNA segment of 185 bp for use as a probe for the detection and confirmation of amplified products.

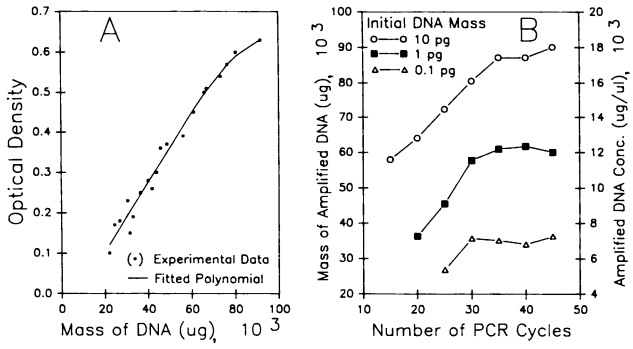

FIG. 2. (A) Optical density of the DNA bands in the negative photographic plate of the agarose gel as a function of DNA mass (concentration) of a known molecular weight standard. (B) DNA mass as a function of the number of PCR cycles in determining the amplification profile of *E. risticii* DNA. For each initial DNA mass (0.1, 1, and 10 pg), amplification reached a plateau after about 30 to 35 PCR cycles. Thus, 40 cycles were used for maximum amplification of *E. risticii* DNA.

and its purification was performed by the method of Maniatis et al. (10). This recombinant plasmid containing the *E. risticii* DNA fragment, designated C5, was used as a template for PCR.

Sequencing of template DNA and selection of primers. The C5 DNA was sequenced by Sanger's dideoxy-chain termination method (17) for selecting the PCR primers. A synthetic oligonucleotide primer (M13/pUC), a reverse-sequencing 17-mer (Boehringer Mannheim, Indianapolis, Ind.), specific for DNA cloned at the *Hin*dIII restriction sites, was used for sequencing. Sequencing was performed by using T7 DNA polymerase (Sequenase; U.S. Biochemical, Cleveland, Ohio) and the double-stranded DNA sequencing procedure recommended by the manufacturer. A total of 297 nucleotides were sequenced (Fig. 1). Two primers of 28 and 29 bases from the plus (+) and minus (−) strands were selected on the basis of their 50% G + C content. The 3′ ends of these two primers started with base G, except that the minimum two bases at the 3′ ends were also not complementary to each other. This set of primers, external or amplifying primers (Fig. 1, primers A and B), was used to amplify a target DNA of 247 bp from the *E. risticii* genome. Another set of primers of 18 bases each, internal or probe primers (Fig. 1, primers C and D), was similarly selected from the region of the template internal to the external primers to amplify target DNA of 185 bp for use as a probe DNA. Here the nucleotide sequence complementary to the sequence of the external primers was avoided to preclude any nonspecific hybridization background.

PCR amplification and standardization. A preliminary PCR amplification (16) was performed by using C5 insert DNA, a PCR kit, and the protocol recommended by the manufacturer (Perkin-Elmer Cetus, Norwalk, Conn.). The target sequence was amplified in a total reaction volume of 100 μl for 25 cycles, using an Ericomp double-block thermocycler (Ericomp, Inc., San Diego, Calif.). After successful preliminary amplification of the target sequence from *E. risticii* DNA, optimization of all of the parameters and reagents was performed to achieve maximum amplification. A DNA amplification protocol was set up by using a 50-μl total reaction mixture volume consisting of 29.75 μl of distilled water, 5 μl of 10× reaction buffer (500 mM KCl, 100 mM Tris-Cl [pH 8.3], 15 mM $MgCl_2$, 0.1% [wt/vol] gelatin), 8 μl of deoxynucleoside triphosphate mixture (200 μM (each) of the four), 2 μl of each primer (0.1 μM each), 0.25 μl of *Taq* polymerase (1.25-U assay), and 5 μl of specimen. With this protocol, it was observed that a final magnesium concentration of 1.5 μM in each reaction gave maximum amplification. Following an initial template denaturation step at 94°C for 90 s, the following cycle profile was used: annealing at 52°C for 2 min, extension at 72°C for 3 min, and denaturation at 94°C for 1 min. For determining the optimum number of cycles giving maximum DNA amplification, various starting DNA masses (concentrations) of the target DNA were amplified with a constant enzyme concentration. The DNA mass of the amplified product from different cycles of amplification was estimated by relating the optical densities of the DNA bands of these amplified products in negative photographic plates to the optical densities of the bands of known DNA masses of a molecular weight standard (Fig. 2A). It was observed that for each initial DNA concentration, the amplification reached a plateau after about 30 to 35 PCR cycles (Fig. 2B). Hence, 40 cycles were used to obtain maximum amplification.

Detection and identification of amplified *E. risticii* sequences. The segment of *E. risticii* DNA sequences amplified by PCR was identified by three different methods. In the agarose gel method, 5 μl of amplified material was electrophoresed on 4% low-melting-point NuSieve (FMC BioProducts, Rockland, Maine) agarose minigel for 45 min at a constant 75 V, using TBE buffer (0.089 M Tris, 0.089 M borate, 0.002 M EDTA). Molecular weight markers (Boehringer Mannehein) were run concurrently. The gels stained with ethidium bromide were examined for the presence of a 247-bp band. In the restriction analysis, two enzymes (*Bam*HI and *Cla*I), each having one restriction site in the target DNA sequences (247 bp) as determined by computerized sequence analysis, were selected for the digestion of PCR-amplified products to confirm the specificity of *E. risticii* DNA amplification. Samples (about 7 μl each) of the amplified products were treated with the restriction enzymes for complete digestion and electrophoresed. The gels were examined for the presence of DNA bands of the appropriate

sizes, 143 and 104 bp for *Bam*HI and 27 and 220 bp for *Cla*I digests. In Southern blot and dot blot DNA hybridization, specific signals were obtained when amplified *E. risticii* products were hybridized with a ^{32}P-labeled 185-bp probe DNA.

Preparation of probe and DNA hybridization. The 185-bp amplified product of the internal primers, the probe DNA, was radiolabeled with ^{32}P by the conventional nick translation method, using a nick translation kit (Bethesda Research Laboratories, Inc., Bethesda, Md.) and a modification of the PCR labeling method (1). For the latter method, a 100-μl mixture consisting of 10 μl of 10× reaction buffer, 1 μl of dCTP, 2 μl (each) dATP, dGTP, and dTTP from a 10 mM stock, 10 μl of [^{32}P]dCTP (3,000 Ci/mmol) (Amersham, Arlington Heights, Ill.), 2 μl of each internal primer (final concentration, 0.1 μM), 0.5 μl (2.5-U assay) of *Taq* polymerase, 10 μl of template C5 DNA containing 0.01 ng of DNA, and 58.5 μl of distilled water was prepared. This was amplified, maintaining the standard cycling profile described above. The amplified material was phenol-chloroform extracted and ethanol precipitated, and the DNA pellet was resuspended in 1 ml of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]).

Southern blot and dot blot hybridizations of the PCR-amplified products were performed by the procedures described previously (21).

Detection of *E. risticii* antibodies. *E. risticii* antibodies were detected by the indirect immunofluorescence assay (3) and enzyme-linked immunosorbent assay (5) described previously.

Isolation of *E. risticii* in cell culture. *E. risticii* was isolated in mouse macrophage cell line P388D (American Type Culture Collection, Rockville, Md.) from the buffy coat cells (peripheral-blood leukocytes) collected from heparinized blood samples of horses by the procedure described previously (3).

Specimen collection and processing for PCR amplification. Infected cell culture specimens and clinical specimens from horses were collected and processed by the phenol extraction (21) and rapid lysis (7) methods.

For cell culture specimens, human histiocyte (ATCC U937) and mouse macrophage cell lines were infected with *E. risticii*, the resulting infection was monitored, and the cells containing the organism were quantitated by acridine orange staining (3). For phenol extraction (21), a 5-ml sample of the infected cell culture containing approximately 1.5×10^6 infected cells per ml was centrifuged at 500 × *g* for 10 min. The cell pellet was subjected to three cycles of freezing and thawing, treated with lysozyme (2 mg/ml) for 30 min at room temperature, and then treated with sodium dodecyl sulfate (1%) and proteinase K (1 mg/ml) at 60°C for 1 h. DNA was extracted with phenol and chloroform, precipitated with ethanol, and then resuspended in 1 ml of TE buffer. For the rapid lysis method, the cell pellets were suspended in 1 ml of lysis buffer (50 mM KCl, 10 mM Tris HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.1 mg of gelatin per ml, 0.4% Nonidet P-40, 0.45% Tween 20) containing 0.6 μg of proteinase K per 100 μl of solution. Proteinase K was finally inactivated by boiling the lysate at 95°C for 10 min. To determine the minimum number of *E. risticii*-infected human histiocyte cells required for the detection of infection by the PCR amplification method, samples (of about 5 ml each) of infected cell cultures with over 95% of the cells containing the organism, harvested on day 5 postinfection, were pelleted at 500 × *g* for 10 min, washed with TE buffer (pH 7.5), and resuspended to 2×10^6 cells per ml with TE buffer. The cell suspension was serially diluted with TE buffer, to which an equal volume of 2× lysis buffer was added and processed separately by the rapid lysis method prior to amplification.

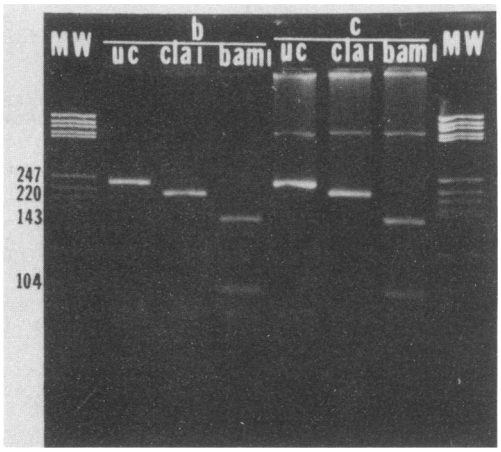

FIG. 3. Restriction enzyme patterns of the PCR-amplified products from target (C5) DNA (b lanes) and the phenol-extracted DNA of buffy coat cells from experimentally infected horses (c lanes). uc and MW denote undigested products and molecular weight standards, respectively. *Cla*I digestion produced bands of 220 and 27 bp, whereas *Bam*HI digestion produced bands of 143 and 104 bp (27-bp band is not visible because of the low intensity). A nonspecific amplified product (upper bands) in the c lanes was not digested with these enzymes.

For clinical specimens, buffy coat cells from horses experimentally infected with *E. risticii* and from field cases of PHF were used. From a previous study of experimentally infected horses (4), the buffy coat cells had been separated by sedimentation from the heparinized blood that had been collected on days 12 to 14 postinoculation, frozen, and stored at −70°C in 7.5% dimethyl sulfoxide medium (Whittaker, Walkersville, Md.). These infected horses had displayed the typical clinical disease. *E. risticii* had been isolated in cell cultures from the buffy coat cells of these horses and their serum samples collected 4 weeks postinoculation were positive for *E. risticii* antibodies by indirect immunofluorescence assay and enzyme-linked immunosorbent assay. Prior to use, the frozen buffy coat cells were thawed quickly and washed two times with RPMI 1640 medium at 500 × *g* for 10 min to remove the dimethyl sulfoxide. Heparinized blood samples from suspected field cases of PHF were collected immediately after the first appearance of clinical signs, and the buffy coat cells were removed from the blood. The cells were pelleted at 500 × *g* for 10 min, and the cell pellets were frozen and stored at −20°C. The buffy coat cell specimens were processed by the phenol extraction and rapid lysis methods as described above.

RESULTS

Specificity and sensitivity of PCR amplification of *E. risticii* DNA. The specificity of the amplified DNA using external primers was determined by its unique size of 247 bp upon electrophoresis in 4% NuSieve agarose gel and by the exact matching of the projected restriction profile for *Bam*HI and *Cla*I of the amplified target DNA (Fig. 3). The specificity of the amplified product was also confirmed from the specific signals obtained by the Southern blot and dot blot hybrid-

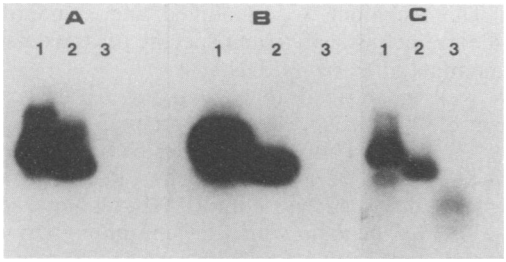

FIG. 4. Autoradiograms of Southern blots hybridized with probes, showing the specificity of internal target DNA probe. Lanes: 1, amplified products of external primers; 2, amplified products of internal primers; 3, excess external primers. The primers were hybridized with ^{32}P-labeled (nick translation) internal target DNA probe (A), PCR-labeled internal target DNA probe (B), and ^{32}P-labeled external probe (C). Signal background was observed for excess external primers hybridized with an external probe (lane 3 of panel C), while such signal was not observed with hybridization with an internal probe (lanes 3 of panels A and B).

izations using the internal DNA probe (185 bp) labeled with ^{32}P by nick translation and PCR labeling methods (Fig. 4). The 185-bp size of the amplified DNA produced by using internal primers further confirmed the specificity of amplification. The external primers failed to amplify nonspecific DNAs from various sources; namely, pUC13 plasmid vector, λgt11, pUCC7 (insert DNA from another *E. risticii* recombinant in pUC13 vector), human histiocyte cells, normal horse buffy coat cells, and *E. canis*. An amplification of 10^5 to 10^6-fold of the target DNA (purified *E. risticii* DNA and C5 DNA) was achieved, thus enabling detection of 0.1 pg or less of *E. risticii* DNA.

Detection of *E. risticii* in cell culture specimens. *E. risticii* DNA was detected at 3 h postinfection in in vitro-infected human histiocyte and mouse macrophage cell cultures by detecting a 247-bp amplified segment of the *E. risticii* genome (Fig. 5). There was a steady increase in *E. risticii* DNA concentration in the infected cell culture specimens up to 72 h postinfection. During this period, a significant number of infected cells was not detected by acridine orange staining. This suggested that *E. risticii* DNA could be detected in the infected cells by the PCR amplification method prior to detection of *E. risticii* in the infected cells by acridine orange staining. It was determined that *E. risticii* could be detected from as few as two to three infected human histiocyte cells.

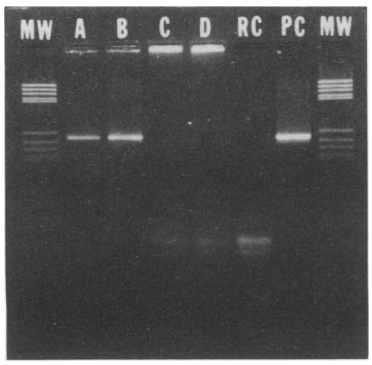

FIG. 5. Agarose gel electrophoregram of PCR-amplified products stained with ethidium bromide. Lanes A, B, C, and D were loaded with 5-μl samples of amplified products from mouse macrophage cells, human histiocyte cells, mouse macrophage cells infected with *E. risticii*, and human histiocyte cells infected with *E. risticii*, respectively. All specimens were processed by a phenol extraction method. Reagent control (RC), positive control (PC), and molecular weight standards (MW) were run simultaneously.

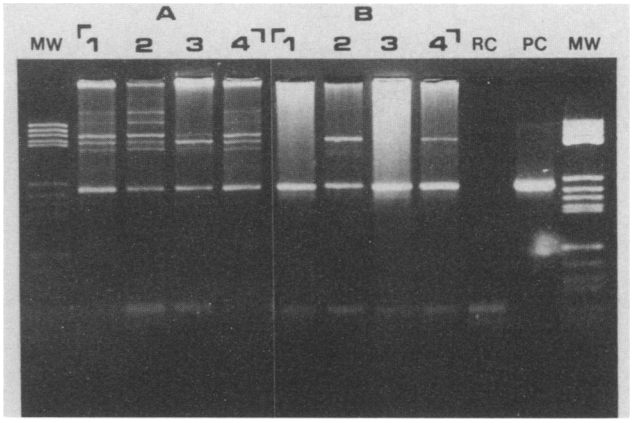

FIG. 6. Ethidium bromide-stained agarose gels containing PCR-amplified products from four clinical specimens (lanes 1 to 4) collected from experimentally infected horses and processed by the rapid lysis method (A) and phenol extraction method (B). RC, PC, and MW denote reagent control, positive control, and molecular weight standards, respectively. About four to five nonspecific bands, in addition to a specific band, are observed in all the lanes with rapid lysis material (panel A), whereas only one nonspecific band is observed in lanes 2 and 4 with phenol extraction material, giving a higher amplification of target DNA (panel B).

Detection of *E. risticii* in clinical specimens. *E. risticii* was detected by PCR amplification in the buffy coat cells of all 11 horses experimentally infected with *E. risticii*. There was efficient amplification of the target part of the *E. risticii* genome, so that 5-μl samples of the amplified products were sufficient to show a clear target band in agarose gel. To compare the efficiency of the rapid lysis and phenol extraction methods, when equal concentrations of infected buffy coat cells were processed by both methods simultaneously and the products were brought to the same final volume and then amplified, efficient amplification occurred in both cases. However, for rapid lysis (Fig. 6A), several (five to six) nonspecific bands in addition to the specific band were observed, whereas the phenol extraction material (Fig. 6B) presented a comparatively negligible number of nonspecific bands and showed higher amplification of the target DNA.

The specificity of the amplified product from the clinical specimens was further confirmed by the specific signals exhibited from the amplified target portion of *E. risticii* DNA by Southern blot hybridization of the gel (Fig. 7A) with a ^{32}P-labeled internal probe (Fig. 7B). Similarly, all 11 PCR-amplified specimens exhibited strong signals in dot blot hybridization with the probe (Fig. 8), whereas the amplified products from uninfected horse buffy coat cells did not generate any signal. Unamplified material of known positive specimens failed to generate any signal.

Of 16 suspected field cases of Potomac horse fever, *E. risticii* was detected in buffy coat cells by PCR amplification from two horses (Fig. 8). PHF for these two horses was confirmed by the isolation of *E. risticii* in cell culture from the buffy coat cells of one horse and by the detection of *E. risticii* antibodies in both horses. In the remaining field cases, *E. risticii* organisms were not isolated and there was

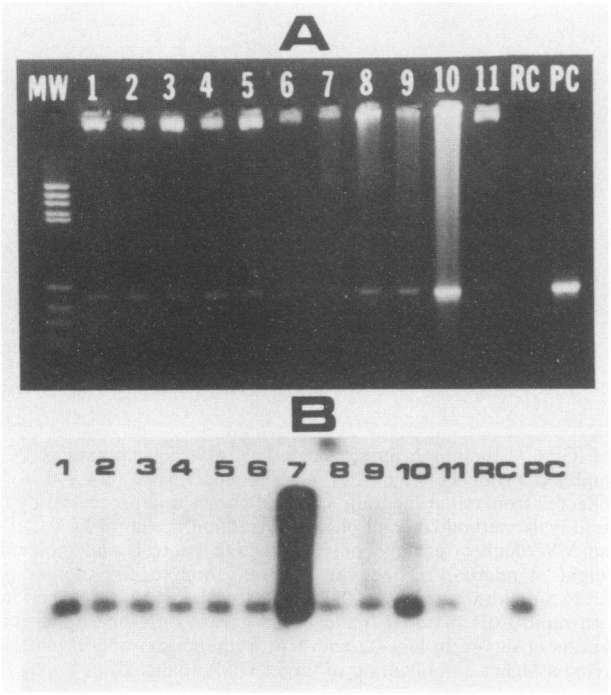

FIG. 7. PCR-amplified products of buffy coat cells from 11 experimentally infected horses (lanes 1 to 11). (A) Agarose gel electrophoregram showing a 247-bp target DNA band in all 11 lanes. (B) Corresponding Southern blot hybridized with ^{32}P internal probe. (Smearing of amplified DNA in lane 7, for some unknown reason, allowed the probe to bind and generated a signal throughout the path of electrophoresis.) RC and PC denote reagent control and positive control, respectively.

no seroconversion for *E. risticii* antibodies, substantiating the absence of target DNA by PCR amplification.

DISCUSSION

In the last few years, the PCR technology has been widely used for amplification of nucleic acids for diagnostic purposes. The technique was modified and standardized to make it extremely sensitive and specific for the detection of small amounts of *E. risticii* DNA.

It is well established that *E. risticii* is the causative organism of PHF. Also, there are other *Ehrlichia* species, namely, *E. sennetsu* and *E. canis*, which are pathogens for humans and animals, respectively. Thus, in order to develop a PCR-based diagnostic system for PHF, a unique sequence of the *E. risticii* genome which had no homology with the genomes of other known *Ehrlichia* species (21) was used for generating species-specific primers. A separation of 190 bases between the two primers, which provided a 247-nucleotide product of the target DNA, was maintained during selection of amplification primers. This had an optimum size for easy handling after amplification and also ensured better visualization after electrophoresis in 4% NuSieve low-melting-point agarose gel.

To detect the amplified target product (247 bp) by dot blot hybridization, an internal sequence of 185 bp which has no sequence homology with the two external primers used for amplification was chosen. Generally, a conventional diagnostic approach of the PCR ensures high sensitivity and specificity by detecting amplified target product, using a part of the target DNA internal sequence as a probe. This internal sequence used for the probe is specifically chosen so that it is not a part of the sequences of the two primers used for amplification. Usually, to generate such a probe, short stretches of oligomer (20 to 28 bases) are synthesized by a gene synthesizer. However, this process is time-consuming and expensive. Also, for the purpose of developing a routine diagnostic procedure, a continuous source of synthesized oligomer is needed to replenish the stock. This procedural difficulty of constant replenishment was solved by selecting and synthesizing two new internal primers which could amplify an internal sequence of 185 bp from the target part. This internal target amplification product bears no sequence homology with the external primers. Once the synthesis of these primers has been done, one can then take advantage of the PCR amplification process itself for a generous supply of the internal amplified product (185 bp). Another advantage of this method is that one can radiolabel the probe during PCR amplification itself without using nick translation or

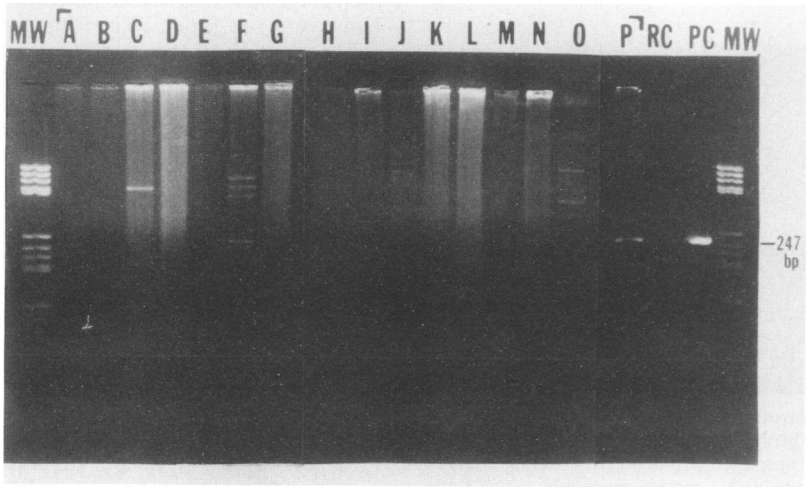

FIG. 8. PCR-amplified products of buffy coat cells from 16 (lanes A to P) suspected field cases of PHF. Agarose gel electrophoregram showing a target DNA band in two lanes (lanes F and P) identified by its specific size of 247 bp, indicating these two samples are positive for *E. risticii*. Lanes C, F, J, and O show nonspecific amplification for which the product sizes are completely different from the target band. RC, PC, and MW denote reagent control, positive control, and molecular weight standards, respectively.

other labeling methods. This feature saves the time and expenditure involved in the generation of a probe for routine diagnostic evaluation of the amplified product.

It was shown that regardless of the initial DNA concentration amplification always reached a plateau after 30 to 35 PCR cycles. It was assumed that the enzyme activity (1.25 U of enzyme in the assay), which was the same for different initial DNA concentrations, degraded to an insignificant level after that many cycles. However, to ensure maximum amplification of DNA, 5 more cycles were used, for a total of 40 PCR cycles.

The specificity of DNA amplification makes PCR a very attractive method for this DNA-based diagnostic method for pathogens. Amplification from a known quantity of *E. risticii* DNA was estimated to be about 10^5- to 10^6-fold. During all cycles of amplification from a very small quantity of DNA, specificity is very important in each cycle. Since it was not possible to check the specificity at the end of each cycle, the final amplified product was subjected to restriction enzyme analysis for confirmation of the specificity. The result of this restriction analysis revealed that amplified bands from different templates (denoted here as nonspecific amplification) did not possess the restriction sites for *Bam*HI and *Cla*I, which were designated restriction sites for the target DNA as well as its amplified product. The specificity of the amplified product was further checked by hybridization, using a ^{32}P-labeled internal probe. An added advantage of this method is an increase in the detection sensitivity of the amplified product. Dot blot hybridization was a rapid and sensitive method for diagnostic purposes.

The PCR method was highly sensitive, detecting *E. risticii* DNA from as few as two to three infected cells from cultures. Also, PCR was successfully used to detect the organism directly from the blood buffy coat cells of the infected horses. The omission of the laborious step of Ficoll-Hypaque separation of mononuclear cells (21) allowed this method to be faster and simpler, thereby increasing its efficacy as a diagnostic tool. Of the two methods used for the preparation of samples from the buffy coat cells of infected horses, the phenol extraction method was time-consuming and laborious. Therefore, for routine diagnostic purposes, the rapid lysis method was selected because it was simple and required not more than 2 h of sample preparation time. It was estimated that buffy coat cells obtained from less than 1 ml of blood from infected horses was adequate for the detection of *E. risticii*. Thus, the technique of PCR amplification of *E. risticii* DNA promises to provide a rapid, highly sensitive, and specific method to study various aspects of PHF.

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