Detection of *Ehrlichia risticii* from Feces of Infected Horses by Immunomagnetic Separation and PCR†

BISWAJIT BISWAS, RAMESH VEMULAPALLI, AND SUKANTA K. DUTTA*

Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, Maryland 20742

Received 27 December 1993/Returned for modification 2 February 1994/Accepted 9 June 1994

Potomac horse fever, caused by *Ehrlichia risticii*, is an important disease of equines. The major features of the disease are fever, leukopenia, and diarrhea. The organism has been detected from the blood mononuclear cells of infected horses, but its presence in the feces has not been known. A method for immunomagnetic separation of *E. risticii* from the feces of infected horses was developed, and the separated organisms were detected by PCR. Coating immunomagnetic beads (Dynabeads) with a 1:5 dilution of rabbit anti-*E. risticii* serum and incubating the Dynabeads with fecal samples for 25 min at room temperature gave optimum results. *E. risticii* was detected from the feces during the course of diarrhea from two experimentally infected horses. In horse 1, watery diarrhea occurred from days 11 to 16 postinfection (p.i.), after which the feces became soft on day 17 p.i. and then returned to normal. The organisms were first detected from the feces on day 11 p.i., peaked on day 13 p.i., and then gradually decreased until day 16 p.i., after which they became undetectable. In horse 2, first, on day 12 p.i., there was soft feces which continued and progressed to diarrhea on day 17 p.i. The feces became normal after day 18 p.i. The organisms in the feces of this horse were first detected on day 12 p.i. and peaked on day 14 p.i., after which they declined until day 16 p.i. and then became undetectable. In both horses, the number of organisms in the mononuclear cells peaked on days 10 and 11 p.i., respectively, 3 days prior to the respective peaks in the feces. *E. risticii* was not detected from the plasma samples obtained from these horses. There was a drastic reduction in PCR amplification of *E. risticii* DNA for fecal samples stored frozen at −20°C in comparison with those stored at 4°C. The presence of the organism in the feces only during the soft- or diarrheal-feces phase supports the previous hypothesis that the diarrhea is caused by the organisms replicating in cells lining the intestines. This rapid and simple method of detection of the organisms from the feces will be helpful in diagnostic and epidemiologic studies of Potomac horse fever.

This paper presents the separation of *E. risticii* from the feces of experimentally infected horses by an immunomagnetic method and detection of *E. risticii* by PCR.

**MATERIALS AND METHODS**

Experimental infection of horses and collection of clinical samples. Two horses free of detectable antibodies to *E. risticii* were inoculated with 5 × 10⁷ *E. risticii*-infected mouse macrophage cells P388D1 (American Type Culture Collection), as described previously (5). One horse (horse 1) was inoculated with a variant strain of *E. risticii* (6), and another horse (horse 2) was inoculated with the standard strain of the organism (4). The horses were monitored regularly for the clinical and hematological features of PHF, and clinical samples were collected.

Fecal samples were collected from the horses prior to infection and after infection on alternate days before the onset and after cessation of diarrhea and daily during the diarrheal phase for 40 days postinfection (p.i.). Heparinized whole blood was collected at intervals from these horses for 40 days p.i., and the mononuclear cells were separated on a Histopaque-1077 (Sigma) gradient by the procedure recommended by the manufacturer. Blood plasma samples were obtained by centrifugation of heparinized blood at 1,700 × g for 15 min and stored at 4°C. Serum samples were collected weekly for the detection of anti-*E. risticii* antibodies.

Immunomagnetic separation of *E. risticii* from the feces and release of genomic DNA. Solid or semisolid feces samples were suspended 1:2.5 (wt/vol) in phosphate-buffered saline (PBS), pH 7.2, and filtered through several layers of cotton gauze. Soft and diarrheal fecal samples, without dilution, were squeezed or

* Corresponding author. Mailing address: Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, MD 20742. Phone: (301) 935-6063, ext. 113. Fax: (301) 935-6079.

† Scientific article A6535, contribution 8746, from the Maryland Agriculture experiment station.
filtered through several layers of cotton gauze. Several fecal samples of different consistencies from apparently normal horses and horses with clinical conditions unrelated to PHF were included as a control, and they were similarly processed. The filtrates were clarified twice at 3,000 rpm for 10 min, and the supernates were used for immunomagnetic separation of *E. risticii* by the procedure recommended by Dynal with some modifications (12). Magnetic beads coated with sheep anti-rabbit immunoglobulin G (Dynabeads M-280; Dynal) were washed twice with PBS (pH 7.2)-bovine serum albumin (0.1%) (PBS-BSA) by magnetic force by using a Dynal Magnet (Dynal) and then resuspended with PBS-BSA to the original volume. A 20-μl volume (or multiples) of Dynabeads was mixed with 100 μl (or multiples) of various dilutions in PBS of rabbit anti-*E. risticii* serum, having an immunofluorescence assay (IFA) titer of 1:5,120 and enzyme-linked immunosorbent assay (ELISA) titer of approximately 1:50,000. Dynabeads alone and Dynabeads coated with normal rabbit serum were kept as controls. Following incubation with continuous shaking for 30 min at room temperature, the beads were washed four times for 5 min each with PBS-BSA and then resuspended with PBS-BSA to the original volume (coated Dynabeads). A 20-μl volume of coated Dynabeads was mixed with 1 ml of undiluted fecal supernates or various dilutions in PBS of fecal supernates and incubated at room temperature with continuous shaking for 5-min time increments for from 5 to 45 min. The coated Dynabeads were recovered by magnetic force, washed very gently once with PBS-BSA, and then resuspended in 20 μl of distilled water. The suspension was boiled for 7 min to release the *E. risticii* genomic DNA.

The plasma samples were centrifuged twice at 1,700 × g for 15 min, and the final supernate was collected. A 100-ml volume of plasma was centrifuged at 27,000 × g for 45 min, and the pellet was resuspended in 1 ml of PBS. An immunomagnetic separation procedure similar to the one described above was followed.

**Direct extraction of *E. risticii* DNA from the feces.** The supernates from the fecal samples, after clarification as described above, were centrifuged at 17,000 × g for 30 min, and the pellets were resuspended in EDTA (5 mM)–sodium dodecyl sulfate (SDS) (1.5%)–proteinase K (1 mg/ml) buffer to a 20× concentration and incubated at 56°C for 1 h. The suspensions were boiled for 5 min and centrifuged at 27,000 × g for 30 min, after which the supernates were subjected to the standard procedure of phenol-chloroform extraction and ethanol precipitation and the pellets were suspended in 50 μl of distilled water.

**PCR.** Feces and plasma preparations for the PCR have been described above. Processing of peripheral blood mononuclear cells was performed as described previously (1). The mononuclear cells were pelleted and lysed with a lysis buffer at 100 μl/10 × 10⁶ cells. A 5-μl volume of the sample was used in all cases for PCR amplification.

PCR was performed according to the procedure described previously (1). Two specific primers of 28 and 29 bases were used to amplify a target sequence of 247 bp. The amplified products were then subjected to agarose gel electrophoresis for detection of *E. risticii* DNA. The optical densities of the DNA bands were determined by a scanning densitometer (video densitometer model 620; Bio-Rad) by using negative photographic plates of the agarose gels.

**Restriction enzyme analysis.** The PCR-amplified products from the positive fecal samples were digested with 5 U of *BamHI* for 1 h at 37°C and then subjected to agarose gel electrophoresis according to the standard procedures. Amplified product from the *E. risticii* genomic DNA was used as a positive control.

**DNA hybridization.** For the preparation of DNA probe, an internal 180-bp fragment of the target sequence (247 bp) of *E. risticii* was amplified by using an internal primer pair (nested set) as described previously (1). A 50-μl volume of the amplified product was purified by using the GeneClean Kit (BIO 101) and resuspended in 10 μl of distilled water. A 5-μl volume of this purified product was labelled with 32P by a random-primer labelling method (catalog no. U1100; Promega Corporation), and the unincorporated nucleotide was removed by using the Stratagene Pusicholumn Kit (catalog no. 400700). A conventional hybridization was performed by using this probe according to the procedure described previously (1).

**Isolation of *E. risticii*.** Isolation of the organism from the mononuclear cells in P388D1 cell cultures was performed according to the procedure described previously (4).

**IFA and C-ELISA.** The IFA and competitive ELISA (C-ELISA) were performed according to procedures described previously (4,18).

**RESULTS**

**Disease features in horses.** The horses exhibited typical clinical and hematological features of the disease (Table 1; Fig. 1). In horse 1, the disease was very severe. The horse had a fever starting on day 6 p.i. with a temperature of 38.6°C, reaching a peak of 39.8°C on day 10 p.i. and declining to 38.7°C on day 12 p.i. before the temperature returned to normal. Leukopenia occurred between days 11 and 14 p.i. with a peak drop to 6.4 × 10⁸/mm³, a 30% reduction in leukocyte count, on day 12 p.i. The horse was considerably depressed and anorectic for several days. Diarrhea started on day 11 p.i. and was severe and watery. This watery diarrhea continued until day 16 p.i., and then the feces became soft on day 17 p.i., after which they became normal. Horse 2 had a classical case of the disease (5) which was relatively mild. There was biphasic fever with a first fever peak on day 6 p.i. The second fever started on day 10 p.i., peaked at 39.3°C on day 11 p.i., and then dropped to a normal temperature after day 15 p.i. Leukopenia occurred between days 9 and 12 p.i. and peaked on day 10 p.i. with a 30% reduction in leukocyte count. The horse had soft feces starting on day 12 p.i. and became diarrheic on day 17 p.i., and then the feces became soft on day 18 p.i. before returning to normal.

**Detection of *E. risticii* in feces.** The organisms were detected in the feces of infected horses by the immunomagnetic separation method and PCR. Rabbit anti-*E. risticii* serum at a 1:5

**TABLE 1. Range and peak times of observation of clinical-hematological features of PHF and *E. risticii* detection in horses 1 and 2**

<table>
<thead>
<tr>
<th>Horse no.</th>
<th>Day(s) p.i. (range/peak) when indicated clinical-hematological feature observed</th>
<th>Day(s) p.i. (range/peak) of <em>E. risticii</em> detection in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fever</td>
<td>Leukopenia</td>
</tr>
<tr>
<td>1</td>
<td>6–12/10</td>
<td>11–14/12</td>
</tr>
<tr>
<td>2</td>
<td>10–15/11</td>
<td>9–12/10</td>
</tr>
</tbody>
</table>
FIG. 1. Profiles of *E. risticii* detection from the feces and mononuclear cells in correspondence with the clinical-hematological features of the disease for horse 1. Diarrhea was graded on a scale of 0 to 5, 0 being negative and 5 being highest, representing severe watery diarrhea. PCR-amplified products were evaluated by direct determination of optical densities of the bands in the negative photographic plates of the agarose gel (Fig. 2A) with a scanning densitometer.

dilution was found to be suitable for coating the Dynabeads. In determining the optimum time for incubating the coated Dynabeads with the fecal samples, PCR amplification was observed at a low level for 5-, 10-, and 15-min incubations and increased to a higher level for 20-, 25-, 30-, and 35-min incubations, with a maximum level at 25 min. The level of amplification was decreased considerably when the incubation was 40 min or longer. Thus, a 1:5 dilution of the rabbit anti-*E. risticii* serum for coating Dynabeads and incubation of fecal samples with coated Dynabeads for 25 min were subsequently used in the procedure.

In horse 1, *E. risticii* was first detected from fecal samples on day 11 p.i., the first day of severe watery diarrhea. The concentration of organisms in the feces, as determined by the intensity of the band of the PCR-amplified product in an agarose gel, peaked on day 13 p.i. and then gradually declined until day 16 p.i., after which it became undetectable (Fig. 2A). *E. risticii* was first detected in the feces of horse 2 on day 12 p.i., the first day that soft feces were observed. The concentration of the organism in the feces increased on subsequent days, peaked on day 14 p.i., and then became undetectable after day 16 p.i. *E. risticii* was not detected from the feces during the times of peak diarrhea on day 17 p.i. and soft feces on day 18 p.i. (Fig. 2B). From the fecal samples of days 13 and 14 p.i. from horses 1 and 2, respectively, *E. risticii* was detectable up to a dilution of 1:64. The *Bam*HI restriction profile of the 247-bp amplified DNA product from the fecal samples was identical to that of the amplified product of the *E. risticii* genomic DNA. Also, there was specific hybridization of the amplified DNA obtained from the feces with the internal DNA probe. These results established the specificity of the amplified product from the feces and thereby the detection of *E. risticii*.

The amount of PCR-amplified product from fecal samples stored at −20°C was much less than that from the same samples stored at 4°C. The fecal samples were positive to about the same level even after storage for over 4 months at 4°C. Dynabeads alone or Dynabeads coated with normal rabbit serum incubated with *E. risticii*-containing fecal samples showed a low level of DNA amplification, as determined by a faint specific band in the agarose gel. Preparations of direct DNA extract of fecal samples were negative by PCR. Control fecal samples were negative by PCR amplification.

**Detection and isolation of *E. risticii* from the mononuclear cells.** *E. risticii* was detected from the mononuclear cells of the
FIG. 2. Agarose gel electrophoresis of PCR-amplified products of immunomagnetically separated preparations from the fecal samples of infected horses. (A) Horse 1. Lanes 1 through 7 represent days 10, 11, 12, 13, 14, 15, and 16 p.i., respectively. Day 13 p.i. feces produced maximum amplification. (B) Horse 2. Lanes 1 through 7 represent days 11, 12, 13, 14, 15, 16, and 17 p.i., respectively. The amplified product of the undiluted supernate of the day 12 p.i. feces sample did not show any band (lane 2), possibly because of the presence of inhibitors in the feces. However, the amplified product from a 1:6 dilution of the same fecal supernate showed a specific band, indicating that the sample was positive (data not shown). Day 14 p.i. feces produced maximum amplification. In all cases (except for lane 2 of panel B), undiluted fecal supernates were used for immunomagnetic separation. Arrows indicate the positions of the specific DNA bands. For the positive control (lane 8 in both panels), E. risticii DNA was used as a template. The negative control was without E. risticii DNA template, and a specific band was not observed (data not shown).

infected horses by PCR (Table 1; Fig. 1). For horse 1, the organism was first detected on day 7 p.i. Peak detection occurred on day 10 p.i., with the detection level gradually declining until day 21 p.i., and E. risticii was undetectable on day 28 p.i. For horse 2, E. risticii was first detected on day 7 p.i., peaked on day 11 p.i., maintained a high level up to day 17 p.i., gradually declined until day 21 p.i., and was undetectable on day 28 p.i. E. risticii was isolated in cell culture from the mononuclear cells obtained on different days during fever and leukopenia from both the horses.

Detection of E. risticii from plasma. E. risticii was not detected from both straight and 100× concentrated preparations of the plasma samples, including the plasma collected on the day when the mononuclear cells showed a peak concentration of E. risticii.

Serum E. risticii antibodies. Both horses produced high titers of E. risticii antibodies. Antibodies were detected by IFA in serum taken 1 week p.i. with a titer of 1:20. The titers increased to 1:640 and 1:320 for horses 1 and 2, respectively, in 3 weeks. For both horses, the titer peaked to 1:5,120 at 6 weeks and then gradually declined to 1:2,560 at 8 weeks and 1:1,280 at 16 weeks p.i. The sera were positive for specific antibodies by C-ELISA.

DISCUSSION

It is important to know whether E. risticii is present in the feces of horses suffering from PHF. By immunomagnetic separation and PCR methods, the organisms were detected in the feces from the infected horses. For the immunomagnetic separation procedure, rabbit anti-E. risticii serum at a 1:5 dilution was suitable for coating the Dynabeads. Incubation of coated Dynabeads with the fecal samples gave a maximum amplification in 25 min. The amplification was considerably decreased with samples incubated for 40 min or longer. This decrease may be due to the dissociation of organisms from the coated Dynabeads as a result of shaking for a longer period of time. This phenomenon with respect to the binding affinity was also observed with other organisms (11a). The specificity of detection from the feces was established from the appropriate size and restriction profile of the PCR-amplified target DNA and by DNA hybridization. A low degree of DNA amplification observed with fecal samples incubated with uncoated Dynabeads or Dynabeads coated with normal rabbit serum may be due to nonspecific adhering of a few organisms to the magnetic beads. Similar nonspecific adherence and separation of M13 phage particles with uncoated magnetic beads have been reported elsewhere (21). Storing of fecal samples at 4°C was suitable for detection, but when samples were stored frozen at −20°C there was a drastic reduction in PCR amplification. The most logical explanation for this is that freezing and thawing damaged or ruptured the organisms, thereby exposing the organisms’ DNA to nucleases and inhibitors present in the feces, causing degradation of DNA, and reduced the specific templates for PCR amplification. The DNA extracted directly from the fecal samples was negative by PCR amplification, possibly because of the presence in the fecal extracts of substances like bile salts and bilirubin which cause inhibition of PCR (20).

For both horses, the peak presence of the organism in the mononuclear cells was during peak fever and leukopenia or thereabouts, at which time the organisms were not detected from the feces. The peak presence of E. risticii in the feces of both horses occurred 3 days later, during the soft- or diarrheal- feces phase. E. risticii was first detected from the feces on the first day of diarrhea or soft feces. This supports previous evidence that the organisms are primarily responsible for causing diarrhea (14, 16). Thus, it appears that soon after ehrlichia the organisms invade the cells lining the intestines, causing diarrhea, and are shed in the feces. E. risticii was not detected from the plasma samples obtained from these horses, including the samples obtained on the day of peak presence of the organism in the mononuclear cells, indicating that the ehrlichia is possibly cell associated.

The shedding of E. risticii in the feces may have implications for the diagnosis and epidemiology of the disease. Because of the presence of the organism in the feces, detection of E. risticii can be used as a simple, quick, and sensitive method for the diagnosis of the disease. As yet, the mode of transmission of E. risticii remains unknown. Epidemiological evidence and the seasonality of the disease in summer months strongly suggest that a vector(s) may be involved in the transmission of PHF (13). To date, all attempts to identify a blood-feeding arthropod vector have been consistently unsuccessful (17). It may be that the feces containing the organisms are the source for the transmission of the disease, possibly involving a vector. In that case, the detection of E. risticii in the feces, as presented here, will facilitate the study of the transmission of E. risticii in PHF.

ACKNOWLEDGMENTS

We are grateful to Orjan Olsvik for his expert advice on the magnetic separation technique and thank Patricia Pope for excellent technical support.

REFERENCES


