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Pathogenic, Immunologic, and Molecular Differences between Two *Ehrlichia risticii* Strains†

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Ehrlichia risticii **is the causative agent of Potomac horse fever (PHF), an acute infectious disease of horses. In the last few years, there have been several reports of PHF cases occurring even in vaccinated horses. We isolated a new strain of *E. risticii* (90-12 strain) from a vaccinated horse suffering from clinical PHF. The major pathogenic, immunologic, and molecular differences between the 90-12 strain and the 25-D strain, which was originally isolated during the outbreaks in 1984, were studied. The 90-12 strain was more pathogenic for mice and horses compared with the 25-D strain. In enzyme-linked immunosorbent assay and immunofluorescence assay with mouse and horse antisera of both the strains, two- to fourfold differences were observed between homologous and heterologous antigens. The differences in antigen profiles were demonstrated by Western blot (immunoblot) with mouse and horse antisera and also with the recombinant clone-specific antibodies. Though several antigens were similar in both the strains, there were significant differences between them in the 110-, 85-, 70-, 51-, and 33-kDa antigens. The 85-kDa antigen was present only in the 90-12 strain but cross-reacted with a 50-kDa antigen of the 25-D strain. The 51-kDa antigens of both strains had different migration patterns. Southern blot hybridization of the genome from both the strains with DNA probes made from the 51-, 55-, and 85-kDa recombinant clones of the 90-12 strain showed a similar pattern with probes of the 51- and 55-kDa clones for both the strains, whereas the probe of the 85-kDa clone showed a completely different pattern. The 16S rRNA gene sequences from the two strains were identical. Neither strain replicated in gamma interferon-treated mouse peritoneal macrophages. In in vitro neutralization assay, sera from the 25-D strain-infected horse neutralized the homologous strain but did not neutralize the 90-12 strain, whereas sera from the 90-12 strain-infected horse neutralized both the strains. In mouse protection experiments, there was complete homologous protection. But in cross-protection, mice immunized with the 25-D strain were only partially protected against challenge with the 90-12 strain, whereas mice immunized with the 90-12 strain were completely protected against the 25-D strain challenge. These results clearly indicate that there are major differences between the 90-12 and 25-D strains which may have implications regarding the vaccine failure for PHF and the development of an efficient vaccine.**

Ehrlichia risticii, a rickettsial organism, is the causative agent of Potomac horse fever (PHF) (7, 13, 23). This disease was initially identified in 1979 in areas along the Potomac River in Maryland and Virginia. Since then, PHF has been recognized in many states in the United States and in Canada. Serological evidence also indicates the prevalence of the disease in parts of Europe and other regions of the world (15, 30). PHF affects horses of all ages, and the typical clinical signs include fever, leukopenia, depression, anorexia, and diarrhea. The mortality rate is as high as 20 to 25%. The disease occurs most commonly during the summer months. The mode of transmission of this disease remains unknown (15).

At present, vaccination with commercially available inactivated vaccines is widely practiced, especially in areas where the disease is endemic. In spite of this, there have been many reports of PHF cases even in vaccinated horses. In the 1994 season alone, in a limited study in our laboratory, 20 horses with clinical PHF were confirmed. Of these 20 horses, 16 horses had been vaccinated earlier (unpublished data). This vaccine failure may be, in addition to other causes, due to emergence of a variant strain(s) of the organism. In the 1990 season, a new strain (90-12 strain) of *E. risticii* was isolated from a horse suffering from severe clinical PHF, even though the horse was carrying a high titer of antibodies from previous PHF vaccinations. The antigenic composition of this new strain was shown to be considerably different from that of the original strain (25-D strain) (10) isolated in 1984 (7).

This paper presents the pathogenic, immunologic, and molecular differences between the 25-D and 90-12 strains of *E. risticii* and demonstrates the difference in neutralization characteristics between them.

MATERIALS AND METHODS

E. risticii **strains.** The 25-D and 90-12 strains of *E. risticii* were propagated in mouse macrophage (MM) cell line P388D1 (American Type Culture Collection) or human histiocyte cell line U937 (American Type Culture Collection) with RPMI 1640 medium supplemented with 10% fetal bovine serum and 4 mM L-glutamine. Organisms grown in human histiocyte cells were purified by the Renografin density gradient method as described previously (9) for preparing antigens for use in enzyme-linked immunosorbent assay (ELISA) and also for DNA extraction. For immunofluorescence assay (IFA), and for experimental infection of mice and horses, organisms grown in MM cells were used. The growth of *E. risticii* in cell culture was monitored by observing acridine orange-stained smears under a UV microscope.

Cell-free *E. risticii* organisms were prepared from the infected MM cell cultures. Three days after the infection when more than 50% of the cells showed infection, the cells from five T150 flasks were pooled (150 ml) and centrifuged at 500 × *g* for 10 min. The supernatant was saved, and the pellet was resuspended in 30 ml of RPMI 1640 medium and homogenized in a Sorvall Omni mixer. The homogenate was centrifuged at 500 × *g* for 10 min, and the supernatant was collected and pooled with the supernatant from the earlier centrifugation. The pooled supernatant was centrifuged at 17,000 × *g* for 30 min, and the pellet

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containing the cell-free ehrlichiae was resuspended in 6 ml of cryopreservative medium with 7.5% dimethyl sulfoxide and 15% fetal bovine serum. Aliquots of 1 ml of this cell-free *E. risticii* preparation were stored in liquid nitrogen until used.

Experimental infection of mice and horses. Two groups of 35 3-week-old CF1 mice (Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) were inoculated, intraperitoneally, with 10^5 *E. risticii*-infected MM cells of the 25-D and 90-12 strains. The 25-D strain-infected MM cells had a 40% and the 90-12 strain-infected cells had a 35% infected cell population. A third group of 30 mice was kept as an uninoculated control. All mice were observed for 3 weeks for clinical signs and mortality. The severity of the clinical infection was scored on a scale of 1 to 3 based on the clinical signs of ruffled hair, hunched back, squinty eyes, and lethargy while the moribund state was scored as 4. Two mice from each group were sacrificed for collecting sera on days 9, 14, and 21 postinfection (p.i.). The recovered mice from the two infected groups were used in the challenge infection study (described below). At the time of challenge infection, six mice from the uninoculated group were inoculated with 1.5×10^6 *E. risticii*-infected MM cells of each of the two strains, having an infected cell population of 40 and 35% for the 25-D and 90-12 strains, respectively. The mice were observed for 3 weeks for clinical signs, which were scored as described above, and for mortality. These mice served as positive controls in the challenge infection study.

Two horses, free of antibodies against the two strains of *E. risticii* as shown by IFA and their mononuclear cells negative for *E. risticii* DNA as shown by PCR (3), were experimentally infected intravenously with 5×10^7 *E. risticii*-infected MM cells for each strain. One horse was used for each strain. The cell culture materials of the two strains were the same as those used for mouse inoculation. The horses were monitored for clinical signs of a rise in body temperature, leukopenia, anorexia, depression and diarrhea, both in severity and in duration. Serum samples were collected at weekly intervals p.i. from both horses.

Serological methods and Western blotting (immunoblotting). ELISA and IFA were performed according to the procedure described previously (7, 9). Various dilutions of horse and pooled mice antiserum samples, collected at different time intervals p.i. for each strain, were tested against the antigen of the same strain (homologous) and also against antigen of the other strain (heterologous) in both ELISA and IFA.

Western blotting was performed according to the procedure described previously (25) with mouse and horse sera collected at 3 and 6 weeks p.i., respectively. Western blotting was also performed with the specific recombinant clone-specific antibodies prepared by eluting the absorbed rabbit anti-ER 90-12 antibodies from a nitrocellulose membrane containing the recombinant λZAP phage expressing the *E. risticii* antigens according to the previously described procedure (11).

Southern blotting. Southern blotting was done with the genomic DNA of both the strains and the probes made from the inserts of λZAP clones expressing the 85-, 55-, and 51-kDa antigens of the 90-12 strain by the previously described procedure (11). The DNA of each strain was extracted from the Renografin-purified organisms as described earlier (12). The probes were prepared by randomly labelling the inserts in the presence of [^{32}P]dCTP. The genomic DNA was digested with the restriction enzymes *Eco*RI, *Hin*dIII, and *Sau*3AI. The digested fragments were separated on a 1% agarose gel and transferred onto a nylon membrane according to standard procedures. The membrane was hybridized with the ^{32}P-labelled probe and exposed to an X-ray film. After each hybridization, the membrane-bound probe was removed by boiling in 1 M NaOH for 30 min and then reused for hybridizing another probe.

16S rRNA gene amplification and sequencing. The 16S rRNA genes of the two *E. risticii* strains were amplified by PCR from the infected cell culture materials with the universal primer pair EC 9 and EC 12 (1). The infected cell culture material was processed for PCR as previously described (3). The PCR was performed in a 50-μl volume with the previously described reaction conditions and amplification parameters (3). The amplified products (~1,450 bp) were cloned in a pCR II vector with the TA cloning system (Invitrogen, Inc.), and the inserts were sequenced with a Sequenase T7 DNA polymerase kit (United States Biochemical, Cleveland, Ohio).

Gamma interferon (IFN-γ) sensitivity. The sensitivity of the *E. risticii* strains in interferon-treated mouse peritoneal macrophages was tested according to published procedures (20). To obtain peritoneal macrophages, 8- to 10-week-old male CF1 mice were injected intraperitoneally with 1 ml of 5% thioglycolate broth (Difco Laboratories, Detroit, Mich.). After 5 days, the mice were euthanatized by cervical dislocation, and peritoneal cells were collected following intraperitoneal infusion of 10 ml of sterile phosphate-buffered saline. The cells were centrifuged at $500 \times g$ for 5 min and resuspended at 1.2×10^6 cells per ml in RPMI 1640 medium containing 10% fetal bovine serum, 4 mM L-glutamine, and 1% antibiotic-antimycotic mixture. The cells were dispensed in a Lab-Tek eight-chamber slide (Nunc, Inc., Naperville, Ill.), 0.5 ml per chamber, and incubated at 37°C in a 5% CO_2 incubator. After 24 h, the nonadherent cells were removed by washing twice with RPMI 1640 medium. One hundred microliters of thawed, cell-free *E. risticii* suspension was placed onto the monolayer of mouse peritoneal macrophages in each chamber and incubated at 37°C. After 3 h, the inoculum from each chamber was removed and 0.5 ml of fresh media containing different concentrations of recombinant murine IFN-γ (rMuIFN-γ; Genzyme Corporation, Boston, Mass.) was added and incubated at 37°C for 5 days. Infection of the cells was determined by staining the cells with acridine orange. Experiments were performed with triplicate samples.

TABLE 1. Experimental infection of mice with the 25-D and 90-12 strains of *E. risticii*

Days p.i.	% Clinical infection (% mortality)[a]			
	25-D strain		90-12 strain	
	1×10^5-cell inoculum (35 mice)	1.5×10^6-cell inoculum (6 mice)	1×10^5-cell inoculum (35 mice)	1.5×10^6-cell inoculum (6 mice)
4		0 (0)		0 (0)
5		16 (0)		66 (0)
6		16 (0)		100 (0)
7	10 (0)	66 (0)	0 (0)	100 (0)
8	12 (0)	100 (0)	37 (0)	100 (16)
9	12 (0)	100 (0)	55 (0)	100 (50)
10	100 (0)	100 (0)	100 (0)	100 (50)
11	100 (0)	83 (0)	100 (3)	100 (66)
12	100 (0)	83 (0)	100 (11)	100 (83)
13	82 (0)	83 (0)	97 (14)	100 (83)
14	14 (0)	66 (0)	88 (14)	100 (83)
15	0 (0)	66 (0)	46 (14)	100 (83)
16		16 (0)		100 (83)
17		0 (0)		83 (83)
21	0 (0)		3 (14)	

[a] Percent clinical infection represents the percentage of sick mice in a group on a particular day p.i., and percent mortality represents the accumulative percentage of dead mice in a group on the given days p.i.

In vitro neutralization assay. In vitro neutralization of *E. risticii* with horse sera was performed according to the published procedure (24) with some modifications. One hundred microliters of horse sera was mixed with 100 μl of cell-free *E. risticii* suspension and incubated at 37°C. After 1 h, the mixtures were transferred to each chamber of the Lab-Tek eight-chamber slide containing 1-day-old MM cells. Following 3 h of incubation at 37°C, the chambers were washed twice with RPMI 1640 medium to remove the unbound *E. risticii* organisms and 0.5 ml of fresh medium was added to each chamber. The chamber slides were then incubated for 5 days at 37°C, and the infection was monitored by staining the cells with acridine orange and observing them under a UV microscope. Experiments were performed with triplicate samples.

Mouse immunization and challenge infection. The recovered mice from experimental infection with 10^5 *E. risticii*-infected MM cells of each strain (described for the infection experiment) were challenge infected on day 30 p.i. with 1.5×10^6 *E. risticii*-infected MM cells of either of the two strains. The infected cell population for the 25-D strain and 90-12 strain was 40 and 35%, respectively. Mice from each of the two recovered groups were divided into three subgroups of six mice each. One subgroup was inoculated with the homologous strain, the second subgroup was inoculated with the heterologous strain, and the third subgroup was kept as an unchallenged control. The uninoculated group of mice was similarly divided into three subgroups of six mice each, two subgroups were infected with the 25-D and 90-12 strains as positive controls (as mentioned for the infection experiment), and the third subgroup was kept as an uninoculated negative control. The mice were observed for 3 weeks for clinical signs, which were scored as described above, and for mortality.

RESULTS

Pathogenicity in mice. The 90-12 strain was more pathogenic for mice than the 25-D strain, and in both cases, the severity of the disease is dose related. The 25-D strain did not cause any mortality with either a 1×10^5- or a 1.5×10^6-infected-cell inoculum, but the 90-12 strain caused mortality with both concentrations of the cell inoculum. With a 1.5×10^6-cell inoculum, the 90-12 strain caused 100% morbidity by day 6 p.i. and 83% mortality by day 12 p.i., whereas the 25-D strain caused 100% morbidity by 8 days p.i., but without any mortality (Table 1).

Pathogenicity in horses. A significant difference in pathogenicity was observed between the two horses infected with each strain. The horse infected with the 90-12 strain had a fever for 7 days, from day 6 to day 12 p.i. with a peak temperature of

TABLE 2. Difference in antibody titers between homologous and heterologous reactions with the mouse antisera against the two strains of *E. risticii*

E. risticii strain/ antiserum	ELISA						IFA	
	9-day-p.i. serum		14-day-p.i. serum		21-day-p.i. serum		21-day-p.i. serum	
	Antibody titer[a]	Difference in titer	Antibody titer[a]	Difference in titer	Antibody titer[a]	Difference in titer	Antibody titer[a]	Difference in titer
90-12/90-12	12,800	4-fold	25,600	2-fold	51,200	2-fold	4,800	2-fold
90-12/25-D	3,200		12,800		25,600		2,400	
25-D/25-D	12,800	4-fold	25,600	4-fold	102,400	4-fold	4,800	2-fold
25-D/90-12	3,200		6,400		25,600		2,400	

[a] The titers are expressed as reciprocal of serum dilution.

103.8°F on day 10 p.i. The typical biphasic fever was not observed in this horse. Diarrhea lasted for 10 days and was profuse and watery for 6 days (day 11 to day 16 p.i.). The horse was severely depressed and anorectic for 7 days, so much so that supportive fluid therapy had to be administered twice during the course of infection. The horse infected with the 25-D strain suffered a milder disease. The first rise in body temperature occurred on day 6 p.i., and second fever lasted for 6 days from days 10 to 15 p.i. with a peak temperature of 102.8°F on day 11 p.i. The horse had soft feces for 5 days and diarrhea for 1 day, on day 17 p.i. The horse was mildly depressed for 1 day but continued to consume feed throughout the course of the disease.

Antigenic differences. The antibody titers of sera by IFA and ELISA showed a difference of two- to fourfold between the homologous and the heterologous antigens. The difference was more pronounced with early mice antisera of day 9 p.i. and horse antisera of 1 week p.i. (Tables 2 and 3).

Difference in antigenic composition. The Western blot antigenic profile of the 90-12 strain differed considerably from that of the 25-D strain with both mouse (Fig. 1) and horse (Fig. 2) antisera. Though several major antigens, namely, the 68-, 49-, and 28-kDa antigens, are similar in both the strains, there are several differences. The main differences between them are as follows: (i) the 85-kDa antigen is present only in the 90-12 strain but reacts with the 25-D strain antisera; (ii) the 55- and 51-kDa antigen bands in the 90-12 strain migrate close together (labelled 55/51-kDa band), whereas in the 25-D strain they are well separated; and (iii) the 33-kDa antigen band of each strain shows less color intensity with the heterologous antisera compared with homologous antisera. Additionally, antisera from mice infected with the 25-D strain reacted with the 110- and 70-kDa antigens of the 25-D strain. These two antigens are not present in the 90-12 strain, and also they did not react with the 90-12 strain antisera.

In Western blotting, the recombinant clone-specific antibodies of the 55-kDa antigen of the 90-12 strain reacted with the same-molecular-mass proteins in both the strains, whereas the 51-kDa clone-specific antibodies of the 90-12 strain reacted with the 51-kDa antigen of the 25-D strain and the 51-kDa part of the 55/51-kDa antigen band of the 90-12 strain. The clone-specific antibodies of the 85-kDa antigen of the 90-12 strain reacted with the same-molecular-mass protein of the 90-12 strain but with a 50-kDa antigen of the 25-D strain (Fig. 3), indicating that these two antigens are counterparts of their respective strains. In Western blots developed with polyclonal sera, 50-kDa antigen of the 25-D strain is not distinguishable as a separate band, as it overlaps with the 51-kDa antigen band.

Genomic differences. In Southern blotting, the probes made with the inserts of the 55- and 51-kDa recombinant clones of the 90-12 strain hybridized with the same-size fragments in each of the three restriction enzyme digests of both strains. The 85-kDa probe of the 90-12 strain hybridized with different-size fragments in each of the restriction enzyme digests for both strains (Fig. 4). The 25-D strain showed a low-intensity hybridization signal with all the probes.

16S rRNA gene sequences. The nucleotide sequences of the 16S rRNA genes of the two strains were identical to the previously described sequence of the ATCC strain of *E. risticii* (GenBank accession no. M21290).

Interferon sensitivity. The rMuIFN-γ treatment made the thioglycolate-induced mouse peritoneal macrophages refractory to infection with either of the strains (data not shown). At a 10-U/ml concentration of rMuIFN-γ, there was incomplete

TABLE 3. Difference in ELISA and IFA antibody titers between homologous and heterologous reactions with horse antisera against the two strains of *E. risticii*

Assay and *E. risticii* strain/ antiserum	1-wk-p.i. serum		2-wk-p.i. serum		3-wk-p.i. serum		4-wk-p.i. serum		5-wk-p.i. serum		6-wk-p.i. serum	
	Antibody titer[a]	Difference in titer	Antibody titer[a]	Difference in titer	Antibody titer[a]	Difference in titer	Antibody titer[a]	Difference in titer	Antibody titer[a]	Difference in titer	Antibody titer[a]	Difference in titer
ELISA												
25-D/90-12	1,600	4-fold	6,400	2-fold	12,800	2-fold	25,600	2-fold	51,200	2-fold	51,200	2-fold
90-12/90-12	6,400		12,800		25,600		51,200		102,400		102,400	
25-D/25-D	6,400	4-fold	12,800	2-fold	25,600	2-fold	51,200	2-fold	102,400	2-fold	51,200	2-fold
90-12/25-D	1,600		6,400		12,800		25,600		51,200		25,600	
IFA												
25-D/90-12			320	2-fold	320	2-fold	640	2-fold	1,280	2-fold	1,280	2-fold
90-12/90-12			640		640		1,280		2,560		2,560	
25-D/25-D			320	2-fold	640	2-fold	1,280	2-fold	2,560	2-fold	1,280	2-fold
90-12/25-D			160		320		640		1,280		640	

[a] The titers are expressed as reciprocal of serum dilution.

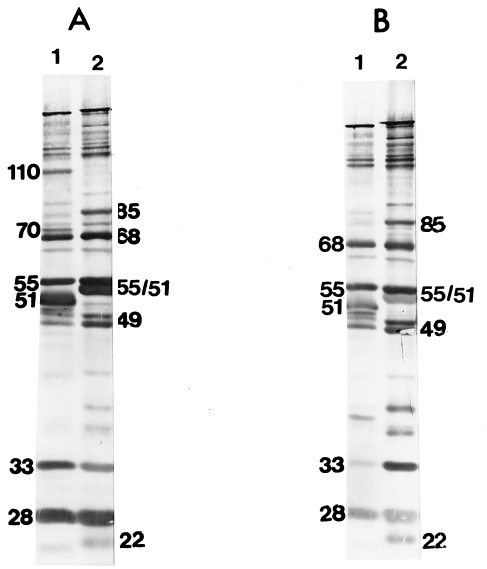

FIG. 1. Western blot profiles of the two *E. risticii* strains with mouse antisera. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-separated antigens of the 25-D strain (lanes 1) and the 90-12 strain (lanes 2) were reacted with sera from the 25-D strain-infected mice (A) and 90-12 strain-infected mice (B). The numbers indicate approximate molecular masses in kilodaltons.

(95%) inhibition of *E. risticii* infection, whereas at 25- and 100-U/ml concentrations there was complete inhibition of *E. risticii* replication. When added after 3 days p.i., rMuIFN-γ at all concentrations (10, 25, and 100 U/ml) did not affect the *E. risticii* infection (data not shown).

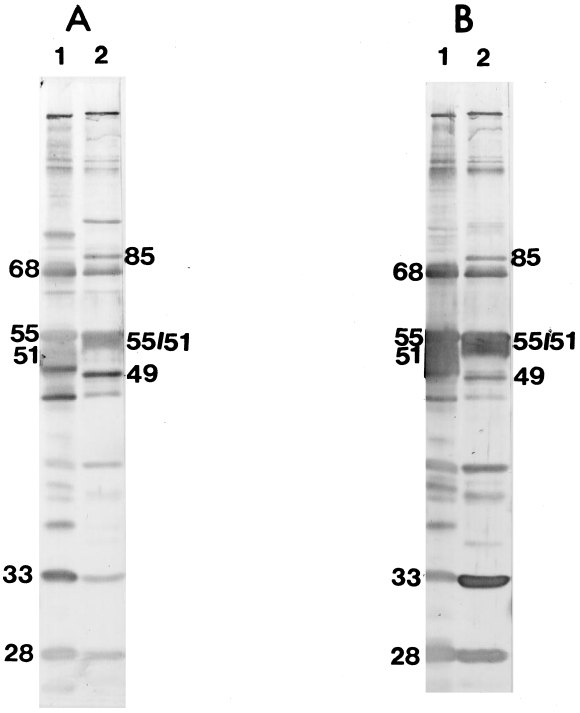

FIG. 2. Western blot profiles of the two *E. risticii* strains with horse antisera. The SDS-PAGE-separated antigens of the 25-D strain (lanes 1) and 90-12 strain (lanes 2) were reacted with sera from the 25-D strain-infected horse (A) and 90-12 strain-infected horse (B). The numbers indicate approximate molecular masses in kilodaltons.

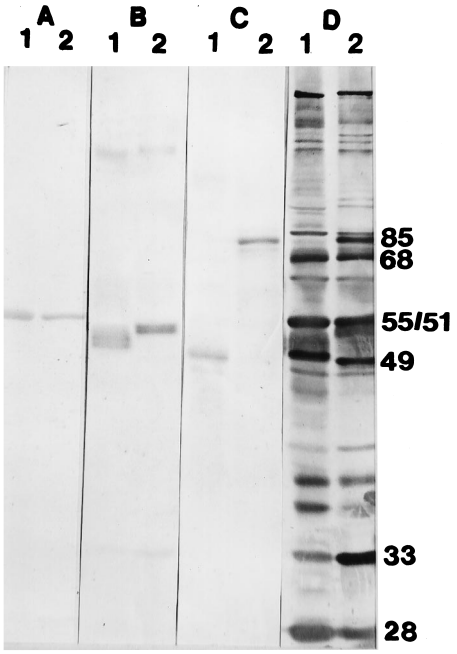

FIG. 3. Western blot reactivities of the recombinant clone-specific antibodies with antigens of the two *E. risticii* strains. Lanes 1 contain the antigens from the 25-D strain of *E. risticii*, and lanes 2 contain the antigens from the 90-12 strain of *E. risticii*. Blots in panels A, B, and C were reacted with the 55-, 51-, and 85-kDa recombinant clone-specific antibodies of the 90-12 strain, respectively. The blot in panel D was reacted with antiserum from mice infected with the 90-12 strain. In panel B, the recombinant clone-specific antibodies also reacted with a high-molecular-weight and a low-molecular-weight protein in both the strains. Numbers at right are molecular masses in kilodaltons.

In vitro neutralization. The first-week-p.i. sera from both of the horses did not show any neutralization activity. The second-, third-, fourth-, fifth-, and sixth-week-p.i. sera from the horse infected with the 90-12 strain showed complete neutralization (more than 90% of inhibition) of both the 25-D and 90-12 strains. The 2-week-p.i. sera from the 25-D-infected horse showed 80% inhibition of the 25-D strain, and the third-, fourth-, fifth-, and sixth-week-p.i. sera from this horse completely neutralized the 25-D strain. None of the serum samples from this horse neutralized the 90-12 strain (Table 4).

Mouse immunoprotection. There was complete homologous immunoprotection with both the strains. In cross-challenge, the 90-12 strain-immunized mice were completely protected against 25-D strain challenge, whereas the 25-D strain-immunized mice were only partially protected against the challenge with the 90-12 strain. These mice showed mild clinical signs during the early period after challenge infection, and the percentage of infected mice was low. There was no mortality (Table 5).

DISCUSSION

Studies presented in this paper clearly demonstrate the major differences between the two strains of *E. risticii*. The observation that the 90-12 strain is highly pathogenic in mice as well as in horses in conjunction with the fact that this strain was isolated from a sick horse with PHF even though that horse received two vaccinations against PHF emphasizes the importance of this variant strain's role in the occurrence of clinical PHF. Also, the comparative mouse and horse infection studies suggest that the 90-12 strain is more pathogenic than the 25-D

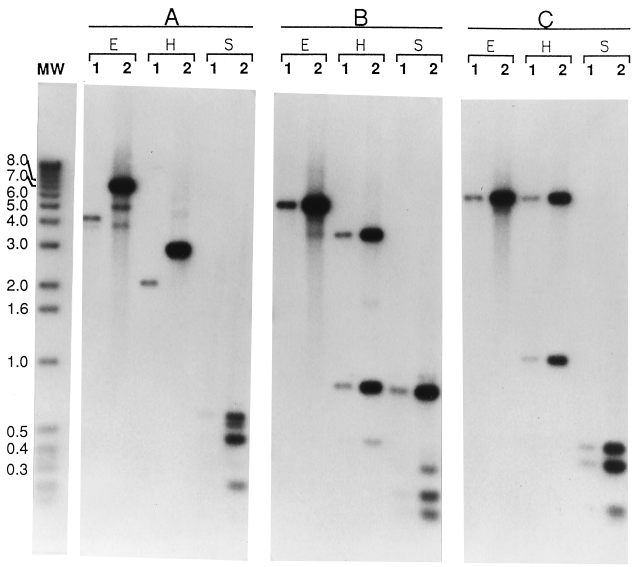

FIG. 4. Southern blot profiles of genomic DNA of the two *E. risticii* strains with probes of recombinant clones expressing the 90-12 strain antigens. The genomic DNA of the 25-D strain (lanes 1) and 90-12 strain (lanes 2) was digested with *Eco*RI (E), *Hin*dIII (H), and *Sau*3AI (S) and hybridized with probes made from the 90-12 strain clones expressing the 85-kDa antigen (A), 55-kDa antigen (B), and 51-kDa antigen (C). Lane MW contains the molecular size markers, and the numbers indicate the molecular size in kilobase pairs.

strain. Although the pathogenicity in horses seems to parallel the differences in pathogenicity in mice, these results must be reproduced with more horses to confirm the higher pathogenicity of the 90-12 strain. It should be mentioned that there was a difference in the number of cell culture passages between the two strains at the time of inoculation: the 25-D strain had 30 cell culture passages whereas the 90-12 strain had only 20. But to date, to the best of our knowledge, there is no report of a decrease in pathogenicity due to increased cell culture passages for rickettsial organisms. Also, even at low passage number, the 25-D strain showed the same extent of less pathogenicity in our previous mouse experiments as observed in the present study (unpublished data). Thus, in this study, the difference in cell culture passage number between the two strains probably had no influence on the pathogenicity of the organisms.

TABLE 4. In vitro neutralization of the two strains of *E. risticii* by the sera from experimentally infected horses

Serum	90-12 strain		25-D strain	
	% Infection[a]	% Inhibition[b]	% Infection[a]	% Inhibition[b]
Normal serum	88 ± 5		86 ± 4	
Anti-90-12				
1 wk p.i.	95 ± 2	0	91.3 ± 1.9	0
2 wk p.i.	6.9 ± 1.7	92	7.9 ± 2.2	90
3 wk p.i.	9.3 ± 2.1	89	6.2 ± 2.7	92
4 wk p.i.	5.1 ± 1.9	94	7.3 ± 1.7	91
5 wk p.i.	2.4 ± 3.1	97	4.2 ± 3.4	93
6 wk p.i.	3.5 ± 2.7	96	5.3 ± 2.6	93
Anti-25-D				
1 wk p.i.	89.7 ± 4.1	0	94.1 ± 2.1	0
2 wk p.i.	90.2 ± 1.4	0	1.7 ± 4.9	80
3 wk p.i.	93.1 ± 1.4	0	2.8 ± 1.8	96
4 wk p.i.	90.7 ± 3.0	0	4.7 ± 1.4	94
5 wk p.i.	78.8 ± 5.2	10[c]	3.2 ± 2.4	96
6 wk p.i.	84.3 ± 4.9	4[c]	3.9 ± 2.8	95

[a] Expressed as a mean ± the standard deviation ($n = 3$).

[b] % inhibition = (mean percent infected cells with normal serum − mean percent infected cells with antiserum)/(mean percent infected cells with normal serum) × 100.

[c] The mean percent infection values in these assays and that of normal serum control were not significantly different by analysis of variance ($P > 0.1$), and the calculated percent inhibition values were considered to be not significant.

TABLE 5. Homologous and heterologous immunoprotectivity between the 25-D and 90-12 strains of *E. risticii* in mice

Day p.i.	% Clinical infection (% mortality)[a]					
	25-D strain challenge infection			90-12 strain challenge infection		
	Unimmunized	25-D strain immunized	90-12 strain immunized	Unimmunized	25-D strain immunized	90-12 strain immunized
4	0 (0)	0	0	0 (0)	0 (0)	0
5	16 (0)	0	0	66 (0)	16 (0)	0
6	16 (0)	0	0	100 (0)	50 (0)	0
7	66 (0)	0	0	100 (0)	50 (0)	0
8	100 (0)	0	0	100 (0)	50 (0)	0
9	100 (0)	0	0	100 (50)	66 (0)	0
10	100 (0)	0	0	100 (50)	50 (0)	0
11	83 (0)	0	0	100 (66)	16 (0)	0
12	83 (0)	0	0	100 (83)	16 (0)	0
13	83 (0)	0	0	100 (83)	16 (0)	0
14	66 (0)	0	0	100 (83)	0 (0)	0
15	66 (0)	0	0	100 (83)	0 (0)	0
16	16 (0)	0	0	100 (83)	0 (0)	0
17	0 (0)	0	0	83 (83)	0 (0)	0

[a] Percent clinical infection represents the percentage of sick mice in a group on the particular day p.i., and percent mortality represents the accumulative percentage of dead mice in a group on the given days p.i. Six mice were used in each group.

The difference in antibody titers between the homologous and heterologous systems indicates an antigenic difference between the two strains. This antigenic difference was further substantiated from the Western blot profiles. The 85-kDa antigen of the 90-12 strain and its counterpart, the 50-kDa antigen of the 25-D strain, were very different in their sizes and also in their gene sequences as indicated by the Southern blot results (Fig. 3 and 4). In the case of the 51-kDa antigen, though the Western blotting with clone-specific antibody showed a size difference in the proteins, the Southern blot did not show any difference in the hybridization pattern. This can possibly be explained in two different ways: the insert used as the probe may be in the invariable region of the gene or the size difference in the protein may be due to posttranslational modifications, like proteolytic cleavages. In Southern blotting, the low intensity of the hybridization signal for the 55- and 51-kDa probes in the 25-D strain was due to a lesser amount of DNA, but not due to a difference in nucleotide sequences, which we at present know, whereas in the 85-kDa antigen gene of the 90-12 strain and its corresponding 50-kDa antigen gene of the 25-D strain there is considerable sequence variation (4).

Sequence comparison of 16S rRNA genes is the frequently used method for determining the phylogenetic relationships of prokaryotic organisms (19). Differences in the 16S rRNA gene sequences of different strains of the same species of rickettsial organisms vary from 0 to 0.1% (1, 26). The absence of nucleotide sequence difference in 16S rRNA genes of the two *E. risticii* strains indicates that these two strains are very closely related, and the observed differences are most likely due to changes in the important antigens involved in the pathogenicity and immune response.

rMuIFN-γ has been shown to inhibit the growth of many facultative and obligate intracellular bacteria in macrophages (2, 5, 14, 17, 27). Inhibition of *E. risticii* infection in mouse peritoneal macrophages by rMuIFN-γ has been shown previously (20, 21). The presence of IFN-γ-resistant strains has been documented in *Rickettsia prowazekii* (28, 29). Though it is possible that IFN-γ-resistant strains of *E. risticii* are present in nature, our study with the two strains did not show any difference in their sensitivity to rMuIFN-γ.

Evidence, including passive protection of mice with horse antisera (24) and purified immunoglobulin G from mouse antisera (16), and the fact that horses develop neutralizing antibodies to *E. risticii* after infection (24), strongly suggests that humoral immunity plays a major role in host defense against PHF. The in vitro neutralization of *E. risticii* organisms by the anti-*E. risticii* horse serum was shown to be due to the specific interaction between the surface-exposed antigen(s) of the organism and the immunoglobulin G antibodies (18). In neutralization assays, the 25-D strain-infected horse sera neutralized the homologous strain but did not neutralize the 90-12 strain, whereas 90-12 strain-infected horse sera neutralized both the 90-12 and 25-D strains. This one-way cross-neutralization is an important difference between the two strains, and the reason for this difference can be understood only after the antigen(s) of *E. risticii* involved in the neutralization process is identified and characterized. The specificity of neutralizing characteristics of the 90-12 strain indicates a significant role for variant strains in causing clinical PHF in vaccinated horses. In Western blotting, the 25-D strain-infected horse sera reacted with all of the major antigens of the 90-12 strain, and there was no difference between the 90-12 strain Western blots developed with anti-25-D strain horse sera or anti-90-12 strain horse sera (Fig. 2). This observation, in conjunction with the one-way cross-neutralization, suggests that a certain epitope(s), but not a unique antigen, is involved in the neutralization process and that the difference in this epitope(s) may be responsible for the one-way cross-neutralization.

In cross-protection experiments, mice immunized with the 25-D strain were only partially protected against challenge with the 90-12 strain, whereas mice immunized with the 90-12 strain were completely protected against the 25-D strain challenge. This result can be correlated with the result of in vitro cross-neutralization assays. The lack of complete protection of the 25-D strain-immunized mice against the 90-12 strain challenge may be due to the absence of neutralizing antibodies against the challenging 90-12 strain at the time of infection. Perhaps infection in these mice was established during the early period of challenge infection, but it did not develop to a full infection because of the host's cell-mediated immunity response and subsequent humoral response to the challenging *E. risticii* strain.

Strain variation in *E. risticii* is not unexpected as the existence of different strains has already been clearly established in other rickettsial organisms (31). Recently, biological diversity of nine clinical isolates of *E. risticii* was reported (6). Until recently, our knowledge about the molecular biology and immune responses of *E. risticii* was restricted to the original isolates from different laboratories, and we do not know whether there is any variation in the pathogenicity and immunoprotectivity level between these isolates. In *Rickettsia tsutsugamushi*, it was shown that immunity against heterologous strains is short lived compared with that against the homologous strain (22). Similar differences in the immune response may also be present in *E. risticii* strains as well. A systematic comparison among all the presently available isolates from different geographical regions would be helpful in understanding the extent of variation in *E. risticii* and its implication in vaccine failure. Identification of different strains involved in the field cases and studies concerning their cross-immunoprotectivities are very important for the development of an efficient vaccine(s) for PHF.

REFERENCES

1. **Anderson, B. E., J. E. Dawson, D. C. Jones, and K. H. Wilson.** 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J. Clin. Microbiol. **29:**2838–2842.
2. **Bhardwaj, N., T. Nash, and M. A. Horowitz.** 1986. Gamma interferon-activated human monocytes inhibit the intracellular multiplication of *Legionella pneumophila*. J. Immunol. **137:**2662–2669.
3. **Biswas, B., S. K. Dutta, D. Mukherjee, and B. L. Mattingly-Napier.** 1991. Diagnostic application of polymerase chain reaction for detection of *Ehrlichia risticii* in equine monocytic ehrlichiosis (Potomac horse fever). J. Clin. Microbiol. **29:**2228–2233.
4. **Biswas, B., R. Vemulapalli, and S. K. Dutta.** 1994. Cloning and analysis of randomly repeated sequences of the genes for a cross-reacting major antigen with different molecular weights from two strains of *Ehrlichia risticii*, abstr. p. 22–23. *In* 11th meeting of the American Society for Rickettsiology and Rickettsial Diseases, St. Simons Island, Georgia, September 1994.
5. **Byrne, G. I., L. K. Lehmann, and G. J. Landry.** 1986. Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular *Chlamydia psittaci* replication in T24 cells. Infect. Immun. **53:**347–351.
6. **Chaichanasiriwithaya, W., Y. Rikihisa, S. Yamamota, S. Reed, T. Crawford, L. E. Perryman, and G. Palmer.** 1994. Antigenic, morphologic and molecular characterization of the new *E. risticii* isolates. J. Clin. Microbiol. **38:**3026–3033.
7. **Dutta, S. K., A. C. Myrup, R. M. Rice, M. G. Robel, and R. C. Hammond.** 1985. Experimental reproduction of Potomac horse fever in horses with a newly isolated ehrlichia organism. J. Clin. Microbiol. **22:**265–269.
8. **Dutta, S. K., B. E. Penney, A. C. Myrup, M. G. Robl, and R. M. Rice.** 1988. Disease features in horses with induced equine monocytic ehrlichiosis (Potomac horse fever). Am. J. Vet. Res. **49:**1747–1751.
9. **Dutta, S. K., R. M. Rice, T. D. Hughes, P. K. Savage, and A. C. Myrup.** 1987. Detection of serum antibodies against *Ehrlichia risticii* in Potomac horse fever by enzyme-linked immunosorbent assay. Vet. Immunol. Immunopathol. **14:**85–92.
10. **Dutta, S. K., B. Shankarappa, and B. L. Mattingly-Napier.** 1991. Antigenic analysis of *Ehrlichia risticii* isolates, p. 61–65. *In* W. Plowright, P. D. Rossdale, and J. F. Wade (ed.), Equine infectious disease. VI. Proceedings of the Sixth International Conference 7 to 11 July 1991. R & W Publication (Newmarket) Limited, Newmarket, Suffolk, United Kingdom.
11. **Dutta, S. K., B. Shankarappa, and B. L. Mattingly-Napier.** 1991. Molecular cloning and analysis of recombinant major antigens of *Ehrlichia risticii*. Infect. Immun. **59:**1162–1169.
12. **Dutta, S. K., B. Shankarappa, S. R. Thaker, and B. L. Mattingly-Napier.** 1990. DNA restriction endonuclease cleavage pattern and protein antigen profile of *Ehrlichia risticii*. Vet. Microbiol. **25:**29–38.
13. **Holland, C. J., M. Ristic, A. I. Cole, P. Johnson, G. Baker, and T. Goetz.** 1985. Isolation, experimental transmission and characterization of the causative agent of Potomac horse fever. Science **277:**522–526.
14. **Kagaya, K., K. Watanabe, and Y. Fukazawa.** 1989. Capacity of recombinant gamma interferon to activate macrophages for *Salmonella*-killing activity. Infect. Immun. **57:**609–615.
15. **Kahler, S.** 1989. Transmission is unsolved mystery of equine monocytic ehrlichiosis (news). J. Am. Vet. Med. Assoc. **12:**1681–1687.
16. **Kaylor, P., T. B. Crawford, T. R. McElwain, and G. H. Palmer.** 1991. Passive transfer of antibody to *Ehrlichia risticii* protects mice from ehrlichiosis. Infect. Immun. **59:**2058–2062.
17. **Kemmerich, B., G. J. Small, and J. E. Pennington.** 1986. Relation of cytosolic calcium to the microbicidal activation of blood monocytes by recombinant γ-interferon. J. Infect. Dis. **154:**770–777.
18. **Messick, J. B., and Y. Rikihisa.** 1994. Inhibition of binding, entry, or intracellular proliferation of *Ehrlichia risticii* in $P388D_1$ cells by anti-*E. risticii* serum, immunoglobulin G, or Fab fragment. Infect. Immun. **62:**3156–3161.
19. **Olsen, G. J., and C. R. Woese.** 1993. Ribosomal RNA: a key to phylogeny. FASEB J. **7:**113–123.
20. **Park, J., and Y. Rikihisa.** 1991. Inhibition of *Ehrlichia risticii* infection in murine peritoneal macrophages by gamma interferon, a calcium ionophore, and concanavalin A. Infect. Immun. **59:**3418–3423.
21. **Park, J., and Y. Rikihisa.** 1992. L-Arginine-dependent killing of intracellular *Ehrlichia risticii* by macrophages treated with gamma interferon. Infect. Immun. **60:**3504–3508.
22. **Rights, F. L., J. E. Smadel, and E. B. Jackson.** 1948. Studies on scrub typhus (tsutsugamushi disease). III. Heterogeneity of strains of Rickettsia tsutsugamushi as demonstrated by cross vaccination studies. J. Exp. Med. **87:**339–351.

23. **Rikihisa, Y., and B. D. Perry.** 1985. Causative ehrlichial organisms in Potomac horse fever. Infect. Immun. **49:**513–517.
24. **Rikihisa, Y., R. Wada, S. M. Reed, and S. Yamamoto.** 1993. Development of neutralizing antibody in horses infected with *Ehrlichia risticii*. Vet. Microbiol. **36:**139–147.
25. **Shankarappa, B., and S. K. Dutta.** 1989. Production and characterization of monoclonal antibodies to *Ehrlichia risticii*. Am. J. Vet. Res. **50:**1145–1149.
26. **Stothard, D. R., J. B. Clark, and P. A. Fuerst.** 1994. Ancestral divergence of *Rickettsia bellii* from the spotted fever and typhus groups of *Rickettsia* and antiquity of the genus *Rickettsia*. Int. J. Syst. Bacteriol. **44:**798–804.
27. **Turco, J., and H. H. Winkler.** 1986. Gamma-interferon-induced inhibition of the growth of *Rickettsia prowazekii* in fibroblasts cannot be explained by the degradation of tryptophan or other amino acids. Infect. Immun. **53:**38–46.
28. **Turco, J., and H. H. Winkler.** 1989. Isolation of *Rickettsia prowazekii* with reduced sensitivity to gamma interferon. Infect. Immun. **57:**1765–1772.
29. **Turco, J., and H. H. Winkler.** 1990. Selection of alpha/beta interferon- and gamma interferon-resistant rickettsiae by passage of *Rickettsia prowazekii* in L929 cells. Infect. Immun. **58:**3279–3285.
30. **Van der Kolk, J. H., W. F. Bernadina, and J. R. Visser.** 1991. A horse seropositive for *Ehrlichia risticii* in the Netherlands. Tijdschr. Diergeneeskd. **116:**69–72.
31. **Weisburg, W. G., M. E. Dobson, J. E. Samuel, G. A. Dasch, L. P. Mallavia, O. Baca, L. Mandelco, J. E. Sechrest, E. Weiss, and C. R. Woese.** 1989. Phylogenetic diversity of the rickettsiae. J. Bacteriol. **171:**4202–4206.