

Antigenic, Morphologic, and Molecular Characterization of New *Ehrlichia risticii* Isolates

WIWAT CHAICHANASIRIWITHAYA,¹ YASUKO RIKIHISA,^{1*} SHIZUO YAMAMOTO,^{1†} STEPHEN REED,² TIMOTHY B. CRAWFORD,³ LANCE E. PERRYMAN,³ AND GUY H. PALMER³

Department of Veterinary Pathobiology¹ and Department of Veterinary Clinical Science,² The Ohio State University, Columbus, Ohio 43210-1093, and Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040³

Received 4 April 1994/Returned for modification 23 June 1994/Accepted 1 September 1994

Ehrlichia risticii causes an acute infectious disease in horses called Potomac horse fever. To investigate the biological diversity of *E. risticii* organisms, nine *E. risticii* isolates derived from the peripheral blood monocytes of clinically sick horses in Ohio and Kentucky during the summers of 1991 and 1993 were compared with Illinois and Virginia isolates originally obtained from horses in Maryland in 1984. Seven of the nine isolates (081, 606, 380, 679, As, Co, and Ov) formed large morulae (tightly packed inclusions of ehrlichial organisms). The remaining isolates, including 1984 isolates, were individually dispersed or formed small morulae in the cytoplasm of P388D₁ cells. In Western blot (immunoblot) analysis with four equine and one rabbit polyclonal anti-*E. risticii* sera, these recent *E. risticii* isolates showed patterns of antigenic proteins distinct from those of the 1984 isolates and could be divided into three groups: (i) 081; (ii) 606, 022, 067, 380, and 679; and (iii) As, Co, and Ov. By indirect fluorescent antibody labeling with two panels of murine anti-*E. risticii* (Illinois and Maryland isolates) monoclonal antibodies, isolate 081 was not labeled with any of 20 monoclonal antibodies tested. The remaining isolates were not labeled with several monoclonal antibodies. The digestion pattern with one of the restriction enzymes, *Ava*II, of the PCR-amplified partial 16S rRNA gene of *E. risticii* from all Kentucky isolates (As, Co, and Ov) was different from that of Illinois, Virginia, and six Ohio isolates. These results indicate the presence of distinct variants of *E. risticii* which vary significantly in morphology, antigenic composition, and the base sequence of the 16S rRNA gene.

The tribe *Ehrlichieae* belongs to the family *Rickettsiaceae* and consists of small, gram-negative, round, obligate-intracellular bacteria (19). Potomac horse fever (PHF), caused by *Ehrlichia risticii* (1, 4, 14), is an acute infectious disease of equidae. It was first recognized in 1979 in areas along the Potomac River in Maryland and Virginia (6), and three isolates were obtained in 1984 from horses in this area (the Virginia isolate [14], the Illinois isolate [4], and the Maryland isolate [1, 23]). The disease is characterized by fever, depression, loss of appetite, explosive diarrhea, dehydration, leukopenia, and laminitis (6). The severity and nature of clinical disease, however, vary widely from death to subclinical infection. The case mortality rate is 5 to 30%. The disease has been documented in many states of the United States (9, 18). It has also been reported in Europe (21) and possibly in India (17). Most of the clinical cases occurred during the summer season, with a peak incidence from July to September (10, 15). The transmission mode of the disease is still unknown. Arthropod vectors, including ticks, so far have not been found to transmit *E. risticii* (20).

On the basis of the published literature on the Illinois, Maryland, and Virginia isolates, they do not appear significantly different. The purpose of this study is to examine recent *E. risticii* isolates and whether there are strains of *E. risticii* which vary significantly in antigenic composition, morphology, and 16S rRNA base sequence. Three of the new *E. risticii*

isolates included in this study were isolated from horses which developed typical clinical signs of PHF despite previous vaccination.

MATERIALS AND METHODS

***E. risticii* 1984 isolates.** The Illinois isolate of *E. risticii* (strain HRC-IL [ATCC VR-986]) (4) was obtained from the American Type Culture Collection (ATCC; Rockville, Md.). The Virginia isolate of *E. risticii* (14) was previously propagated in a human monocyte cell line, U-937 (ATCC). Both isolates, kept frozen in a liquid nitrogen tank since 1985, were thawed and used for this study to compare them during early passage.

Isolation of ehrlichiae from horse blood. Blood samples (100 to 200 ml) from six horses in Ohio and three horses in Kentucky having positive (>1:80) indirect fluorescent antibody (IFA) titers against *E. risticii* (Table 1) were aseptically collected in sterile heparinized (5 U/ml) syringes and centrifuged at 1,600 × *g* for 10 min. All nine horses had clinical signs (fever, depression, anorexia, diarrhea, dehydration, and/or laminitis) compatible with PHF (Table 1). After removal of plasma, the buffy coat was aspirated and layered on Histopaque 1077 (Sigma Chemical Co., St. Louis, Mo.) and centrifuged at 800 × *g* for 15 min at room temperature. The interface containing mononuclear cells was collected, washed in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing a 1% antibiotic mixture (10⁴ U of penicillin per ml, 1 mg of streptomycin per ml, and 25 U of amphotericin B per ml; GIBCO), and centrifuged at 1,000 × *g* for 5 min. The cell pellet containing horse leukocyte fraction was overlaid on a monolayer of P388D₁ (ATCC) murine macrophage cells in tissue culture flasks (25 cm²; Corning Glass Works, Corning,

* Corresponding author. Mailing address: Department of Veterinary Pathobiology, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Rd., Columbus, OH 43210-1093. Phone: (614) 292-9677.

† Present address: Department of Immunology, Azabu University, Sagami, Japan.

TABLE 1. Clinical record of horses from which *E. risticii* was isolated

Horse	Clinical signs ^a	Location ^b	Date of vaccination ^c	IFA titer (date) ^d	Date of <i>E. risticii</i> isolation
022	A, F, Di, Deh, L	Troy, Ohio	ND	1:640 (7/2/91)	7/10/91
067	A, De, Di, Deh	Alexandria, Ohio	ND	1:640 (7/5/91)	7/25/91
081	A, F, De, Di	Findley, Ohio	ND	1:160 (7/8/91)	7/29/91
				1:640 (7/15/91)	
606	A, F, Deh, L	Dover, Ohio	2/91	1:2,560 (8/8/91)	8/30/91
380	A, F, Di	Hudson, Ohio	4/93	1:1,280 (8/17/93)	8/30/93
679	A, F, Di, L	Athens, Ohio	3/93	1:5,120 (9/15/93)	9/20/93
Co	A, F, De, Deh, Di, L	Versailles, Ky.	ND	1:320 (9/21/93)	10/4/93
Ov	A, F, Di, De, Deh	Versailles, Ky.	ND	1:640 (9/21/93)	10/4/93
As	A, F	Versailles, Ky.	ND	1:320 (9/22/93)	9/30/93

^a Clinical signs at the time of blood collection. A, anorexia; F, fever; De, depression; Deh, dehydration; Di, diarrhea; L, laminitis.

^b Location of the horse during the month preceding isolation of the strain.

^c ND, not vaccinated with PHF-VAX (Schering Animal Health). Dates are given as month/year.

^d Date of blood collection (month/day/year).

N.Y.) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 2 mM L-glutamine (GIBCO) without antibiotics at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. After 1 or 2 days, floating lymphocytes were discarded. Samples of cultured cells were taken from each flask and examined daily after centrifugation (Cytospin 2; Shandon Inc., Pittsburgh, Pa.) and Diff-Quik staining (Baxter Scientific Products, Obetz, Ohio) until cultures became positive for *E. risticii* growth. Infection and absence of contamination were confirmed by transmission electron microscopy and IFA labeling. *E. risticii*-infected cultures were continuously passed in P388D₁ cells or frozen at -80°C. All isolates used for this study were cultured for fewer than 10 passages. The passage numbers for the indicated strains were 4 to 6 for As; 5 to 7 for Illinois, Virginia, 022, 067, 606, Co, and Ov; 6 to 8 for 380 and 679; and 8 to 10 for 081.

Western immunoblot analysis. Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis of nine *E. risticii* isolates purified by Sephacryl S1000 chromatography was performed as described previously (11). *E. risticii* was dissolved in 0.125 M Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.05% pyronine Y at a concentration of 2 mg of protein per ml by being heated at 100°C for 3 min. Solubilized *E. risticii* isolates (2 mg/ml) were applied at 20 μl per lane to a 12% polyacrylamide gel. A mixture of proteins of known molecular weights (Bio-Rad Laboratories, Richmond, Calif.) was electrophoresed on one lane of each gel. Electrophoresis was performed at constant amperage (30 mA per gel) for 4 h in 0.025 M Tris-HCl (pH 8.3) containing 0.192 M glycine and 0.1% SDS. After electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane filter (Schleicher & Schuell, Keene, N.H.) by electrophoresis in a semidry electroblotter (Integrated Separation Systems, Hyde Park, Mass.) at constant amperage (130 mA) for 45 min. After being immersed in 5% (wt/vol) nonfat dried milk in phosphate-buffered saline (PBS) at 37°C for 30 min, the membrane filters were incubated with pony 19 anti-*E. risticii* (Virginia isolate) serum, horse 067 antiserum collected when isolate 067 was isolated, horse 081 antiserum collected when isolate 081 was isolated, horse 606 antiserum collected when isolate 606 was isolated, and rabbit antiserum to *E. risticii* (Maryland isolate) at a 1:20 or 1:50 dilution in PBS-nonfat dried milk at 37°C for 2 h. The rabbit anti-*E. risticii* serum was raised by intradermal inoculation of 500 μg of β-propiolactone-inactivated protein of chromatographically purified *E. risticii* with Saponin adjuvant for a total of four times with

2-week intervals between injections into two rabbits as previously described (13). The rabbit serum was absorbed with a lysate of 10⁶ uninfected P388D₁ cells per 10 ml of serum at room temperature for 1 h before use. After three 10-min washes in PBS-0.002% Tween 20 and a 20-min wash in PBS, the membrane filters were incubated in alkaline phosphatase-conjugated affinity-purified goat anti-horse or goat anti-rabbit immunoglobulin G (heavy and light chains) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) at a 1:1,000 dilution in PBS-nonfat dried milk at 37°C for 2 h. The membrane filters were washed as before and immersed in substrate solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Bio-Rad) for 30 min. The enzymatic reaction was terminated by rinsing of the membranes in distilled water.

Monoclonal antibody production. The Washington State University (WSU) monoclonal antibodies were raised against *E. risticii* (Illinois isolate). BALB/c mice were inoculated with 10⁴ *E. risticii*-infected viable P388D₁ cells and monitored daily for illness. All mice became sick, exhibiting ruffled fur, squinty eye, inactivity, weight loss, and/or diarrhea. Seven days following complete recovery from clinical signs of illness, all five mice were challenged with 10⁶ infected viable P388D₁ cells. All mice were protected from this challenge and did not show any clinical signs. Sera from the mice were titrated, and a mouse with a high titer (>1:2,500) was selected for fusion with SP/2 cells (ATCC). This mouse given an intravenous booster inoculation with 10³ infected P388D₁ cells, and 3 days later the spleen was collected for fusion.

The Ohio State University (OSU) monoclonal antibodies were raised against *E. risticii* (Maryland isolate). Four- to five-week-old BALB/c mice (Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) were injected intraperitoneally with 10⁶ *E. risticii*-infected P388D₁ cells resuspended in 0.1 ml of RPMI 1640 medium per mouse, and given booster injections four times at 2-week intervals. After confirmation of the development of antibody of high titer (1:5,120) by IFA in the blood collected from the retro-orbital plexus every 2 weeks, mice were sacrificed and spleens were collected. Spleen cells from inoculated mice were fused with myeloma cells (P3X63-Ag8.653). Initial screening was performed by enzyme-linked immunosorbent assay on partially purified *E. risticii* organisms to select for monoclonal antibodies against surface antigens. Subsequent screening was done by IFA.

Determination of molecular sizes of antigens of *E. risticii* to which monoclonal antibodies were directed. *E. risticii* (Illinois

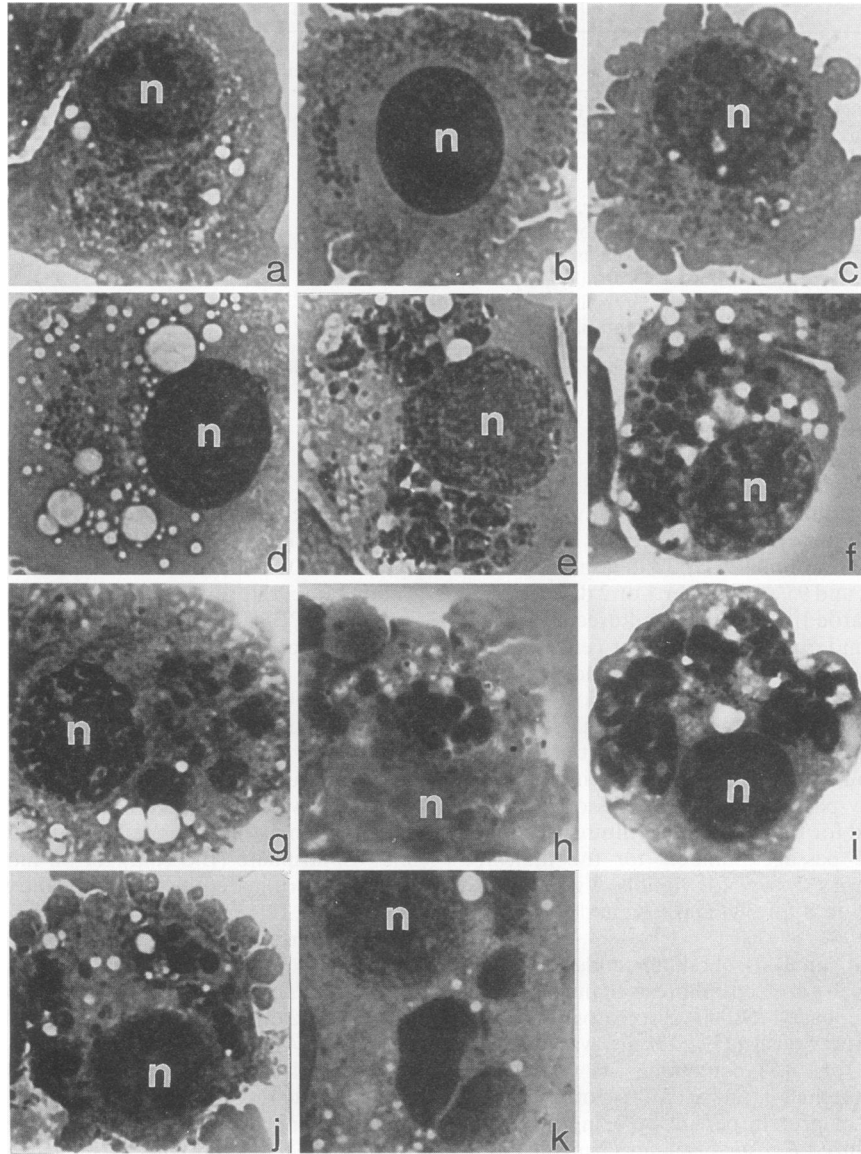


FIG. 1. Light micrographs of Diff-Quik-stained *E. risticii* isolates in the cytoplasm of P388D₁ (a and c to k) and U937 (b) cells in culture. Isolates: Illinois (a), Virginia (b), 022 (c), 067 (d), 081 (e), 606 (f), 380 (g), 679 (h), As (i), Co (j), and Ov (k). n, nucleus. Magnification, $\times 1,700$.

isolate) was labeled with [³⁵S]methionine by addition of [³⁵S]methionine at 60 μ Ci/ml to *E. risticii*-infected P388D₁ cells and incubation in the presence of 10 μ g of cycloheximide per ml at 37°C for 48 h as previously described (5). [³⁵S]methionine-labeled cells were disrupted by suspension in 50 mM Tris (pH 8.0) buffer containing 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*- α -p-tosyl-L-lysine chloromethyl ketone, 10 mM dithiothreitol, and 1% Nonidet P-40 (lysis buffer). The antigen was sonicated, incubated at 37°C for 2 h, sonicated again, and centrifuged at 130,000 $\times g$ for 1 h. Samples with 250,000 cpm of trichloroacetic acid-precipitable ³⁵S-radiolabeled organisms were added to 10 μ l of 500- μ g/ml monoclonal antibodies and immunoprecipitated as described previously (5). Antigens eluted from the protein G-Sepharose were boiled in SDS-polyacrylamide gel electrophoresis sample buffer and applied to polyacrylamide gels. Gels were fixed, vacuum dried, and

exposed to Kodak XAR-2 X-ray film with an intensifying screen at -80°C .

IFA labeling with murine polyclonal and monoclonal antibodies. P388D₁ cells infected with each *E. risticii* isolate at 10³ cells per well were placed on 12-well teflon-coated glass slides (Cel-Line Associates Inc., Newfield, N.J.) and fixed in acetone. Cells were incubated with murine polyclonal anti-*E. risticii* (Maryland isolate) (1:5,120 titer) and monoclonal antibodies to *E. risticii* (Illinois or Maryland isolate) (100 to 460 μ g of protein per ml at a 1:20 dilution). The cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Organon Teknika Corporation, West Chester, Pa.) at a 1:100 dilution, and slides were read under an epifluorescence microscope (Nikon Inc., Garden City, N.Y.).

***E. risticii* DNA purification.** The DNA extraction was performed by lysis of approximately 10⁵ *E. risticii*-infected P388D₁ cells resuspended in 1 ml of TE buffer (40 mM Tris-1 mM

TABLE 2. Approximate distribution of *E. risticii* organisms as individuals and as small and large morulae in P388D₁ cells

Isolate	% of <i>E. risticii</i> organisms in indicated distribution pattern ^a		
	Individual	Small morula ^b	Large morula ^c
Illinois	100	0	0
Virginia	89 ± 17	8 ± 11	3 ± 6
022	54 ± 27	46 ± 27	0
067	51 ± 18	49 ± 18	0
081	17 ± 5	48 ± 4	36 ± 5
606	39 ± 21	57 ± 19	5 ± 4
380	14 ± 1	49 ± 9	36 ± 10
679	36 ± 12	35 ± 8	29 ± 10
As	6 ± 4	41 ± 21	53 ± 19
Co	7 ± 6	52 ± 6	41 ± 11
Ov	4 ± 2	51 ± 7	45 ± 9

^a Values are means ± standard deviations (n = 3). Fifty cells were counted on each of three slides derived from three cultures at different passage numbers.
^b Percentage of *E. risticii* in small morulae (diameter < 2 µm), with an average number of 30 organisms per morula.
^c Percentage of *E. risticii* in large morulae (diameter, 3 to 10 µm), with an average number of 150 organisms per morula.

EDTA, pH 8.0) in the presence of 1% SDS–20 µg of proteinase K (Sigma) for 2 h at 50°C. The lysed suspension was extracted twice with an equal volume of phenol-chloroform (phenol-chloroform-isoamyl alcohol, 25:24:1) and once with an equal volume of chloroform (chloroform-isoamyl alcohol, 24:1). The top layer, the aqueous phase, was transferred to a fresh tube each time. DNA was subsequently precipitated from the resulting aqueous phase by the addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold 95% ethanol and washed with ice-cold 70% ethanol after a brief centrifugation at 10,000 × g. Following the centrifugation, the pellet was air dried for 2 min and resuspended in 50 µl of distilled water. The DNA concentration was determined on the basis of readings of A₂₆₀ and A₂₈₀ with a DU-70 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Amplification of the 16S rRNA gene by PCR. Oligonucleotides ER 5-3 (5' ATTTGAGAGTTTGATCCTGG 3', specific for *E. risticii*) and ER 3-2 (5' GTTTTAAATGCAGTTCCTGG 3', specific for all *Ehrlichia* species), produced on the basis of the 16S rRNA gene sequence of *E. risticii* (Illinois isolate) (22), were used at 50 pmol per reaction mixture to prime amplification of 597 bp of the 5' one-third of the 16S rRNA gene in a thermal cycler (Minicycler; MJ Research, Inc., Watertown, Mass.). Template DNAs at 0.1 µg per reaction mixture were amplified by hot-start PCR including 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. A volume of 10 µl from the resulting PCR products was electrophoresed through an agarose gel (40 mM Tris-acetate, 1 mM EDTA buffer, pH 8.0, containing 3% agarose [Nusieve; FMC Bioproducts, Rockland, Main] and 0.5 µg of ethidium bromide per ml) in a gel electrophoresis unit (International Biotechnologies, Inc., New Haven, Conn.) at a constant amperage (20 mA) for at least 30 min. The ethidium bromide-stained DNA was visualized with UV illumination and photographed (Foto/Phoresis I; Fotodyne Inc., New Berlin, Wis.).

Restriction enzyme digestion pattern of the amplified 16S rRNA gene. PCR-amplified products (597 bp in length) of the Illinois and Virginia isolates and of nine new isolates were purified by using Wizard PCR Prep (Promega, Madison, Wis.) and subjected to digestion with three endonucleases (*Hae*III, *Sau*3AI, and *Ava*II; GIBCO BRL, Gaithersburg, Md.) at 37°C overnight according to the manufacturer's instructions. The

TABLE 3. Molecular sizes of reacting antigens of *E. risticii* isolates as determined by Western immunoblotting^a

<i>E. risticii</i> isolate	Molecular mass(es) (kDa) of antigen(s)
Illinois.....	103, 80, 55, 46, 37
Virginia.....	103, 80, 55, 46, 37
022.....	103, 80, 55, 46, 37
067.....	103, 80, 55, 46, 37
081.....	55
606.....	80, 55, 46
380.....	103, 80, 55, 46, 37
679.....	103, 80, 55, 46, 37
As.....	55
Co.....	55, 44, 37
Ov.....	55, 44, 37

^a The primary antibody used was pony 19 serum (titer, 1:1,280) at a 1:50 dilution.

restriction enzyme digestion pattern of the 5' third of the 16S rRNA gene was observed after agarose gel electrophoresis and ethidium bromide staining with UV illumination.

RESULTS

Clinical signs, IFA titers, and vaccination status of horses examined. All nine isolates were obtained during acute stages of the disease. Six horses had been located in different areas of Ohio, and three Kentucky horses had been located in the same farm and became sick around the same time. All nine horses showed the clinical sign(s), anorexia, fever, dehydration, depression, diarrhea, and/or laminitis. All nine horses were seropositive by IFA with *E. risticii* (Maryland isolate) as the antigen. The titer varied from 1:160 to 1:5,120 at the time of blood collection. Three horses in Ohio had been vaccinated and had higher antibody titers than nonvaccinated horses. Antibody titers of these horses after vaccination but prior to infection were unknown. Approximately 5 to 22 days were required to cultivate *E. risticii* from the blood specimens (Table 1).

Light microscopy of *E. risticii* isolates. All isolates were small cocci, stained bluish purple in the cytoplasm of P388D₁ macrophages by Diff-Quik stain. The Illinois and Virginia isolates appeared primarily as individual organisms, as previously described (4, 14). New isolates made morulae (inclusions densely packed with microorganisms) of variable sizes. Four Ohio isolates (081, 606, 380, 679) and all three Kentucky isolates (As, Co, Ov) made relatively large (3 to 10 µm in diameter) morulae (Fig. 1; Table 2). By light microscopy, individual organisms could not be distinguished in morulae, since they were so tightly packed. The remaining isolates (022 and 067) were present individually or as small morulae but did not make large densely-packed morulae (Fig. 1).

Western immunoblot profiles of *E. risticii* isolates. Isolates 022 and 067, as well as isolates 380 and 679, had almost identical strong Western immunoblot profiles regardless of the antiserum (four equine and one rabbit antisera) used to probe the antigenic proteins (Fig. 2). Isolate 081 lacked most of the antigenic peptides found in other isolates with the four horse and one rabbit antisera, including the homologous anti-081 isolate serum, although the reactivity of isolate 081 antigen to the homologous antiserum was stronger than that to other equine sera. All Kentucky isolates were also similar among themselves but distinct from remaining isolates in patterns, reacting with all antisera tested, except for isolate As, which showed weaker reaction with pony 19 antiserum and horse 067

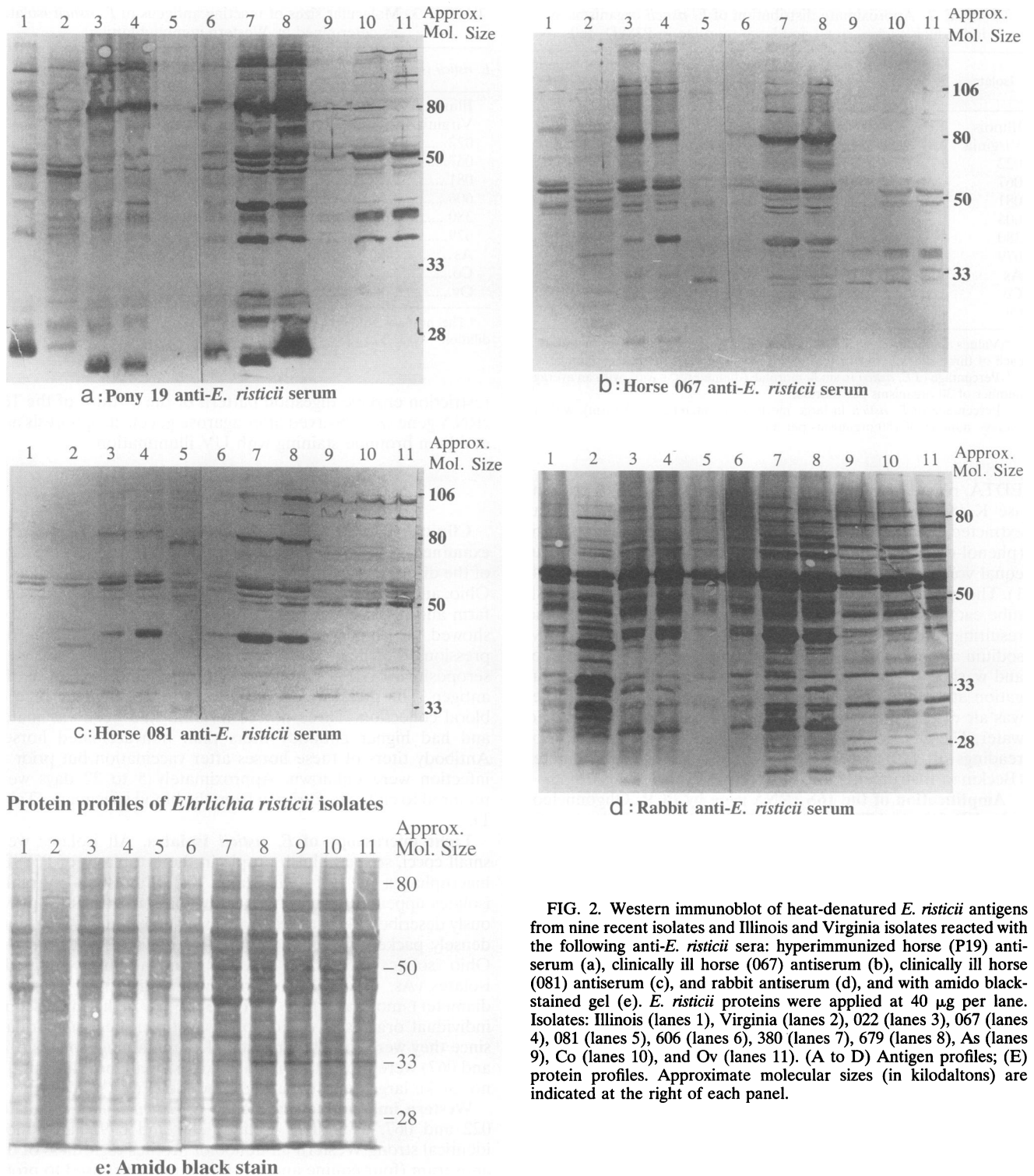


FIG. 2. Western immunoblot of heat-denatured *E. risticii* antigens from nine recent isolates and Illinois and Virginia isolates reacted with the following anti-*E. risticii* sera: hyperimmunized horse (P19) antiserum (a), clinically ill horse (067) antiserum (b), clinically ill horse (081) antiserum (c), and rabbit antiserum (d), and with amido black-stained gel (e). *E. risticii* proteins were applied at 40 μ g per lane. Isolates: Illinois (lanes 1), Virginia (lanes 2), 022 (lanes 3), 067 (lanes 4), 081 (lanes 5), 606 (lanes 6), 380 (lanes 7), 679 (lanes 8), As (lanes 9), Co (lanes 10), and Ov (lanes 11). (A to D) Antigen profiles; (E) protein profiles. Approximate molecular sizes (in kilodaltons) are indicated at the right of each panel.

antiserum than did isolates Co and Ov. Isolate 606 also showed poor reaction with four equine antisera; however, its general reaction pattern was similar to those of isolates 022, 067, 380, and 679.

With pony 19 serum, common antigenic proteins of 103, 80,

55, 46, and 37 kDa were detected in all Ohio isolates except isolate 081 (Table 3; Fig. 2). Kentucky isolates share only the 55-kDa antigenic protein with Ohio isolates and lacked the 103-, 80-, and 46-kDa antigenic proteins. With horse 067 serum, reactions similar to that of pony 19 serum were seen

TABLE 4. Reactivity of nine recent *E. risticii* isolates and Illinois and Virginia isolates with anti-*E. risticii* murine monoclonal antibodies (WSU)

Monoclonal antibody	Size of recognizing antigen (kDa)	Isotype ^a	Isolate ^b										
			Ill	Va	022	067	081	606	380	679	As	Co	Ov
49/3.6.1	14	IgG2b	+	+	+	+	-	+	+	+	-	+	+
49/35.8.2	14	IgG3	+	+	-	-	-	-	-	-	-	-	-
49/67.14.2	27	IgG2a	+	+	-	-	-	-	+	-	-	-	+
49/63.5.1	53	IgG2a	+	+	-	-	-	-	+	+	-	-	-
49/66.22.4	53	IgG2a	+	+	+	+	-	+	+	+	-	+	-
49/68.10.4	53	IgG2a	+	+	-	-	-	+	+	+	+	+	+
49/70.12.2	53	IgG2a	+	+	+	+	-	+	+	+	+	+	+
49/71.1.2	53	IgG2a	+	+	-	-	-	+	+	+	+	+	+
49/4.3.2	62	IgG2a	+	+	-	-	-	-	-	-	-	-	-
49/25.1.10	62	IgG2a	+	+	+	+	-	+	+	+	-	+	+
49/40.15.2	62	IgG2a	+	+	-	-	-	-	+	-	-	-	-
49/16.7.2	70	IgG2a	+	+	+	+	-	+	+	+	+	+	+
Mouse anti- <i>E. risticii</i> (Maryland isolate) serum (1:100)		Polyclonal	+	+	+	+	+	+	+	+	+	+	+

^a IgG, immunoglobulin G.

^b Ill, Illinois; Va, Virginia. Reactivity was determined by IFA test; +, positive staining; -, negative staining.

(Fig. 2). An antigenic polypeptide of 55 kDa was shared among all isolates except isolate 081. The antigens of 80, 55, and 46 kDa were dominant especially in isolates 022, 067, 380, and 679, indicating that these four isolates were closely related (Fig. 2). With horse 606 serum, results essentially similar to those with horse sera 067 and 19 were observed. Isolate 606 showed the weakest reaction, even to homologous serum, indicating that isolate 606 had the ability to induce good humoral immune responses to each major polypeptide in the horse but failed to react with the antiserum after the Western blot procedure.

With horse 081 serum, isolates 022, 067, 380, and 679 demonstrated the same dominant common antigens of 80, 55, and 46 kDa. Isolate 081 reacted with this antiserum more strongly than with other antisera tested but lacked these major common antigens of the remaining Ohio isolate. Rabbit antiserum reacted more strongly and with more numbers of proteins with all isolates than did the four horse sera. Polypeptides of 55, 46, and 33 kDa were found with isolates 022, 067, 380, and 679, but antigenic polypeptides smaller than 50 kDa were mostly missing or weak in isolate 081. Three Kentucky isolates were identical to each other but distinct from Ohio isolates on the basis of reactivity with rabbit antiserum (Fig. 2). A major protein of approximately 60 kDa was common among all 11 isolates. As three Kentucky isolates had identical protein profiles, so also did isolates 380 and 679, isolates 022 and 067, and isolates 081, 606, and Illinois have identical protein profiles. But these four groups were different from each other in their protein profiles (Fig. 2).

Reactivity of two panels of monoclonal antibodies to *E. risticii* isolates. Molecular sizes of *Ehrlichia* proteins recognized with monoclonal antibodies as determined by [³⁵S]methionine metabolic labeling and immunoprecipitation are shown in Fig. 3. The Illinois isolate against which 12 WSU monoclonal antibodies were made reacted with all monoclonal antibodies tested. Monoclonal antibodies 49/35.8.2 and 49/4.3.2 did not react with any new *E. risticii* isolates. Although isolate 081 reacted with polyclonal mouse anti-*E. risticii* serum, this isolate did not react with any of the tested monoclonal antibodies. The remaining isolates did not react with various monoclonal antibodies (Table 4). Isolates 022 and 067 reacted with the same sets of monoclonal antibodies. Isolates 380 and 679 reacted with the same sets of monoclonal antibodies, except for two of them (49/67.14.2 and 49.15.2).

The Illinois and Virginia isolates and all the Ohio isolates except for isolate 081 reacted with all 10 OSU monoclonal anti-*E. risticii* (Maryland isolate) antibodies tested. Isolate 081 did not react with any OSU monoclonal antibodies tested. Kentucky isolates reacted with only one to three of the OSU monoclonal antibodies (data not shown).

PCR amplification and restriction enzyme digestion pattern of the 16S rRNA gene. According to PCR protocol, 597 bp of the 16S rRNA gene predicted from the *E. risticii* (Illinois isolate) sequence was amplified with the ER 5-3 and ER 3-2 primers and 0.1 µg of DNA was extracted from all *E. risticii* isolates as templates (Fig. 4). On the basis of the published sequence, *Hae*III and *Sau*3AI digestions of the partial 16S rRNA gene of *E. risticii* (Illinois isolate) produce fragments of 34, 151, 199, and 213 bp and of 16, 24, 83, 227, and 247 bp, respectively (22). *Hae*III digestion of the PCR-amplified 597 bp of the 16S rRNA gene of all 11 isolates, including the Illinois isolate, yielded two identical bands corresponding approximately to 151 and to 199 and 213 bp. The latter two fragments could not be distinguished, and the 34-bp fragment

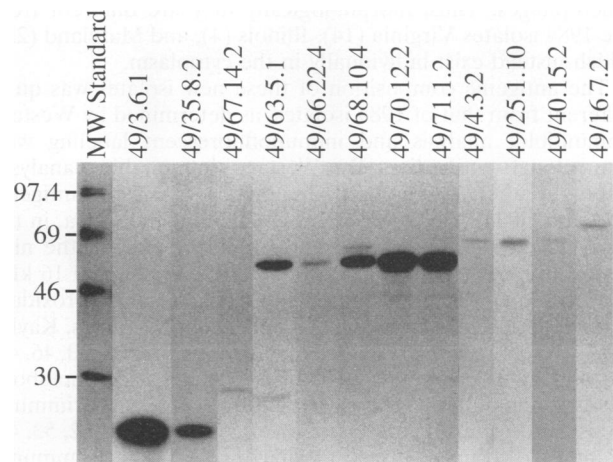


FIG. 3. *E. risticii* antigens recognized by WSU murine monoclonal antibodies. *E. risticii* was metabolically radiolabeled with [³⁵S]methionine and immunoprecipitated with each monoclonal antibody. Molecular weights (in thousands) of standards are indicated at the left.

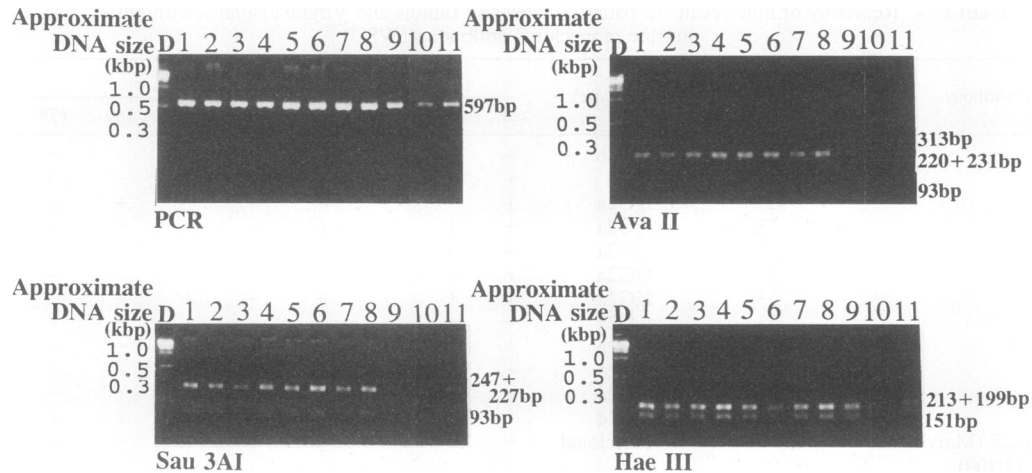


FIG. 4. PCR-amplified products of the *E. risticii* 16S rRNA gene and their endonuclease digestion patterns from nine recent isolates and the Illinois and Virginia isolates, as follows: Illinois (lanes 1), Virginia (lanes 2), 022 (lanes 3), 067 (lanes 4), 081 (lanes 5), 606 (lanes 6), 380 (lanes 7), 679 (lanes 8), As (lanes 9), Co (lanes 10), and Ov (lanes 11).

could not be detected by the agarose gel electrophoresis. *Sau3AI* digestion yielded two bands corresponding to 83 and to 227 and 247 bp (Fig. 4). The latter two fragments could not be distinguished, and 16- and 24-bp fragments could not be detected. Digestion with *AvaII* produces fragments of 53, 93, 220, and 231 bp of 16S rRNA DNA of *E. risticii* (Illinois isolate) (22). *AvaII* digestion yielded two bands corresponding to fragments of 93 and 220+231 bp in the Illinois and Virginia isolates and all six Ohio isolates, whereas all three Kentucky isolates yielded two bands corresponding to fragments of 231 and 313 bp, indicating that they lack the site to yield a 93-bp band (Fig. 4). A band of 53 bp could not be detected.

DISCUSSION

Among various *Ehrlichia* species, *Ehrlichia canis* is known to make the largest morulae, *Ehrlichia chaffeensis* makes smaller morulae, and *Neorickettsia helminthoeca* makes a mixture of individually localized organisms and morulae (12). The present study revealed that of nine new Ohio and Kentucky isolates of *E. risticii*, seven made densely packed morulae in P388D₁ macrophages. Thus, morphologically they are different from the 1984 isolates Virginia (14), Illinois (4), and Maryland (23), which instead exist individually in the cytoplasm.

The antigenic composition of these new isolates was quite different from that of 1984 isolates, as determined by Western immunoblot analysis and immunofluorescent labeling with monoclonal antibodies. By Western immunoblot analysis, Dutta et al. (3) identified nine major *E. risticii* antigenic proteins, of 110, 86, 70, 55, 51, 49, 44, 33, and 28 kDa, in the Maryland isolate with horse anti-*E. risticii* sera. Of the nine major antigens those of 110, 55 or 51, 44, 33, 28, and 16 kDa are located on the surface, on the basis of ¹²⁵I-lactoperoxidase surface preferential labeling. With the Illinois isolates, Kaylor et al. (5) reported the presence of antigens of 87, 62, 50, 46, 44, 33, and 27 kDa on the basis of [³⁵S]methionine metabolic labeling and radioimmunoprecipitation with a hyperimmune mouse antiserum. Of these seven antigens, those of 62, 53, 40, 33, and 27 kDa are surface localized. Our Western immunoblot analysis revealed that major antigenic polypeptides of similar molecular sizes (103, 80, 55, 46, and 37 kDa) were identified in four Ohio isolates by use of any equine antiserum. Isolate 081 lacked most of these antigenic polypeptides. In

addition, isolates 606 and As showed weaker reactions with equine sera than did other isolates. In comparison between rabbit and equine antisera, the rabbit antiserum apparently recognized more common epitopes of *E. risticii* proteins than did equine antisera. By comparison of overall reacting polypeptide patterns with the four equine serum specimens and the one rabbit serum specimen, nine *E. risticii* isolates can be divided into three serogroups: (i) 081; (ii) 022, 067, 380, 649, and 606; and (iii) the Kentucky isolates. Dutta et al. also reported one isolate which differs from the Maryland isolate on the basis of Western immunoblotting with a polyclonal equine serum (2).

Results of IFA labeling with monoclonal antibody correlate with Western immunoblot results with polyclonal anti-*E. risticii* sera. Especially, isolate 081 was unique and did not react with any of 22 monoclonal antibodies tested. Two monoclonal antibodies, 49/35.8.2 and 49/4.3.2, were 1984 isolate-specific and did not react with any new isolates tested. OSU monoclonal antibodies preferentially bind to Ohio isolates over Kentucky isolates, except for isolate 081. Thus, monoclonal antibodies are very useful in differentiating various *E. risticii* isolates. Monoclonal antibody reactivity results support the notion of three serogroups identified by Western immunoblot analysis. We intend to study more isolates from various parts of the United States to further learn the extent and nature of antigenic diversity among *E. risticii* isolates.

Palmer reviewed the efficacy of one PHF vaccine. The efficacy of the vaccine is perceived as marginal in both experimental and field trials (8). The humoral immune response appears to have an important role in host defense in PHF. Development of neutralizing antibodies was demonstrated in vivo (5, 16) and in vitro (16), and the presence of antibody-dependent cell-mediated cytotoxicity has been demonstrated in vitro (7). Mice (13) and horses (18) immunized with killed *E. risticii* organisms were protected from challenge with the homologous isolate. Although the reasons for the vaccine failure can be numerous (e.g., inactivation of protective antigenic epitopes by formalin fixation used to inactivate the organism, poor immune responses to the vaccine in a given horse population, and/or rapid waning of immunity), this broad diversity of *E. risticii* serotypes may partially account for the current vaccine failure in the field. In fact, three of nine isolates tested in this study were isolated from previously

vaccinated horses. It is unlikely that these horses had been infected with PHF prior to or at the time of vaccination, since the usual incubation time for PHF is 7 to 10 days (14).

The Kentucky isolates are different even at the 16S rRNA base sequence level in the hypervariable region, suggesting that this diversity did not evolve recently. The 16S rRNA sequence analysis of these unique isolates is in progress to compare the sites of base difference and percentages of homology among these isolates. Since all isolates were derived from acutely sick horses, all isolates appear to be virulent in horses. However, our other study in progress revealed that, in mice, isolate 081 and the Kentucky isolates were not virulent but the remaining Ohio isolates were virulent (4a). This study will help in selecting the most-immunogenic isolate(s) for the development of an improved vaccine and of a serodiagnosis and a vaccine coherent with the *E. risticii* strain prevalent in the area of disease outbreak in the future.

ACKNOWLEDGMENTS

The work at OSU was supported in part by USDA Section 1433 Animal Health and Disease Formula funds. The work done at WSU was supported by USDA CSRS grant WNP 00839 and the AVMA Foundation.

The isolation of the four Ohio and one Kentucky isolates was facilitated by Jim Laird and Holly Ferrell and by Zafar Iqbal, respectively. Blood samples from horses in Kentucky were provided by William Bernard, Lexington, Ky. We also express our appreciation to Yang Yu for initial instruction in PCR procedure and for synthesizing primers and to Kathy Lester and Takeo Sugiura for technical assistance in monoclonal antibody production and screening.

REFERENCES

- Dutta, S. K., A. C. Myrup, R. M. Rice, M. G. Robl, 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.
- Dutta, S. K., B. Shankarappa, and B. 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 diseases. VI. Proceedings of the Sixth International Conference 7-11 July 1991*. R & W Publication (Newmarket) Limited, Suffolk, United Kingdom.
- 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.
- 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.
- Jason, M., and Y. Rikihisa. Unpublished data.
- Kaylor, P. S., T. B. Crawford, T. F. McElwain, and G. H. Palmer. 1991. Passive transfer of antibody to *Ehrlichia risticii* protects mice from ehrlichiosis. *Infect. Immun.* **59**:2058-2062.
- Knowles, R. C., D. W. Anderson, W. D. Shipley, R. H. Whitlock, B. D. Perry, and J. P. Davidson. 1983. Acute equine diarrhea syndrome (AEDS): a preliminary report. *Proc. Am. Assoc. Equine Pract.* **29**:353-357.
- Messick, J. B., and Y. Rikihisa. 1993. Characterization of *Ehrlichia risticii* binding, internalization, and proliferation in host cells by flow cytometry. *Infect. Immun.* **61**:3803-3810.
- Palmer, J. E. 1989. Prevention of Potomac horse fever. *Cornell Vet.* **79**:201-205. (Editorial.)
- Palmer, J. E., R. H. Whitlock, and C. E. Benson. 1986. Equine ehrlichial colitis (Potomac horse fever): recognition of the disease in Pennsylvania, New Jersey, New York, Ohio, Idaho, and Connecticut. *J. Am. Vet. Med. Assoc.* **189**:197-199.
- Perry, B. D., J. E. Palmer, H. F. Troutt, J. B. Birch, D. Morris, M. Ehrlich, and Y. Rikihisa. 1986. A case-control study of Potomac horse fever. *Prev. Vet. Med.* **4**:69-82.
- Rikihisa, Y. 1991. Cross-reacting antigens between *Neorickettsia helminthoeca* and *Ehrlichia* species, shown by immunofluorescence and Western immunoblotting. *J. Clin. Microbiol.* **29**:2024-2029.
- Rikihisa, Y. 1991. The tribe *Ehrlichieae* and ehrlichial diseases. *Clin. Microbiol. Rev.* **4**:286-308.
- Rikihisa, Y. 1991. Protection against murine Potomac horse fever by an inactivated *Ehrlichia risticii* vaccine. *Vet. Microbiol.* **28**:339-350.
- Rikihisa, Y., and B. D. Perry. 1985. Causative ehrlichial organisms in Potomac horse fever. *Infect. Immun.* **49**:513-517.
- Rikihisa, Y., S. M. Reed, R. A. Sams, J. C. Gordon, and C. I. Pretzman. 1990. Serosurvey of horses with evidence of equine monocytic ehrlichiosis (Potomac horse fever). *J. Am. Vet. Med. Assoc.* **197**:1327-1332.
- 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.
- Ristic, M. 1990. Current strategies in research on ehrlichiosis, p. 136-153. *In* J. C. Williams and I. Kakoma (ed.), *Ehrlichiosis: a vector-borne disease of animals and humans*. Kluwer Academic Publishers, Boston.
- Ristic, M., C. J. Holland, and T. E. Goetz. 1988. Evaluation of a vaccine for equine monocytic ehrlichiosis, p. 89-100. *In* Proceedings of a symposium on potomac horse fever. Veterinary Learning Systems, Lawrenceville, N.J.
- Ristic, M., and D. L. Huxsoll. 1984. Tribe II. *Ehrlichieae* Philip 1957, 948^{AL}, p. 704-711. *In* N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore.
- Schmidtmann, E. T., R. M. Rice, M. G. Roble, and A. F. Azad. 1988. Search for an arthropod vector for *Ehrlichia risticii*, p. 9-16. *In* Proceedings of a symposium on potomac horse fever. Veterinary Learning Systems, Lawrenceville, N.J.
- Vidor, E., G. Bissuel, Y. Moreau, J. L. Madec, and J. L. Cadore. 1988. Serologie positive a *Ehrlichia risticii* chez une jument presentant un tableau d'ehrlichiose equine. *Pratique Veterinaire Equine* **20**:5-10.
- 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.
- Weiss, E., G. A. Dasch, Y.-H. Kang, and H. N. Westfall. 1988. Substrate utilization by *Ehrlichia sennetsu* and *Ehrlichia risticii* separated from host constituents by renografin gradient centrifugation. *J. Bacteriol.* **170**:5012-5017.