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Cross-Reacting Antigens between *Neorickettsia helminthoeca* and *Ehrlichia* Species, Shown by Immunofluorescence and Western Immunoblotting

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Dogs orally infected with *Neorickettsia helminthoeca* developed immunoglobulin G titers against *Erlichia risticii*, *Erlichia sennetsu*, and *Erlichia canis* similar to those against *N. helminthoeca* antigen, as determined by immunofluorescence. Western immunoblotting showed that the major common antigens shared among the microorganisms were 80- or 78-kDa and 64-kDa polypeptides. In contrast, horse anti-*E. risticii* and anti-*E. sennetsu* and dog anti-*E. canis* sera reacted more weakly to *N. helminthoeca* antigen than to homologous antigens in both immunofluorescence and Western immunoblotting. Antisera raised in other species of animals, i.e., mouse anti-*E. canis* and rabbit anti-*E. risticii* and anti-*E. sennetsu* sera, however, all reacted with the 64-kDa antigen of *N. helminthoeca*. This strong antigenic cross-reactivity and similarity in Western immunoblotting reaction profiles indicate that *N. helminthoeca* is antigenically closely related to *E. risticii* and *E. sennetsu* and less so to *E. canis*. In both immunofluorescence and Western immunoblotting, *E. canis* shared fewer common antigens with *E. risticii* and *E. sennetsu* than *N. helminthoeca* did. It is reasonable to conclude that these results may have both diagnostic and taxonomic significance.

Neorickettsia helminthoeca is an obligate intracellular bacterium parasitic for a fluke. When a dog eats the salmonid fish encysted with the fluke harboring *N. helminthoeca*, *N. helminthoeca* is transmitted to the dog from the fluke and causes salmon poisoning disease (4). *N. helminthoeca* is classified in the tribe *Ehrlichieae* (12).

N. helminthoeca infects macrophages of dogs and ultrastructurally resembles members of the genus *Ehrlichia* (11). The antigenic relationship of *N. helminthoeca* to members of the genus *Ehrlichia* is unknown, except for two studies which reported that there is no cross-reactivity between *N. helminthoeca* and *Ehrlichia sennetsu*, a human pathogen (6), and between *N. helminthoeca* and *E. canis*, another canine pathogen (1a, 14), in the fluorescent-antibody test.

E. canis is the etiologic agent of canine ehrlichiosis, or tropical canine pancytopenia (12). An *E. canis*-like agent was seen by Maeda et al. (7) and isolated (2) from a human patient exhibiting the clinical signs of human ehrlichiosis in the United States. Laboratory diagnosis of both diseases has been made by indirect immunofluorescence with *E. canis* as the antigen. Immunologic cross-reactivity was reported in immunofluorescence between *E. canis* and *E. sennetsu* (13); between *E. risticii*, an equine pathogen, and *E. sennetsu* (5); and between *E. canis* and *E. risticii* (5). In fact, *E. sennetsu* and *E. risticii* were classified in the genus *Ehrlichia* chiefly on the basis of this immunofluorescence cross-reactivity in addition to morphologic and some biologic characteristics.

Among the members of the tribe *Ehrlichieae*, so far antigenic comparison by Western immunoblotting has been made only between *E. risticii* and *E. sennetsu* (10). Since it has been difficult to propagate both *N. helminthoeca* and *E. canis* in sufficient quantities, antigenic polypeptides recognized by the natural host (dogs) or experimentally immunized animals have not been analyzed for these two microorganisms. Furthermore, as far as we know, the cross-reacting antigenic polypeptides of *N. helminthoeca* and *E. canis* have never been compared with those of other members of the tribe *Ehrlichieae*. My colleagues and I have recently succeeded in culturing *N. helminthoeca* (11) and *E. canis* (3) in a continuous canine monocyte cell line, DH82.

In this work an antigenic comparison among *N. helminthoeca*, *E. canis*, *E. risticii*, and *E. sennetsu* was made by immunofluorescence and Western immunoblotting. Results obtained with anti-*E. equi* serum are also included.

MATERIALS AND METHODS

***N. helminthoeca* and *Ehrlichia* cultures.** *N. helminthoeca* was originally isolated from dogs which had developed salmon poisoning disease after they were fed metacercaria-infested kidneys from salmon caught in an area endemic for salmon poisoning disease and was cultured in a dog macrophage cell line, DH82 (11). *E. risticii* was cultured in a murine macrophage cell line, $P388D_1$ (10), or DH82 cells. *E. sennetsu* Miyayama was cultured in $P388D_1$ cells (10). *E. canis* Oklahoma was propagated in DH82 cells (3). Infected and uninfected DH82 cells were cultured in 150-cm^2 plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.) with Dulbecco's minimum essential medium (DMEM; GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 2 mM L-glutamine (GIBCO) at 37°C in a humidified atmosphere of 5% CO_2–95% air (3). Infected and uninfected $P388D_1$ cells were cultured in RPMI 1640 medium (GIBCO) instead of DMEM (10). The cells were harvested when they were more than 90% infected, as assessed by Diff-Quik staining as previously described (3, 10, 11).

Purification of *N. helminthoeca* and *Ehrlichia* organisms. A typical preparation consisted of 5 to 10 150-cm^2 flasks of infected DH82 or $P388D_1$ cells. Infected cells were dislodged from the growth surface by rapping the side of the flasks by hand. The suspensions were pooled in 250-ml flat-bottom polycarbonate centrifuge bottles (Fisher Scientific Co., Cincinnati, Ohio). The remaining adherent cells were harvested

with a rubber policeman and added to the pooled suspensions.

The cultured cell suspensions were centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant fluids were discarded. The cell pellets were evenly suspended at 5×10^6 cells per ml in DMEM or RPMI 1640, and the suspensions were divided into 5-ml aliquots each in 15-ml centrifuge tubes (Corning) and disrupted at power setting 2 for 5 min at 20 kHz with the microtip of ultrasonic processor W-380 (Heat Systems Ultrasonics, Farmingdale, N.Y.). Unbroken cells and nuclei were sedimented by centrifugation at 1,500 × *g* for 30 min, and the supernatants were decanted, pooled, and kept at 4°C. The pellets were evenly resuspended in 5 ml of DMEM or RPMI 1640, sonic lysis was repeated twice, and the supernatants were again kept at 4°C. The final pellets were discarded. The supernatants were pooled and centrifuged at 15,000 × *g* for 10 min at 4°C. The pellets were suspended in 1.5 ml of 2× phosphate-buffered saline (PBS) (2 mM KH_2PO_4, 6 mM Na_2HPO_4, 2 mM KCl, 136 mM NaCl [pH 7.4]). The suspension was applied to the top of a packed chromatography column (20 by 2 cm) of Sephacryl S-1000 (Pharmacia, Uppsala, Sweden) and eluted with 2× PBS at 0.7 ml/min. Flowthrough fractions collected from A_{280} peak 1 contained *Ehrlichia* organisms and were pooled and centrifuged at 15,000 × *g* for 10 min at 4°C to pellet the *Ehrlichia* organisms. The pellets were suspended in a minimal volume of distilled water and stored at −70°C. The presence and purity of *Ehrlichia* organisms were routinely evaluated after Diff-Quik staining of the fractions by light microscopy. By electron microscopy, various degrees of membrane contamination, presumably of host origin, were noted.

Western blot analysis. Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis of *N. helminthoeca*, *E. risticii*, *E. sennetsu*, and *E. canis* purified from infected cells was performed as described previously (10). Microorganisms were dissolved at a concentration of 4 mg of protein per ml in 0.125 M Tris hydrochloride (pH 6.8) containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.05% pyronine Y by being heated at 100°C for 2 min. Solubilized microorganisms were applied at 8 μl per lane to precast 4 to 20% polyacrylamide gradient gels (Integrated Separation Systems, Hyde Park, Mass.). A high-molecular-mass protein mixture (200,000, 116,250, 97,400, 66,200, and 45,000 Da) and a low-molecular-mass protein mixture (97,400, 66,200, 45,000, 31,000, 21,500, and 14,400 Da) (both from Bio-Rad, Richmond, Calif.) were electrophoresed on a portion of each gel. Uninfected DH82 cells and P388D$_1$ cells were electrophoresed as a control. Electrophoresis was performed for 5 h at a constant amperage (25 mA per gel) in 0.025 M Tris hydrochloride buffer (pH 8.3) containing 0.192 M glycine and 0.1% SDS. After electrophoresis, proteins were transferred to nitrocellulose membrane filters (Schleicher & Schuell, Inc., Keene, N.H.) by electrophoresis in a semidry electroblotter (Integrated Separation Systems) at a constant amperage (170 mA per gel) for 40 min in accordance with the manufacturer's instructions. Lanes with molecular mass standards were separated, and the proteins were stained with amido black. The remaining filters were immersed in 5% (wt/vol) nonfat dried milk (Carnation Co., Los Angeles, Calif.) in PBS (PBS-milk) at 37°C for 1 h. After being blocked, the filters were washed in three changes of PBS.

The filters were separated into lanes with a scalpel blade. The strips were immersed in a 1:50 dilution of dog, rabbit, horse, or mouse sera in PBS-milk and incubated at 37°C for 2 h. After three successive 5-min rinses in PBS–0.002% Tween 20, the strips were immersed in alkaline phosphatase-labeled affinity-purified goat anti-dog, -rabbit, -horse, or -mouse immunoglobulin G (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) diluted to 50 ng/ml in PBS-milk and incubated at 37°C for 2 h. The strips, washed as described above, were immersed in a substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium (Bio-Rad) for approximately 10 min. The enzymatic conversion of the substrate was stopped by immersion of the strips in water.

TABLE 1. Indirect-fluorescent antibody titers of dog antisera against *N. helminthoeca* and other ehrlichial antigens

Dog antisera	Titer against the following antigen:			
	N. helminthoeca	*E. risticii*	*E. sennetsu*	*E. canis*
NH1	1:640	1:160	1:320	1:160
NH2	1:1,280	1:160	1:2,560	1:640
NH3	1:160	1:80	1:320	1:40

Antisera used for Western blotting. Antisera against *N. helminthoeca* were obtained from three dogs which had developed clinically and microbiologically proven salmon poisoning disease after they were fed metacercaria-infested kidneys from salmon caught in an area endemic for salmon poisoning disease in Oregon (11). Pooled mouse sera against *N. helminthoeca* were obtained by two intraperitoneal injections of five BALB/c mice each with 1 mg of protein from an *N. helminthoeca*-infected dog lymph node homogenate, with 3 weeks between injections.

Experimental dog sera were obtained by inoculation of two German shepherd dogs each with 10^7 *E. canis*-infected DH82 cells. The dogs developed acute canine ehrlichiosis, characterized by fever, anorexia, lethargy, and thrombocytopenia (9). Pooled mouse sera against *E. canis* were obtained by three intraperitoneal inoculations of five BALB/c mice each with 10^5 *E. canis*-infected DH82 cells, with 3 to 4 weeks between injections.

Sera against *E. risticii* were produced by intravenous inoculation of 4×10^7 *E. risticii*-infected P388D$_1$ cells into ponies. The animals developed acute Potomac horse fever, characterized by fever, anorexia, depression, and diarrhea (8, 10). Mouse and rabbit anti-*E. risticii* sera were obtained similarly by three intraperitoneal inoculations of 10^5 and three subcutaneous inoculations 10^6 *E. risticii*-infected P388D$_1$ cells into 10 BALB/c mice and 3 rabbits, respectively, with 2 to 4 weeks between injections. Rabbit sera were preabsorbed three times for 3 h each time with 10^6 uninfected P388D$_1$ cells per ml of serum at room temperature before use. Rabbit anti-*E. sennetsu* sera were obtained by three subcutaneous inoculations of 10^6 to 10^7 *E. sennetsu*-infected P388D$_1$ cells into two rabbits, with 2 to 4 weeks between injections. The rabbit sera against *B. sennetsu* were kindly provided by C. I. Pretzman, Ohio Department of Health, Columbus. Equine sera against *E. sennetsu* were obtained by intravenous inoculations of *E. sennetsu* Miyayama into two ponies as previously described (10). Equine anti-*E. equi* sera were kindly provided by J. Madigan, University of California, Davis. As controls, preimmune sera from experimentally infected animals (except for anti-*E. equi* sera) were tested by Western blot analysis.

Indirect immunofluorescence titration of the sera was done as previously described (10) with cell-cultured *E. risticii*, *E. sennetsu*, *E. canis*, and *N. helminthoeca* as the antigens. The sera were screened at a 1:20 dilution, and

TABLE 2. Indirect-fluorescent antibody titers of antisera against *E. risticii*, *E. sennetsu*, and *E. canis* for the *N. helminthoeca* antigen

Antisera	Titer against the following antigen:	
	Homologous	*N. helminthoeca*
Anti-*E. risticii* (pony 58)	1:1,280	<1:20
Anti-*E. sennetsu* (pony 41)	1:640	1:40
Anti-*E. canis* (dog EC1)	1:1,280	1:80

those with a positive result were titrated by use of serial twofold endpoint dilutions. This initial low dilution was chosen to detect weak reactions and to make a comparison with the weak antigenic cross-reactivity among ehrlichial organisms reported by others (1a, 6). Within-test variability was reduced as much as possible by having all of the slides read by one person, who was not informed as to the specific sera being tested. Initially, serial twofold dilutions were made in triplicate, and there was less than a one-well variation in the results.

RESULTS

In indirect immunofluorescence, sera from three *N. helminthoeca*-infected dogs distinctly and reproducibly reacted with *N. helminthoeca*, *E. sennetsu*, *E. risticii*, and *E. canis* antigens (Table 1). The dogs were seronegative against these four ehrlichial organisms prior to being infected with *N. helminthoeca*. These dog sera did not show a nonspecific reaction with uninfected P388D$_1$ or DH82 cell controls. In contrast, the titers of antisera against *E. risticii*, *E. sennetsu*, and *E. canis* for *N. helminthoeca* were at least 16-fold lower than those for homologous antigens in immunofluorescence (Table 2). A summary of the relative antigenic cross-reactivities of the four monocytic ehrlichial organisms is shown in Table 3.

TABLE 3. Antigenic cross-reactivities among monocytic ehrlichial organism

Antisera (animal species)	Relative immunofluorescence (Western blotting) reactivity against the following antigen[a]:			
	E. risticii	*E. sennetsu*	*E. canis*	*N. helminthoeca*
E. risticii (horse)	4 (4)	3 (3)	1 (1)	1 or 0 (2)
E. sennetsu (horse)	4 (4)	4 (4)	1 (1)	1 (2)
E. canis (dog)	1 (1)	1 (1)	4 (4)	2 (1)
N. helminthoeca (dog)	3 (4)	4 (4)	3 (4)	4 (4)

[a] Relative immunofluorescence reactivity compared with the homologous antigen: 4, same or higher titer; 3, 2- to 4-fold lower titer; 2, 4- to 8-fold lower titer; 1, 8- to 16-fold titer; 0, no reaction at 1:20. Relative Western blotting reactivity was based on visual observation: 4, strong; 3 moderate; 2, weak; 1, marginal; 0, negative.

In Western immunoblotting, dog anti-*N. helminthoeca* sera reacted with all four species of ehrlichial antigens in a similar manner (Fig. 1C and 2A). The commonly reacting polypeptides were 78 or 80 kDa and 64 kDa. In contrast, dog anti-*E. canis* sera showed a strong reaction with *E. canis* but not with the remainder of the microorganisms (Fig. 1A). Horse anti-*E. risticii* and anti-*E. sennetsu* sera showed reciprocal cross-reactivity (Fig. 1B and E and Fig. 2B and C, respectively). Both anti-*E. risticii* and anti-*E. sennetsu* sera moderately reacted with a few proteins of *N. helminthoeca* but showed a weak to negative reaction with *E. canis* (Fig. 1B and E and Fig. 2B and C, respectively). Horse anti-*E. equi* sera reacted mainly with the *E. risticii* 160- and 58-kDa proteins and the *E. sennetsu* 58-kDa protein; however, a weak to negative reaction with *N. helminthoeca* or *E. canis* was seen (Fig. 1D).

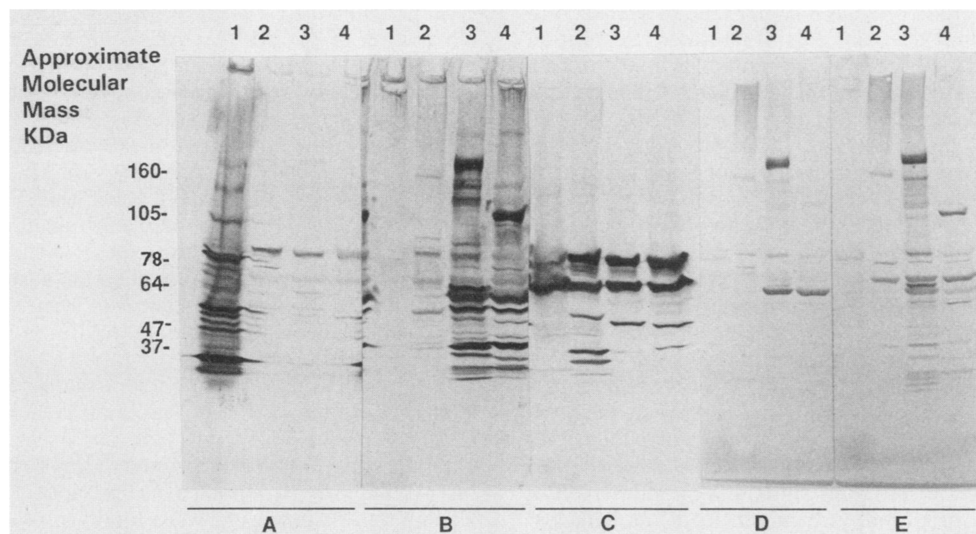

FIG. 1. Western blotting analysis of *N. helminthoeca*, *E. risticii*, *E. sennetsu*, and *E. canis* antigens with various antisera. Lanes contained antigens from *E. canis* grown in DH82 cells (lanes 1), *N. helminthoeca* grown in DH82 cells (lanes 2), *E. risticii* grown in DH82 cells (lanes 3), and *E. sennetsu* grown in P388D$_1$ cells (lanes 4). Panels contained the following antisera: A, dog EC1 antisera against *E. canis* grown in DH82 cells (indirect-fluorescent antibody [IFA] titer against *E. canis*, 1:5,120); B pony 41 antisera against *E. sennetsu* grown in P388D$_1$ cells (IFA titer against *E. sennetsu*, 1:640); C, dog NH2 antisera against *N. helminthoeca* in metacercaria (IFA titer against *N. helminthoeca*, 1:640); D, horse antisera against *E. equi*; E, pony 58 antisera against *E. risticii* grown in P388D$_1$ cells (IFA titer against *E. risticii*, 1:1,280).

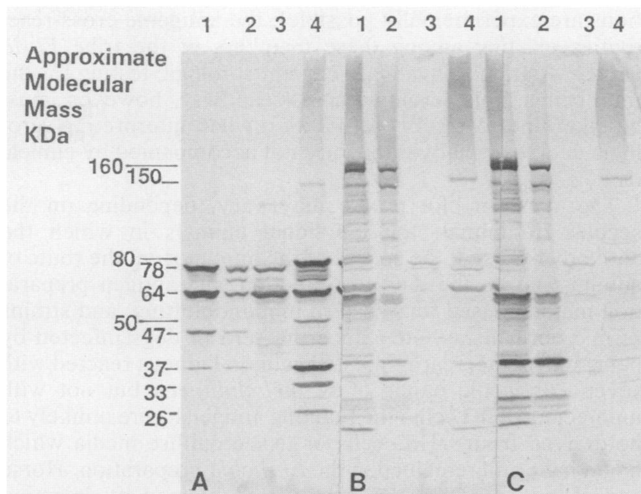

FIG. 2. Western blotting analysis of *N. helminthoeca*, *E. risticii*, and *E. canis* antigens with antisera against *N. helminthoeca*, *E. risticii*, and *E. sennetsu*. Lanes contained antigens from *E. risticii* grown in DH82 cells (lanes 1), *E. risticii* grown in P388D_1 cells (lanes 2), *E. canis* grown in DH82 cells (lanes 3), and *N. helminthoeca* grown in DH82 cells (lanes 4). Panels contained the following antisera: A, dog NH2 antisera against *N. helminthoeca* in metacercaria (indirect-fluorescent antibody [IFA] titer against *N. helminthoeca*, 1:640); B, pony 58 antisera against *E. risticii* grown in P388D_1 cells (IFA titer against *E. risticii*, 1:1,280); C, pony 41 antisera against *E. sennetsu* grown in P388D_1 cells (IFA titer against *E. sennetsu*, 1:640).

To evaluate the influence of host cell components which still may have existed in these ehrlichial antigen preparations, Western immunoblotting results for *E. risticii* propagated in P388D_1 cells and DH82 cells were compared. There were no significant differences in reacting bands with anti-*N. helminthoeca*, anti-*E. risticii*, or anti-*E. sennetsu* sera (Fig. 2). The host cells for the original organisms used to obtain these antisera were flukes in the fish for *N. helminthoeca* and P388D_1 cells for *E. risticii* and *E. sennetsu*.

All antisera showed no reaction with uninfected P388D_1 or DH82 cells in Western blotting. Most of these antisera were consecutively obtained. Preimmune sera from the animals were negative for four microorganisms in either immunofluorescence or Western blotting. A summary of the molecular masses of the reacting polypeptides is shown in Table 4.

Since the common antigens recognized by anti-*N. helminthoeca* sera were either weakly or not recognized by dog anti-*E. canis* and horse anti-*E. risticii* or anti-*E. sennetsu* sera, the reactions of antisera raised in mice and rabbits against these organisms were examined. As shown in Fig. 3A, mouse anti-*E. canis* and rabbit anti-*E. risticii* and anti-*E. sennetsu* sera strongly reacted with the 64-kDa antigen of *N. helminthoeca*, although mouse anti-*E. canis* sera still did not react with *E. risticii* (Fig. 3B).

DISCUSSION

Like *N. helminthoeca*, *E. canis*, *E. risticii*, and *E. sennetsu* are monocytic ehrlichial organisms which in nature infect dogs, horses, and humans, respectively (8, 11, 12). Because of difficulty in detecting or isolating these organisms, serologic tests are of great value (8). However, I found a distinctly positive reaction of anti-*N. helminthoeca* sera against *N. helminthoeca*, *E. sennetsu*, *E. risticii*, and *E. canis* in immunofluorescence. It is not clear why the cross-reactivity was not detected in two previous studies that included antigenic work (1a, 6). The use of peritoneal cell smears from infected mice or lymph node smears from an infected dog as the antigen (6) rather than heavily infected tissue-cultured organisms like those used in the present study may have made the interpretation of immunofluorescence slides more difficult. This study further demonstrated that with the anti-*N. helminthoeca* serum, approximately 78- or 80-kDa and 64-kDa polypeptides were the major antigens shared by *N. helminthoeca*, *E. risticii*, *E. sennetsu*, and *E. canis*.

Humoral immune responses to these common antigens by infected or immunized animals varied among different combinations of animal species and ehrlichial species. Dogs infected with *N. helminthoeca* produced antibodies which almost exclusively reacted with the common or group-specific antigens. On the contrary, dogs infected with *E. canis* produced antisera which primarily reacted with *E. canis* species-specific antigens and poorly reacted with antigens of the other members of the tribe *Ehrlichieae* tested. Ponies infected with *E. risticii* or *E. sennetsu* produced antisera which reacted with the antigens common to these two *Ehrlichia* species but not shared with the rest of the members of the tribe *Ehrlichieae*. When rabbits or mice were immunized with these other members of the tribe *Ehrlichieae*, however, their immune systems responded to these common antigens. The lack of response due to a deficiency in the B-cell repertoire for the common antigens in a particular species of animals, since dogs can respond intensely when infected with *N. helminthoeca* but not at all when infected with *E. canis*.

The fact that the sera from the dogs infected with *N. helminthoeca* reacted with *E. canis* may pose problems for the serodiagnosis of canine ehrlichiosis and salmon poisoning disease in an area such as the Northwestern Pacific coastal region, where salmon poisoning disease is endemic (4). Since the reaction of dog anti-*E. canis* sera with *N.*

TABLE 4. Antigenic cross-reactivities among ehrlichial organisms in Western immunoblotting

Antisera (animal species)	Approx molecular mass(es), in kDa, of the following antigen(s)[a]:			
	E. risticii	*E. sennetsu*	*E. canis*	*N. helminthoeca*
E. risticii (horse)	160, 145, 120, 95, 80, 64, 58, 37	105, 80, 64, 58, 37	78, 58	150, 76, 64, 37
E. sennetsu (horse)	160, 145, 120, 95, 80, 58, 37	105, 53, 37, 35, 30	(78, 58)	150, 80, 64, 51, 37
E. canis (dog)	(78, 64)	(78, 64)	160, 100, 78, 74, 64, 47, 40, 30, 27, 24, 21	(78, 64)
N. helminthoeca (dog)	78, 64, 47, 37	78, 64, 47	78, 64	150, 80, 75, 71, 64, 50, 37, 33, 26
E. equi (horse)	160, 78, 58	78, 58	(75)	(150, 78, 71)

[a] Parentheses indicate a weak reaction.

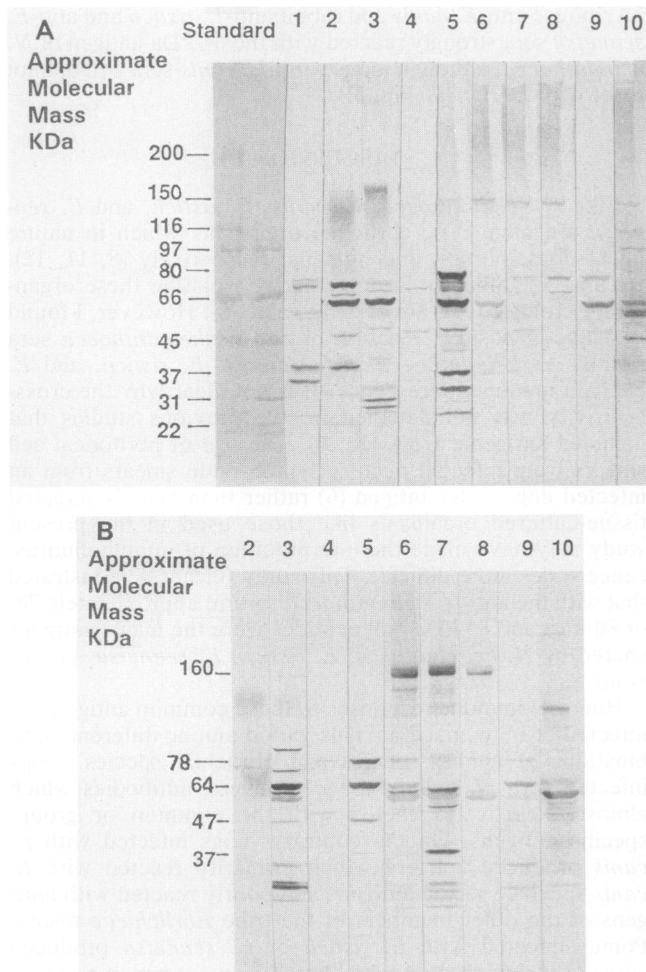

FIG. 3. Western blotting analysis of various sera against *N. helminthoeca* (A) or *E. risticii* (B) antigens from DH82 cultures separated in 4 to 20% SDS-polyacrylamide gradient gels. Lanes contained the following antisera: 1, mouse antisera against *E. risticii* grown in $P388D_1$ cells (indirect-fluorescent antibody [IFA] titer against *E. risticii*, 1:5,120); 2, mouse antisera against *E. canis* grown in DH82 cells (IFA titer against *E. canis*, 1:320); 3, mouse antisera against *N. helminthoeca* in a dog lymph node (IFA titer against *N. helminthoeca*, 1:160); 4, dog EC1 antisera against *E. canis* grown in DH82 cells (IFA titer against *E. canis*, 1:1,280); 5, dog NH2 antisera against *N. helminthoeca* in metacercaria (IFA titer against *N. helminthoeca*, 1:640); 6, pony 58 antisera against *E. risticii* grown in $P388D_1$ cells (IFA titer against *E. risticii*, 1:1,280); 7, pony 41 antisera against *E. sennetsu* grown in $P388D_1$ cells (IFA titer against *E. sennetsu*, 1:640); 8, horse antisera against *E. equi*; 9, rabbit antisera against *E. risticii* grown in $P388D_1$ cells (IFA titer against *E. risticii*, 1:1,280); 10, rabbit antisera against *E. sennetsu* grown in $P388D_1$ cells (IFA titer against *E. sennetsu*, 1:640).

helminthoeca was minimal to negative, the immunofluorescence test with both *E. canis* and *N. helminthoeca* as the antigens can be used to distinguish *E. canis* and *N. helminthoeca* infections of dogs serologically. Moreover, Western blotting with *E. canis* as the single antigen also was effective in distinguishing these two types of canine infections. Since natural infections of horses with *N. helminthoeca*, *E. canis*, or *E. sennetsu*, of dogs with *E. risticii* or *E. sennetsu*, and of humans with *N. helminthoeca* or *E. risticii* have not been reported (although some of these combinations are experimentally possible), the antigenic cross-reactivities existing among these members of the tribe *Ehrlichieae* would not invalidate current serologic testing for the time being. This serologic cross-reactivity, however, must be taken into careful consideration when interpreting serologic data, especially if they are not accompanied by clinical data.

The Western blot profile may vary, depending on the species of animal, the individual animals in which the antibody is raised, the source of the immunogen, the route of administration, the day postinfection, the antigen preparation methods used for Western immunoblotting, and strains of microorganisms, etc. Since the sera of dogs infected by being fed metacercaria-infested salmon kidneys reacted with several antigenic bands of *N. helminthoeca* but not with uninfected DH82 cells, the reacting antigens were unlikely to be derived from DH82 cells or tissue culture media which might have still remained in the *Ehrlichia* preparation. Horse and rabbit anti-*E. risticii* sera were obtained by injection with *E. risticii* cultured in murine $P388D_1$ cells and did not react with uninfected $P388D_1$ or DH82 cells. Additionally, there was no significant difference in Western blot analyses depending on whether *E. risticii* was cultured in $P388D_1$ cells or in DH82 cells. Thus, reacting common antigens of *N. helminthoeca* and *Ehrlichia* spp. were unlikely to have been derived from host cells. Furthermore, the sera of dogs immunized with *E. canis* in DH82 cells did not show any strong reaction to uninfected DH82 cells or *N. helminthoeca* antigens purified from infected DH82 cells. Thus, infection did not induce an immune response to the host cells, either. Preimmune sera from experimentally infected or immunized animals did not react with any bands, including these ehrlichial common antigens. Furthermore, the reactions were consistent and not randomly seen in these animals, suggesting that this immune response was specific and not due to prior exposure to environmental microorganisms or vaccination. It is possible, however, that some of these common antigens may be heat shock proteins commonly found in other bacteria and strongly recognized in dogs orally infected with *N. helminthoeca*. Thus, I conclude that strong common antigens exist among *N. helminthoeca*, *E. risticii*, *E. sennetsu*, and *E. canis*.

In Western blotting, anti-*E. equi* serum reacted most strongly with *E. risticii* and then with *E. sennetsu* but reacted poorly with *N. helminthoeca* and *E. canis*. More sera, however, especially samples from well-defined experimentally infected horses, should be analyzed to clarify the antigenic relationship of *E. equi* with the other *Ehrlichia* species. Moreover, it would be ideal to have culture methods developed for growing sufficient quantities of *E. equi*. Purified *E. equi* antigens could then be used for comparison of *E. equi* with the other species in the tribe *Ehrlichieae*.

On the basis of the overall Western blot reaction patterns, *N. helminthoeca* appears to be more closely related to *E. risticii* and *E. sennetsu* than to *E. canis*. The antigenic relationships among *E. risticii*, *E. sennetsu*, and *E. canis* were in agreement with the recent 16S rRNA gene sequencing data of Anderson et al. (1). Morphologically, *N. helminthoeca* resembles *E. risticii* and *E. sennetsu* more than it does *E. canis* (8, 11). *N. helminthoeca* does not develop extremely tightly packed morulae like *E. canis* does, although it does develop morulae (11). *E. canis* is extremely pleomorphic or physically distorted and is embedded in abundant capsulelike substances in the membrane-lined vacuole (3). On the contrary, most *E. risticii*, *E. sennetsu*, and *N. helminthoeca* organisms are individually enveloped in the

host membrane. There is almost no space between the host membrane and the ehrlichial outer membrane for any significant amount of capsule to exist (8). Although this study has provided new taxonomic information, 16S rRNA gene sequence analysis is needed to better clarify the classification of *N. helminthoeca*.

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REFERENCES

1. **Anderson, B., J. E. Dawson, K. Wilson, and S. A. Ewing.** 1991. Recognition of two new species of *Ehrlichia* as determined by comparative sequencing of the 16S rRNA gene. Abstr. 9th Sesquiannu. Meet. Am. Soc. Rickettsia Rickettsial Dis., Galveston, Tex., abstr. 2, p. 15.

1a. **Brown, J. L., D. L. Huxsoll, M. Ristic, and P. K. Hildebrandt.** 1972. In vitro cultivation of *Neorickettsia helminthoeca*, the causative agent of salmon poisoning disease. Am. J. Vet. Res. **33:**1695–1700.

2. **Dawson, J. E., B. Anderson, D. Fishbein, J. Sanchez, C. Goldsmith, K. Wilson, and C. Duntley.** 1991. Cultivation of an *Ehrlichia* from a patient with human ehrlichiosis. Abstr. 9th Sesquiannu. Meet. Am. Soc. Rickettsia Rickettsial Dis., Galveston, Tex., abstr. 11, p. 20.

3. **Dawson, J. E., Y. Rikihisa, S. A. Ewing, and D. B. Fishbein.** 1991. Serologic diagnosis of human ehrlichiosis using two *Ehrlichia canis* isolates. J. Infect. Dis. **163:**564–567.

4. **Gorham, J. R., and W. J. Foreyt.** 1984. Salmon poisoning disease, p. 538–544. *In* C. E. Green (ed.), Clinical microbiology and infectious diseases of the dog and cat. The W. B. Saunders Co., Philadelphia.

5. **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 **227:**522–524.

6. **Kitao, T., R. K. Farrell, and T. Fukuda.** 1973. Differentiation of salmon poisoning disease and Elokomin fluke fever: fluorescent antibody studies with *Rickettsia sennetsu*. Am. J. Vet. Res. **34:**927–928.

7. **Maeda, K., N. Markowitz, R. C. Hawley, M. Ristic, D. Cox, and J. E. McDade.** 1987. Human infection with *Ehrlichia canis*, a leukocyte rickettsia. N. Engl. J. Med. **316:**853–856.

8. **Rikihisa, Y.** 1991. The tribe *Ehrlichieae* and ehrlichial diseases. Clin. Microbiol. Rev. **4:**286–308.

9. **Rikihisa, Y.** 1991. Western immunoblot analysis of *Ehrlichia canis* antigens shared with other ehrlichial organisms. Abstr. Annu. Meet. Am. Soc. Microbiol. 1991, C193, p. 374.

10. **Rikihisa, Y., C. I. Pretzman, G. C. Johnson, S. M. Reed, S. Yamamoto, and F. Andrews.** 1988. Clinical, histopathological, and immunological responses of ponies to *Ehrlichia sennetsu* and subsequent *Ehrlichia risticii* challenge. Infect. Immun. **56:**2960–2966.

11. **Rikihisa, Y., H. Stills, and G. Zimmerman.** 1991. Isolation and continuous culture of *Neorickettsia helminthoeca* in a macrophage cell line. J. Clin. Microbiol. **29:**1928–1933.

12. **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.

13. **Ristic, M., D. L. Huxsoll, N. Tachibana, and G. Rapmund.** 1981. Evidence of a serologic relationship between *Ehrlichia canis* and *Rickettsia sennetsu*. Am. J. Trop. Med. Hyg. **30:**1324–1328.