Antigenic and Genomic Relatedness among *Ehrlichia risticii*, *Ehrlichia sennetsu*, and *Ehrlichia canis*†

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Antigenic and genomic relatedness among *Ehrlichia risticii*, *E. sennetsu*, and *E. canis* was analyzed by enzyme-linked immunosorbent assay, Western blotting (immunoblotting) and DNA-DNA hybridization. *E. risticii* and *E. sennetsu* were serologically related, and their Western blot antigen profiles were nearly identical. Two antigens of *E. sennetsu* corresponding to the 28- and 51-kDa antigens of *E. risticii* were apparently larger than the *E. risticii* antigens, and the 55-kDa antigen of *E. risticii* appeared to be unique to this species. The 110-, 70-, and 44-kDa antigens of these two species were identical, as determined by the use of monospecific antibodies. DNA homology between these two species was high. On the other hand, *E. canis* was antigenically least reactive with the antisera to *E. risticii* and *E. sennetsu*. However, a dog convalescent-stage *E. canis* antiserum recognized antigens in the other two species which were different from those recognized by their homologous antisera. Similarly, homology between the DNA of *E. canis* and the DNAs of the other two species was very minimal. These results indicate that *E. risticii* and *E. sennetsu* are closely related both at the genomic and antigenic levels and that the relationship of these two species with *E. canis* is minimal.

Members of the family *Ehrlichieae* belong to a group of obligate intracellular parasites with complex mechanisms of growth and multiplication which are poorly understood. *Ehrlichia risticii* was identified in 1985 as the causative agent of Potomac horse fever (3, 8, 16). It has recently been reported that antibodies to *E. risticii* are common in the United States and Europe (7a, 18). *E. sennetsu* is the causative agent of human sennetsu fever, found mainly in western Japan, while *E. canis* causes canine ehrlichiosis (tropical canine pancytopenia) and occurs worldwide (17, 20). One common feature among these ehrlichieae is their predilection for the monocytic series of cells in their respective hosts (17).

The renewed interest in the study of *Ehrlichieae* is due to the recent identification of *E. risticii*, serologic evidence of an association of human ehrlichiosis with *E. canis* (7, 11, 14), and recent isolation of the etiologic agent from humans with ehrlichiosis (1a). The ability of *E. risticii* and *E. sennetsu* to experimentally infect nonhuman primates (22a) and the economic considerations of Potomac horse fever and canine ehrlichiosis are the other reasons for this interest.

Present knowledge of the relatedness among *Ehrlichieae* is based on similarity in morphologic and biologic characteristics and cross-reactivities of antisera (8, 15, 19). The use of modern approaches in the identification of cross-reacting antigens and quantitation of serum cross-reactivity allows a reevaluation of the relatedness among these species in greater detail. Because the analysis of phenotypic expression involves less than 20% of the total genomes of the bacteria (1), it is necessary and now possible to evaluate these relationships at the genomic level. Toward these goals, we report the reactivities of species-specific sera by enzyme-linked immunosorbent assay (ELISA), comparative analyses of the protein-antigen composition, and the relatedness of DNA among the three species of *Ehrlichia*.

MATERIALS AND METHODS

Ehrlichial organisms and purification. *E. risticii*, isolated in our laboratory (3), and *E. sennetsu* (Miyayama strain; provided by G. Dasch, Naval Medical Research Institute, Bethesda, Md.) were propagated in human histiocyte (ATCC U-937) and mouse macrophage (ATCC P-388D₁) cells, respectively, in RPMI 1640 medium supplemented with 10% fetal calf serum and 4 mM L-glutamine. *E. canis* (Florida strain; provided by C. Holland, University of Illinois, Urbana) was grown in primary canine monocytic cells in medium 199 supplemented with 20% dog serum (12). The in vitro infection process was monitored by acridine orange (10) and Giemsa staining.

The method of purifying organisms over a 20 to 45% Renografin linear density gradient (25, 26), as previously described for *E. risticii* (4), was adhered to with slight modification. This modification included digestion of the preparations with DNase I (final concentration, 1 µg/ml; Bethesda Research Laboratories, Gaithersburg, Md.) for 10 min prior to loading it on the gradient. This procedure eliminated host cell DNA strands in the gradient, which were otherwise present. Both *E. sennetsu* and *E. canis* yielded a Renografin banding pattern similar to that of *E. risticii*. However, for *E. canis*, because of limitations of the total yield of infected primary canine monocyte cultures and the lack of previous knowledge of the banding pattern, fractions collected from the gradient were analyzed by conducting an indirect ELISA (22) using dog anti-*E. canis* serum (provided by C. Holland). The peaks of immune reactivity corresponded to the banding patterns observed for other ehrlichieae, and these peak fractions were pooled and processed as described previously (4). The protein concentration of each of the purified organism preparations was estimated by a bicinchoninic acid protein assay system (Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as the standard. For DNA studies, the organisms were suspended in TNE buffer (10 mM Tris [pH 8], 150 mM NaCl, 1 mM EDTA).

Antisera. Production of rabbit anti-*E. risticii* serum has been described previously (6). Antisera to *E. sennetsu* and *E. canis* were produced by injecting rabbits intramuscularly

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with purified organisms by the following slight modification to this protocol. The first injection contained 400 µg of the antigen mixed in Freund's complete adjuvant. The second injection, administered 2 weeks later, contained 200 µg of the antigen mixed in Freund's incomplete adjuvant, and the third injection, given at 4 weeks, contained 200 µg of the antigen alone. Test bleedings were done to evaluate the antibody reactivities by ELISA and Western blot (immuno-blot), and final bleedings were done at 6 weeks postimmunization. A Western blot analysis for *E. canis* was first done with the pre- and postinfection dog antisera provided by C. Holland to accurately determine the antigen profile of *E. canis*. A convalescent-stage dog antiserum to *E. canis* (provided by E. H. Stephenson, University of Maryland, College Park), used in all further analyses, was validated by ascertaining the Western blot reactivity that was identical to that observed with the postinfection antiserum provided by Holland.

Affinity-purified recombinant antigen-selected monospecific antibodies to the 110-, 70-, and 44-kDa antigens of *E. risticii* were prepared by using rabbit anti-*E. risticii* serum and the lambda gt11 recombinant clones expressing these antigens (5, 21a). Monospecific antiserum to the 55-kDa antigen was obtained from mice immunized with the purified recombinant antigen (5; unpublished data).

Extraction of DNA. Chromosomal-DNA extraction from the purified organisms was done as described previously (6) with few modifications. Prior to DNA extraction, MgSO₄ to 10 mM and DNase I (BRL) to 1 µg/ml were added, and the mixture was incubated at 37°C for 30 min. Next, the DNase was inhibited by the addition of EDTA to 20 mM, and the DNA was extracted by using sodium dodecyl sulfate (SDS), proteinase K, phenol-chloroform extraction, and RNase T₁ treatments. The DNA was quantitated with a TKO 100 DNA fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.).

PAGE and Western blotting. Polyacrylamide gel electrophoresis (PAGE) and Western blotting have been described previously (22). For use in Western blotting, identical amounts of the total protein from each species were loaded, and each of the antisera was diluted 1:50 in casein solution. Uninfected human histiocyte, mouse macrophage, and canine monocyte cells were used for Western blots with each of the antisera to determine reactivity with host cell components. Monospecific antibodies were evaluated for their reactivities with the three ehrlichial species in the Western blot assay as previously described (5).

ELISA. The cross-reactivities of native antigens were evaluated by ELISA using purified ehrlichial preparation as the antigen by the procedure described previously (22). Titers of each serum with respect to homologous and heterologous antigens were determined. Optimal binding with the respective homologous systems observed at a serum dilution of 1:100 was used to evaluate the homologous and heterologous sensitivities at lower antigen concentrations.

DNA-DNA hybridizations. Blotting and hybridization experiments were conducted on GeneScreen Plus membranes according to the procedures suggested by the manufacturer (NEN Products, Boston, Mass.). The chromosomal DNA was denatured in 0.25 N NaOH and diluted in 0.125 N NaOH-0.125× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) to obtain twofold dilutions starting with 100 ng. Salmon sperm DNA (Sigma) and DNA from U-937 and P-388D₁ cells were used as controls. Radiolabeling of DNA was performed by nick translation using a commercial kit (Bethesda Research Laboratories) and [α -³²P]dCTP (Amer-

FIG. 1. PAGE of *E. risticii* (lane 1), *E. sennetsu* (lane 2), and *E. canis* (lane 3) stained with Coomassie blue. Numbers on the right indicate the position of molecular size markers in kilodaltons.

sham, Arlington Heights, Ill.). A specific activity of 10^8 cpm/µg of DNA was obtained for *E. risticii*, and a specific activity of 3×10^7 cpm/µg of DNA was obtained for the other two species because of a lower input of DNA. Prehybridization and hybridization solutions contained $5 \times$ SSC, 5% dextran sulfate, $1 \times$ Denhardt's solution, 1% SDS, and 100 µg of nonhomologous DNA per ml. Following hybridizations at 65°C for 36 h, the membranes were washed with $2 \times$, $0.5 \times$, and $0.1 \times$ SSC containing 0.1% SDS for 30 min each. The wet membranes were exposed to X-Omat autoradiograph film (Kodak, Rochester, N.Y.) with intensifying screens for various time intervals.

RESULTS

Ehrlichia purification. Renografin gradient centrifugation for the purification of *E. risticii* (4), based on the technique for the purification of rickettsiae (25, 26), was found to be efficient for the purification of *E. sennetsu* and *E. canis*. Because of the difficulty in visualizing the bands with *E. canis*, the fractions were tested for the presence of antigen by ELISA with anti-*E. canis* dog serum and pooled.

Protein and antigen profiles. The electrophoretic patterns of ehrlichial proteins appeared to be distinctive for each species (Fig. 1). The Coomassie blue-stained components were relatively more similar in *E. risticii* and *E. sennetsu* than in either of these species and *E. canis*. Western blotting analyses with homologous and heterologous antisera identified the profile of the antigenic components of each organism (Fig. 2). Sixteen antigens ranging in size from 24 to 120 kDa were identified in *E. risticii* with the homologous antisera, while nine such antigens were identified in *E. sennetsu*. *E. canis* antisera identified nine antigens in *E. canis* ranging in size from 30 to 138 kDa.

The Western blot reactivities of all antisera were tested

FIG. 2. Western blot analysis of *E. risticii* (lanes 1), *E. sennetsu* (lanes 2), and *E. canis* (lanes 3) with rabbit *E. risticii* antisera (A), rabbit *E. sennetsu* antisera (B), and dog *E. canis* antisera (C). Numbers indicate molecular sizes in kilodaltons.

with the host cells as internal controls (results not shown). The *E. risticii* antisera reacted with one U-937 cell protein that was slightly larger than the 70-kDa antigen, three P-388D₁ cell proteins, and none of the canine monocyte proteins. Similarly, *E. sennetsu* antisera reacted with several of the P-388D₁ cell proteins but none of the other host cells. The dog anti-*E. canis* serum reacted extensively with P-388D₁ and a few U-937 cell proteins. However, none of the antigenic protein bands mentioned above were comparable to those for antigens identified in the Western blots of purified-organism preparations. Further, these antigens identified in the host cells were apparently diluted out in the purification process and hence were not identifiable in Western blots using the purified organisms.

The antigens identified in *E. risticii* (6) and *E. sennetsu* by using *E. risticii* antiserum were almost identical except for differences in the 51- to 55- and 28- to 33-kDa regions (Fig. 2A). A similar antigen profile was observed in the reactivity of *E. sennetsu* antiserum except for the difference in relative mobilities of antigens in the 55- to 70-kDa region (panel B). Antisera to both species identified similar differences between *E. risticii* and *E. sennetsu* in the antigen profiles at the 28- to 33-kDa region, and neither antiserum was reactive with *E. canis* components.

E. canis exhibited a distinctive antigenic profile (Fig. 2C). The antigenic components identified by using dog anti-*E. canis* serum were fewer for *E. canis* than for the other two species with their respective antisera. This dog antiserum also reacted quite extensively with both *E. risticii* and *E. sennetsu*. But the molecular weights of the antigens identified in these two species were different from those observed in *E. canis* and were not comparable to those observed with the respective homologous antisera (Fig. 3A and B). The rabbit antisera produced against purified *E. canis* reacted with *E. risticii* proteins migrating at 51 and 28 kDa, while no such reactivity was seen with *E. sennetsu* (results not shown). However, a majority of the antigens identified in *E. canis* with this serum were analogous to those observed in the host canine monocytic cell components (results not shown), and this serum was therefore not used in further

FIG. 3. Western blot analyses of *E. risticii* (lanes 1), *E. sennetsu* (lanes 2), and *E. canis* (lanes 3) using monospecific antibodies to recombinant antigens of *E. risticii* (numbers correspond to the molecular sizes of the antigens in kilodaltons). Antibody to the 55-kDa antigen was obtained from mice immunized with this recombinant antigen. All the other antibodies were affinity purified by using the rabbit *E. risticii* antisera and the recombinantly expressed antigen. A nonspecific band was observed in the region between the 55- and 70-kDa antigens in many blots.

analyses. Normal dog and rabbit sera were not reactive with any ehrlichial species.

The reactivities of monospecific antibodies to the 110-, 70-, and 44-kDa recombinant antigens of *E. risticii* indicated the presence of identically sized proteins in *E. sennetsu* (Fig. 3). However, monospecific antisera to the 55-kDa antigen of *E. risticii* failed to react with any antigen of *E. sennetsu*, and none of these monospecific antibodies reacted with *E. canis*.

ELISA reactivity. The ELISA results with homologous and heterologous systems are presented in Table 1. Both anti-*E. risticii* and anti-*E. sennetsu* sera exhibited high homologous titers along with an extensive cross-reactivity between them. The magnitude of homologous ELISA titers of these sera indicated the presence of species-specific determinants, while the extent of cross-reactivity was an indication of the presence of a large pool of shared determinants. Antisera to both of these organisms had minimal cross-reactivity with *E. canis*, with *E. sennetsu* antisera exhibiting a slightly higher titer. Dog anti-*E. canis* serum reacted moderately with *E. risticii* and to a fourfold-lesser titer with *E. sennetsu*. Rabbit anti-*E. canis* serum exhibited a slight reactivity with both *E. risticii* and *E. sennetsu*.

TABLE 1. Serologic relatedness among *E. risticii*, *E. sennetsu*, and *E. canis*

Antiserum	Titer for antigen from ^a :		
	<i>E. risticii</i>	<i>E. sennetsu</i>	<i>E. canis</i>
Rabbit anti- <i>E. risticii</i>	102,400	12,800	<50
Rabbit anti- <i>E. sennetsu</i>	25,600	102,400	200
Rabbit anti- <i>E. canis</i>	800	800	51,200
Dog anti- <i>E. canis</i>	6,400	1,600	51,200 ^b

^a Numbers represent the reciprocal dilution of the serum when the reactivity was equivalent to the background optical density.

^b A higher background was observed because of the use of canine monocyte-derived antigen and anti-dog immunoglobulin second antibody.

FIG. 4. Comparison of ELISA reactivities of rabbit *E. risticii* (ER) (A), rabbit *E. sennetsu* (ES) (B), and dog *E. canis* (EC) (C) antisera with limiting dilutions of each organism. The organisms were serially diluted and reacted with a 1:100 dilution of each antiserum.

When the ehrlichial antigens were serially diluted to determine the lower limits at which each serum could detect the homologous and heterologous antigens, the ELISA patterns were similar (Fig. 4) to those observed in the ELISA described above (Table 1). Distinguishing between *E. risticii* and *E. sennetsu* was easier with antisera to *E. risticii*. Both of these sera reacted minimally with *E. canis*. The dog anti-*E. canis* serum exhibited a hierarchy in its ability to detect a smaller amount of *E. risticii* than of *E. sennetsu*.

DNA relatedness. DNA-DNA dot hybridization studies (Fig. 5) demonstrated extensive homology between *E. risticii* and *E. sennetsu*. At concentrations of DNA used in these blots, hybridization signals obtained from the labeled *E. risticii* DNA with *E. canis* DNA were only faintly visible in the autoradiogram (Fig. 5A), while no similar signal was observed with labeled *E. sennetsu* DNA. Because of the low specific activity of the *E. canis* DNA probe, the reciprocal hybridization signals could not be identified.

DISCUSSION

While comparative structural studies on the rickettsial group of organisms have been reported (13, 24), such studies with ehrlichial organisms are lacking. Most of the studies on *E. canis*, which is the type species (20), were stimulated by the severe epizootic of canine ehrlichiosis in military dogs in Vietnam from 1968 to 1970 (17). The recent emergence of Potomac horse fever and the isolation of the etiological agent of human ehrlichiosis signify the need to study these species in greater detail.

Our demonstration of cross-reactivity between *E. risticii* and *E. sennetsu* supports the reported serologic relationship between these two species (9, 15, 19). Also, we have identified the antigenic constituents apparently responsible for this cross-reactivity and demonstrated equivalent DNA homologies. The antigen profile of *E. sennetsu* is in general

agreement with one report identifying 100-, 80-, 52-, and 46-kDa antigens of *E. sennetsu* with patient sera (23). While similar antigenic profiles were observed with *E. risticii* and *E. sennetsu*, the differences in this antigenic profile in the 51- to 70- and 28- to 33-kDa regions (Fig. 3A and B) suggest an important distinction. From the pattern of reactivity of *E. risticii* antiserum with the 51-, 49-, and 28-kDa *E. risticii* antigens and their apparent counterparts in *E. sennetsu*, it appears that analogous proteins are present in *E. sennetsu* but are slightly larger. Furthermore, this pattern has been identical in the 28- to 33-kDa region with both antisera. However, a monoclonal antibody to the 28-kDa *E. risticii* antigen was not reactive with *E. sennetsu* (22). The use of monospecific antibodies has demonstrated the analogous reactivities and sizes of the 110-, 70-, and 44-kDa antigens in *E. risticii* and *E. sennetsu*, while such a relationship was not observed for the 55-kDa antigen.

It is possible that some of the differences identified in the reactivities of antisera can be attributed to qualitative differences in purified ehrlichial preparations. Because of differences in the growth of *E. risticii* in suspension culture compared with that of monolayer and primary cell cultures of other ehrlichiae, it is possible to obtain a highly enriched preparations of *E. risticii* compared with preparations of the other species. However, we believe that such differences are only minor and do not contradict any of the observed results.

Species-specific antibodies to *E. risticii* and *E. sennetsu* failed to substantiate the relationship between these species and *E. canis* to the extent implied in previous studies (19, 21). While the normal dog sera did not react with any ehrlichiae, the dog *E. canis* antiserum has shown an interesting reactivity with other species. Because this cross-reactivity is with antigens that were not identified by the homologous antisera and is not observed with normal dog and rabbit *E. canis* hyperimmune sera, we believe this cross-reactivity is of nonspecific origin. In a previous study,

FIG. 5. DNA-DNA dot blot hybridization analysis of *E. risticii* (ER), *E. sennetsu* (ES), and *E. canis* (EC) DNAs. Numbers at the top indicate the total amount of DNA (in nanograms) applied to the membrane. Radiolabeled *E. risticii*, *E. sennetsu*, and *E. canis* DNAs were used as probes in panels A, B, and C, respectively. *E. canis* DNA was added to the one 100-ng well, was absent in the 50-ng wells, was added to all other wells in panel C, and was added to all wells in panels A and B.

we had identified antibodies in the preinfective sera of horses that were reactive with SDS-denatured antigens in Western blots of *E. risticii* (2). Hence, we believe more studies are needed to assess the presence of normal antibodies reactive with various ehrlichial species prior to unambiguous interpretation of cross-reactivity.

The relationship between *E. risticii* and *E. sennetsu* observed in their DNAs substantiates the extent of relationship observed by using the antibodies. Similarly, a lack of extensive DNA homology of these species with *E. canis* is analogous to the antigenic relationship observed with the antisera to purified organisms. In conclusion, it appears that *E. risticii* and *E. sennetsu* are highly related at both the genomic and the antigenic levels, while *E. canis* seems to be minimally related to these species.

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