Ehrlichia risticii sp. nov.: Etiological Agent of Equine Monocytic Ehrlichiosis (Synonym, Potomac Horse Fever)

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The etiological agent of equine monocytic ehrlichiosis (synonym, Potomac horse fever) was isolated and propagated in vitro by using techniques similar to those established for some members of the genus *Ehrlichia*. The organisms, which are contained within a cytoplasmic vacuole, are intermediate in size between *Ehrlichia canis* and *Ehrlichia sennetsu*, ranging from 0.4 to 0.75 μm wide and from 0.5 to 1.5 μm long. The agent is antigenically related to *E. sennetsu* and *E. canis* but also possesses unique antigenic determinants, thereby distinguishing it as a new species of the genus *Ehrlichia*. The name *Ehrlichia risticii* is proposed for this new agent. The type strain is strain HRC-IL (= ATCC VR-986).

Equine monocytic ehrlichiosis (EME; synonym, Potomac horse fever) is a relatively recently recognized disease of horses. Since the first major outbreak in 1979 within a narrow zone adjacent to the Potomac River in Montgomery County, Md., the geographical distribution and incidence of this disease have increased, with confirmed cases reported in other regions of Maryland, as well as in Virginia, Pennsylvania, New Jersey, and, most recently, Idaho (3; J. E. Palmer, personal communication). Numerous clinically diagnosed but serologically unconfirmed cases have also been reported in many other states and abroad (3).

Clinical symptoms associated with EME vary markedly and may include any or all of the following: fever, depression, anorexia, leukopenia, distal edema of the limbs, laminitis, colic, and mild to severe diarrhea. The mortality rate is more than 30% in confirmed cases. Preliminary experimental studies have determined that horses which have recovered from the disease are refractory to subsequent infection for an undetermined length of time, although relapse of the disease has been observed (J. E. Palmer, Am. Assoc. Equine Pract. Newsl., p. 63, 1983).

Previous studies revealed that EME is infectious and is transmissible to horses by intravenous inoculation of whole blood obtained from horses during the acute stage of the disease (A. L. Jenny, Am. Assoc. Equine Pract. Newsl., p. 64-65, 1983). In an effort to identify the etiological agent, blood was passaged from infected to susceptible horses on a continuous basis (A. L. Jenny, personal communication). Microscopic examination of infected blood and tissue specimens obtained during the acute phase of the disease, along with numerous bacterial and viral isolation attempts and serological studies, failed to identify a possible causative agent (1, 3, 6; Jenny, Am. Assoc. Equine Pract. Newsl., p. 64-65, 1983).

The possibility of a rickettsial etiology of the disease was suspected after the microscopic observation of a rickettsia-like organism in the leukocytes of a horse affected with EME (J. Sessions, personal communication). Sera from both naturally and experimentally infected horses were sent to the laboratory of M. Ristic at the University of Illinois College of Veterinary Medicine to test for antibodies to three agents belonging to the genus *Ehrlichia* (in particular, *Ehrlichia*

equi, the causative agent of equine ehrlichiosis) (11). All of the serum samples were negative for antibodies to *E. equi* (3). However, some reacted at low titers with *Ehrlichia canis*, the causative agent of canine ehrlichiosis. All serum samples obtained from convalescent horses reacted moderately well against *Ehrlichia sennetsu*, the agent responsible for human sennetsu rickettsiosis, a disease which is well documented in Japan and may occur in other regions of Southeast Asia (3, 11). By contrast, none of the serum samples obtained from horses before experimental infection and from other clinically normal horses which were not known to have experienced the disease reacted with any of the antigens described above (3). Based upon these serological findings, attempts were undertaken at the University of Illinois to isolate the causative agent from the blood of an experimentally infected horse. By using techniques previously developed in the laboratory of M. Ristic for the isolation and in vitro cultivation of *E. canis* and *E. sennetsu* (2, 4, 8), an organism resembling members of the genus *Ehrlichia* was isolated in monocyte cell cultures obtained from the blood of this animal (C. J. Holland and M. Ristic, Abstr. Conf. Res. Workers Anim. Dis. 1984, p. 34). Inoculation of the cultured organism into a susceptible pony induced a disease which was clinically and pathologically consistent with EME syndrome (3, 5; Jenny, unpublished data). The organism was reisolated in monocyte cultures prepared from the blood of this animal, thereby fulfilling Koch's postulates (3; Holland and Ristic, Abstr. Conf. Res. Workers Anim. Dis. 1984). Almost simultaneously, Rikihisa et al. (10), using electron microscopy, observed intracytoplasmic microorganisms resembling *Ehrlichia* within macrophage-like cells in the connective tissue of the large colon of an experimentally infected horse. Rikihisa and Perry (9) also reported preliminary observations on the growth of the agent in human histiocytes. More recently, the agent was isolated from an infected horse by using an established mouse macrophage cell line (P388D₁) by one of us (E.W., unpublished data) and by S. K. Dutta of the University of Maryland (personal communication). The organisms isolated by these investigators are morphologically indistinguishable from the original blood isolate (3).

Based upon morphological and serological studies between the isolate and established members of the genus *Ehrlichia* and the genus *Rickettsia*, we confirmed that the

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etiological agent of EME belongs to the genus *Ehrlichia* (3, 9, 11) in the order *Rickettsiales* (12). Cross-adsorption serological studies (Table 1) confirmed that the etiological agent of EME possesses unique antigenic determinants, warranting its classification as a new species of *Ehrlichia*. For this new species, we propose the name *Ehrlichia risticii* sp. nov. (ris. ti. ci' i. N.L. gen. n. *risticii* in honor of Miodrag Ristic for his many valuable contributions to the field of rickettsiae and rickettsial diseases).

Description of the species. *Ehrlichia risticii* possesses all of the general phenotypic characteristics common to other members of the genus *Ehrlichia* (3, 5, 9, 11). The organisms are pleomorphic, appearing as round, oval, or elongated sausage-like structures surrounded by a double plasma membrane and a highly rippled cell wall. Their internal components are highly electron dense and are presumed to consist of deoxyribonucleic acid and ribosomes (3, 10). The sizes of individual cells are intermediate between the sizes of *E. canis* and *E. sennetsu* cells, ranging from 0.4 to 0.75 μm wide and from 0.5 to 1.2 μm long (3, 10, 11). The organisms are contained within a cytoplasmic vacuolar membrane and may occur singly or multiply as inclusion bodies (3, 10). Division occurs by binary fission (Fig. 1). The organisms are gram negative and stain bluish purple with Wright Giemsa stain. They cannot be propagated in cell-free media. In vitro cultivation has been established in primary equine and canine monocyte cultures (3) and with murine macrophage cell lines P388D₁ and MDH-SP in medium 199 (GIBCO Laboratories) or RPMI 1640 medium (GIBCO; Quality Biological, Inc.) supplemented with 20% fetal bovine serum. The optimal growth temperature is 37 to 38°C under normal atmospheric conditions (3), but the organism may also be cultured in an humidified atmosphere of 5% CO₂ in air. It can be successfully stored at -100°C as a suspension of infected host cells in tissue culture medium containing 8 to 10% dimethyl sulfoxide. The organism requires L-glutamine for growth and is severely inhibited by low concentrations of antibiotics, such as tetracycline, penicillin, streptomycin, and gentamicin. As has been reported for *E. sennetsu* (7), mice are susceptible to infection by *E. risticii* when they are infected intraperitoneally with the blood or buffy coat from an acutely infected horse or with cultured organisms (Jenny, personal communication; A. I. Cole and M. Khondowe, unpublished data; G. S. Miranpuri and W. Burgdorfer, unpublished data).

The susceptibility of other mammalian species and the

FIG. 1. Ultrathin section of an inclusion body of *E. risticii* which is contained by a cytoplasmic vacuolar membrane. One of the organisms is at the stage of binary fission (arrow). Bar = 0.1 μm .

mechanisms by which this organism is transmitted in nature are not known. A blood-sucking arthropod, such as ticks, is strongly suspected in transmission of the disease since experimental infections have only been produced thus far by blood transfusion or intravenous inoculation of cultured organisms.

Within the genus *Ehrlichia*, *E. risticii* is more closely related to *E. sennetsu* and *E. canis*, which are also monocytic parasites, than to *E. equi* and, presumably, *Ehrlichia phagocytophila*, which multiply in granulocytes (11). The guanosine-plus-cytosine contents of the major species of *Ehrlichia* are currently being investigated.

Description of the type strain. The type strain of *E. risticii* has been deposited with the American Type Culture Collection as strain ATCC VR-986; this strain is the first isolate of *E. risticii* (= strain HRC-IL) and is described in this paper and elsewhere (3, 5).

Serum samples from naturally and experimentally infected horses were received from J. E. Sessions, Glenvilah Veterinary Clinic, Potomac, Md., A. L. Jenny, National Veterinary Services Laboratory, Ames, Iowa, and R. Whitlock, University of Pennsylvania, New Bolton Center, Kennett Square. We thank M. Khondowe, J. Dawson, and I. Abeygunawardena for skillful technical assistance. We gratefully acknowledge D. L. Huxsoll, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md., for his part in the initiation of these studies.

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TABLE 1. Antigenic differentiation between *E. risticii* and *E. sennetsu*, as demonstrated by the indirect fluorescent antibody test before and after cross-adsorption of antiserum with homologous and heterologous antigen^a

Antisera	Adsorbing antigen	Indirect fluorescent antibody titer with the following antigens: ^b	
		<i>E. risticii</i>	<i>E. sennetsu</i>
Anti- <i>E. risticii</i>	None	160, 320, 640, 1,280	20, 40, 40, 80
Equine	<i>E. sennetsu</i>	40, 160, 640, 640	N, N, N, N
Four sera	<i>E. risticii</i>	10, 10, 20, 40	N, N, N, N
Anti- <i>E. sennetsu</i>	None	40	640
Canine	<i>E. sennetsu</i>	N	40
One serum	<i>E. risticii</i>	N	320

^a Undiluted sera were adsorbed with predetermined optimal concentrations of antigen at 37°C for 30 min and centrifuged at 10,000 × g for 20 min. The supernatant sera were diluted in phosphate-buffered saline (pH 7.2).

^b Expressed as the reciprocal dilution of each serum sample examined. N, Negative at a dilution of 1:10.

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