

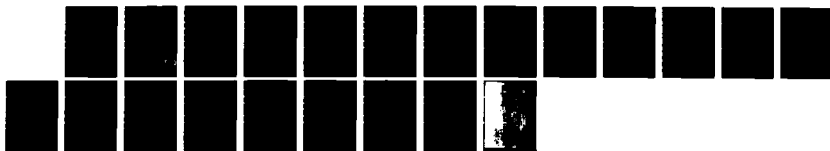
AD-A127 958

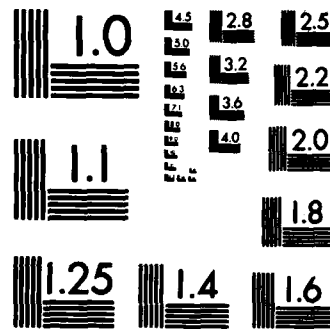
CONTROL OF HEMOTROPIC DISEASES OF DOGS(U) ILLINOIS UNIV 1/1
AT URBANA DEPT OF PATHOBIOLOGY M RISTIC DEC 79
DADA17-70-C-0044

UNCLASSIFIED

F/G 6/5

NL





MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

ADA 127958

DTIC FILE COPY

ANNUAL REPORT

January, 1979 through December, 1979

CONTROL OF HEMOTROPIC DISEASES OF DOGS

U.S. Army DADA 17-70-C-0044

by

Miodrag Ristic

Department of Pathobiology

College of Veterinary Medicine

University of Illinois

Urbana, Illinois 61801

Approved for public release;
distribution unlimited

DTIC
ELECTE
MAY 12 1983
S D
A

83 04 15 107

DISCLAIMER NOTICE

**THIS DOCUMENT IS BEST QUALITY
PRACTICABLE. THE COPY FURNISHED
TO DTIC CONTAINED A SIGNIFICANT
NUMBER OF PAGES WHICH DO NOT
REPRODUCE LEGIBLY.**

TABLE OF CONTENT

I. Introduction	3
II. Pertinent Information	6
A. Sennetsu Rickettsiosis	6
B. <u>Rickettsia sennetsu</u>	7
III. Research Accomplished by the Applicant's Laboratory	8
A. Cultivation of <u>R. sennetsu</u> in Human and Canine Blood Monocytes	8
B. Electron Microscopy of Cultured <u>R. sennetsu</u>	9
C. Serologic Relationship Between <u>R. sennetsu</u> and <u>E. canis</u> . .	10
D. Infection of Dogs with <u>R. sennetsu</u>	11
E. Detection of Serum Antibodies to <u>R. sennetsu</u> in Human Populations in Malaysia	11
Tables and Figures	13
References	19

Classification For	
1. CRA&I	☒
2. TAB	☐
3. Unrecorded	☐
4. Classification	



Distribution/
Availability Codes
Available for
Distribution
A-23

I. INTRODUCTION

Scientific interest into human sennetsu rickettsiosis and its causative agent Rickettsia sennetsu was generated through long term studies of the applicants laboratory into canine ehrlichiosis, better known as tropical canine pancytopenia (TCP). The study of TCP and the etiologic agent Ehrlichia canis was initiated in collaboration with the U. S. Army scientists in 1968 when the disease caused severe losses among military dogs in Viet Nam. This collaborative research effort resulted in the development of methods for in vitro propagation of E. canis which led to the development of serologic procedures for diagnosis of TCP and means of controlling the disease. Other studies included pathogenesis of the disease and elucidation of immunopathologic mechanism of thrombocytopenia, development of E. canis in, and transmission by, the vector tick, biologic and morphologic properties of the agent.

It is a close morphologic resemblance between E. canis and R. sennetsu and their antigenic uniqueness with reference to other common rickettsia that instigated our specific interest into the latter agent. Peripheral blood monocytes serve as host cells of both agents. Unlike most classic rickettsiae, these two agents develop and multiply within a well-defined vacuolar membrane rather than free in the cytoplasm of the host cell. Investigations in our laboratory on antigenic properties of E. canis and similar studies on R. sennetsu in several other laboratories showed that these two agents do not share antigenic properties with other human rickettsial pathogens.

A crucial finding that, in addition to their morphologic resemblance, E. canis and R. sennetsu are also closely antigenically related has prompted our further interest in Sennetsu rickettsiosis syndrome.

These studies demonstrated that sera of patients convalescing from Sennetsu rickettsiosis and those of dogs with canine ehrlichiosis reacted strongly in the indirect fluorescent antibody (IFA) test for E. canis and R. sennetsu, respectively. In addition it was demonstrated that similarly to E. canis, R. sennetsu can also be efficiently propagated in cultures of peripheral blood monocytes. Of special interest was the finding that canine blood monocyte cultures were superior to similar cultures of human origin as a substrate for propagation of R. sennetsu. Finally it was demonstrated that dogs are susceptible to infection with R. sennetsu. Infected animals, however, do not show clinical and hematologic abnormalities suggesting that the dog may be an asymptomatic carrier and consequently a possible source of infection for humans. (see beyond).

By again utilizing the experience acquired from the study of E. canis, we developed an IFA test for sennetsu rickettsiosis in which the organism derived from canine monocyte cultures served as an antigen. The IFA test was effectively used to detect and titrate anti-R. sennetsu antibodies in serum from convalescing patients from Japan and to monitor experimental infections in dogs caused by this agent.

At this stage of our investigation of R. sennetsu we were ready to embark on sero-epidemiologic studies aimed at determining if infections with this agent may be occurring in human populations residing in areas of South East Asia other than Japan. Unfortunately, our research grant proposal to that effect to the U. S. Army Medical Research and Development Command was not funded. Nevertheless, being committed to completion of thesis research of two graduate students, we initiated these studies with our own resources and with kind cooperation of the U.S. Army medical research unit in Kuala Lumpur, Malaysia.

Four hundred "blind" human serum samples from Malaysia were received at the University of Illinois and tested for the presence of R. sennetsu antibodies using the IFA test. After the tests were completed and results reported it was revealed that these samples were derived from two medically different populations, each represented by approximately 200 individuals. Group 1 consisted of randomly collected samples from apparently healthy subjects. Group 2 were samples from patients who suffered from fever of unknown origin and on whom no causal diagnosis had been established using multiple laboratory techniques for malaria, scrub typhus, murine typhus, epidemic typhus, leptospirosis, malicoidosis, typhoid and paratyphoid fevers, and group A and B of arbovirus infections.

The results of the above serologic survey revealed no reactors among samples of the group 1 (apparently healthy individuals) and 29% reactors among samples of group 2 (individuals with fever of unknown origin). From this preliminary but well controlled study

it was concluded: 1) that R. sennetsu and/or R. sennetsu-like agents may be occurring in the Malaysian population, 2) that this agent is associated with clinical disease.

Based on the findings of this preliminary study it is deemed appropriate to confirm these initial results through additional serologic studies and all out efforts to isolate the causative agent from selected clinical cases.

II. PERTINENT INFORMATION

A. Sennetsu Rickettsiosis: Sennetsu rickettsiosis earlier referred to as infectious mononucleosis, glandular fever, Hyuga fever and Kagami fever is an infectious disease of man caused by Rickettsia sennetsu. The organism was originally isolated by inoculation of mice with blood, bone marrow, and lymph node samples of a patient with acute signs of the disease (Misao and Kobayashi, 1954).

Clinical forms of sennetsu rickettsiosis may vary from mild headache, slight back pain, and a low-degree fever to a severe form of the disease characterized by persistent high fever, anorexia, lethargy, lymphadenopathy, and prominent hematologic abnormalities. Because of the multiplicity and variation of the intensity of clinical symptoms, misdiagnosis is possible. In Japan, for many years, the disease was identified as viral infectious mononucleosis. In other southeast Asian Countries, i.e., Malaysia, confirmed cases of infectious mononucleosis are found only among the European population. The occurrence of signs and symptoms compatible with infectious mononucleosis in

non-European patients, however, is common (Huxsoll, 1979). Under the circumstances, the incidence of infections with R. sennetsu may have wider geographic distribution but has remained undetected because of the unavailability of specific and practical serodiagnostic procedures suitable for epidemiologic studies.

Sennetsu rickettsiosis is widely distributed in western Japan, however, the mode of transmission is not known. An association between sennetsu rickettsiosis and eating or catching certain types of seasonal fish has been suggested by some Japanese workers. Attempts have failed, however, to recover the organism from the fish. Also, no relationship between the salmon poisoning agent, Neorickettsia helminthoeca, and R. sennetsu was demonstrated (Kitao et al., 1973). More recent observations revealed that within a Japanese family which ate fish, only the member who actually caught the fish developed the disease (Tachibana, 1979). Thus, it appears that sennetsu rickettsiosis, similar to many other rickettsial diseases including canine ehrlichiosis, is a vector-borne disease. Questions regarding identity of the vector, animal reservoir, and prevalence of the disease in other areas of Southeast Asia, however, remain unknown.

B. Rickettsia sennetsu: Although R. sennetsu exhibits certain rickettsial properties, no serologic relationship to other rickettsial agents has been established. Thus until its biological properties are better known, it is regarded as a species incertae sedis (Weiss and Moulder, 1974). Light and

electron microscopic studies of R. sennetsu showed that the agent differs morphologically from most other classic rickettsiae. Unlike the latter organisms, R. sennetsu does not occur freely in the host cell cytoplasm but rather single or multiple forms of the organism are contained in a membrane-like vacuole (Anderson et al., 1965). The organism used in the latter study was propagated in African green monkey kidney cells (BS-C-1). Results of similar studies by Tanaka and Hanoaka (1961) using tissues of infected mice differ, however, from those by Anderson, et al. (1965). Tanaka and Hanaoka (1961) found small clusters of the organism occurring apparently freely in the cytoplasm of peritoneal mouse macrophages. The authors admitted that at first glance the rickettsiae seem to possess a double limiting membrane of their own located within the cytoplasmic ground substance of the host. However, they concluded that the outer surface belonged to the smooth endoplasmic reticulum of the host cell. In vitro cell cultures used to propagate R. sennetsu include the above described African green monkey kidney cells (BS-C-1) (Anderson et al. 1965) and human amnion tissue-derived (FL) cell line (Minamishima, 1965).

III. RESEARCH ACCOMPLISHED BY THE APPLICANT'S LABORATORY

A. Cultivation of R. sennetsu in Human and Canine Blood Monocytes:

Microscopic examination of cultured human (Hoilien et al., 1980; Hoilien, 1980) and canine (Holland, 1979) blood monocytes infected with Rickettsia sennetsu and stained by the Giemsa method revealed

the presence of various organismal growth forms in the cytoplasm of infected cells. The growth forms observed were (1) loosely scattered individual organisms, (2) clusters of organisms, (3) various sizes of dense inclusion bodies in intact and vacuolated cytoplasm, and (4) organisms in proximity to disintegrated monocytes. The appearance and the morphology of these R. sennetsu growth forms were similar to those of E. canis propagated in canine monocytes. Specific identification of R. sennetsu was made by staining cultured monocytes with fluorescein-conjugated globulins extracted from pooled sera of patients convalescing from sennetsu fever. Mice infected with the cultured organism developed gross-pathologic changes indicative of infection and the organism was demonstrated in their spleens, peritoneal macrophages, and mononuclear blood cells.

B. Electron Microscopy of Cultured R. sennetsu: The ultrathin sections of R. sennetsu in human and canine monocytes confirmed earlier work of Anderson et al., (1965) that the individual as well as clusters of R. sennetsu organisms are confined to a membrane-lined vacuole. The number of organisms in each vacuole varied from 1 to 20. The individual organisms or elementary bodies, as they are called in the literature, were quite pleomorphic, ranging from round to oval to oblong, and sometimes twisted in shape. The elementary bodies in the individual vacuoles, however, were generally similar in shape, that is, round bodies were found in one vacuole, and oblong, twisted bodies were found in another vacuole. Individual cells frequently contained more than one

vacuole and some cells were packed with vacuoles. Those packed cells appeared to be less active macrophages, as evidenced by a lack of peripheral pseudopods. On the other hand, cells containing only a few vacuoles appeared as healthy activated macrophages with stimulated mitochondria and out-stretched pseudopods.

The organism was readily identified by its electron-dense structure bounded by an inner plasma membrane and an outer rippled cell wall. The two membranes were generally separated by an electronlucid space. At times these tri-layered membranes were out of focus, but the vacuolar membrane surrounding the individual organisms was always clearly defined. A remarkable morphologic resemblance between Ehrlichia equi, the causative agent of equine ehrlichiosis, and R. sennetsu is shown in figures 1 and 2. Individual organisms of both agents are bound by a characteristic rippled cell wall and surrounded by a membrane-lined cytoplasmic vacuole. Experimentally, E. equi was shown to infect several animal species, including monkeys. A close antigenic relationship between R. sennetsu and E. equi was recently demonstrated in our laboratory.

C. Serologic Relationship Between R. sennetsu and E. canis:

Results of examination in the E. canis IFA test of sera from 5 human patients in Japan infected with R. sennetsu are given in Table 1. Serum No. 5 which was received in lyophilized form reacted at a titer of 1:80 while the remaining 4 sera received

frozen in dry ice sera reacted at 1:160. None of the sera from apparently normal human beings reacted at a serum dilution of 1:5. In a homologous IFA test using R. sennetsu antigen, serum No 3 showed titer of 1:640 and the remaining 4 sera reacted at 1:128. None of the 14 specific antisera to 10 common rickettsiae reacted at a 1:5 dilution against E. canis (Table 2).

Results of examination by the R. sennetsu IFA test of 6 canine sera collected before and after infection with E. canis are given in table 3. None of the preinfection sera reacted at a titer of 1:5. All post infection sera reacted with R. sennetsu at titers from 1:80 to 1:320. Homologous titers of these sera were slightly higher, ranging between 1:320 to 1:2560.

D. Infection of Dogs with R. sennetsu: Culture material derived from different subpasses of R. sennetsu infected human and canine monocyte cultures were used to inoculate two dogs of mixed breeds. Blood from one of these dogs was subsequently inoculated into a third dog. Although inoculated dogs failed to develop clinical signs of the disease, the organism was isolated by culturing peripheral blood monocytes from the blood of all 3 dogs one month post-inoculation. Furthermore, these animals showed a serologic response to R. sennetsu in the IFA test. It is suggested that dogs may serve as asymptomatic carriers of R. sennetsu.

E. Detection of Serum Antibodies to R. sennetsu in Human Populations in Malaysia: Four-hundred human serum samples from Malaysia were tested for the presence of R. sennetsu antibodies using the IFA test. The samples were from 2 groups; group 1 included sera from

healthy individuals and group 2, sera from patients with fevers of unknown origin. There were no reactors among sera obtained from apparently healthy individuals. Among sera from patients with fever of unknown origin, however, there were 29% reactors.

It is important to note that the sera were received at the University of Illinois as "blind" samples. After the tests were complete, the identity of the samples were made available. Many of the sera from the second group were paired. In many cases in which paired sera were collected on two different dates both the first and the second were positive. Of those, the titer of the second samples was often higher than that of the first. In addition, in some cases, there was a sero conversion from negative to positive at the time of illness. On the other hand, many other sera remained negative at each testing period. The findings have added to the credibility of the test (table 4).

Based upon the above results it is apparent that R. sennetsu and/or R. sennetsu-like agents may be present in the Malaysian population and that this agent may be responsible for clinical disease.

Table 1

Serologic cross-reaction between human convalescent
Rickettsia sennetsu sera and Ehrlichia canis using
 the Indirect Fluorescent Antibody (IFA) test

Human Serum	IFA titer	
	<u>E. canis</u>	<u>R. sennetsu</u>
1	1:160*	1:320
2	1:160	1:320
3	1:160	1:640
4	1:160	1:320
5	1:80	1:320
(8 normal human sera)	-**	-

*Reciprocal of the final serial serum dilution showing reaction in the test.

**No reaction at 1:5 serum dilution

Table 2

Specificity of Ehrlichia canis Indirect Fluorescent Antibody (IFA) test.

Agent	Immune animal species	Homologous IFA titer	IFA for <u>E. canis</u> (1:5 serum dilution)
<u>Rickettsia tsutsugamushi</u>	human	1:200 (Karp strain)	-*
		1:200 (Gilliam strain)	-
<u>Rickettsia tsutsugamushi</u>	human	1:400 (Karp strain)	-
		1:800 (Gilliam strain)	-
<u>Rickettsia rickettsii</u>	human	1:640	-
<u>Rickettsia typhimurium</u>	human	1:2,560	-
<u>Rickettsia tsutsugamushi</u>	guinea pig	1:20,000	-
<u>Rickettsia rickettsii</u>	guinea pig	1:20,000	-
<u>Rickettsia canada</u>	guinea pig	1:20,000	-
<u>Rickettsia burnetti</u>	guinea pig	1:20,000	-
<u>Rickettsia mooseri</u>	guinea pig	1:20,000	-
<u>Rickettsia akari</u>	guinea pig	1:20,000	-
Psittacosis agent	guinea pig	1:20,000	-
Tatlock agent (rickettsia-like) agent of guinea pig origin	guinea pig	1:20,000	-

*No reaction

Table 3

Serologic cross-reaction between canine anti-Ehrlichia canis
sera and Rickettsia sennetsu antigen using Indirect
Fluorescent Antibody (IFA) test

Canine Serum	IFA titer	
	<u>E. canis</u>	<u>R. sennetsu</u>
1	1:2560*	1:160
2	1:1280	1:160
3	1:1280	1:80
4	1:320	1:80
5	1:2560	1:320
6	1:1280	1:160
Normal sera	-**	-

*Reciprocal of the final serial serum dilution showing reaction in the test.

**No reaction at 1:5 serum dilution

Table 4

Results of Indirect Fluorescent Antibody (IFA) test
for Rickettsia sennetsu with human sera from Malaysia

Group/Description	IFA*Results	#Positive/ #Tested)	Titer of Positive Sample
1 Apparently healthy Individuals	0% positive	(0/200)	
2 Patients with fever of unknown origin	29% positive	(58/200)	1:10**7% (14/200) 1:20 13.5% (27/200) 1:40 5% (10/200) 1:80 3% (6/200) 1:640 0.5% (1/200)

*Indirect Fluorescent Antibody (IFA) test used as an antigen R. sennetsu propagated in
in canine blood monocyte cultures.

**Reciprocal of the final serial serum dilution. The beginning dilution of the
serum was 1:10.

Legends:

Figure 1. An intact vacuole with R. sennetsu organisms occurring extracellularly, X27,000. The upper right corner reveals a closer view of two R. sennetsu organisms. Note the characteristic rippled cell wall (arrow) X63,000.

Figure 2. Neutrophil containing several Ehrlichia equi inclusions (I). Several single organisms are tightly bound by the membrane of the vacuole (S). Vesicles (V) are contained within and around the vacuoles. A rippled cell wall (C) and plasma membrane (P) are evident around the organisms, X41,000.



REFERENCES

- Anderson, D.R., Hopps, H.E., Barile, M.F., and Bernheim, B.C. 1965. Comparison of the ultrastructure of several rickettsiae, ornithosis virus, and mycoplasma in tissue culture. *J. Bact.*, 90: 1387-1404.
- Hoilien, C., 1980. Cultural, morphologic, and immunologic properties of Rickettsia sennetsu propagated in primary human blood monocyte cell culture. M. S. Thesis, University of Illinois Urbana, IL 61801.
- Hoilien, C.A., Ristic, M., Huxsoll, D.L., Rapmund, G., and Tachibana, H., 1980. Rickettsia sennetsu in human blood monocyte cultures: similarities to the grown cycle of Ehrlichia canis. Submitted to *Amer. J. Trop. Med. and Hygiene*.
- Holland, C. 1979. Rickettsia sennetsu in canine monocyte cell culture: Development of a serodiagnostic test using culture-derived antigens. M. S. Thesis, University of Illinois, Urbana, IL 61801.
- Huxsoll, D.L., 1979. Personal communication, U.S. Army Medical Unit, Kuala Lumpur, Malaysia
- Kitao, T., Farell, K.P., Fukuda, T. 1973. Poisoning Disease and Elokomin Fluke Fever: Fluorescent Antibody Studies with Rickettsia sennetsu. *Am. J. Vet. Res.*, 34: 927-929
- Minamishima, Y. 1965. Persistent infection of Rickettsia sennetsu in cell culture system. *Jap. J. of Microbiology*. 9: 75-86.
- Misao, T. and Kobayashi, Y. 1955. Studies on infectious mononucleosis. I. Isolation of etiologic agent from blood, bone marrow, and lymph node of a patient with infectious mononucleosis by using mice. *Tokyo Iji Shinshi*, 71: 685-686.
- Tachibana, N. 1979. Personal communication.
- Tanaka, H. and Hanaoka, M. 1961. Ultrastructure and taxonomy of Rickettsia sennetsu (the causative agent of "sennetsu" or infectious mononucleosis in West Japan) as studied with the electron microscope. *Ann. Report Inst. Virus Res.*, Kyoto Univ., 4: 67-82.
- Weiss, E., and Moulder, J.W. 1974. Genus Rickettsia. In Bergey's Manual of Determinative Bacteriology. R.E. Buchanan and B. E. Gibbons, eds., 8th edition, Williams and Wilkins, Baltimore, MD, pp. 883-885.

END

FILMED

6-83

DTIC