2064 To Dr. G. H. E. Hopkins with the compliments of the author.

STUDIES ON SUCKING LICE (ANOPLURA) IN JAPAN

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BY

Kiyotoshi KANEKO

(Department of Medical Zoology, School of Medicine, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo)

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PART V. EXPERIMENTAL TRANSMISSION OF *RICKETTSIA ORIENTALIS*, *R. MOOSERI* AND *R. PROWAZEKI* WITH MURINE LICE*

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I. INTRODUCTION

For many years it was believed that tsutsugamushi disease (scrub typhus) was one of the endemic diseases, occurring in summer in river basins of restricted areas in three prefectures of Northeastern Japan and was transmitted by tsutsugamushi (trombiculid mite).

After World War II, however, another type of scrub typhus was discovered in the foot hills of Mt. Fuji and in the Izu Seven Islands in the winter season.

Since 1954, rickettsiae from small wild mammals were isolated in almost every part of Japan by members of a research group on rural rickettsiosis headed by Dr. Takeo Tamiya. Furthermore, with the discovery of patients with rickettsiosis in certain localities, rickettsiosis has been found to be distributed widely over the country. To date, the development of epidemiological studies on scrub typhus has gradually revealed the relationship among reservoirs, vectors and man. In these researches, trombiculid mites were most extensively studied in their role of vectors. Until now the only vector of scrub typhus was thought to be trombiculid mites, and epidemiological studies have concentrated on the combination of small wild rodents, trombiculid mites and man.

Murine lice, however, comprise many species, abundantly infesting small rodents and distribute widely throughout Japan. Also some kinds of murine lice were proved to transmit rickettsiae and tularemia as described in the following sentenses. In the experimental transmission of rickettsia with lice, Mooser, Castaneda, Zinsser and other reported in 1931 that *Polyplax spinulosa* transmitted the causative agent of Mexican typhus among rats. In 1932, Kono successfully made an experimental transmission of the rickettsiosis of Manchurian fever and typhus with *Polyplax spinulosa*. In another experiment carried out by Francis & Lake in 1922, *Polyplax serrata* transfered *Pasteurella turalensis*.

Considering those experiments, murine lice are expected to be capable of transmitting *Rickettsia orientalis* among rats as they do *R. mooseri* & *R.*

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prowazeki. And if this assumption is true, it would indicate that scrub typhus can be spread by the vectors among rats as well as by the interaction of rats, trombiculid mites and man.

To determine the epidemiological relationship of murine lice to scrub typhus, the author made an experimental transmission of *R. orientalis* applying murine lice. As a check, *R. mooseri* and *R. prowazeki* were also used for experimental transmission.

II. MATERIALS AND METHODS

Roof Rat (*Rattus rattus*): The roof rats were collected in Tokyo, and weighed from 100 to 150 g., and were kept in the laboratory for 2–3 months before use.

Old World Woods Mouse (*Apodemus speciosus*): Woods mice were first caught in Tochigi Pref. and have been bred in the laboratory for several years. The mice used were the 2nd generation of the wild ones, and weighed from 30 to 35 g.

Murine Lice: The lice used were of three species, *Hoplopleura oenomydis* and *Polyplax spinulosa* collected from Tokyo and *Hoplopleura akanezumi* from Tochigi Pref. The collected lice were reared on the rats and woods mice and were reproduced in the laboratory. Lice of the 2nd and 3rd generations were used in the experiments.

Strains of Rickettsiae*: In these experiments 3 strains of rickettsiae were used; the Wilmington strain of murine typhus (*R.mooseri*), the Breinl strain of typhus (*R. prowazeki*), passed through more than 300 eggs at the Institute for Infectious Diseases, University of Tokyo; and the Ozeki strain of scrub typhus (*R. orientalis* = *R. tsutsugamushi*), passed through about 270 mice at the same Institute.

Methods of Experimental Transmission: The yolk sac in which rickettsiae were cultivated was suspended in an sucrose PG solution of 20 per cent. One ml. of the suspension was intraperitoneally introduced into the roof rats on which a large numbers of lice were previously fed, and 0.5 ml. was applied to the woods mice in the same manner.

A large amount of rickettsia suspension was injected into experimental animals so that the lice on the animal readily have a chance to engorge the agents. The volume of the inoculum was so large compared with other experiments that two rats inoculated with *Rickettsia mooseri* died. After the first trial, the concentration of the suspension was reduced and adjusted to 1 per cent in the experiment with *R. mooseri*. The murine lice were removed several time from the experimental animals during from the 3rd to the 46th day after inoculation of rickettsiae with yolk sac suspension.

The murine lice were detached from the body of the infected animals with a fine needle and put into 70 per cent alcohol solution in small test tubes. In the 70 per cent alcohol solution they were shaken for half a minute and poured on sterile filter-paper, and finally washed with sucrose PG solution added with antibiotics (penicillin G 500 units and dihydrostreptomycin 500 γ per ml).

The washed lice were smashed on hold slides and emulsified with the sucrose PG solution (1.2 ml.). The emulsion was inoculated with 0.3 ml

* Kindly supplied by Ass. Prof. A. Kawamura, of the Institute for Infectious Diseases, University of Tokyo, on September, 16, 1958.

to 2 mice and 0.1 ml. into 5 yolk sacs of six-day-old embryonated eggs. As for *Rickettsia prowazeki*, an inoculum of 0.5 ml. emulsion was used to 2 guinea pigs and 0.1 ml. to 5 yolk sacs of six-day-old eggs. The yolk sacs were examined on the 14 th day after inoculation.

The existence of rickettsiae was microscopically certified with smear specimens stained with Giemsa's solution. At the same time, the yolk sacs were emulsified and inoculated in the mice to differentiate the strain of the rickettsia obtained during the experiment from non-pathogenic rickettsia.

The mice inoculated with the emulsified lice were kept for 2 weeks and then destroyed. After the presence of rickettsia was microscopically examined, spleens and livers of mice were emulsified and inoculated into fresh mice. The next passage was made after 1 week. From the mice of the 2 nd subinoculation, the morophology and the pathogenicity of the rickettsia certified.

The establishment of infection in the case of *R. prowazeki* was determined by the observation of the course of the rectal temperature in guinea pigs and by the complement fixation test, specific to the strain of rickettsia.

Complement Fixation Test: Sources of sera; guinea pig antisera were obtained from animals which had received intraperitoneal injections of infected li ɔe emulsion, and bled on the 46 th day. The separated sera were inactivated at 56°C for 30 minutes. Before use the sera were again inactivated, in order to minimize their anticomplementary action.

Standard serum used for the titration of the antigenic unit was a serum of rabbit immunized by the emulsion of guinea pig testis in which *R. prowazeki* was infected. The results of the block titration soon after the bleeding are shown in Table 1-a. Standard sera and the same lot of antigen as applied in the following experiments were used.

Antigen: The specific antigen of *R. prowazeki* used, was prepared from the infected yolk sacs at the Institute for Infectious Deseases. Its antigenic units were estimated from the results of the block titration with the standard serum, as shown in Table 1-b.

Technique: The CFT procedure was based on the methods of the US Army Medical School, which is a modification of Kolmer's method. Unit volumes of 0.25 ml. were used for the constant dilution of antigen (2 antigenic unit) and for the serial dilution of antisera. Two full units of complement in 0.5 ml. were added and fixation was carried out overnight in the refrigerator (16-18 hours).

The adequacy of the amount of complement was checked by the complement control experiment. Fixation at 3 or greater was considered as positive.

III. Experimental Studies

Experiment I

Transmission of *Rickettsia orientalis*: In order to see when the most extensive rickettsiaemia takes place in a rat infected with *R. orientalis*, rickettsia titration was examined. From three intact *Rattus rattus* intraperitoneally infected with *R. orientalis* in 1 ml. of liver and spleen emulsion of the previously infected mice in a concentration of 10^{-1}, blood samples

TABLE 1

Boxtitlation with standard serum and antigen of *R. prowazeki*

a. Titration of the standard serum.

Antigen \ Antiserum	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	C
1 : 1	4	4	4	4	4	3	2	2
1 : 2	4	4	4	4	3	2	1	0
1 : 4	4	4	3	3	2	2	1	0
1 : 8	3	2	2	1	1	1	±	0
1 : 16	2	2	1	1	0	0	0	0
1 : 32	1	1	0	0	0	0	0	0
Contr.	±	0	0	0	0	0	0	0

b. Titration of the antigen.

Antigen \ Antiserum	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	C
1 : 1	4	4	4	4	4	3	1	0
1 : 2	4	4	4	4	3	2	1	0
1 : 4	4	4	3	2	1	0	0	0
1 : 8	1	1	0	0	0	0	0	0
1 : 16	0	0	0	0	0	0	0	0
1 : 32	0	0	0	0	0	0	0	0
Contr.	0	0	0	0	0	0	0	0

were drawn from the heart 2, 4, 6, 9, 12 and 15 days respectively after the inoculation and were transferred into laboratory mice. During the life period of those mice, being observed for the 2 weeks, the intensity of rickettsiaemia was measured by the death rates of the inoculated mice as illustrated in Table 2. As a result it was found that the rickettsiaemia of the rat occured most extensivly on the 6 th day after inoculation and showed a sudden reduction afterward. Rat No. 5 accidentally died by heart puncture in the course of the experiment.

In the following experiment with *R. orientalis*, murine lice were removed from the infected rats, considering the course of rickettsiaemia revealed in the experiment mentioned above.

Apodemus speciosus on which *Hoplopleura akanezumi* were reared was infected with *R. orientalis*. On the 4th, 7th, 12th 16th and 22nd days, 20 and/or 50 lice were removed from the host and examined to see whether or not infected agents present. The detection of rickettsiae from lice was made by applying an inoculation of lice emulsion to the mice and yolk sacs mentioned above. As a result, the lice were revealed not to transmit *R. orientalis* as shown in Table 3.

Transmission of *Rickettsia mooseri*: In this experiment, three combinations of lice and hosts were employed, as follows; *Rattus rattus* with *H. oenomydis* and with *P. spinulosa* and *Apodemus speciosus* with *H. akanezumi*.

TABLE 2

The course of the grade of rickettsiaemia of rat infected with *R. orientalis*, measured by LD_{50} of mice inoculated rats blood

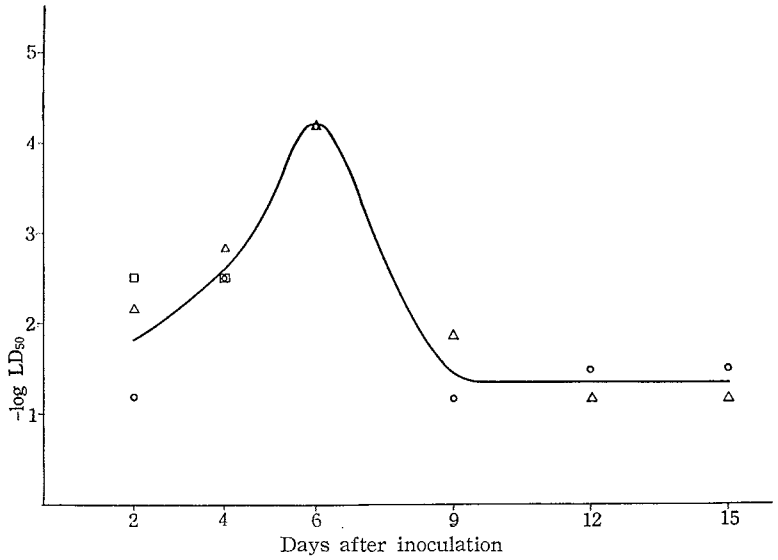


TABLE 3

Isolation of rickettsia from lice reared on the infected rats with rickettsiae

Experiment 1 — Agent: *Rickettsia orientalis*; Host: *Apodemus speciosus*

Days after inoculation / Species of lice	4	7	12	16	22
Nos. of *Hoplopleura akanezumi*	20	20	50	50	50
Isolation of rickettsia	—	—	—	—	—

Agent: *Rickettsia mooseri*; Hosts: *Apodemus speciosus*, *Rattus rattus*

Days after inoculation / Species of lice	3	9	17	42
Nos. of *Hoplopleura akanezumi*	10	20	20	15
Isolation of rickettsia	+	+	+	—
Nos. of *Hoplopleura oenomydis*	20			
Isolation of rickettsia	+			
Nos. of *Polyplax spinulosa*	10			
Isolation of rickettsia	—			

Eggs inoculated with the emulsion of two species of Hoplopleura began to die after 6 days. In the smear of the yolk sacs of those eggs enormous numbers of rickettsia were observed in Giemsa's stain.

In the isolation of rickettsia from lice using the laboratory mice, detection of rickettsia did not yield fixed results.

In the inoculation of the emulsion of *Polyplax spinulosa*, the eggs were killed and no rickettsia was found.

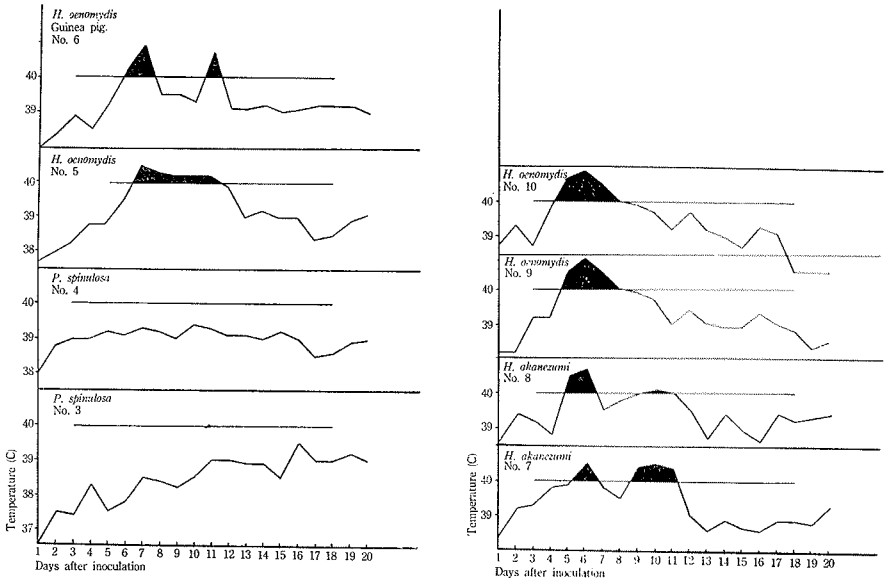
Rickettsia could be isolated from only 10 lice from the woods mice on the 3rd day after inoculation of the emulsion of *H. akanezumi*. On the other hand, no rickettsia was detected from the lice on the 42nd day after injection.

Transmission of *Rickettsia prowazeki*: This experiment employed the same three combinations of lice and hosts as in the transmission of *R. mooseri*. Lice were detached from rats on the 3rd day after the hosts was inoculated with rickettsiae. A temperature rise in 2 guinea pigs (No. 7, 8) inoculated with the emulsion of 10 specimens of *H. akanezumi* was observed on the 5th day. Then, the fever abated temporarily on the 7th or 8th day, rose again on the 9th and lasted for 3 days in both guinea pigs. Of 2 guinea pigs injected with the 20 *H. oenomydis*, one (No. 5) fevered during the period from the 7th to 11th days. While the other (No. 6) fevered on only 2 days, the 7th and 11th days. The characteristic fever was not observed in both guinea pigs (No. 3, 4) infected with 10 *P. spinulosa*.

Of these 3 species of lice, *H. oenomydis* were removed from rats on the 9th and 17th days after the rats were infected. The emulsion of 20 *H. oenomydis* was inoculated in 2 guinea pigs and 5 eggs on the 9th day, and 100 *H. oenomydis* were also inoculated in guinea pigs and eggs on the 17th day. The characteristic fever of the guinea pigs was observed in

TABLE 4

The course of the rectal temperature of guinea pigs after inoculation of lice reared on the rat infected with *R. prowazeki*



the former and not in the latter. In this experiment, however, rickettsia was not found in the smear of the yolk sacs.

The blood of the guinea pigs inoculated with 20 *H. oenomydis* on the 9th day was passed to the fresh guinea pigs after a week. A rise of temperature was observed in these animals.

The guinea pigs used in these experiments, were killed on the 46th day after inoculation, and were applied to the complement fixation test for the serum.

Results of complement fixation test: The results of the rickettsia specific CFT were summarized in Table 5.

Guinea pigs Nos. 3–8 were inoculated with various lice, which were detached from rats injected with rickettsial suspension 3 days before.

One of the two guinea pigs, which were inoculated with removed *P. spinulosa* showed the marked antibody respons. As stated above, both animals showed no typical rise of body temperature.

The guinea pigs inoculated with *H. oenomydis*, had a similar reaction and only one of the two guinea pigs showed the antibody respons.

In both experiments with *H. akanezumi*, one animal died on the 24th day, but the other produced the rickettsia specific antibody.

TABLE 5

Results of complement fixation test on sera from guinea pigs

Days after inoculation	Gui. No.	Species of lice		10	20	40	80	160	320	640	1280	C
3	3	*P. spinulosa*	Antigen	0	0	0	0	0	0	0	0	0
			C	0	0	0	0	0	0	0	0	0
	4	,,	A	4	4	4	3	1	0	0	0	0
			C	0	0	0	0	0	0	0	0	0
	5	*H. oenomydis*	A	4	4	4	3	1	0	0	0	0
			C	0	0	0	0	0	0	0	0	0
	6	,,	A	0	0	0	0	0	0	0	0	0
			C	0	0	0	0	0	0	0	0	0
	7	*H. akanezumi*	A	4	3	0	0	0	0	0	0	0
			C	0	0	0	0	0	0	0	0	0
9	12	*H. oenomydis* (2nd generation)	A	4	4	3	2	0	0	0	0	0
			C	0	0	0	0	0	0	0	0	0
17	13	*H. oenomydis*	A	0	0	0	0	0	0	0	0	0
			C	0	0	0	0	0	0	0	0	0
	14		A	0	0	0	0	0	0	0	0	0
			C	0	0	0	0	0	0	0	0	0
Complement			A	0	0	0	0	0	0	0	0	0
			C	0	0	0	0	0	0	0	0	0
Standard serum			A	4	4	4	4	3	0	0	0	0
			C	0	0	0	0	0	0	0	0	0

Guinea pigs Nos. 9 and 10 were injected with *H. oenomydis* emulsion, which was detached from rats infected 9 days before. Both showed a marked rise of body temperature. The blood specimens of Nos. 9 and 10 were transfurred to fresh guinea pigs Nos. 11 and 12 and they all showed the characteristic rise of temperature. No. 11 died on the 16th day. The serum of No. 12 was found to contain a sufficient amount of specific antibody, as shown in the Table 5.

In order to see how long infected rats can be the infectious source, *H. oenomydis* were detached 17 days after the inoculation. These lice were injected into guinea pigs No. 13 and No. 14, they showed no typical signs and antibodies were not detected.

Experiment 2.

Transmission of *Rickettsia orientalis*: For the experimental transmission in the 1st experiment all results were negative. Similar experiments were repeated, with the additional use of *H. oenomydis* and *P. spinulosa*. In this experiment, the results were similar to the previous experiment and *H. oenomydis* and *P. spinulosa* were not certified to take any role in the transmission of *R. orientalis* among rats.

Transmission of *Rickettsia mooseri*: As *H. akanezumi* was proved to take the role of a vector of *R. mooseri* among rats in the preceeding experiment, *H. oenomydis* and *P. spinulosa* were used in the 2nd experiment. The specimens of *H. oenomydis* which were removed from the rat on the

TABLE 6

Isolation of rickettsiae from lice reared on the infected rats with rickettsiae

Experiment 2

Agent: *Rickettsia orientalis*
Host: *Apodemus speciosus*
Rattus rattus

Species of lice \ Days after inoculation	4	8	10	18	42
Nos. of *Hoplopleura akanezumi*	100	500		100	100
Isolation of rickettsia	—	—		—	—
Nos. of *Hoplopleura oenomydis*	50		15	10	30
Isolation of rickettsia	—		—	—	—
Nos. of *Polyplax spinulosa*	10		10	10	10
Isolation of rickettsia	—		—	—	—

Agent: *Rickettsia mooseri*
Host: *Rattus rattus*

Species of lice \ Days after inoculation	4	15	22	46
Nos. of *Hoplopleura oenomydis*	10	10	15	30
Isolation of rickettsia	+	+	+	—
Nos. of *Polyplax spinulosa*	10	10	15	30
Isolalion of rickettsia	—	—	—	—

4th, 15th, 22nd and 46th days after the rat was inoculated with rickettsia agent, were emulsified and inoculated in the eggs. Rickettsia were detected in great numbers in all eggs except those isolated from the lice specimens on the 46th day. On the other hand, *P. spinulosa* did not transmit rickettsia as shown in Table 6.

TABLE 7

Isolation of rickettsia from lice reared on the infected rats with rickettsiae

Summary

Agent : *Rickettsia orientalis*

Species of lice \ Days after inoculation	4	7	8	10	12	15	18	21	42
Isolation of rickettsia from *H. akanezumi*	−	−	−	−	−	−	−	−	−
Isolation of rickettsia from *H. oenomydis*	−		−	−			−		−
Isolation of rickettsia from *P. spinulosa*	−		−	−			−		−

Agent : *Rickettsia mooseri*

Species of lice \ Days after inoculation	3	4	9	14	16	22	31	46
Isolation of rickettsia from *H. akanezumi*	+		+		+		−	
Isolation of rickettsia from *H. oenomydis*	+	+		+		+		−
Isolation of rickettsia from *P. spinulosa*	−	−		−		−		−

Agent : *Rickettsia prowazeki*

Species of lice \ Days after inoculation	3	6	17
Isolation of rickettsia from *H. akanezumi*	+		
Isolation of rickettsia from *H. oenomydis*	+	+	−
Isolation of rickettsia from *P. spinulosa*	+		

IV. CONCLUSION

In the present paper, the author has given a great deal of attention to the role of murine lice in the transmission of rickettsia among rats in order to find a solution to the epidemiology of rural rickettsiosis in Japan.

On the epidemiological study of tsutsugamushi disease (scrub typhus), the effects have so far been directed only to the relationship among man, trombiculid mites and small rodents. However, if scrub typhus can be trans-

mitted by murine lice, these lice should be considered as an additional factor to the spread of this disease.

With this consideration in mind, the author, attempted an experimental transmission of *Rickettsia orientalis*, *R. mooseri* and *R. prowazeki*, the most important strains in Japan, applying *Hoplopleura oenomydis*, *Polypolax spinulosa* and *H. akanezumi* which were found in great numbers with wild rats and which were distributed throughout the country.

The species of murine lice appearing in reports hitherto published and used for experimental transmission of rickettsia have not been always classified according to their taxonomical proofs. As an incomplete identification of the lice used in the experiments had given confusing results, special attention was payed to the use of proven and purely reared species in the course of the experiment. The identification of murine lice used in the present experiment was certified prior to and in the course of rearing in the laboratory by the author himself who made studies on the taxonomy of murine lice of Japan as presented in the preceeding papers.

In two repetitions of the experiment with *R. orientalis*, rickettsia was not found in 3 species of lice which attacked and fed on the inoculated rats. The experimental transmission of *R. orientalis* with human lice (*Pediculus humanus corporis*) made by Weyer in 1958 indicated that rickettsia was not detected after the rectal inoculation of agents in the body of the lice and that the lice intraperitoneally injected were proved to harbour the enormous numbers of rickettsia in the hemolymph. The results coincided with the author's suggestion that *Rickettsia orientalis* introduced into the alimentary truct of lice was immediately evacuated into the feces or died without proliferation.

In the experiment with *R. mooseri* combined with three species of murine lice, rickettsia was detected in two species of lice which were isolated from infected rats from 3 to 22 days after inoculation; but none was detected in the lice isolated after 1 month. The species with positive results were two in genus Hoplopleura, *H. akanezumi*, living on *Apodemus speciosus* and *H. oenomydis* living on *Rattus* spp.

The negative result of the transmission with *Polyplax spinulosa* in this experiment differred from the results of the transmissions made by Mooser, Castaneda & Zinsser (1931) and from those made by Kono (1932), in which the isolation of rickettsia from lice was successfully proved using guinea pigs. In the present experiment, however, an attempt was made to isolate the rickettsia from the lice by applying mice and eggs. Of course, if a more suitable animal such as the guinea pig had been used for the isolation of rickettsia from the lice, the results would undoubtedly have been positive.

On the three species of lice fed on the infected rats with *R. prowazeki*, rickettsia was found on the 3rd day after inoculation. The experiment with *H. oenomydis* was extended to last for 17 days. The lice detached from hosts, and emulsified and injected into guinea pigs to isolate rickettsia, were found to carry the agents on the 9th day but none on the 17th day after the injection.

From the results mentioned above, it can be concluded that murine lice are not primary vectors of *Rickettsia orientalis* among rats because no positive detection was found from any kind of murine lice through experimental mice, the most sensitive animal to the *Rickettsia orientalis* infection. On the other hand there is no doubt that murine lice did carry *Rickettsia*

mooseri among rats in the present experiment in which numerous proliferations could be found on the eggs infected with the soaked lice, two species of genus *Hoplopleura*.

In spite of the existence of numerous rickettsia like bodies in the stained specimens of the abdominal parts of the murine lice before use, no rickettsia was isolated from the lice through laboratory mice or eggs in the controled experiment in which the same procedure was used for the isolation as in the experimental transmission.

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