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TRANSMISSION, CONTROL AND TREATMENT OF INFECTIOUS DISEASES OF M--ETC(U)
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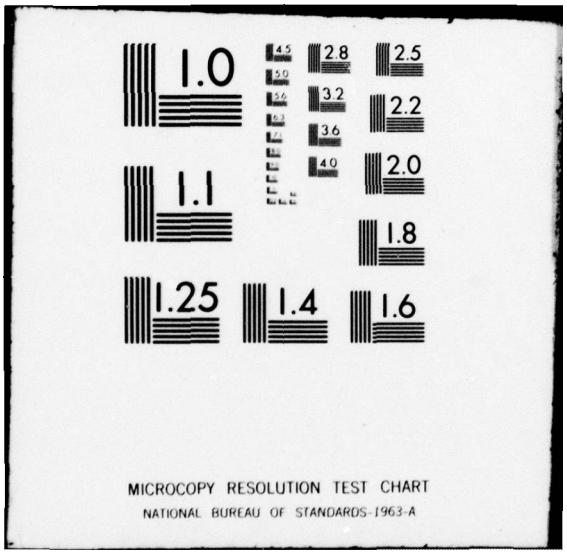
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(6) TRANSMISSION, CONTROL AND TREATMENT OF INFECTIOUS DISEASES OF MILITARY IMPORTANCE IN EQUATORIAL ASIA

(9) ANNUAL REPORT. 1 Oct 74 - 30 Sep 75

Dr. R. Bhagwan Singh
LTC D. L. Huxsoll

1975

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Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D. C. 20314

(11) 1975 (12) 37 p.

(15) Contract No. DAMD17-75-G-9399
Contract No. DAMD17-75-G-9402
(1 October 1974 - 30 September 1975)

U.S. Army Medical Research Unit
Institute for Medical Research
Kuala Lumpur, Malaysia

(16) 3A762759A831 (17) 00

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Transmission, Control and Treatment of Infectious Diseases of Military Importance in Equatorial Asia		5. TYPE OF REPORT & PERIOD COVERED Annual Report 1 October 1974 - 30 Sept. 1975 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) R.B. Singh, D. L. Huxsoll, D. M. Robinson, C. R. Roberts, G. W. Brown, A. L. Dohany, L.W. Roberts, E. Gan, A.H. Sim, G.F. deWitt		8. CONTRACT OR GRANT NUMBER(s) DAMD 17-75-G-9402 ✓ DAMD 17-75-G-9399
9. PERFORMING ORGANIZATION NAME AND ADDRESS Institute for Medical Research ✓ Jalan Pahang Kuala Lumpur, Malaysia		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62759A 3A762759A831.00.101
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Washington, D. C. 20314		12. REPORT DATE 1975 13. NUMBER OF PAGES 33 pages
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

R. tsutsugamushi; Human infections; Laboratory diagnosis; Epidemiology; Ecology; Immunity; Leptotrombidium spp

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

74 10 - 75 09 Local and foreign populations have been found which are sustaining significant infections with R. tsutsugamushi. Rodent hosts of the common vector chiggers have been trapped in the areas where several of the populations are exposed to the disease. Rickettsial isolations have been achieved from humans, rodents, and chiggers; and the strains are being identified. A micro-IFA test which produces equivalent data in a shorter period of time with a saving in reagents has been developed. Passively

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

transferred convalescent sera do not completely protect mice against challenge with homologous scrub typhus strains and are ineffective against heterologous strains. L. L. fletcheri and L. L. arenicola chigger colonies infected with R. tsutsugamushi have been employed to study the effects of the duration of feeding and ambient temperature on transmission of organism. Samples have been shipped to WRAIR to examine the ultrastructure of organism in the vector and the fate of the rickettsia following attempted infections.

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TRANSMISSION, CONTROL AND TREATMENT OF INFECTIOUS DISEASES OF MILITARY IMPORTANCE IN EQUATORIAL ASIA

ANNUAL REPORT

Dr. R. Bhagwan Singh
LTC D. L. Huxsoll

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Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D.C. 20314

Contract No. DAMD17-75-G-9399
Contract No. DAMD17-75-G-9402
(1 October 1974 - 30 September 1975)

U.S. Army Medical Research Unit
Institute for Medical Research
Kuala Lumpur, Malaysia

U.S. ARMY MEDICAL RESEARCH
AND DEVELOPMENT TECHNICAL REPORT

1 October 1974 - 30 September 1975

SUMMARY

Local and foreign populations have been found which are sustaining significant infections with R. tsutsugamushi. Rodent hosts of the common vector chiggers have been trapped in the areas where several of the populations are exposed to the disease. Rickettsial isolations have been achieved from humans, rodents, and chiggers; and the strains are being identified. A micro-IFA test which produces equivalent data in a shorter period of time with a saving in reagents has been developed. Passively transferred convalescent sera do not completely protect mice against challenge with homologous scrub typhus strains and are ineffective against heterologous strains. L. L. fletcheri and L. L. arenicola chigger colonies infected with R. tsutsugamushi have been employed to study the effects of the duration of feeding and ambient temperature on transmission of organism. Samples have been shipped to WRAIR to examine the ultrastructure of organism in the vector and the fate of the rickettsia following attempted infections.

FOREWARD

In conducting the research described in this report, the investigators adhered to the"Guide for the Care and Use of Laboratory Animals, DHEW Publication No. (NIH) 73-23, as prepared by the Institute of Laboratory Animal Resources, National Research Council."

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1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 10 04	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A831	00	101
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Procede with Security Classification Code)* (U) Transmission, control and treatment of infectious diseases of military importance in equatorial Asia

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
010100 Microbiology 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 10	75 09	-	

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: 74 10 EXPIRATION: 75 09
b. NUMBER:* DAMD17-75-G-9399 DAMD17-75-G-9402
c. TYPE: G. Grant d. AMOUNT: $363,000
e. KIND OF AWARD: NEW f. CUM. AMT. $363,000

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	3.0	
	CURRENT 75	3.0	363

19. RESPONSIBLE DOD ORGANIZATION
NAME:* US Army Medical Research & Development Command
ADDRESS:* Washington DC 20314

RESPONSIBLE INDIVIDUAL
NAME: SWANSON, D.R. LTC MC
TELEPHONE: (202)693-7845

21. GENERAL USE

20. PERFORMING ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER: NA

ASSOCIATE INVESTIGATORS
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NAME:

22. KEYWORDS (Procede EACH with Security Classification Code) (U) R. tsutsugamushi; (U) Human infections; (U) Laboratory diagnosis; (U) Epidemiology; (U) Ecology; (U) Immunity; (U) leptotrombidium spp

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23.(U) To investigate the incidence of scrub typhus infection in selected local and foreign populations, to relate this to the prevalence of vector chiggers and their common rodent hosts, to develop improved serological methods, to study the basis for immunity, to define vector-rickettsia relationships, and to produce laboratory animals in support of the mission. Scrub typhus is an incapacitating disease of military importance.

24.(U) Coordinated studies are being conducted at selected field sites in an effort to relate the incidence of scrub typhus in humans to numbers of vector chiggers, rodent hosts, and the strains of R. tsutsugamushi prevalent in each. Standard techniques developed here and in other laboratories are employed in other aspects.

25.(U) 74 10 - 75 09 Local and foreign populations have been found which are sustaining significant infections with R. tsutsugamushi. Rodent hosts of the common vector chiggers have been trapped in the areas where several of the populations are exposed to the disease. Rickettsial isolations have been achieved from humans, rodents, and chiggers; and the strains are being identified. A micro-IFA test which produces equivalent data in a shorter period of time with a saving in reagents has been developed. Passively transferred convalescent sera do not completely protect mice against challenge with homologous scrub typhus strains and are ineffective against heterologous strains. L. L. fletcheri and L. L. arenicola chigger colonies infected with R. tsutsugamushi have been employed to study the effects of the duration of feeding and ambient temperature on transmission of the organism. Samples have been shipped to WRAIR to examine the ultrastructure of organism in the vector and the fate of the rickettsia following attempted infections.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 101, Transmission, Control and Treatment of Infectious Diseases of Military Importance in Equatorial Asia

Investigators:

Principal: Dr. R. Bhagwan Singh

Associate: LTC David L. Huxsoll, VC; LTC David M. Robinson, VC; MAJ Clifford R. Roberts, VC; Maj Grahaem W. Brown, RAMC; MAJ Alexander L. Dohany, MSC; CPT Lyman W. Roberts, MSC; Miss Elsie Gan, B.S.; Mr. Sim Ah Bah, B.S.

1. Epidemiology and Ecology of Rickettsia Tsutsugamushi.

Studies in this unit in the past have added significantly to knowledge of vector chiggers and their rodent hosts. However, with few exceptions, studies on human infections have not be pursued. To completely accomplish the goal of providing sites and means whereby emerging concepts of rickettsial disease can be tested, it became imperative to add studies on the human disease to the research program. Investigations during the past year have included a number of sites and populations which fall into three broad categories: (1) normal populations, (2) febrile patients, and (3) military populations. Although studies at some of the sites have been fully operational for only 2-3 months, our results show that studies on the human disease are feasible.

In-depth studies on the epidemiology and ecology of the disease have been initiated at the various study sites. Studies described below under "Normal Populations" and "Febrile Patients" involve every department in the unit. Further, having identified sites and populations in which studies of the human disease are feasible, field studies on the epidemiology and ecology of the disease have been confined, for the most part to these areas. In addition to yielding important clinical, epidemiological, and ecological information these studies are providing valuable speciments, i.e. sera and isolates, for other studies.

The primary objective of our first year's study was to identify populations in which the incidence of scrub typhus was sufficiently high to warrant detailed studies.

In order to identify infections in which clinical effects may vary from asymptomatic to sever, studies were conducted in three broad population categories:

(1) Normal population
(2) Febrile patients
(3) Military populations

(1) Normal Populations were selected in areas of high endemicity. The groups resided in:

(a) Bukit Lanjan is an Aboriginal village on the jungle fringe near Kuala Lumpur. A population of 250 people live there, and pursue a variety of occupations, both urban and in the jungle.

Capillary blood was collected on filter paper from 186 people in a preliminary survey and screened for antibody at a dilution of 1/50. A total of 81% of the males over 25 and 34% of the total population were positive. In young children, 9% of those up to the age of 9 years were positive; but antibody was detected in several infants under 1 year of age.

The unit has surveyed the small rodents and chiggers in this area over the past several years. See Annual Reports for 1970-1974. As these data were available no additional sub-human work was conducted in the area this year.

(b) Pos Iskandar is the 'administrative center' of a group of Aboriginal villages in a large area of secondary jungle in southern Pahang. It is approximately 80 miles from Kuala Lumpur on the edge of Tasek (Lake) Bera. About 1000 people live in the area, practicing mostly 'slash and burn' shifting cultivation in the surrounding jungle.

Monthly visits are made to the area and samples and data collected. A medical orderly who staffs a small clinic in the area cooperates by keeping a log-book of patients. Multiple sera have been collected from about 350 people, with no difficulty. The sero-positivity rate is 50%, but no sequential results are yet available.

Rodents and chiggers are being collected from the same area, but rickettsial isolation results are not yet complete.

(c) Estate Workers. Elmina Estate is a mixed rubber and oil-palm plantation, about 20 miles west of Kuala Lumpur. There are over 100 workers in each type of plantation, and little interchange takes place. Also, the workers generally have well defined jobs; weeders, harvesters, tappers etc.

A monthly clinico-serological study on the oil-palm group has been started. The hospital assistant who staffs the hospital on the estate assists the collection of clinical data by maintaining a record of all illnesses.

Filter paper spots were collected from rubber workers, and oil-palm workers in a preliminary survey. Fourteen (15%) of the 93 rubber

workers, and 54 (40%) of the 135 oil-palm workers were positive for antibody. None of the rubbers workers had worked previously in oil-palm. A decision was made to continue the survey on only the oil-palm workers and to broaden the scope of the study to include the small rodent and chigger populations.

The trapping of rodents was confined to an area containing fully mature oil-palm trees of about 12 years old. The area, covering 12,800 sq. meters, was divided in a grid system incorporating 200 trapping points spaced out at intervals of 8 meters on an east-west axis and 8 meters on a north-south axis. Trapping of field rats began in December 1974 and will continue for a full 12-month period.

Trapping is being carried out for a full 7 days the first week of each month. On the first 5 days, the captured rats are marked and released the following day to the original point of capture. Rats captured on the last two days are sacrificed. Before the marked rat is released it is examined for ectoparasites, a blood sample drawn to detect rickettsemia, and a filter paper spot prepared for assay of R. tsutsugamushi antibody. The sacrificed animals are processed similarly with the addition of examination of blood and spleen/kidney pools for the presence of rickettsia.

The fluctuation in monthly catches (Table 1) are probably due to physical factors such as rainfall and temperature in the study area. An estimation of the population of the rats can be computed by using the "Lincoln Index" based on the monthly mark-release and recapture. (See Table 1).

Table 1. Estimated population of R. tiomanicus as the Elmina Estate study site.

Month	No. of rats marked	No. of rats recaptured	Total	Estimated population
December 1974	68	4	72	455
January 1975	65	8	73	112
February	37	4	41	132
March	26	8	34	78
April	49	2	51	223
May	68	12	80	373

Chiggers have been collected from these rodents, as well as at 22 established black plating sites within the trapping area. Ten black plates are checked at each site, giving a total of 220 plates from which a relative index of the chigger population can be determined.

only 2 species have been identified in large numbers. The scrub typhus vector chigger L. (L.) deliense represented 68.5% of the total 6394 chiggers identified from Rattus tiomanicus jalorensis, while Ascoschongastia (L) indica totaled 26.6%. The other four species identified to date, in exceedingly small numbers, were: Gahrliepia (Walchia) lewthwaiti (3.9), A. (L.) lorius (0.6), Walchiella impar (0.1%) and L. (L.) fletcheri (0.3%).

R. tsutsugamushi has been isolated from whole blood, tissue pools, or both from several rats collected early in the experiment. Other specimens have been stored and will be processed as time permits.

(2) Febrile Patients

(a) Bukit Mendi Clinic

Bukit Mendi is a FELDA(Federal Land Development Authority), government managed, worker's cooperative. It is located in central Pahang, lying in the middle of a triangle formed by Karak, Temerloh and Kuala Pilah, just south of Kemasul Forest Reserve. The jungle is cleared in stages, and oil-palm is planted. All stages currently co-exist, from mature plantation, to newly-felled but not burned jungle, to undisturbed jungle itself. The total population is approximately 10,000.

Of the 46 cases of scrub typhus reported to the Ministry of Health in 1974, 6 were in settlers on this scheme, and a further 5 came from neighboring oil-palm schemes. (Personal communication, Director of Medical and Health Services, Pahang).

Medical care is furneshed to the settlers by a clinic located on the FELDA scheme and staffed by a hospital assistant and nurses and visited weekly by a doctor. Cases requiring hospitalization are sent to the 2 District Hospitals , at Kuala Pilah and Mentakab.

All patients attending the clinic with a history of fever, cough, headache, or any general malaise (deliberately loosely defined), are entered in the study. Venous blood is drawn, mice immediately inoculated intraperitoneally, and a further whole blood specimen is placed in liquid nitrogen as a back up sample for the isolation attempts. The serum is decanted from the remainder of the blood sample, divided into two aliquots, and frozen.

The patients then receive treatment, as usual, from the health center staff and are asked to return in 2 weeks for a further specimen of blood to be taken. If they do not appear, the technician visits the house to obtain the specimen. Only serum is collected on the second occasion. Cooperation is excellent.

We have collected 93 pairs of sera in the first 9 weeks of this study. When screened at a 1/25 dilution both sera of a pair were negative in 20 individuals (215%); 51 (54,8%) were positive without a significant

rise in titer. In 16 (17.2%) of the pairs, there was a 4-fold or greater rise in titer. In a further 6 (6.5%) there was a rise to positive a 1/50 from negative at the screening dilution. None of these people had eschars or noticeable rashes.

From the 22 patients whose titers rose significantly, we have 8 presumptive isolates.

Recently a field team was sent to Bukit Mendi to collect small rodents and chiggers in areas where humans had been working prior to becoming ill. A total of 47 small rodents were collected and isolation, serological, and chigger infestation results are pending.

(b) Hospitals

Clinical data, plus pairs of sera are collected from febrile patients in anumber of hospitals. As far as is possible, the patients are unselected, i.e., they are not excluded by a diagnosis such as malaria.

(i) Mentakab Hospital is 80 miles from Kuala Lumpur and 40 miles from our study site at Bukit Mendi. It is in an area containing many oil-palm development schemes. One of our technicians collects the sera, and inoculates mice from all febrile patients. Of the first 50 patients studies 28% had a four-fold or greater rise in antibody, and we have 5 presumptive isolates to date.

(ii) Kuala Pilah Hospital is about 60 miles from Kuala Lumpur, and lies south of Bukit Mendi. Together with Mentakab, it provides the hospital care for a large area of Central Malaysia, and for all of our potential patients at Bukit Mendi. The hospital staff collect the sera for us, but are only able (due to pressure of work) to inoculate mice from selected patients.

Data have been collected from 195 patients in the first 10 weeks of the study at Kuala Pilah Hospital. In 8 (4.1%) pairs of sera, there is a 4-fold or greater rise in titer. One of these patients had "classical" scrub typhus, with an eschar, rash and adenopathy. In a further 17 (8.7%) pairs, there was a rise from negative at 1/25 to positive at 1/50.

(3) Military Personnel

(a) Non-indigenous soldiers

We are investigating the incidence of infection with R. tsutsugamushi in soldiers operating in Malaysia, as opposed to the incidence of "classical" scrub typhus.

British and New Zealand soldiers training in jungle areas in Johore for relatively short periods comprise the study groups. Each man completes a questionnaire with personal details and history of previous exposure. Blood is drawn for serology prior to and after

exposure. Blood from selected groups of soldiers are also inoculated into mice for attempted isolation of rickettsia. Medical records of all positives are examined, and most positives are recalled for interview about any symptoms.

Multiple samples at various time intervals have been obtained from about 900 troops. These were screened by the F.A. technique for evidence of infection with R. tsutsugamushi.

In the first group of 375 men, sera were collected from them on arrival in Singapore and at the end of a 6 week period of training in Johore. Twenty-three (6.1%) of the second sera were positive, and only 1 of the corresponding first sera was positive. Thus 22 (5.9%) developed antibody during their training. The presence of antibody in the first specimen from the other man is unexplained as he denied any previous service in endemic areas. Both his sera had antibody at 1/50 dilution to both Karp and Gilliam antigens.

In the second group, of 50 men, three sera were obtained from each man prior to exposure, immediately after 5 weeks of jungle training, and again 4 weeks after the second bleeding. At the time of the second bleeding, pairs of mice were inoculated with each man's blood. A total of 5 (10%) of this group developed antibody during training. Results of the mouse inoculation are not yet available.

A total of 28 (6.6%) of the whole 425 thus developed antibody. Nine (32.1%) of these men admitted some form of illness - cough, fever, malaise, etc. Forty-seven (12%) of the 397 with no antibody admitted similar illnesses.

Pairs of sera were also collected from 264 British and New Zealand soldiers stationed in Singapore, before and after a particular jungle exercise. These men had all been exposed several times in the previous year. A total of 32 (12.1%) of the soldiers had positive sera. Of these, 4 (1.5%) had 4-fold rise over the period of observation. An additional 14 (5.3%) showed a rise from less than 1/25 to 1/50. None of the 32 men had a history resembling "classical" scrub typhus from the questionnaire, medical documents or interview data.

(b) Indigenous soldiers

Sera and clinical data have also been obtained from 280 Malaysian soldiers, at the beginning and end of their recruit training (6 months).

Two battalions of Malaysian soldiers, consisting of 1000 men were bled prior to 3-4 months of jungle operations on the Thai border. A second specimen will be collected following the operation.

No results will be available until the second sera are drawn, and the sera pairs studied by the P A test.

Trans-placental Passage of Antibody/Infection

R. tsutsugamushi can persist in tissues for years following infection; and transplacental infection of foetuses, as measured by raised IgM levels, occurs in Q fever. Therefore we are collecting mother/cord pairs of sera from a group with a high incidence of scrub typhus antibody - Aborigines at Gombak Hospital.

The sera will be examined for IgG and IgM levels using commercial Mancini plates (Behring Co.). Sera will be tested for IgM and IgG specific fluorescent antibody. We thus hope to demonstrate the presence or absence of transplacental infection or passage of maternal antibody. Preliminary results show that a number of the cord specimens are positive when screened against polyvalent antigens, using labeled anti whole human globulin.

From results obtained during the past year we wfeel that several populations have been identified in which the incidence of scrub typhus is sufficiently large to warrant detailed studies.

2. Adaption of a Micro-indirect Immunofluorescence Test to the Detection of R. tsutsugamushi Antibody.

Indirect immunofluorescence (IFA) has been employed to detect antibody conversions (1) and to study the maximum response to the Karp, Kato and Gilliam strains as well as the temporal relationships in the development and persistence of the antibodies (2). In both cases the reaction was specific, reproducible, and more sensitive than the CF tests. In those cases where the infecting strain was known the homologous antibody appeared more rapidly, attained a higher titer, and persisted for a much longer period of time.

A microimmunofluorescence (micro-IF) test has been applied to the study of antibody responses to Chlamydia trachomatis infections in humans (3). This test proved to be specific and in general the infecting strain antigen could be precisely determined (4). We have adapted the micro-If test to a study of antibody produced as a result of natural human R. tsutsugamushi infection to determine its feasibility and reproducibility in dectecting the response to the major and minor antigens present in R. tsutsugamushi strains.

Antigens were prepared by inoculating eggs by the yolk sac route with 0.1 ml of a 10^{-1} or 10^{-2} dilution of the seed which was stored as a 20% yolk sac suspension in sucrose phosphate glutamate buffer (SPG) (5). Yolk sacs were harvested from the live eggs when about 20% of the inoculated eggs were dead. Following freezing and thawing the yolk sacs were diluted to a 20% suspension with 0.01N phosphate buffered saline (PBS) ph 7.6 and disrupted by treating the chilled suspension in an ice bath with an Omni mixer (Ivan Sorvall, Inc., Newton, Connecticut) for 4 intervals of 15 sec each at 25,000 rpm. These intervals were interspersed with 30 sec cooling periods. The

suspension was centrifuged at 450 x g for 10 min. The middle layer was removed with a syringe and needle, and serial 2 fold dilutions of this preparation were examined by means of the micro-IF test employing high titer human sera. The proper concentration was determined and the suspension was diluted with PBS, dispensed with 0.1 ml amounts and stored at -169C.

The slides were prepared with 3 rows of antigen spots. Each row contained 6 groups of spots, and each group was composed of 9 individual spots. Thus each slide contained 162 separate antigen spots. The spots were applied by positioning the slide over the template and touching the spot with a pen nib (Kingsley 2788; Hinks, Wells and Co., England) filled with antigen. The volume of the spot delivered was approximately 0.1 lambda. When the spots were complete the slides were dried at least 30 min at room temperature prior to an 10 min fixation in acetone. The fixed slides were allowed to dry at room temperature and stored at -20C for not longer than two weeks.

The antigen slides were warmed to room temperature in circulating air to remove condensate. Each group of 9 microspots was covered with 5 lambda of the appropriate dilution of a serum. The slides were incubated in a humidified cabinet at 35C for 30 min. Following removal of the bulk of the sera by washing with PBS, the slides were immersed in PBS on a magnetic mixer for 10 min. They were then blotted dry and 5 lambda of the appropriate dilution of conjugated rabbit origin anti-human globulin was placed over each group of spots. During the application of both the test sera and the conjugated antiserum care was taken to ensure no mixing occurred between the groups of spots. Following incubation, washing, and drying as described for the test sera the slides were mounted with 25 lambda of 90% glycerin with 10% 0.1N PBS pH 9.4 and a 22 x 50 mm cover slip. The slides were usually read within 24 hr although they could be stored several days at 4C without affecting the results. The criteria of Gan et al.(6) were employed to determine endpoints.

Table 2 presents the data derived when a number of sera were simultaneously studied at a 1/50 dilution with the IFA and the micro-IF. While the two methods were in agreement on 151 of the 179 samples (85%) there were obvious discrepancies. Although they detected approximately equal numbers of positive sera these did not completely coincide. The IFA detected antibody in 12 sera which were negative in the micro-IF screen while the micro-IF detected antibody in 16 sera which were negative in the IFA. When these 28 sera were retested many results were at variance with the original test. To examine a possible reason for these discrepancies the sera were titrated beginning at a 1/25 dilution. All 28 of the sera were positive at the low dilution, but the reaction at a 1/50 dilution was variable and none of the sera were positive at the higher dilutions.

Table 2. Correlation between the indirect microimmunofluorescence test and the indirect immunofluorescence test when employed as screening tests.

	IFA		
Micro-IFA	Positive	Negative	Total
Positive	68 (38)	16 (9)	84 (47)
Negative	12 (7)	83 (47)	95 (53)
Total	80 (45)	99 (56)	179

Comparison of titers

Figure 1 compares the titers obtained from representative sera by the two techniques. This figure combines both IFA data derived from 2 fold dilutions as well as 4 fold dilutions recommended by Bozeman and Elisberg (1). Only 8 of 126 sera (7%) had titers that differed $\geq$ 4-fold in the two systems. Repeat titrations of these sera in the micro-IFA system yielded titers within 1 dilution of the previous micro-IFA titer rather than the commonly lower value seen with the IFA technique. The proportion of the sera which differed $\leq$ 1 dilution in the two systems was 78% (98/126).

A comparison of the values indicated that while 58% of the titers were higher in the micro-IF test only 21% were lower. The remaining 21% of the sera gave equivalent titers in the two systems. A serum which titered 1/40 in the old system commonly titered 1/50 in the micro-IF; a serum which titered 1/160 in the old commonly 1/200 in the micro-IF. If these sera are not included in the totals as being higher in the micro-If 29% of the sera gave higher titers in the micro-IF test and 21% gave lower titers ($P > 0.05$).

A series of 149 sera were examined by the micro-IF on two occasions two months apart. The titer was the same in 61% (91/149) of the tests, varied from $\geq$ 1 to $\leq$ 2 fold in 36% (53/149), and was 4 fold different in 3% (5/149). In no case were the results greater than 4 fold different.

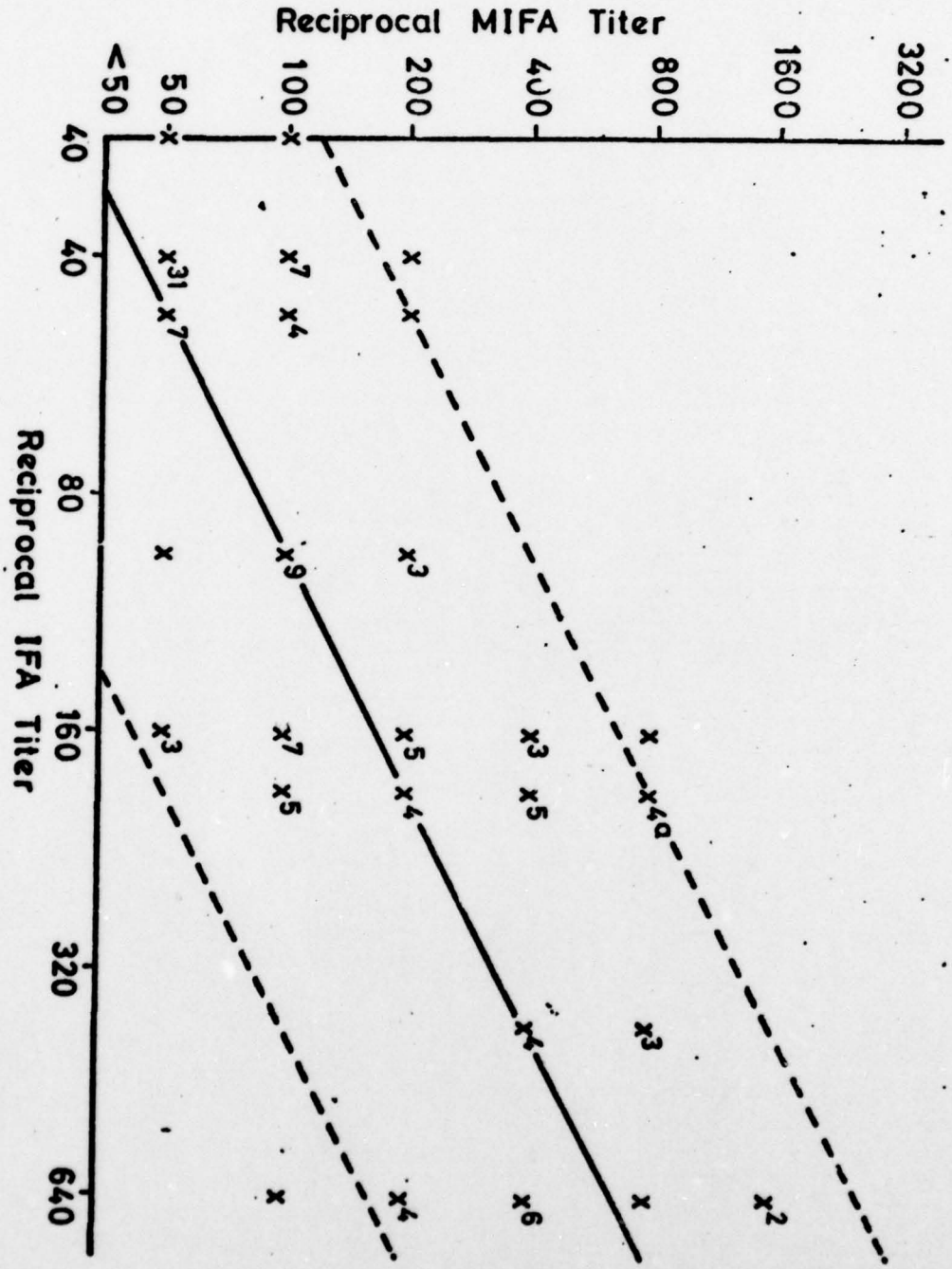
The variation between the procedures occurred in sera containing low titers of antibody; and while endpoints seldom varied more than 1 dilution, the presence of a high proportion of sera with low titers led to greater variation in screening tests. The variation was as prevalent in sera tested repeated by the same system, as when the repeat assay employed the other system. This difference was, therefore, inherent in some groups of sera and little could be done to minimize it. In any case, the variation tended to even itself out yielding an equal proportion of positives in each test. In sera collected from older individuals who lived in hyperendemic areas in which the positive sera generally titered $\geq$ 1/100 no discrepancies were noted between the systems.

Fig. 1 - Comparison of the titers obtained when sera were tested by the microimmunofluorescence test and the immunofluorescence test.

_____ line of equivalent titers; _ _ _ line ± a 4 fold titer from equivalence.

a; 3 sera titered 1/800, one serum titered 1/400 on retest with the micro-IF test.

Figure 1


Reciprocal MIFA Titer
<50
50
100
200
400
800
1600
3200
Reciprocal IFA Titer
40
80
160
320
640

Titers appear somewhat higher in the micro-IF test than in the original system, but since the old system yielded titers of 1/40, 1/160, 1/640, etc., and the new system 1/50, 1/100, 1/200, etc., the number of sera which gave higher titers in the micro-IF test did not differ significantly ($P > 0.05$) from the number which gave lower titers.

Titers derived from repeat titrations in the micro-IF test corresponded with each other more closely than the 93% correspondence within the $\pm < 4$ fold limits which was found with the micro-IF test was compared to the regular IF test. This indicated that the results from the new system were at least as reliable as those from the old system. Although tedious it was possible for a technician to prepare 40 slides a day which was enough to screed 680 sera with controls at a single dilution. This was also sufficient to titer 240 sera through 1/200 or 120 sera through 1/1,600 against any 9 strains. Since sera from certain areas and populations ha d varying peak titers which were a relatively constant property we were able to choose dilutions to encompass these endpoints without wasting reagents.

3. Protection Against Scrub Typhus Infection Engendered by the Passive Transfer of Immune Sera.

The inability of passive antibodies to protect mice has led us to initiate a series of investigations into the mechanisms of protection operable in the resistance to challenge following recovery from infection. Since R. tsutsugamushi represents a mosaic of strains with varying antigens and virulences, we decided to initially define the contribution of humoral antibody to resistance to challenge with both homologous and heterologous strains.

Sera were prepared by the intraperitoneal (IP) inoculation of 100-1,000 median infectious doses for mice (MID_{50}) of the specific strain. The mice were bled by cardiac puncture from 30-60 days following inoculation. Infection was controlled in those strains lethal for mice by adding 2.5 mg of chloramphenicol per ml of drinking water from day 3 to day 24 post inoculation. Sera pools represented no fewer than 50 mice and were inactivated at 56C for 30 min prior to inoculation. All sera were from mice convalescent from 1^{o} infections, and no attempt was made to produce hyperimmune sera.

Since all strains of R. tsutsugamushi were not equally efficacious in stimulating antibody production we titrated the antisera against the several antigens present on the selected strains. The results in Table 1 show that the highest homologous titer was produced by the Karp strain at 1/1,280 while the TA 763 and TC 586 strain were 2 fold lower. A significant reciprocal cross reaction was seen with the Karp and TA 763 strains. The TC 586 strain did not cross with either of the other two strains.

Table 3. Titers of antisera employed

Antigen	Antisera		
	Karp	TA 763	TC 586
Karp	1280[a]	160	< 10
TA 763	320	640	< 10
TC 586	< 10	< 10	640

[a] Reciprocal indirect fluorescent antibody titer.

Figure 2 presents the results obtained when predetermined volumes of convalescent Karp strain sera were inoculated subcutaneously (SQ) 1 hr prior to intraperitoneal (IP) challenge with the homologous strain. Although significant protection ($P < 0.025$) was conferred by the administration of the sera, significant differences ($P > 0.05$) were not detected among the doses within the 4 fold range of volumes of immune sera inoculated. This was true even though the higher volumes of sera prolonged the survival times of the mice. The largest dose, 1 ml of whole immune sera, was capable of protecting only 18% (7 of 40) of the inoculated mice.

To investigate the result of varying the challenge dose a group of mice were inoculated with graded doses of the Karp strain following the inoculation of 0.5 ml of homologous convalescent sera. The results of this experiment are shown in Figure 3. An inoculated dose of $10^{3.4}$ median lethal doses (MLD_{50}) was also employed, but since the values fell between the other two doses they are not shown. The number of survivors was not significantly different ($P > 0.05$) with the three doses employed in the challenge. The smallest challenge dose (250 MLD_{50}) did not kill 100% of the non-passively immunized controls.

To investigate the temporal relationships involved in the transfer of immune sera we inoculated 0.5 ml of Karp immune sera SQ into groups of mice from 2 days before challenge with the Karp strain until 8 days post challenge at 2 day intervals. In those groups which received sera on days -2,0,+2, and +4 from 15 to 26% of the mice survived and no significant differences were detected ($P > 0.05$), but in the groups given sera on days 6 and 8 no mice survived and no differences in death times were noted from the control group given normal sera.

Since we had been successful in protecting a portion of the mice against challenge with a mouse virulent strain by the transfer of immune sera we investigated the response of passively immunized mice

Captions

Fig. 2 - Response of mice to Karp challenge following the administration of graded doses of homologous convalescent sera.

▲ no sera, △ 0.5 ml normal sera, ▲ 0.25 ml convalescent sera, △ 0.5 ml convalescent sera, 1.0 ml convalescent sera.

Fig. 3 - The effect of varying the challenge level on the survival of mice following the administration of normal and convalescent sera.

△---△ normal sera following by $10^{4.4}$ MID_{50} challenge, ▲---▲ immune sera with $10^{4.4}$ MID_{50} challenge, △——△ normal sera with $10^{2.4}$ MID_{50} challenge, ▲——▲ immune sera with 10^{24} MID_{50} challenge.

Figure 2

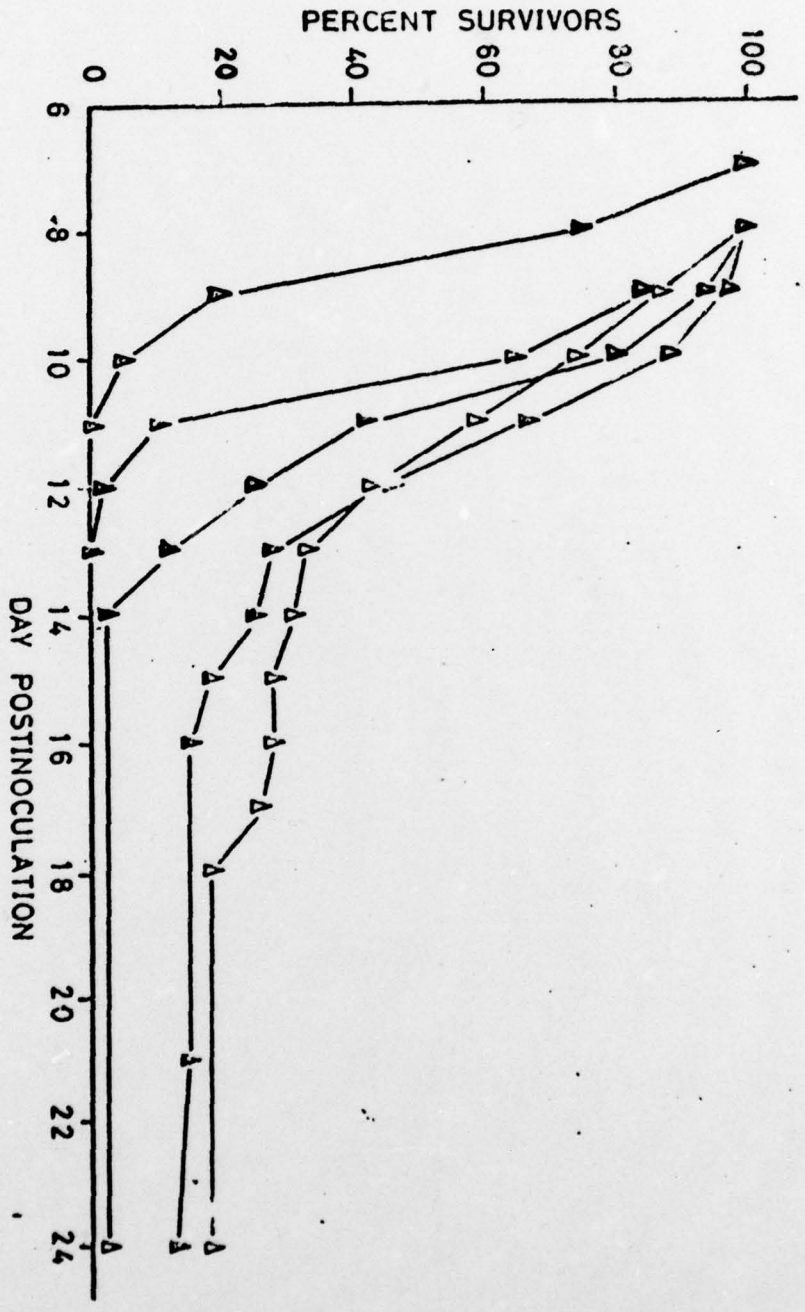
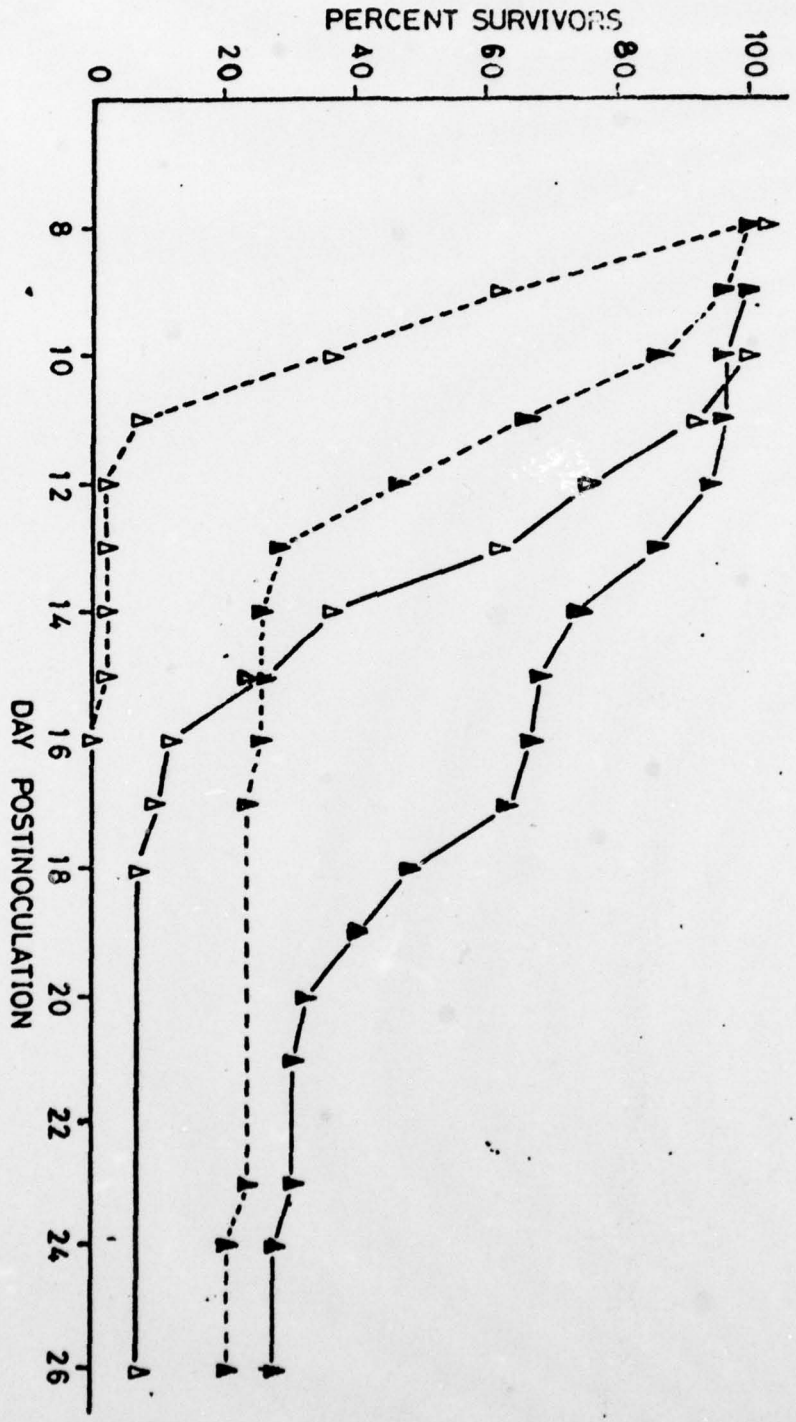

PERCENT SURVIVORS
0
20
40
60
80
100
DAY POSTINOCULATION
6
8
10
12
14
16
18
20
22
24

Figure 3


PERCENT SURVIVORS
0
20
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10
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14
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22
24
26
DAY POSTINOCULATION

to heterologous as well as homologous challenger. The results of these experiments are presented in Table 4. Earlier a challenge dose of 63,000 MID_{50} of the TC 586 strain had killed 85% of the control mice which had been given 0.5 ml of normal mouse sera. A challenge dose of 630 MID_{50} killed 76% of the mice, and survival times were 3-4 days longer. However, the death rate of mice given normal sera again paralleled that of mice given immune sera and no significant differences ($P > 0.05$) could be detected in the survival rates or times. For this reason the data presented were derived from challenge doses of $10^{2.4}$ to $10^{2.8}$ MID_{50} depending on the experiment. Other serial 10 fold doses were employed, but since the data were comparable they are not shown.

Table 4. Response of passively immunized mice to homologous and heterologous challenges.

Challenge strain	Convalescent Sera			
	Karp	TA 763	TC 586	Normal
Karp	5/40[a]	4/40	0/40	0/40
TA 763	1/40	6/40	2/40	1/40
TC 586	4/40	6/40	4/40	6/40

[a]Survivors/total

Karp and TA 763 immune sera were equally effective against Karp challenge. This could be expected because of their close relationship in the fluorescent antibody test (Table 3). TC 586 sera was not effective against either Karp or TA 763 challenge. Again this could be proposed from the lack of relationship in the FA test. However, the complete lack of protection when compared to control values in the homologous TC 586 challenge was surprising. While 10% of theindividuals did not die following administration of TC 586 immune sera and homologous challenge this was no different than the proportions following administration of normal sera followed by challenge or challenge alone. The failure of Karp immune sera to protect against TA 763 challenge also remains unexplained.

A group of mice were inoculated with 0.2 ml containing $10^{2.9}$ LD_{50} of the Karp strain which had been incubated for 30 min at room temperature with 0.5 ml of immune sera. Controls were reacted under the same conditions with normal sera. All of the control mice were dead by day 11 postinoculation, but 85% of the mice inoculated with rickettsia treated with immune sera in vitro were alive at the termination of the experiment on day 28 postinoculation.

Rickets (7) reported that immunity to spotted fever infections could be transferred by cell free sera. Gambril and Wisseman (8) found

that the passive transfer of immune sera from a hyperimmune human was capable of protecting mice from a normally lethal dose of R. mooseri; but neutralization of R. rickettsia or R. australis by convalescent sera could not be demonstrated in a cell culture system (9). These conflicting results were explained by the requirement of coating of the rickettsia with specific antibody as a prerequisite for destruction of the organism following phagocytosis.

In our system the antibody was not capable of preventing death in the majority of individual mice when it was administered passively. However, when the sera were preincubated with the rickettsia the majority of the mice survived inoculation of the sera/organism mixture. The existence of antigen-antibody complexes in vivo can be demonstrated in scrub typhus infections by specific staining of rickettsia in mouse peritoneal fluid with fluorescein isothiocyanate labelled antimouse globulin. The presence of such complexes, persistent rickettsemias in the presence of circulating antibody, and the inability of passively transferred antibody to protect the majority of mice indicates that humoral antibody may not be important in determining the fate of R. tsutsugamushi in vivo. The transfer of peritoneal fluid containing organisms which are coated with antibody results in a course of illness in the recipient indistinguishable from the course produced by the inoculation of equivalent doses of rickettsia of egg yolk sac or cell culture origin. Additionally, sera administered as late as 4 days after challenge was as effective in protecting mice as sera administered simultaneously. These two observations would seem to diminish the importance of coating with antibody to the in vivo fate of R.tsutsugamushi.

The increased protection found when the immune sera was incubated with the microorganism prior to inoculation is unexplained. Several possibilities exist. Kenyon and McManus (9) were not above to demonstrate neutralization of R. rickettsii or R. australis in cell culture employing immune sera alone. However, when a standard amount of anti-globulin was added a decrease in the number of plaques was found with increasing immune sera concentrations. It would appear that we detected neutralization by immune sera alone, but we did not conduct parallel titrations in egg yolk sacs. Therefore, we can't comment on the physical state of the organisms. It is possible that immune sera produces aggregation of the rickettsia and that each aggregate is handled as an ifectious unit which would effectively decrease the titer. Rickettsial agglutination tests have been employed to quantify antibody for several years (10). It is also possible that inoculated rickettsia promptly enter cells and escape the complete effects of being coated with immune sera.

The immune mechanisms operable in the recovery from R. tsutsugamushi infections appear to be analogous to those in intracellular bacteria e.g., tuberculosis, leprosy, tularemia, etc., rather than in typhus organisms where passively administered immune sera is protective and immunity is complete and long lasting. Whether this points to

different evolutionary processed within the rickettsia is a matter of speculation.

4. Colonization of Trombiculid Mites.

The Acarology Section is continuing to maintain colonies of vector and nonvector mites. The infected colony of L. (L.) fletcheri is currently in the 17th and 18th generations, while the colony of infected L. (L.) arenicola is in the 6th generation.

Rapmund (11) presented a detailed study of the first 5 generation of the L. (L.) fletcheri colony, and its progress has been reported in previous USAMBU-M Annual Reports. Much of the rearing after the 5th generation was conducted in pools, with individuals being studied only in the 9th and 10th generations. In the 11th generation, three lines (11158, 11203, and 11408) were established from a single adult (10885) of the 10th generation. Pools are obtained from a common pool of larvae collected from all the adults of the preceding generation. Of 153 pools tested through the 16th generation, 94.8% were positive for Rickettsia tsutsugamushi. In the 17th and 18th generations, negative pools became apparent. Currently, comprehensive data are available only on line 11203, although, to date, tests from line 11408 have produced only negative results. In addition to the change in the rate of infection within the colony, the sex ratio (previously 100% females) has also changed. Table 5 presents the infectiveness and sex ratio data for the 18th generation of line 11203. Of 27 pools obtained from a common egg pool, 26 were positive and one was negative. Of 46 pools derived from single adults, 10 were positive and 36 were negative. The sex ratio of 16 of the 26 positive pools from the common egg pool was determined, as well as the one negative from the same pool. The positive pools contained 163 females and 36 males (81.9% female), while the negative pool contained 8 females and 9 males (47.1% female). Of the pools derived from single adults, two positive and 18 negative pools were available for sex determination. The two positive pools produced 14 females and no males (100% females), while the 18 negative pools produced 85 females and 87 males (49.4% females). The reason for the change within this colony is not known at the present time; however, intensive studies have been initiated in an effort to determine the cause.

Table 5. The infectivity and sex ratio of the 18th generation (Line 11203) of a Rickettsia tsutsugamushi-infected colony of L. (L.) fletcheri.

Transmission during feeding	Total No. of Pools	No. of Pools Sexed	Sex Ratio (♀/♂)	Percent (♀/♂)
		Common Egg Pool		
+	26	16	163/36	81.9/18.1
-	1	1	8/9	47.1/52.9
		Individual Adults		
+	10	2	14/0	100.0/0.0
-	36	18	85/87	49.4/50.6

The filial infection and transovarial rates of the fifth generation of the infected L. (L.) arenicola colony were similar to those reported in the past. 23 F_4 adults were separated for study of their progeny. Approximately 20 larvae from each line were studied. Of 21 lines in which information is currently available, 353 of a total 366 were positive, giving a filial transmission rate of 96.4 percent. All 21 adults transmitte Rickettsia tsutsugamushi transovarially.

Part of the objective of the Acarology section is to gather comprehensive data on the life histories and rickettsial transmission of the three proven vectors in Malaysia; therefore, it is important that an infected L. (L.) deliense colony be obtained. As was reported in the USAMRU-M 1974 Annual Report, infection rates of specific rodents were high at Bukit Lanjan, Selangor, and the only vector collected from that area during previous studies was L. (L.) deliense. Thus, it seemed likely that the infection rates of collections of L. (L.) deliense would also be high. However, this has not been the case. In addition to the negative results reported in the previous annual report, an additional 38 pools, totalling 1,423 chiggers, were fed on white mice. Of these, only 5 were positive. However, due to loss during pool feeding and cannibalism within the postlarval stages, a positive colony was not established from any of these pools.

As reported in the USAMRU-M 1974 Annual Report, over 2,500 specimens of L. (L.) scutellare were collected from a banana grove in the Cameron Highlands, Pahang, in June, 1974. The final mouse passage results showed that, of the 44 pools having a total of 2,200 chiggers that were fed on white mice, no positive pools were obtained. This data corresponds with collections of L. (L.) scutellare previously made by this unit.

5. Duration of Vector Attachment and Transmission of Rickettsia tsutsugamushi to Mice.

Although chiggers usually feed to repletion on their animal hosts, they do not normally do this on humans. Abrasive action of clothing and personal hygiene tend to interrupt their feeding. Even so, humans do contract scrub typhus. Recently, this was demonstrated when two members of the British Army contracted scrub typhus after investigating a helicopter crash in Mersing, Johor, West Malaysia. Both individuals left the crash site each night, giving a hypothetical maximum time between attachment and an evening's bath of 12 hours. It seems apparent that full engorgement is not necessary for transmission.

In an effort to determine the length of attachment necessary for a chigger to transmit scrub typhus, a series of interrupted feedings was conducted with varying lengths of feeding time. Chiggers were allowed to attach to white mice and then were disengaged by gently manipulating them with a sharpened applicator stick. Table 6 presents the data of interrupted feedings of infected chiggers of both L. (L.) fletcheri and L. (L.) arenicola.

Table 6. Duration ofattachment and rate of transmission of Rickettsia tsutsugamushi to mites by two species of Leprotrombidium larvae.

Hours	L. (L.) fletcheri Mouse Feeding		L. (L.) arenicola Mouse Feeding	
	Positive Total	Percent Transmission	Positive Total	Percent Transmission
1	0/5	0	0/5	0
6	0/5	0	0/5	0
12	10/15	67	1/15	7
16	5/10	50	3/10	30
18	15/15	100	-	-
20	7/10	70	6/10	60
24	19/30	63	12/15	80
28	6/10	60	8/10	80
30	13/15	87	-	-
36	10/15	67	-	-
Repletion	7/10	70	10/10	100

6. Effects of Temperature on Rickettsia tsutsugamushi Infections in Naturally Infected Chiggers.

Positive larvae of L. (L.) fletcheri when treated at 40°C for 72 hours did not transmit rickettsia to white mice during feeding (USAMRU-M 1974 Annual Report). Extreme temperatures are known to have both a direct and an indirect effect upon the transmission of numerous disease organisms. Temperatures play a direct role in the incubation

times of arbovirus and in the interaction of blood clotting and infection with the plague bacillus in fleas. Temperatures are indirectly involved in the transmission of diseases by affecting the physiology of vectors, either by extending or decreasing the life cycle or by causing estivation or hibernation to occur. Jameson (12,13) has studied the temperature-development relationships of several Japanese trombiculids, L. (L.) deliense from Malaysia, and Eutrombicula belkini from California. In general, within normal limits, developmental times decreased with an increase in temperature.

Infected pools of L. (L.) fletcheri adults were maintained in controlled environmental chambers of constant temperatures of 22° and 40°C for a period of 30 days or more. Thirty larvae from each temperature were fed singly on mice. At both temperatures, the larvae, when fed on mice, were negative for R. tsutsugamushi.

Larvae of L. (L.) arenicola were maintained at both 22° and 40°C for 24 and 72 hours after which they were fed singly on laboratory mice. In the 22°C experiment, 16 larvae were held for 24 hours and 10 for 72 hours, while in the 40°C experiment 15 larvae were held 24 hours and 13 were held for 72 hours. In all cases, the larvae transmitted the infection during feeding.

As negatives had begun appearing in the infected L. (L.) fletcheri colony, adults of both L. (L.) fletcheri and L. (L.) arenicola were separated from positive pools and were tested for the infectivity. Only known infected adults were used to initiate these temperature experiments. Several temperature treatments have been started; but, as of this report, results are not available. The treatments for both L. (L.) fletcheri and L. (L.) arenicola include: egg-laying adults treated at both 22° and 40°C for over one month; eggs and egg-deutoval stages treated at both 22°and 40°C for the duration of these stages; and additional larval treatments at each temperature. Treatment of post larval stages is planned. Bionomic studies, including developmental rates and egg production, of the temperature-treated mites are also being undertaken.

7. Ultrastructure of Rickettsia tsutsugamushi in Chigger Cells and the Fate of Rickettsiae Taken Up by Previously Uninfected Chiggers.

In an effort to study the ultrastructure of Rickettsia tsutsugamushi in chigger cells and the fate of rickettsiae taken up by previously uninfected chiggers, a cooperative study between the USAMRU-M Acarology, Rickettsia Sections and the WRAIR Division of Pathology has been instigated. USAMRU-M will supply both whole and dissected specimens of variously treated and untreated chiggers, and known positive and known negative specimens of both L. (L.) fletcheri and L. (L.) arenicola. WRAIR Division of Pathology will be responsible for studies utilizing the electron transmission microscope.

The specimens listed below have been fixed and shipped in gluteraldehyde to WRAIR for embedding and study. Additional specimens are being prepared by fixation and embedding at USAMRU-M and will be shipped to WRAIR.

	unfed larvae	engorged larvae	protonymphs	deutonymphs	tritonymphs	adults
L. (L.) fletcheri known positives	20	--	20	20	20	20
L. (L.) fletcheri known negatives	10	8	--	10	10	10
L. (L.) fletcheri Attempted Infections	--	30	20	20	--	--
L. (L.) arenicola Attempted Infections	--	20	22	20	20	11

In addition to the whole specimens listed, specimens of L. (L.) arenicola were dissected and organs and tissues were sent to WRAIR for embedding and study. Twenty specimens, all of larval and post-larval stages, were dissected. The material included:

(a) unfed larvae -- salivary glands, mid-gut
(b) engorged larvae -- salivary glands, mid-gut
(c) protonymph, deutonymphs, tritonymphs, adult -- salivary glands, mid-gut, epidermis, ovaries, excretory tubules.

8. Management of Tropical Laboratory Animal Resources.

The major effort of the Department of Laboratory Animal Medicine is the production and care of ICR outbred mice used in scrub typhus research. Production is a joint project with IMR, and personnel, equipment, and supervisory responsibility are shared. Approximately 90,000 four week old mice were issued to investigators in FY 75, of which 75,000 were issued to USAMRU for scrub typhus related projects. The average USAMRU experimental mouse is kept 30 days, thus, our average experimental mouse population was in excess of 6,000.

Although all of the old mouse cages in the production colony have now been replaced with new cages, the experimental colony still requires approximately 600 new cages.

Serum samples from the mouse production colony were sent to Microbiological Associates in the United States in August 1974, for testing for antibody to 14 indigenous murine viruses. REO III antibody was found in 10% of the samples; all other tests were negative.

Dogs are now being used in small numbers (20-30). They are purchased as puppies to reduce the possibility of natural exposure to selected infectious agents. No spontaneous disease problems have occurred, but some difficulty in getting the dogs to eat ordinary dog food has been encountered. We now require that the dogs be accustomed to dry dog food before purchase.

Project 3A762759A831 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 101, Transmission, Control and Treatment of Infectious Diseases of Military Importance in Equatorial Asia

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