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

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ANNUAL REPORT

Dr. R. Bhagwan Singh
COL Charles R. Webb, Jr.

1973

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(1 October 1972 to 30 September 1973)

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

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ANNUAL REPORT,
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Dr. R. Bhagwan Singh
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US ARMY MEDICAL RESEARCH
AND DEVELOPMENT TECHNICAL REPORT

1 October 1972 - 30 September 1973

SUMMARY

INVESTIGATIONS OF THE DEPARTMENT OF ACAROLOGY

Chiggers and host blood samples on filter paper were collected from 6 areas of North Sumatra, Indonesia. *Leptotrombidium deliense* was the only known vector of scrub typhus found in these areas. Only 13 of 214 mammalian hosts sampled were positive for scrub typhus antibody.

Rattus argentiventer had significantly ($P < .001$) greater numbers of *L. deliense* chiggers per rat than did *Rattus tiomanicus jalorensis*. There was no apparent difference between numbers of chiggers on rats from grassland and those from edge (scrub) habitats. Both host species appeared to be highly efficient as hosts for *L. deliense*. Fluctuations in chigger numbers on these hosts appeared to be correlated with dew-point temperature.

A naturally infected colony of *L. arenicola* was established. The rate of transovarial transmission of *Rickettsia tsutsugamushi* was 100 percent over 3 generations as shown by single feedings of chiggers on mice. The rickettsial strain was found to have Karp and TA 763 as major - and Kato as a minor antigenic component. A partially engorged infected chigger was shown to be capable of transmitting scrub typhus during refeeding on a laboratory mouse. Infection in a *L. arenicola* male was demonstrated.

Rates of spermatophore production by *Leptotrombidium* males were determined. Initial spermatophore deposition occurred an average of 4 days after adult emergence. The average number of spermatophores per day has been 7.9 for *L. arenicola*, 13.0 for *L. deliense* and 11.8 for *L. fletcheri*. Females of one *Leptotrombidium* species did not take up spermatophores from a related species.

Sex ratios of progeny were determined in infected and noninfected colonies of *L. arenicola* and *L. fletcheri*. Infected chiggers produced female offspring almost exclusively, and it appeared that female production and *R. tsutsugamushi* in the chigger are related. Noninfected chiggers had an average ratio of about 2.5:1 (females:male) and even lines producing only females in one generation did not continue to produce females exclusively in the subsequent generation.

Rats of the *R. rajah* group were infested with significantly fewer chiggers than other species in initial field tests. Experimental feedings showed that fewer *L. deliense* chiggers fed successfully on *R. surifer* than on *R. annandalei*. The potential involvement of gamasoid mites in scrub typhus transmission to spiny furred rats was suggested.

INVESTIGATIONS OF THE DEPARTMENT OF BACTERIAL DISEASES

The department continues to provide routine diagnostic support as part of the medical care of U.S. staff and their dependents and locally engaged staff. Support is also afforded to the Aborigine Hospital at Gombak in the form of one technician on detached duty.

The staff of four technicians, only one of whom has received any formal training, was augmented by the appointment, on August 7, of a B.S. degree holder in microbiology. The department remains physically small, occupying one partitioned room (18' x 21').

Much of the year was spent in collecting field material for a reappraisal of the incidence of *Ps pseudomallei* and *Chromobacterium violaceum* in rice fields. Collaborative work in serology and in the antigenic structure of *Ps pseudomallei* has been established with the Department of Viral and Rickettsial Diseases. The results of the rice field survey indicate a significant reduction in incidence of *Ps pseudomallei* from former years, most likely due to ecologic change; antibody incidence closely parallels recovery rates of the organism, titers of 1:20 or even 1:10 being considered as evidence of prior exposure in the population of an endemic area. The incidence of *Chromobacterium violaceum* is nine-fold greater than that of *Ps pseudomallei*.

Good support from the Department of Laboratory Animal Resources has been received for the above work and in the development of the Silvered Leaf-Monkey (SLM) as a model for melioidosis. The results of this latter project show that disease in the SLM reflects the human disease in all its various forms.

The work on leeches of genus *Hirudinaria*, as vectors of *Ps pseudomallei*, has not produced any final conclusion. Tentatively it can be stated that the annelid and the bacterium can co-exist in the same environment but that, in the environment of the leech gut, *Ps pseudomallei* seems incapable of multiplication: this despite, or perhaps because of, the fact that the normal inhabitant of the leech gut is of the same bacterial family. This work is not complete.

Continuing work on a serologic test for *Chromobacterium violaceum* is proposed. Bacterial skin disease in troops has been found to be a major problem in Vietnam and currently poses a problem of morbidity in troops of Australian, New Zealand and United Kingdom (ANZUK) origin in Singapore. This forms the basis for the proposed comparative studies of Malaysian and ANZUK troops during the next fiscal year.

INVESTIGATIONS OF THE DEPARTMENT OF ECOLOGY

Studies of vertical distribution of mammals and their parasites and pathogens within the rainforest with the use of canopy transect walkways are in progress. In this area over 2000 animals have already been captured marked and released. Arboreal species here and elsewhere do not become infected with *Rickettsia tsutsugamushi*, however, blood parasites such as *Plasmodium* and *Hepatocystis* are more common in arboreal than in terrestrial hosts. Large differences in the rates of infections with malarial parasites occur within species of arboreal hosts which seem to be correlated with the habitat types of populations. Nests of arboreal mammals are rich with populations of mesostigmatid mites and other parasites. *Rickettsia tsutsugamushi* in terrestrial mammals appears to be most frequent in forest and lalang grass *(Imperata cylindrica)* habitats. Detailed studies using enclosures in different habitats to test sentinel animals are in progress.

In areas surveyed in Sabah, and especially Sarawak, the rates of transmission of *Rickettsia* seemed to be lower than in Peninsular Malaysia. In arbovirus studies in conjunction with the forest canopy transect walkways, 5 isolates (from 3 arboreal and 2 terrestrial host species) have been made from 823 mammals tested. Serological results are available for 201 samples, with 3 HI positives for Group B arboviruses. Studies of population dynamics have shown that some arboreal species in the primary forest have extremely slow population turnover, infrequent breeding cycles with periods of as long as 17 months with no reproduction at all, and small litters. Data for man-altered habitats is available but not yet analyzed. Several taxonomic and systematic studies remain to be written up.

INVESTIGATIONS OF THE DEPARTMENT OF MEDICAL ENTOMOLOGY

Mosquito surveys in conjunction with chloroquine resistance studies have been conducted at five rubber estates in Pahang and Negri Sembilan States, and in the Kuala Brang area of Trengganu. Collections of anophelines have been low at all rubber estates. *Anopheles maculatus*, the presumptive vector of malaria has been collected at all estates. One oocyst-positive *An. maculatus* was found at Paroi estate. In Trengganu, *An. aconitus* was the predominant anopheline collected. In this area the vector situation is very vague and it would be difficult to incriminate any single species at this time.

A total of 30551 mosquitoes were identified, then pooled and screened for arboviruses. From 323 pools, 11 were found positive. The positive isolates will be sent to the U.S. Army Component, SEATO Laboratory, Bangkok, Thailand for further typing.

Other studies in progress include: mosquito surveys in a Federal Land Development Authority (FLDA) scheme to determine the changes in species over a period of years, with special emphasis on

the introduction of *Aedes aegypti* in such an area. A study of *An. balabacensis* in Southeast Asia and its relation,if any, to chloroquine resistant malaria.

Future plans include experiments on Ultra Low Volume (ULV) ground aerosol spray evaluations against mosquito populations and possibly malaria incidence. In conjunction with these experiments mosquito life history data will be collected and analyzed and mosquito collection techniques evaluated.

INVESTIGATIONS OF LABORATORY ANIMAL RESOURCES

New cages have been provided for the hamster, rat, suckling mouse, and 25% of the mouse breeding colonies.

Studies have been completed which show the locally produced animal chow to be deficient in ascorbic acid for guinea pigs and in vitamin A for breeding hamsters. The feed processor has been requested to revise his formulations to provide an adequate ration.

Studies have been partially completed to determine growth rate curves for the species bred in this laboratory.

An accounting system has been devised to give the cost of laboratory animals produced in Malaysia. Use of the system should allow more accurate budgeting for laboratory animal expenses.

Redesign of the proposed new animal facility has been accomplished, and construction is now scheduled to begin in January, 1974.

INVESTIGATIONS OF THE DEPARTMENT OF PARASITOLOGY

The major effort of this department during the reporting period has been geographical studies of chloroquine resistant *Plasmodium falciparum* and simultaneous searches for evidence of resistant *P. vivax.* Previous field studies in Peninsular Malaysia and Thailand have employed a 7-day follow-up, WHO-recommended in vivo assessment method, and occasionally an in vitro technique. Little resistance had been demonstrated in Malaysia, however past experience here has indicated that the great majority of what resistance there is is the late recrudescing R I type, which would have been missed by follow-up limited to 7 days. We undertook a 28-day follow-up, in vivo, in situ assessment with provisions for estimating the attack rate for new *P. falciparum* infection. The resistance rates found in the studies completed to date have been quite different from past estimates and have been as high as 50%.

The department was invited by Dr. A.N. Lewis and Dr. J.T. Ponnampalam (University of California ICMR and Department of Malaria Research,respectively of the Institute for Medical Research, Kuala Lumpur) to participate in analyzing the results of

chloroquine suppression in two groups of control subjects in a prophylaxis study of a different drug. The groups were on either weekly chloroquine suppression or on placebo. In the study area where at least 85% of the *P. falciparum* infections responded to chloroquine treatment, chloroquine suppression appeared completely ineffective against this parasite. However it still protected against *P. vivax*.

Studies on chloroquine resistance are in progress in North Sumatra, Indonesia, in collaboration with Dr. Kwo Eh Hoa, University of North Sumatra, Medan, Professor (Dr.) J. Sulianti Saroso, National Institute for Medical Research, Jakarta, and Capt. P.F.D. Van Peenan of NAMRU-2, Jakarta, Indonesia.

A preliminary visit was made to Sabah (North Borneo), Malaysia, to assess the feasibility of a chloroquine resistance study there in areas where resistance is strongly suspected. This participation was as a member of an Institute for Medical Research Team, requested by the Director of Medical Services, Sabah.

Long term monthly blood surveillance with treatment of parasitemias has been underway on a rubber estate in Peninsular Malaysia to determine 1) the seasonality of *P. falciparum* and *P. vivax* incidence in this area; 2) whether a 20% malaria rate (point prevalence) at any given time actually means that closer to 100% of the people are infected at some time during the year; 3) whether there is a sub-group who are repeatedly infected (and to demograph such a group) or whether new infections occur throughout the entire group; 4) malaria histories on a group of people as background for subsequent chloroquine resistance studies on these subjects. This work is in conjunction with the USAMRU-M Department of Medical Entomology, who are conducting parallel entomological surveillance and in collaboration with Dr. D.R. O'Holohan (Kelinik O'Holohan, Seremban).

Miscellaneous studies in progress, completed, or discontinued during the reporting year include modification of qualitative urine tests for chloroquine, comparison of the relative merits of several described urine tests under field conditions, modification of *in vitro* malaria culture techniques for testing of drug resistance, analysis of data collected on filariasis during an assignment at the Institute for Medical Research prior to Active Duty at USAMRU-M, and compiling a review of certain filariasis studies in Southeast Asia.

INVESTIGATIONS OF THE DEPARTMENT OF VIRAL AND RICKETTSIAL DISEASES

The program of the department was divided into three broad areas viz.:

1. support of projects designed and initiated in other departments,
2. combined projects conducted with one or more other departments and
3. projects conducted wholely within the department.

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The support of projects of other departments consists of assay of sera or filter paper blood spots for antibody to scrub typhus and isolation of rickettsia from specimens of blood, tissue or vectors. Generally, the specimens for isolation (except for vectors) were harvested by a member of this department. Standard techniques described in prior annual reports (1971; 136-139) were employed for serology, isolation, and identification. Projects of this nature have included the vertical and horizontal distribution of scrub typhus in common Malaysia habitats and support of the chigger colony. Filter paper blood spots submitted from Thailand and Indonesia are also in this category, as is technical support to the HAA project of the IMR.

Combined projects initiated with other departments have included the study of the incidence of arbovirus infection in a circumscribed area (Department of Medical Entomology and Department of Parasitology). Rodents, mosquito pools, and human sera have been processed by suckling mouse inoculation and yielded isolates. These isolates have not been definitively identified, but both group A and B arboviruses are represented. (See Department of Medical Entomology Sec.

A study to prepare a purified *Pseudomonas pseudomallei* antigen has been initiated with the Department of Bacteriology. Precipitating antigens have been prepared by three different methods. All contain protein, but differ at least in molecular size. The physical, chemical and immunological relationships of these fractions will be studied during the next year.

The identification of mosquito blood meals was conducted with the Department of Medical Entomology. The project was initiated to determine human feeding rates which could be an aid in explaining the spread of mosquito borne infections. The technique which evolved was based on polyacrylamide gel electrophoresis of the macerated whole mosquito. Results indicate that the technique has advantages over those presently in use.

Projects conducted wholely within the department include the fractionation of purified *Rickettsia tsutsugamushi*. Attempts to adapt the organism to growth in locally available subtrates (duck eggs, cell lines, and primary cell cultures) are continuing. The production of cell cultures free of antibiotics, which is required for rickettsial growth, will be enhanced by laminar flow hoods (on order). Several strains have been serially passed in duck egg yolk sacs and are exhibiting sufficient growth for the yolk sacs to be used in purification procedures. Future work will be centered on DEAE absorption of contaminants followed by ether extraction and/or molecular sieve column chromotography.

The response of silvered leaf-monkey (SLM) to various strains and doses of *R. tsutsugamushi* will be continued. Results indicate that clinical signs are mild following inoculation of single strains compared to multiple strain inoculations (Annual Reports 1971, and 1972). Future work will elucidate the specific immunoglobulin

response of virgin and experienced monkeys to challenge with a spectrum of strains. Challenges in experienced monkeys will be chosen to include homologous, related, and unrelated strains as assayed by fluorescent antibody techniques. Of particular interest will be the response of experienced SLM to challenge strains unrelated to the originally inoculated strain.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Principles of Laboratory Animal Care as established by the National Society for Medical Research."

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Commander	-	COL Charles R. Webb, Jr., MC
Clinical Pathologist	-	LTC James R. Donaldson, RAMC
Medical Officer	-	MAJ Timothy J. Dondero, Jr., MC
Veterinary Officers	-	MAJ David M. Robinson, VC
		MAJ Clifford R. Roberts, VC
Entomologists	-	MAJ Ray E. Parsons, MSC
		CPT Lyman W. Roberts, MSC
Ecologist	-	MAJ Illar Muul, MSC
Laboratory Officer	-	Miss Elsie Gan, B.A. (Serologist)
Adjutant	-	CPT Craig R. Lewis, MSC

INTRODUCTION

This Annual Progress Report has been altered in format for the simple purpose of making it easier to prepare. Instead requiring each research department chief to compose a special report, we have merely adapted the reports of completed (published or publishable) studies and the protocols of research in progress (data added). In some cases it has been appropriate to add a section which could be entitled the "Journal of Negative Results", for the purpose of setting on record information which might alert others to our less profitable undertakings. "Plans for the Future" is a fourth section for those departments with plans for significant changes in emphasis or direction.

During this year we have dealt with problems of currency devaluation as well as inflation within our host country. We have attempted to improve upon chronic administrative problem situations and to anticipate and avoid problems that threaten to trouble our successors. Separate reports will deal with those efforts.

The unique opportunities for communicable disease research in Malaysia have been potentiated for us by the consultive and material assistance rendered to us by the Director of the Malaysian Institute for Medical Research, Dr. R. Bhagwan Singh, and his staff. We are particularly grateful to Dr. Lim Teong Wah, Mr. Lim Boo Liat, Mr. M. Nadchatram, and Mr. Cheong Weng Hooi.

During this past year we have continued the efforts of our predecessors to encourage jointly prepared protocols, joint research and mutually supportive research. The coming years would seem to offer unlimited opportunities for mutually valuable research in tropical communicable disease.

Chas R. Webb Jr

CHARLES R. WEBB, Jr.
Colonel, MC
Commander

RESEARCH GOALS AND ASSOCIATED PROJECTS

FY 1973

I. To correlate the distribution of mammalian hosts and potential hosts of zoonotic pathogens and their parasites and selected pathogens (*Angiostrongylus cantonensis*, *Rickettsia tsutsugamushi*, various arboviruses, including tick-borne viruses, and other zoonotic agents appropriate for study), to develop predictive capabilities to anticipate hazards to humans in particular ecological situations.

Objectives

A. *Angiostrongylus*:

1) to determine the prevalence of this parasite in rats which are pests of oil palm estates (one study completed in Layang Layang, Johore another in progress at Elmina Estate, Selangor).

2) to determine the general prevalence of this parasite in mammals collected.

B. *Rickettsia tsutsugamushi*:

1) to determine the vertical distribution of *Rickettsia* in forests, (complete, except for new areas such as Sumatra).

2) to determine the habitat distribution of *Rickettsia*; studies in progress in four habitat types at Bukit Lanjan, other studies to be proposed e.g. in Thailand, Sumatra, etc.

C. Arbovirus studies: to determine the vertical distribution of selected arboviruses in mammals (study in progress at Bukit Lanjan, Selangor in conjunction with Lim Teong Wah, another study in conjunction with Dave Robinson proposed at the new canopy transect).

D. Tick-borne viruses: to determine the vertical distribution of tick-borne viruses (proposed studies at the proposed new transect).

E. Plague: (proposed) to study in conjunction with Lim Teong Wah and Dan Cavanaugh the possibility of highland foci of undetected, strictly enzootic plague (as highland populations build up if such foci are present, epidemics may result (e.g. as is the case in Bojolali, Indonesia).

F. General: to determine the distribution of various mammals and ascertain which taxa fill given ecological niches which may serve the requirements of various zoonotic pathogens.

II. To correlate aspects of ecological niches of hosts and potential hosts of zoonotic pathogens with the prevalence of zoonotic infections, and to determine which species among the hundreds known in various areas could be significant in the enzootic or epizootic cycles of zoonotic pathogens.

Projects:

A. Vertical distribution:

1) to determine the vertical distribution of host and potential hostsspecies of mammals with the use of the canopy transect systems (old & proposed new one).

2) to determine the vertical distribution of various zoonotic pathogens and parasites in arboreal compared with terrestrial hosts.

B. Temporal distribution: to determine the correlation between presence of parasites (e.g. *Plasmodium)* and periodicity in activity of mammals (one aspect nearly finished: the distribution of *Plasmodium* and other blood parasites in selected canopy mammals).

C. Feeding habits: to determine the correlation between the presence of internal parasites (such as *Angiostrongylus, Capillaria,* etc) and diets of mammals. Stomach contents of mammals are being collected and will be analyzed.

D. Nesting habits: to determine the correlation between the nesting habits of various mammals with the presence of ectoparasites, particularly vectors of scrub typhus. (Proposed studies with Nadchatram).

III. To correlate aspects of population dynamics of hosts and potential hosts of zoonotic pathogens with their degree of importance in transmission cycles, to ascertain when and under what circumstances susceptible individuals are most abundant. Selected species of mammals will be used as prototypes for developing methodology applicable to applied epidemiological studies.

A. Population dynamics: to determine the dynamics of population size and age structure to correlate infections with age cohorts in populations.

B. Seasonality of reproduction: to determine temporal aspects of the influx of new (susceptible) individuals into populations (Some studies already complete (Johore & Selangor) and others in progress).

C. Reproductive Frequency & Litter Size: to determine the potential of various species to produce new (susceptible) individuals; to obtain measurements of population turnover. (All specimens collected are examined to obtain pertinent data).

D. Longevity: to determine life spans and life expectancies of species (this is related to 3A - the determination of population age structure). This type of information is important in evaluating the potential of various species for perpetuating infections with zoonotic pathogens (data for this comes largely from the mark & release studies at Bt. Lanjan, Bt. Mandol, and more will come from the new proposed studies in Johore).

IV. To determine the nature of seasonal phenomena in tropical ecosystems which may be correlated with fluctuations in enzootic and epizootic infections, particularly scrub typhus.

Projects:

A. Seasonal patterns of mammalian reproductions. (Data from all collections).

B. Phenological studies: to determine availability of food to host and potential host species of mammals (data from Bt. Lanjan).

V. To develop species association indices. Such indices would provide predictability in applied epidemiological surveys of overall species diversity on the basis of statistics derived from small samples, employing a single collecting technique. (This will be done on the basis of large amounts of data already collected on statistical associations of species in various types of habitats).

VI. To describe and evaluate zoogeographic boundaries in terms of their relationships to disease distribution. Such boundaries, whether on a faunistic level or sub-specific level provide insight into limits of host and parasite distributions; they also suggest the nature and position of barriers to dispersal of species. This would give epidemiologists convenient putative boundaries for checking the spread of diseases, in that control measures would be chosen to be consonent with environmental barriers.

VII. To determine what ecological factors can be measured in order to evaluate the potential for disease endemicity and epidemicity in various areas within the Sunda Region. Periodic field trips to various places within the region facilitate accumulation of data for comparisons with the baseline of data on hand from West Malaysia (to see if principles derived there have wider applicability).

VIII. To determine the systematic, taxonomic, and zoogeographic status of various mammalian hosts and potential hosts in order to ascertain the species, the discreet functional genetic units, and to relate them to similar forms in the region (extensive museum work has already been completed, several papers need only to be written up, other data are ready for collation and other museum studies are planned).

DEPARTMENT OF PARASITOLOGY

Chloroquine Resistant Malaria

I. To delineate regions of high resistance and of low resistance. This may provide opportunity to study the mechanisms of development, transmission, geographical migration, and vector associations of drug resistant strains.

Objective

A. To establish the presence and extent of chloroquine resistant falciparum malaria in various areas of Malaysia by means of modified WHO 28 day in vivo testing in Trengganu (completed), Johore, Pahang, Sabah and possibly in Sumatra (Indonesia) and elsewhere in the Malay Peninsula. All studies will be coordinated as far as feasible and appropriate with concurrent entomologic studies of vectors active near the study sites. All studies will in so far as is possible be coordinated with similar studies conducted by USAMC-S.

II. To determine whether drug "resistance" or"sensitivity" of falciparum parasites with reduced drug sensitivity as assessed in vivo is affected by the immune state of the host.

Objective

A. To determine in people exposured to malaria over variable periods of time, whether host experience may contribute to apparent drug resistance or sensitivity in the parasite. The effect of host age, duration of malarial infection, previous experience with malaria, measurable antibody will also be assessed.

III. To establish optimal methods to assess body fluids for the presence of chloroquine, suitable for large-scale usage under field conditions.

Objectives

A. To compare established techniques for urine analysis for chloroquine to determine the best simple qualitative test for use in the field and the best, most sensitive quantitative method for use in the laboratory.

B. To modify old and develop new procedures for analysis of chloroquine in the urine.

C. To develop a micro-method for quantitative analysis for chloroquine in serum or plasma (blood obtained from finger prick).

IV. To assess whether the addition of pyrimethamine actually improves the suppressive or therapeutic qualities of chloroquine in chloroquine resistant (R-I to R-II) malaria. (low priority).

Objective

A. Insert Darachlor into chloroquine resistance studies and/or **Fansidar** prophylaxis studies (VIDE INFRA).

V. To develop a means to assess intrinsic drug - resistance/sensitivity in malaria parasites.

Objective

A. Compare in vivo (WHO) methods and in vitro methods (Rieckmann; Diggs) of measuring drug resistance.

Micro-epidemiology of Malaria

VI. To identify and demograph any "malaria-prone" segment of a population chronically experiencing malaria and to compare such a sub-group to the rest of that population. This would be done in coordination with entomological studies of vectors and transmission.

Objective

A. To follow an entire estate population with monthly smears (and treatment of patent parasitemias) for 18-24 months, determining and recording age, sex, occupation, activity and behavior relating to malaria exposure, individual attractiveness to mosquitoes, and previous illness by history.

Prophylaxis

VII. To continue work designed to evaluate the efficacy of Fansidar as an antimalarial chemoprophylactic drug.

Objectives

A. To extend the Fansidar study (Lewis and Ponnampalam) by employing once-monthly Fansidar on two geographically distant rubber estates surveying for parasitemia at 60-day or 90 day intervals.

In vitro Culture of Intrahepatic Stages of Malaria Parasites

VIII. To devise means of maintaining living monkey liver cells as a means for in vitro culture of malaria parasites.

Objectives

A. To confirm and extend preliminary experiments by Cadigan and Kyser in which explanted monkey liver tissue seemed to support the primary exoerythrocytin stage of *P. cynomolgi*.

B. To determine whether the radiobiochemical indicators of liver parenchymal metabolism are valid.

DEPARTMENT OF MEDICAL ENTOMOLOGY

I. To identify mosquito vector variables that may relate to a gradient in chloroquine resistance over the length of the Malay Peninsula, if such a gradient does exist.

Objectives

A. To measure the prevalence of putative and potential vector species and to establish whether these are, or may be vectors. (Studies to be made in conjunction with chloroquine resistance studies in order to detect whether differential vector prevalence may be associated with variations in chloroquine resistance.

B. To determine the total mosquito species complex in each area studied and to determine whether vector species prevalence of chloroquine resistance.

C. To relate changes, if any, in mosquito species prevalence to spray programs.

II. To determine the relation of vector species to malaria case incidence (transmission).

Objectives

A. To determine whether location of malaria cases may be related to vector species prevalence - as measured by spot collections in the village chosen.

B. To determine which malaria collection techniques are the most appropriate for studies of malaria transmission.

C. To determine the validity of observations indicating that malaria cases may occur in large numbers while putative vectors are present only in low concentrations.

D. To determine whether the transmission of malaria depends primarily on a single vector species or on different species at different times of the year.

E. To determine the most expeditious and practical means to accumulate vector prevalence data.

F. To determine whether local village people can be trained to do field collections of mosquitoes, under varied amounts of supervision.

G. To determine whether variations in total mosquito populations may affect malaria vector densities and malaria transmission.

H. To determine whether total mosquito populations can be related to the effectiveness of malaria vectors feeding on humans.

I. To determine if there is a competitive or other interrelation between species, including anophelines and culicines that may effect vector densities on total mosquito densities.

J. To determine whether malaria transmission may be related quantitatively to the prevalence of vector species.

III. Studies of *Anopheles b. balabacensis* in South East Asia.

To determine the importance of *An. b. balabacensis* in the transmission of malaria in Malaysia.

Objectives

A. To determine the geographical boundaries of this mosquito in Malaysia.

B. To determine whether the local species is an efficient vector of malaria.

C. To determine if *An. b. balabacensis* may actually be a complex of similar species; and to determine whether taxonomic differences may be found to exist between various strains of these mosquitoes.

D. To determine whether locally collected strains will cross with known vector strains and produce vector offspring.

E. To determine what types of mosquito control are effective in suppressing this species.

IV. To determine the effect of Federal Land Development Activities on the mosquito fauna of areas involved; and to relate these effects to the phenomena of malaria transmission.

Objectives

A. To measure the effect of land development on species variations and prevalence in the area under development.

B. To relate changes in mosquito populations to malaria rates.

C. To determine the nature and effects of various mosquito control measures being used by villagers.

V. To determine if insecticides, alone, applied by aerial or ground application equipment can control malaria transmission. (An island or an isolated estate would be used for the evaluation).

Objectives

A. To determine by random samples prior to and after spraying the malaria incidence in the area (a parasitology project).

B. To determine by pre-spraying and post-spraying surveys, the vector densities in the area.

C. To determine if such a method would be feasible for areas other than islands or estates.

VI. Arthropod-Borne Virus Studies

To do virus isolates (in conjunction with Virology) on samples of mosquitoes collected during malaria and/or general mosquito studies; and determine by laboratory techniques the species and distribution of viruses in West Malaysia.

Objectives

A. To relate specific viruses to mosquito species.

B. To determine the arthropod-borne viruses in malarious areas.

C. To relate mosquito bionomics to specific viruses.

VII. To collect and classify the fleas of Malaysia.

Objective

To determine the host-flea relationships from animals trapped (by Ecology) throughout Malaysia.

I. Hepatitis with Dr. Kamath

To define the distribution of known antigenic types of hepatitis B virus in Malayan populations.

Interim Requirements:

purify pools of ad and ay antigen

produce antisera to these pools

adsorb & determine purity of antisera

tag antigen with ^{125}I by chloramine T method

utilize these reagents to test sera from acute and chronic patients, blood donors, and *Orang Asli.*

Objectives

A. To characterize the distribution of known serotypes in Malaysia and compare it to distributions reported in Singapore, Thailand and the U.S.

B. To determine whether the w and r antigens found in antigens from Thailand occur in Malaysia.

C. To determine whether the RIA procedure can detect more cases of ab or antigenemia than currently employed assays, without producing false positives.

II. Scrub Typhus Vector Studies with Dr. L. Roberts

To determine why scrub typhus is rarely found in vectors caught in the wild when the infected *L. akamushi* colony produces nearly 100% infected offspring.

Objectives

A. To establish additional infected vector strains.

B. To evaluate the phenomena involved in "curing" and then reinfecting the present infected strain.

C. To evaluate potential vectors by feeding on uncommon hosts.

D. To evaluate the effects of feeding infected vectors on immune hosts.

III. Silvered Leaf-monkey Studies

To support development of a scrub typhus vaccine. The priority of this work is directly related to WRAIR's persuit or non-persuit of a vaccine.

Objectives

A. To infect monkeys with dilutions of single strains of *R. tsutsugamushi* and compare response to that published in man.

B. To determine the role of delayed hypersensitivity to *R. tsutsugamushi*, in relation to protection (with MAJ Donaldson).

C. To determine the sequence of IgM and IgG production after initial inoculation and subsequent challenge with closely and distantly related strains.

IV. To determine, by modern molecular methods, the occurrence of the antigens prevalent in *R. tsutsugamushi*.

Approaches:

Grow organism in silvered leaf-monkey kidney cell culture

Purify by pelleting and subsequent equilibrium density gradient centrifugation in Spinco

To fractionate antigens by SDS or other chemical fractionation (remove intact nucleic acid?)

To separate fractions on polyacrylanide gel

Objectives

A. To determine whether the antigens measured by FA all that are present or actually only represent surface antigens.

B. To determine if surface antigens are covering other antigens common to several strains.

V. Transect Support with Drs. Parsons, Roberts, Muul and Lim.

To describe completely the occurrence of specific diseases in an area, on the basis of the prevalences of the micro-organisms, the vectors, the susceptible hosts, and the amplifiers if they can be identified.

Approach:

determine antibody status of mammals

determine infection rate of the vector

detect "silent" carriers

determine what strain or strains are active in the area.

VI. Isolate and identify arthropod-borne disease agents. If this identification can't be completed locally the strain will be sent to Dr. Shope at Yale Arbovirus Research Unit.

Objectives

A. To determine if the tick typhus reported from Malaysia is caused by a strain related to Indian Tick Typhus strains.

B. To coordinate isolation of agents from ticks with comparable work at SEATO.

DEPARTMENT OF ACAROLOGY

I. To describe the bionomics, behavior and ecology of vector mites and to relate these factors to scrub typhus transmission.

Specific Field Oriented Studies

A. To determine effects of host conditions, weather and seasonality on *Leptotrombidium (L.) deliense* and other chiggers:

1) To determine relative numbers of chiggers on the ground and on selected rodents at Bukit Lanjan (coordinated with Department of Ecology).

2) To determine relative numbers of chiggers on semiarboreal and ground mammals from an undisturbed primary forest habitat (Gombak Transect Study) (coordinated with Department of Ecology).

B. To identify food sources of adult and nymphal stages (with emphasis on discovering additional ways in which vectors may become infected with rickettsia):

1) To determine if one or more other arthropod species are usually present where adults and nymphs are found.

2) To determine additional natural food sources that could be used in colony maintenance.

C. To collect additional known and potential vectors from a variety of locations and different habitats in West Malaysia and surrounding areas (in support of laboratory projects):

1) To obtain potentially infected vectors.

2) To collect various genetic "strains" of vectors, such as white, orange and yellowish *deliense*.

3) To map distribution of infected mites in West Malaysia.

Specific Laboratory Oriented Studies

D. To obtain bionomic data on selected species, determining: Rates of spermatophore deposition and uptake; oviposition rates and duration of egg stage; sex ratios of offspring of infected and uninfected lines; and reproductive longevity.

E. To determine responses of each life stage to stimuli including: responses of engorged and unengorged larvae, nymphs, adults to combinations of light, temperature and humidity; effects of humidity and temperature on length of time chiggers remain on the host (time required for engorgement); roles of CO_2, host odor and substrate factors on host location and attachment by chiggers.

F. To determine why rates of scrub typhus in field collected chiggers are lower than expected (field rates will be determined as part of objective 1)(cooperative study with Department of Microbiology)

1) To establish rates of transovarial transmission for naturally infected *L. (L.) deliense*, *L. (L.) arenicola* and additional *L. (L.) fletcheri (=akamushi)*.

2) To determine if infected chiggers are naturally cleared of *R. tsutsugamushi* in some way (e.g. by taking up antibodies from immunized hosts);

3) To determine if certain combinations of chigger strains, rickettsial strains and hosts can result in scrub typhus transmission to chiggers.

II. To develop improved methods and materials for establishing and maintaining chigger colonies for scrub typhus studies.

A. To determine optimum temperature-humidity-photoperiod combinations for chigger rearing considering combinations for maximum offspring production and combinations needed to simulate particular field conditions.

B. To determine the most practical rearing containers for each research need considering containers for individual chigger rearing, containers for mass rearing, effects of container on temp humidity photoperiod, modifications to reduce mold contamination problem; optimum substrate for containers, and special requirements of each species.

C. To develop improved feeding methods and material in order to determine the most practical natural foods for nymphs and adults, to develop and evaluate better feeding and recovery techniques for larvae, and to evaluate artificial feeding techniques on a limited scale.

III. To identify the viral and rickettsial agents carried by ticks in West Malaysia and to study the epidemiology of these diseases in vectors and laboratory animals (cooperative study with Departments of Microbiology, and Ecology)

Objectives

A. To collect ticks of various species for colonization (in support of lab. studies).

B. To determine the important reservoir hosts on which ticks can become infected with viral and rickettsial agents.

C. To determine if Malaysian tick typhus strains are similar to Indian tick typhus strains.

D. To determine rates of transovarial passage of Malaysian tick typhus in naturally infected ticks.

E. To infect normal ticks of several species on artificially infected hosts.

F. To establish multiple strain and multiple agent (virus and rickettsia) infections.

G. To determine which stage of tick development is most likely to transmit viral and rickettsial agents.

H. To determine natural infection rates for each species of vector.

DEPARTMENT OF BACTERIAL DISEASES

I. Diarrheal studies - no further work is planned.

II. To develop and evaluate the Silvered Leaf-monkey as an animal model for the study of melioidosis.

Objectives

A. To determine the effects of leech bite, by *Hirudinaria spp.*, in conjunction with exposure to water containing *Ps. pseudomallei*.

B. To compare the geographic distributions of soil salinity, aquatic leeches and prevalence of human infection with *Ps. pseudomallei* in Malaysia.

IV. In conjunction with Department of Viral and Rickettsial Diseases, to compare the antibody response in scrub typhus with the level of plasmacytoid cells in the peripheral blood of SLM.

V. To provide some useful military information by documenting the incidence of the various forms of viral respiratory disease in Malaysia.

Objectives

A. To obtain clinical specimens from jungle-dwelling people in epidemic situations.

B. To transmit these specimens to:

1) The virus laboratory in the IMR.

2) The virus laboratory in RAM College, London for comparison of results.

DEPARTMENT OF LABORATORY ANIMAL RESOURCES

I. Laboratory Reproduction of Wild Rodents

Objectives

A. Design and procurement of an adequate cage system.

B. Determination of the time relationships of the phases of the reproductive cycle.

C. Production of the numbers required for approved research protocols.

II. Diseases of Laboratory Animals in Malaysia

Objective

Identification and control of those diseases occurring in the IMR-USAMRU animal colony.

III. Animal Facility Management

Objectives

A. Maintenance of effective liaison with architect of the new animal facility.

B. Purchase of adequate animal chows.

C. Replacement of inadequate animal caging.

D. Institution of an effective employee training program.

INVESTIGATIONS OF THE DEPARTMENT OF ACAROLOGY

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY* 30 06 73			REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY 30 06 72	4. KIND OF SUMMARY	5. SUMMARY SCTY* U	6. WORK SECURITY*	7. REGRADING* N/A	8a. DISP'N INSTR'N NL	8b. SPECIFIC DATA-CONTRACTOR ACCESS ☐ YES ☐ NO		9. LEVEL OF SUM A. WORK UNIT
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY		3A062110A831						
b. CONTRIBUTING								
c. CONTRIBUTING								

11. TITLE (Precede with Security Classification Code)*

Investigations of the Department of Acarology

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

Tropical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
10 72	9 73		

17. CONTRACT/GRANT DADA17-73-G-9368
a. DATES/EFFECTIVE: 10 72 EXPIRATION: 10 73
b. NUMBER:*
c. TYPE: Y. Grant d. AMOUNT: 263
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	1.0	23.4
	CURRENT 74	1.0	26.6

19. RESPONSIBLE DOD ORGANIZATION

NAME:*
ADDRESS:* US Army Medical Research Unit
Institute for Medical Research
Kuala Lumpur, Malaysia

RESPONSIBLE INDIVIDUAL
NAME: Dr. R. Bhagwan Singh, Director
TELEPHONE: Institute for Medical Research

20. PERFORMING ORGANIZATION

NAME:*
ADDRESS:* Institute for Medical Research
Kuala Lumpur, Malaysia

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:*
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE

22. KEYWORDS (Precede EACH with Security Classification Code) Chiggers, mites, *Leptotrombidium deliense, L. arenicola, L. fletcheri*, scrub typhus, *Rickettsia tsutsugamushi*

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

23.(U) Technical Objectives: To determine factors affecting numbers of chiggers on selected hosts; to investigate the prevalence of scrub typhus vectors in N. Sumatra, Indonesia; to determine the efficiency of transovarial transmission of scrub typhus in *Leptotrombidium arenicola*; to investigate spermatophore production by *Leptotrombidium* males; to determine the status of the *Rattus rajah* rats as hosts of scrub typhus vectors; to make bionomic comparisons of infected and noninfected chiggers.

24.(U) Approach: Chigger numbers will be monitored on 2 host species over a 16 month period at Bukit Lanjan, Selangor, Malaysia; collections of medically important chiggers will be made in 6 areas of N. Sumatra; the efficiency of transovarial transmission of scrub typhus in an infected colony of *L. arenicola* will be determined; Spermatophore production will be monitored in 3 species of scrub typhus vectors; *Rattus rajah* and related species will be compared to other rat species as hosts for scrub typhus vectors; comparisons will be made of sex ratios of infected and noninfected chigger colonies.

25.(U) Progress: Chiggers and host blood samples on filter paper were collected from 6 areas of N. Sumatra, Indonesia. *Leptotrombidium deliense* was the only known vector of scrub typhus found in these areas. Only 13 of 214 mammalian hosts sampled were positive for scrub typhus antibody.

Rattus argenitiventer had significantly ($P < .001$) greater numbers of *L. deliense* chiggers per rat than did *Rattus tiomanicus jalorensis*. There was no apparent differences between numbers of chiggers on rats from grassland and those from edge (scrub) habitats. Both host species appeared to be highly efficient as hosts for *L. deliense*. Fluctuations in chigger numbers on these hosts appeared to be correlated with dew-point

*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.

temperature.

A naturally infected colony of *L. arenicola* was established. The rate of transovarial transmission of *Rickettsia tsutsugamushi* was 100 percent over 3 generations as shown by single feedings of chiggers on mice. The rickettsial strain was found to have Karp and TA 763 as major - and Kato as a minor antigenic component. A partially engorged infected chigger was shown to be capable of transmitting scrub typhus during refeeding on a laboratory mouse. Infection in a *L. arenicola* male was demonstrated.

Rates of spermatophore production by *Leptotrombidium* males were determined. Initial spermatophore deposition occurred an average of 4 days after adult emergence. The average number of spermatophores per day has been 7.9 for *L. arenicola*, 13.0 for *L. deliense* and 11.8 for *L. fletcheri*. Females of one *Leptotrombidium* species did not take up spermatophores from a related species.

Sex ratios of progeny were determined in infected and noninfected colonies of *L. arenicola* and *L. fletcheri*. Infected chiggers produced female offspring almost exclusively, and it appeared that female production of *R. tsutsugamushi* in the chigger are related. Noninfected chiggers had an average ratio of about 2.5:1 (females: male), and even lines producing only females in one generation did not continue to produce females exclusively in the subsequent generation.

Rats of the *R. rajah* group were infested with significantly fewer chiggers than other species in initial field tests. Experimental feedings showed that fewer *L. deliense* chiggers fed successfully on *R. surifer* than on *R. annandalei*. The potential involvement of gamasoid mites in scrub typhus transmission to spiny furred rats was suggested.

COMPLETED STUDIES

Chigger Collections and a Survey of Scrub Typhus Antibody in Small Mammals from Areas of North Sumatra

by

Lyman W. Roberts & M. Nadchatram

Department of Acarology, USAMRU & Division of Acarology, IMR.

The land masses of Sumatra and Peninsular Malaysia are separated by less than 25 miles and probably were connected in the recent geologic past. They have many species of plants and animals in common, but comparisons of the chigger fauna of the 2 regions have been limited by the lack of data on Sumatran chiggers. Walch (1925) made a survey of chiggers in lowland and coastal areas of North Sumatra. The vector *L. (L.) deliense* was described from specimens collected near Medan, Sumatra, but little has been published on the prevalence of this vector in Sumatra. Rates of scrub typhus antibody in small mammals from Sumatra have not been investigated previously.

To gain information on the chigger species and the potential threat of scrub typhus in North Sumatra, a survey was made at 6 locations during March and April, 1973. Specific objectives were to determine: (1) what known vectors were present in lalang, scrub, secondary and disturbed primary forest habitats at different elevations; (2) for each habitat, the number of hosts captured that had detectable scrub typhus antibody levels.

METHODS

Figure 1 shows the areas surveyed in North Sumatra. Chiggers were collected from bird and mammal hosts and on black plates (Hubert and Baker 1963). Approximately 200 wire basket traps and 50 "snap" traps were used per night in each area. Birds and bats were caught with mist nets. The hosts were transported in cloth bags from the point of capture to a field processing site. Chiggers and other ectoparasites were removed from the hosts and preserved in 95 percent alcohol or kept alive for laboratory studies. Plastic vials, each having a hardened layer of plaster of Paris and charcoal in the bottom, were used as containers for the live chiggers.

To determine if scrub typhus antibody was present in the mammalian hosts, a blood sample was taken from all mammals captured, except bats. The samples were spotted on filter paper and allowed to dry. Each blood spot was screened several weeks later by the indirect fluorescent antibody test (IFAT), using the methods described by Gan *et al.* (1972). A polyvalent antigen was used which consisted of yolk sac suspensions of the Karp, Gilliam and Kato strains of *Rickettsia tsutsugamushi*.

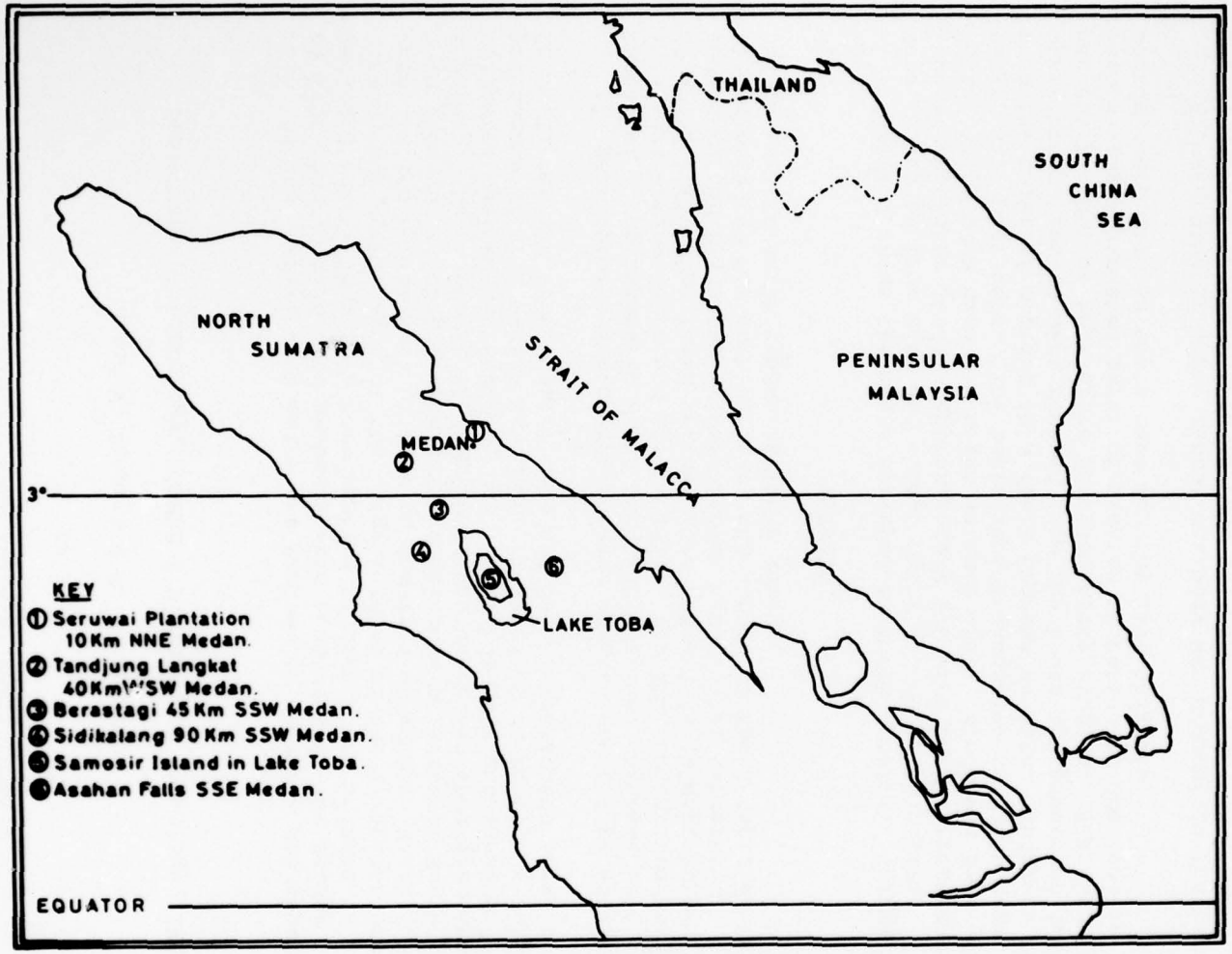


FIG. 1 COLLECTION SITES IN NORTH SUMATRA, INDONESIA.

RESULTS

Lalang and Scrub Habitats: Tables 1-4 show the species of chiggers collected from hosts captured in lalang and scrub habitats from 4 locations in North Sumatra. *L. (L.) deliense* was the only vector collected from these areas, although the lalang grass habitats appeared suitable for *L. (L.) fletcheri* or *L. (L.) akamushi*. No chiggers were collected in black plate attempts in pure stands of lalang, but in predominantly lalang-mixed habitats, *L. (L.) deliense* was occasionally collected by this method. *L. (L.) deliense* chiggers were present on 113 of 145 rodents captured in lalang and scrub habitats. Relatively few *L. (L.) deliense* were collected from hosts trapped in lalang at the coconut plantation (Table 1), and of the 18 rats examined from this area none had detectable scrub typhus antibody. The presence of *Blankaartia acuscutellaris* chiggers on a crow pheasant was unusual since this species is typically found on birds living in aquatic habitats. This species has been reported to bite man but is not known to be a disease vector. Greatest numbers of *L. (L.) deliense* were collected from rodents captured near Tandjung Langkat. *Rattus tiomanicus jalorensis*, a common host for *L. (L.) deliense*, was trapped in greatest numbers near Tanjung Langkat. IFAT results showed that 5 of the 67 *R. t. jalorensis* and one of the 7 *Rattus r. diardii* captured near Tandjung Langkat were positive for scrub typhus antibody. A single *Rattus bowersii* trapped in scrub in the Asahan Falls reserve was positive for scrub typhus antibody but carried no proven vector species at the time of capture. One of two *Tupaia minor* captured in scrub near Sidikalang was also positive by IFAT. *Rattus exulans* was caught in all four areas, but few chiggers were collected from this species. Of the 27 *R. exulans* captured in lalang and scrub habitats, none were shown to be infected. Only 4 *R. argentiventer* were captured in the areas sampled. This species commonly carries *L. (L.) fletcheri* chiggers in Malaysia.

Secondary Forest Habitats: Chigger species collected from mammals trapped in the 3 secondary forest areas are given in Tables 5-7. Numbers of *L. (L.) deliense* collected from hosts were lower in these areas than in the lalang and scrub habitats sampled. Of the 75 hosts examined, 44 were infested with *L. (L.) deliense*. None of these hosts were positive for scrub typhus antibody. *Leptotrombidium (L.) keukenschrijveri* was present in all three areas, and *Leptotrombidium (L.) pilata* was collected in the mixed secondary forest on Samosir Island. Neither of these species are proven vectors of scrub typhus, but *L. (L.) keukenschrijveri* reportedly has been found on man (Walch 1925).

Disturbed Primary Forest Habitats: Tables 8 and 9 show the species of chiggers collected from mammals trapped in the 2 disturbed primary forest habitats. Although only 35 hosts were trapped, large numbers of *L. (L.) deliense* were found on individual hosts. One *Rattus muelleri* male from the Asahan Falls Reserve was infested with 1218 *L. (L.) deliense* chiggers. Three hosts from each of the

Table 1

Chiggers collected from bird and rodent hosts captured in a lalang and coconut tree habitat* (Seruwai Plantation), 10 KM NE Medan, Sumatra, 28-29 March 1973

Hosts	Number Examined	Chigger Species					
		Leptotrombidium (L.) deliense	*Ascoschoengastia (Laurentella) indica*	*A. (L.) lorius*	*Blankaartia acuscutellaris*	*Heslipa gateri*	*Gahrliepia (Walchia) disparanguis pingue*
Rattus argentiventer	3	6	7	2	-	-	8
Rattus r. diardii	9	11	-	-	-	-	-
Rattus exulans	6	13	-	1	-	-	-
Bustard quail	1	-	-	-	-	-	-
Crow pheasant	1	1	-	-	66	3	-
Total	20	31	7	3	66	3	8

* Elevation 0-20m

Table 2

Chiggers collected from rodents captured in lalang and scrub habitats* near Tandjung Langkat, 40 KM WSW Medan, Sumatra, 31 Mar-2 Apr 1973

Host	No. Examined	Chigger Species: *Leptotrombidium (L.) deliense*	*Gahrliepia (Walchia) lewthwaitei*	*Walchiella oudemansi*
Rattus argentiventer	1	79**	-	-
Rattus r. diardii	7	224**	-	-
Rattus exulans	5	142	-	-
Rattus t. jalorensis	67	1917**	1	1
Rattus whiteheadi	1	3	-	-
Total	81	2365	1	1

* Elevation 10-50m

** Chiggers kept alive for scrub typhus transmission studies.

Table 3

Chiggers collected from rodents captured in lalang and scrub habitats*, Asahan Falls Reserve, Sumatra, 15-17 April 1973

Hosts	No. Examined	Chigger Species					
		Leptotrombidium (L.) deliense	*Ascoschoengastia (Laurentella) gressitti*	*Gahrliepia (Walchia) disparanguis pingue*	*G. (W.) turmalis*	*Walchiella impar*	*W. oudemansi*
Lariscus insignis	1	-	-	-	-	-	-
Rattus bowersii	1	-	-	25	10	-	-
Rattus r. diardii	5	146	-	-	-	-	-
Rattus exulans	2	15	-	-	-	-	2
Rattus t. jalorensis	12	736	3	-	3	36	-
Total	22	897	3	25	13	36	2

* Elevation 500-600m

Table 4

Chiggers collected from mammals captured in a lalang and scrub habitat*
6 KM NNW Sidikalang, Sumatra 9-10 April 1973

Hosts	No. Examined	Chigger Species												
		Leptotrombidium (L.) deliense	*L. (L.) keukenschrijveri*	*Ascoschoengastia (Laurentella) globosa*	*A. (L.) gressitti*	*A. (L.) lorius*	*Doloisia (Traubacarus) brachypus*	*D. (T.) domrowi*	*D. (T.) jadini*	*Microtrombicula spicea*	*Gahrliepia (Walchia) disparanguis pingue*	*G. (W.) turmalis*	*Walchiella impar*	*W. oudemansi*
Hylomys suillus	1	23	-	-	-	-	-	-	-	-	-	-	-	-
Rattus bowersii	3	26	-	1	5	4	-	41	8	-	26	11	-	-
Rattus r. diardii	3	123	3	-	-	-	-	2	-	-	-	-	-	-
Rattus exulans	14	2	-	-	1	-	13	-	-	-	278	-	-	-
Rattus niniventer	1	40	8	-	-	-	-	-	-	-	-	-	-	-
Tupaia minor	2	45	-	-	-	188	-	-	-	3	-	-	7	5
Total	24	259	11	1	6	122	13	43	8	3	304	11	7	5

* Elevation 1000-1100m

Table 5

Chiggers collected from mammals captured in a secondary forest habitat*, Asahan Falls Reserve, Sumatra, 15-17 April 1973

Hosts	No. Examined	Chigger Species									
		Leptotrombidium (L.) deliense	*L. (L.) keukenschrijveri*	*Ascoschoengastia (Laurentella) lorius*	*Gahrliepia (Gahrliepia) fletcheri*	*Gahrliepia (Walchia) disparanguis pingue*	*G. (W.) turmalis*	*Microtrombicula spicea*	*Susa sp.*	*Walchiella impar*	*W. oudemansi*
Lariscus insignis	2	1	-	-	-	-	-	-	-	-	-
Rattus t.jalorensis	1	62	-	-	-	-	-	-	-	-	2
Rattus niniventer	3	4	-	-	-	-	-	-	-	-	-
Rattus sabanus	3	-	-	-	65	-	-	-	-	-	-
Rattus surifer	2	-	-	-	-	4	-	-	8	-	-
Tupaia glis	5	22	-	5	214	-	1	2	-	5	27
Tupaia minor	1	4	-	-	-	-	-	-	-	-	-
Tupaia tana	2	42	2	-	3	-	-	-	-	-	23
Total	20	135	2	5	282	4	1	2	8	5	52

* Elevation 500-600m

Table 6

Chiggers collected from mammals captured in a secondary forest habitat* near Berastagi, 45 KM SSW Medan, Sumatra, 4-6 April 1973

Hosts	No. Examined	Chigger Species						
		Leptotrombidium (L.) deliense	*L. (L.) keukenschrijveri*	*Ascoschoengastia (Laurentella) globosa*	*A. krishnani*	*Doloisia (Traubacarus) dommowi*	*Gahrliepia (Gahrliepia) granulata*	*Gahrliepia (Gahrliepia) sp.*
Lariscus insignis	2	42	-	-	-	-	-	-
Rattus r. diardii	1	51	20	-	-	-	-	-
Rattus fulvescens	5	-	14	35	5	63	-	-
Rattus infraluteus	1	1	19	-	-	-	166	-
Rattus surifer	1	-	-	-	-	62	-	3
Tupaia minor	1	-	-	-	-	-	-	-
Total	11	94	53	35	5	125	166	3

* Elevation 1400-1500m

Table 7

Chiggers collected from mammals captured in a mixed secondary forest habitat*
on Samosir Island, Sumatra, 12-14 April 1973

Hosts	No. Hosts Examined	Chigger Species													
		Leptotrombidium (L.) deliense	*L. (L.) keukenschrijveri*	*L. (L.) pilata*	*Ascoschoengastia (Laurentella) globosa*	*A. (L.) gressitti*	*A. (L.) krishnani*	*A. (L.) lorius*	*Doloisia (Traubacarus) brachypus*	*D. (T.) domowi*	*Gahrliepia (Walchia) disparanguis pingue*	*G. (W.) turmalis*	*Walchiella impar*	*W. oudemansi*	*Teinocoptes sp.*
Chironax melanocephalus	2	-	-	-	-	-	-	-	-	-	-	-	-	-	10
Rattus fulvescens	39	56	383	-	700	3	13	-	1	801	92	37	1	3	-
Rattus exulans	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rattus whiteheadi	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tupaia glis	1	137	138	26	-	-	-	1	-	-	-	-	41	18	-
Total	44	193	521	26	700	3	13	1	1	801	92	37	42	21	10

* Conifer and upper dipterocarp forest, elevation 1400-1500m.

Table 8

Chiggers collected from mammals captured in a disturbed primary forest habitat*,
Asahan Falls Reserve, Sumatra, 15-17 April 1973

Hosts	No. Examined	*Leptotrombidium (L.) deliense*	*L. (L.) bodense*	*L. (L.) keukenschrijveri*	*Ascoschoengastia (Laurentella) globosa*	*A. (L.) gressitti*	*A. (L.) lorius*	*Doloisia (Traubacarus) domrowi*	*Gahrliepia (Gahrliepia) fletcheri*	*Gahrliepia (Walchia) disparanguis pingue*	*G. (W.) ewingi*	*G. (W.) marshi*	*G. (W.) tumalis*	*Microtrombicula spicea*	*Siseca rara*	*Walchiella impar*	*W. lacunosa*	*W. nadchatrami*	*W. oudemansi*
Rattus muelleri	1	1218	-	-	-	-	-	-	5	-	-	-	-	-	-	-	-	1	-
Rattus sabanus	6	85	2	6	-	1	-	3	223	4	2	1	-	-	-	-	-	45	-
Rattus surifer	3	-	-	-	-	4	-	20	-	2	-	-	-	-	-	-	-	2	-
Rattus whiteheadi	1	-	-	101	4	-	-	2	-	-	-	-	-	-	-	-	-	-	-
Tupaia glis	7	313	1	-	-	-	8	-	380	1	-	-	-	67	4	5	-	-	5
Total	18	1616	3	107	4	5	8	25	608	7	2	1	-	67	4	5	4	48	5

* Elevation 500-600m.

Table 9

Chiggers collected from rodents captured in a disturbed primary forest habitat*,
10 KM WNW Sidikalang, Sumatra, 7-11 April 1973

Hosts	No. Examined	Chigger Species																	
		Leptotrombidium (L.) deliense	*Leptotrombidium (L.) keukenschrijveri*	*Ascoschoengastia (Laurentella) globosa*	*A. (L.) gressitti*	*A. (L.) krishnani*	*Doloisia (Traubacarus) domrowi*	*D. (T.) brachypus*	*Gahrliepia (G.) fletcheri*	*G. (G.) granulata*	*G. (G.) insigne*	*G. (G.) picta*	*Gahrliepia (G.) sp.*	*Gahrliepia (Walchiella) disparanguis pingue*	*G. (W.) turmalis*	*Schoengastiella birella*	*Susa reidi*	*Walchiella impar*	*W. nadchatrami*	*W. oudemansi*
Rattus fulvescens	4	17	8	9	7	-	4	-	-	-	-	-	1	2	-	-	1	-	3	-
Rattus infraluteus	2	4	1	-	-	-	-	-	7	34	-	3	-	1	-	-	-	-	-	-
Rattus sabanus	5	551	2	22	-	38	-	-	431	1	2	2	5	11	2	-	-	8	-	44
Rattus surifer	3	2	-	-	-	41	137	9	-	-	-	-	-	18	-	-	-	-	-	-
Rattus whiteheadi	3	1	-	-	-	-	46	-	-	-	43	-	1	83	-	1	-	-	-	-
Total	17	575	11	31	7	79	187	9	438	35	45	5	7	115	2	1	1	8	3	45

* Elevation 1000-1100m

disturbed primary forest areas were positive for scrub typhus antibody by IFAT. These were 2 *Tupaia glis* and one *Rattus sabanus* from the Asahan Falls area and 2 *Rattus surifer* and one *Rattus whiteheadi* from the area near Sidikalang. The positive hosts in the Asahan Falls area were infested with 20 or more *L. (L.) deliense*, but 2 of the positive hosts from the area near Sidikalang were not infested, and the other carried only 2 *L. (L.) deliense* at the time of capture.

DISCUSSION

Although the collections were limited, it appears that *L. (L.) deliense* is the most common if not the only known vector present in the areas sampled. As in Malaysia and elsewhere this species apparently has a relatively broad ecological niche in North Sumatra. The absence of grassland vector species, *L. (L.) fletcheri* or *L. (L.) akamushi*, can not be established from the limited collections made. However, the collections by Walch from Sumatra did not include these species, and Audy (1963) stated that there was no record of *L. (L.) akamushi* west of Burma and Malaysia. Small numbers of *L. (L.) fletcheri* were collected in Sabah (USAMRU Annual Report 1970). If present in North Sumatra, it seems likely that *L. (L.) fletcheri* or *L. (L.) akamushi* are localized in distribution and are recently introduced species. The apparent scarcity of *Rattus argentiventer*, a common host for these vectors, may be a limiting factor in North Sumatra.

Even though the vector *L. (L.) deliense* was present in all areas sampled, the numbers of hosts with IFAT - demonstrable scrub typhus antibody was relatively low (13 positives of 214 sampled). Walker *et al.* (in press) found much higher rates of antibody positive hosts in similar habitats in Malaysia. Of the areas sampled, highest rates of antibody positive hosts occurred in the 2 disturbed primary forest habitats.

The absence of proven vectors on hosts with detectable scrub typhus antibody levels is not unusual. The host could have been infected at some indefinite time in the past. Conversely a host lacking detectable antibody could be carrying infected vectors at the time of capture. But some species e.g. *Rattus surifer* rarely become infested with known vectors, and the possibility of unrecognized vectors arises.

Species of chiggers collected and the hosts in each of the habitats corresponded closely to those in similar habitats in Peninsular Malaysia. The ecological groups of chiggers proposed by Nadchatram (1970) for Malaysian chiggers appear to apply equally well to our collections from North Sumatra. As in Malaysia, the longstanding forest habitats in Sumatra had more species of chiggers than did the grassland, scrub or young forest habitats.

Additional surveys in Sumatra would be necessary to make detailed comparisons of Malaysia and Sumatra in terms of scrub typhus prevalence, vector distribution and chigger fauna. This study has provided chigger species and host records and information on numbers of animals with detectable scrub typhus antibody for Sumatran habitats not previously surveyed.

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Numbers of Vector Chiggers on Two Species of Maintaining Hosts

by

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BACKGROUND

Although the vectors of scrub typhus can utilize a wide range of hosts, Harrison and Audy (1951) listed *Rattus argentiventer* and *Rattus tiomanicus jalorensis* as important maintaining hosts for vector chiggers in Malaysia. Maintaining hosts are those which return successive generations of engorged larvae to various foci within their home ranges (Audy 1961). Both species of rats are considered to be commensals, but *R. argentiventer* is usually confined to grassland and ricefield areas, while *R. t. jalorensis* is found in secondary forest and forest edge (scrub) vegetation in addition to the grassland habitat (Harrison 1956, 1957). Of the 2 species, Harrison suggested that *R. t. jalorensis* was less efficient as a host of trombiculid mites because it was a partly arboreal species.

As part of a larger investigation of scrub typhus vector ecology (see Annual Report 1972) numbers of chiggers on *R. argentiventer* and *R. t. jalorensis* were monitored at Bukit Lanjan, Selangor, Malaysia. In the present study, comparisons were made to determine: (1) if there were significant differences between the 2 hosts in numbers of chiggers; (2) when and why fluctuations occurred in numbers of chiggers on these 2 hosts.

MATERIALS AND METHODS

The study area consisted of a sloping grassland habitat as cleared beneath an electrical power line, with edge or scrub vegetation on both sides. There were some herbaceous plants in the grassland habitat, but the predominant plant species was lalang grass *(Imperata cylindrica)*. At the bottom of the slope, the grassland was interrupted by crops of tapioca and sweet potato that were associated with an aboriginal village. The edge vegetation included wild banana, ginger and other herbaceous plants, vines, shrubs and saplings. One hundred traps were placed in each habitat at intervals of about 5 m. The traps were galvanized wire mesh cages, 30 x 15 x 15 cm, and the rats were trapped alive 5 nights per week over a 16 month period. The rats were transported to the laboratory and chiggers were removed manually. After the rats were marked by toe clippings, they were released near the point of capture. For recaptured rats, only collections taken at intervals of 3 or more days between captures were considered. This was necessary because some rats apparently returned to the traps within a few hours after release and thus did not have sufficient opportunity to become infested with chiggers. Analysis of variance was used to

determine if there were significant differences in the numbers of chiggers on the 2 hosts. The Malaysian Weather Bureau provided weather data from the Kuala Lumpur International Airport Station which was located about one mile from the study area.

RESULTS

Although some of the *R. argentiventer* examined were captured in the edge habitat, most were taken from the grassland. Since this host is not found in areas where there is no grassland, it was assumed that even those rats captured in the edge vegetation had home ranges in the grassland. While more *R. t. jalorensis* were captured or recaptured in the edge habitat, over 42 percent were caught in the grassland. Thus, for comparative purposes the average numbers of chiggers on *R. t. jalorensis* from edge and from grassland were considered separately.

Leptotrombidium (L.) deliense was the only known vector collected in the Bukit Lanjan area, and no other chigger species were abundant enough for comparison. Table 1 shows the average numbers of *L. (L.) deliense* collected each month from *R. argentiventer* and *R. t. jalorensis*. Analysis of variance showed that *R. argentiventer* had significantly greater numbers of chiggers than *R. t. jalorensis*, regardless of habitat ($P < .001$). *R. t. jalorensis* from grassland and those from edge vegetation did not differ significantly in numbers of chiggers attached. The greatest numbers of chiggers on *R. argentiventer* were collected during May 1972. The numbers of chiggers on *R. t. jalorensis* also increased during this same month, but proportionally the increase was less than that on *R. argentiventer*.

Comparisons were made to determine if any increases or decreases in chigger numbers could be attributed to weather factors. In Figure 1 the average numbers of chiggers per month are compared to monthly fluctuations in the dew-point temperature. The dew-point temerpature is the temperature to which the air must be lowered to become saturated, assuming that the mass of water vapor in it remains constant. If the temperature of the air and the pressure remain constant, an increase in the dew-point temperature will reflect an increase in the atmospheric moisture level. The average air temperature during the 16 month period was 79.1°F, and the average temperature from one month to the next differed by less than 2°F. The chigger numbers on *R. argentiventer* appeared to increase and decrease with dew-point temperature. The correlation was less apparent with numbers of chiggers on *R. jalorensis*.

DISCUSSION

The collections from *R. t. jalorensis* indicate that the chigger numbers in grassland and edge habitats are similar. This may explain why unpublished data show no significant difference in scrub typhus isolation rates for these 2 habitats at Bukit Lanjan. In addition to behavioral differences, e.g. *R. t. jalorensis* being partly arboreal, the size difference in the 2 host species may explain the larger numbers of

Table 1

Monthly collections of *Leptotrombidium (L.) deliense* from *Rattus argentiventer* and *Rattus tiomanicus jalorensis* captured or recaptured* at Bukit Lanjan, Selangor, Malaysia

Month	*R. argentiventer* (Grassland)		*R. jalorensis* (Grassland)		*R. jalorensis* (Scrub)	
	No. Examined	$\bar{x}$ Chiggers Per Rat	No. Examined	$\bar{x}$ Chiggers Per Rat	No. Examined	$\bar{x}$ Chiggers Per Rat
Aug(1971)	10	72.9	6	62.8	-	-
Sep	21	92.5	63	57.8	35	73.0
Oct	45	141.6	56	71.9	75	52.5
Nov	34	189.1	50	84.8	32	46.3
Dec	50	152.2	66	72.0	50	54.5
Jan(1972)	23	131.5	54	70.5	54	63.0
Feb	25	113.0	18	61.2	32	60.8
Mar	24	134.4	12	62.0	69	80.0
Apr	19	171.6	10	57.5	63	54.1
May	14	395.1	21	103.3	56	100.8
June	18	214.8	28	74.2	55	93.7
July	40	193.8	33	119.7	39	104.0
Aug	63	167.5	37	88.5	58	59.6
Sep	54	117.1	15	51.9	28	34.7
Oct	29	204.5	28	92.7	39	97.1
Nov	18	249.6	34	118.4	40	94.4

* The minimum interval between collections from recaptured rats was 3 days.

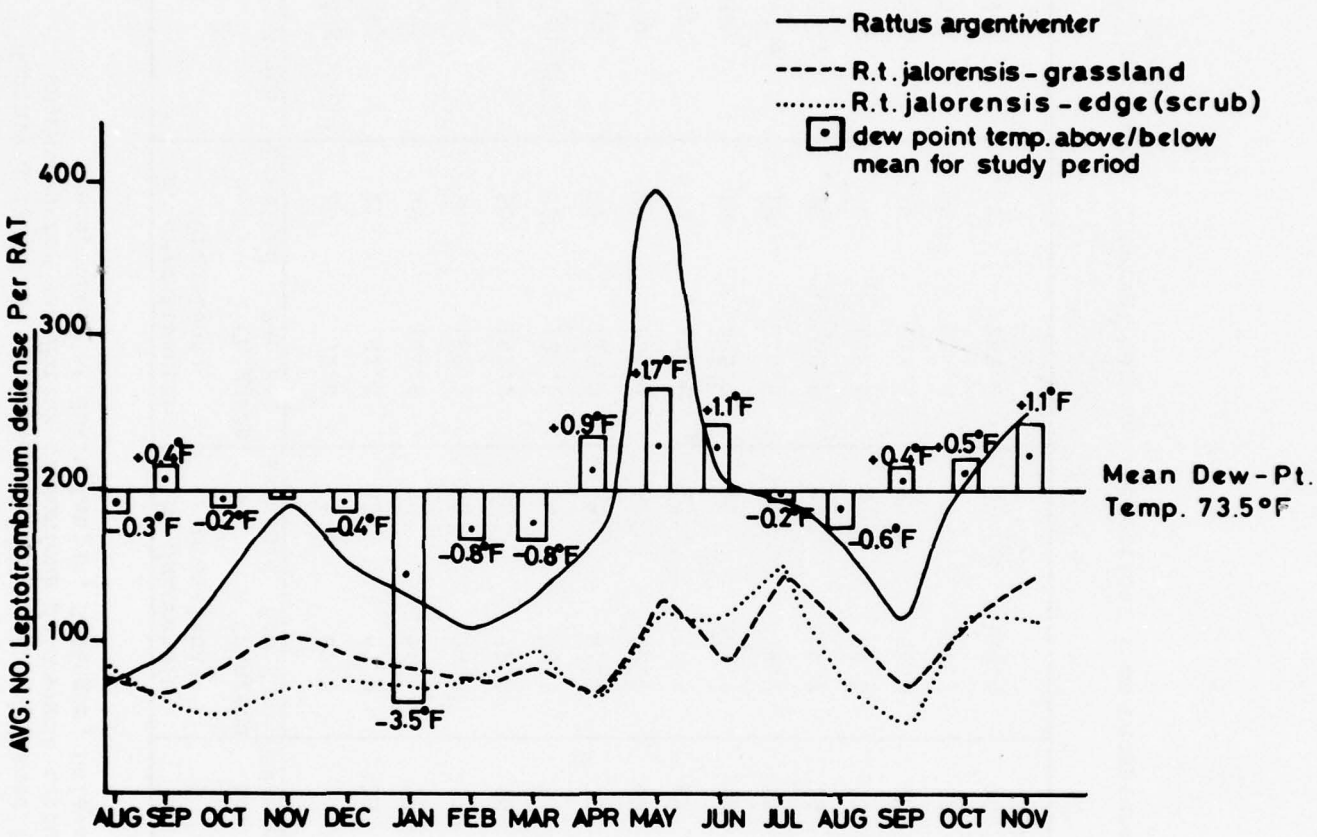


Figure 1. Association of Dew Point Temperature with Relative Numbers of *Leptotrombidium (L.) deliense* on 2 Species of Rats.

chiggers on *R. argentiventer*. Harrison (1957) stated that the average *R. argentiventer* weighed 150 g while the average *R. t. jalorensis* weighed only 90 g. Mohr (1961) theorized that "host preference" among certain ectoparasites may be the result of a difference in the amount of host area available for infestation. Also, a larger host animal would probably generate more CO_2 than a smaller animal with a similar metabolic rate, resulting in greater attraction of chiggers to the larger animal.

The efficiency of both species as hosts for *L. (L.) deliense* was clearly quite high. If the average time for chigger engorgement on a host is about 3 days (Harrison 1954), the turnover in vector chiggers on an average rat may have been 10 times the average number of chiggers per rat for any given month. During May 1972, an average *R. argentiventer* may have been the host for nearly 4,000 chiggers. Eventhough the average numbers of chiggers on *R. t. jalorensis* were lower than on *R. argentiventer*, the total numbers of chiggers fed per month by *R. jalorensis* as a species was often higher since these rats were more abundant than *R. t. argentiventer* in the Bukit Lanjan area. For example, in May 1972, almost 8,000 chiggers were taken in 77 collections from *R. t. jalorensis*. The total taken from *R. argentiventer* was less than 5,600, because only 14 collections could be made from this host species. With such large turnovers in numbers of vector chiggers, the use of systemic insecticides fed to the host rats might be considered as a means of vector control.

The apparent correlation between dew-point temperature and numbers of chiggers on rats is not surprising since several authors have stated that dryness is a limiting factor for vector chiggers. It is likely that many other factors, such as rainfall, cloud cover, ground cover and the number of maintaining hosts available contribute to the number of chiggers per rat collected in any given month. But, increasing dew-point temperature as a reflection of increasing atmospheric moisture level probably is an indicator of favorable weather conditions for chiggers.

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STUDIES IN PROGRESS

Efficiency of *Leptotrombidium (Leptotrombidium) arenicola* as a Vector of Scrub Typhus

by

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Traub (1960) noted that *Rickettsia tsutsugamushi* had been isolated from apparently pure pools of *Leptotrombidium (L.) arenicola* larvae collected from Malaysian beaches. Rapmund *et al.* (1972) demonstrated transovarial passage of *R. tsutsugamushi* through 2 laboratory generations of *L. (L.) arenicola*; however, the infected colony was lost after the second generation and data were insufficient to determine a rate of transovarial transmission. Our objectives were to reconfirm *L. (L.) arenicola* as a scrub typhus vector, and to determine the transovarial transmission rate in an infected colony over several laboratory generations.

MATERIALS AND METHODS

In March 1972, approximately 800 engorged or partially engorged *L. (L.) arenicola* larvae were collected from 16 *Rattus tiomanicus jalorensis* rats. The rats were captured in a beach scrub habitat near Mersing, Johore State, Peninsular Malaysia. Partially engorged larvae were refed on laboratory mice either singly or in groups of 5 to 15. Larvae that were sufficiently engorged at the time of collection were allowed to complete development, and F_1 progeny were fed singly or in groups on mice. Chigger identifications were made from cast larval skins. Except during feeding of larvae, all chiggers were held in humidified rearing containers at an average temperature of 26 C.

The technique for rickettsial strain isolation and verification of *R. tsutsugamushi* was similar to that described by Rapmund *et al* (1969). Mice were killed 14 days after larval feeding, and liver and spleen extracts were passed into a second group of mice. Chiggers were considered infected if the second or third passage mice that were treated with Chloramphenicol for 28 days survived challenge with the Karp strain of *R. tsutsugamushi* at a dosage of 10^2 to 10^3X mouse LD_{50}.

RESULTS

Three infected F_1 progeny were produced in a group of chiggers that had engorged fully at the time of collection. One of these offspring was a male, and spermatophores from this male were taken up by 5 separate noninfected females, but no "trans-spermatophore" transmission occurred. The other two F_1 chiggers were females but one produced only noninfected female offspring and the other died without producing offspring.

One group refeeding of 15 partially engorged, field collected chiggers resulted in transmission of *R. tsutsugamushi*. This group was separated and F_1, larvae were taken from each individual female to determine which mites were infected. The larvae from one female in this group were infected. Although only 4 offspring were produced, all were infected females, and infected F_2 and F_3 progeny have since been obtained. Table 1 summarizes the transovarial transmission in this infected line through three laboratory generations. The rate of transovarial transmission was 100 percent as shown by single feedings of 4 F_1, 20 F_2 and 59 F_3 chiggers. Only female progeny have been produced by infected females in this colony.

A series of conjugates supplied by the Walter Reed Army Institute of Research were used to identify the strain of *R. tsutsugamushi* present in the colony. The strain was found to have Karp and TA 763 as major components and Kato as a minor component. The strain has been mouse lethal and the mice upon which individual larvae had fed usually showed clinical signs of scrub typhus.

DISCUSSION

Our results reconfirm that *L. (L.) arenicola* is capable of vectoring scrub typhus. As in the infected *L. (L.) fletcheri (=akamushi)* colony described by Rapmund *et al.* (1969), *L. (L.) arenicola* has demonstrated a high potential efficiency in transovarial transmission of scrub typhus rickettsiae. *L. (L.) arenicola* is widely distributed along the sandy coastline of Peninsular Malaysia (Upham *et al.* 1971), and since large concentrations of infected chiggers could arise from a single infected female, there is a high potential risk of scrub typhus infection in these areas.

Of potential epidemiological importance is the transmission of *R. tsutsugamushi* during refeeding by a partially engorged *L. (L.) arenicola* larva collected from a wild rat. If a natural host carrying infected chiggers were to die in an area frequented by man, the partially fed chiggers might reattach on a human host and transmit scrub typhus. In unpublished work by this laboratory and by the University of Maryland (Baltimore), larval chiggers of several vector species have been successfully refed under artificial conditions.

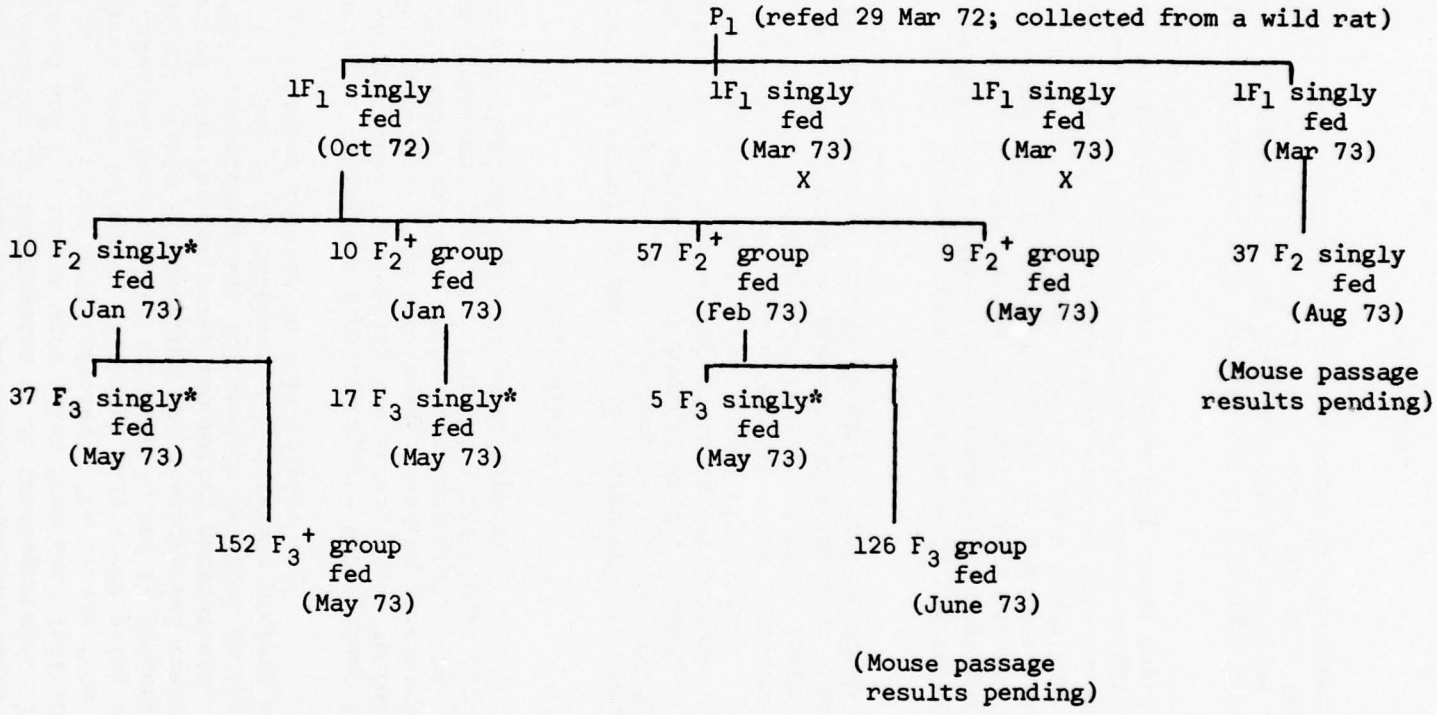
The single infected male chigger was of interest since only one previous infected male has been produced by colonies at this laboratory, and it, too, was an *L. (L.) arenicola* male (Rapmund *et al.* 1972). The sex ratio of vector mites is discussed elsewhere in this report.

FUTURE WORK

Transovarial transmission rates will be followed in this colony at least through the F_5 generation. Ten larvae from 20

Table 1

Scrub typhus transmission in a colony of *L. (L.) arenicola* through three laboratory generations



X Chiggers died without producing F_2 progeny.

\+ Individual or group transmitted *R. tsutsugamushi* during feeding.

* All individuals infected.

infected females will be fed singly in each generation to provide additional data on potential efficiency of transovarial passage in *L. (L.) arenicola*.

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Spermatophore Production by *Leptotrombidium* Males (Acarina: Trombiculidae)

by

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Knowledge of the reproductive characteristics in vector chiggers is essential for determining the feasibility of sterile male release or similar means of vector control. Lipovsky *et al* (1957) first demonstrated that chiggers accomplish insemination with spermatophores and described male deposition and female uptake of spermatophore in the North American Species *Eutrombicula (E.) splendens*. The Lipovsky study included a morphological description of the spermatophores of two *Leptotrombidium* species from Korea, but no data were presented on spermatophores production by these species. Objectives of the present study were to (1) determine the rate of spermatophore deposition by *Leptotrombidium (L.) arenicola, L. (L.) deliense* and *L. (L.) fletcheri*; (2) determine the average duration of viable spermatophore productivity in each species; (3) determine if females of one species will utilize spermatophores from a closely related species; (4) determine if the presence of the female influences spermatophore deposition by male chiggers.

MATERIALS AND METHODS

Chiggers were obtained from laboratory colonies in the teliophane (tritonymph) stage and held individually in humidified rearing containers (Nadchatram 1968). As adulst emerged, males were identified by initial spermatophore production. Twenty males of each species were randomly selected for observations. Spermatophores deposited by each male were counted and removed from the container at 2 day intervals until the male died. Spermatophore viability tests were made at 45 day intervals from the time males were 120 days old. Spermatophores were counted as previously but were left in the containers as they had been deposited. Males were transferred to alternate containers and one uninseminated female was added to each of the original containers with the spermatophores. Subsequent observations were made over a 2 day period to establish that one or more spermatophores had been taken up by each female. Production of offspring indicated that the spermatophores were viable, since isolated, uninseminated females from our colonies have never reproduced parthenogenetically.

Additional groups of males and uninseminated females were used to determine if females of one vector species would attempt to utilize spermatophores of another species. Five individual females of each species were placed in containers having a known number of

spermatophores from the other 2 species of vectors. The females were transferred to fresh containers with spermatophores each day for 2 weeks, and the old containers were examined to determine if spermatophores had been taken up by the females.

Another group of uninseminated females were used to determine if the presence of the female influenced the rate of spermatophore deposition. The female mites were put in 1½ mm x 1 mm diameter glass cylinders. The ends of the cylinders were covered with fine mesh cloth. One cylinder was placed in each of 5 rearing containers with individual males. Five other individual males were used as controls. Spermatophore deposition in the 2 groups of males had been similar during pretest observation.

RESULTS

Figure 1 shows the rates of spermatophore deposition by individual males of each species. Rates for *L. (L.) deliense* and *L. (L.) fletcheri* were similar, and these rates were notably higher than that for *L. (L.) arenicola* during the first 110 days of observation. There was a sharp increase in the rate of spermatophore production in *L. (L.) arenicola* between 110 and 120 days after they became adults. As evidenced by production of viable offspring, spermatophores produced at 120 days were viable in all 3 species, and in *L. (L.) arenicola* and *L. (L.) deliense* at 165 days. Since the observations of *L. (L.) fletcheri* males were started later, only one viability test has been completed. Maximum spermatophore production in a two day period by individual males has been 82 for *L. (L.) arenicola* 113 for *L. (L.) deliense* and 115 for *L. (L.) fletcheri*. But the average per two day period has been 15.8 for *L. (L.) arenicola* 25.9 for *L. (L.) deliense* and 23.6 for *L. (L.) fletcheri*.

No instance was observed of a *Leptotrombidium* female of one species utilizing a spermatophore from a male of another species. None of the females oviposited during a 60 day period after exposure to these spermatophores. The females were later allowed to take up spermatophores from males of the same species, and oviposition occurred within 19 days in all but one *L. (L.) deliense* female.

There was no evidence that the close proximity of females influenced spermatophore production by male chiggers. No definite responses of males to the containers of females were observed.

DISCUSSION

If spermatophore deposition in the absence of females occurs normally, the relatively high rate of spermatophore deposition is understandable, since many spermatophores may not be found by a female. Also, unpublished data on sex ratios in our noninfected colonies of *L. (L.) fletcheri* show a greater frequency of females over 5 generations (2.54 females:male). There appeared to be considerable individual variation in rates of spermatophore deposition.

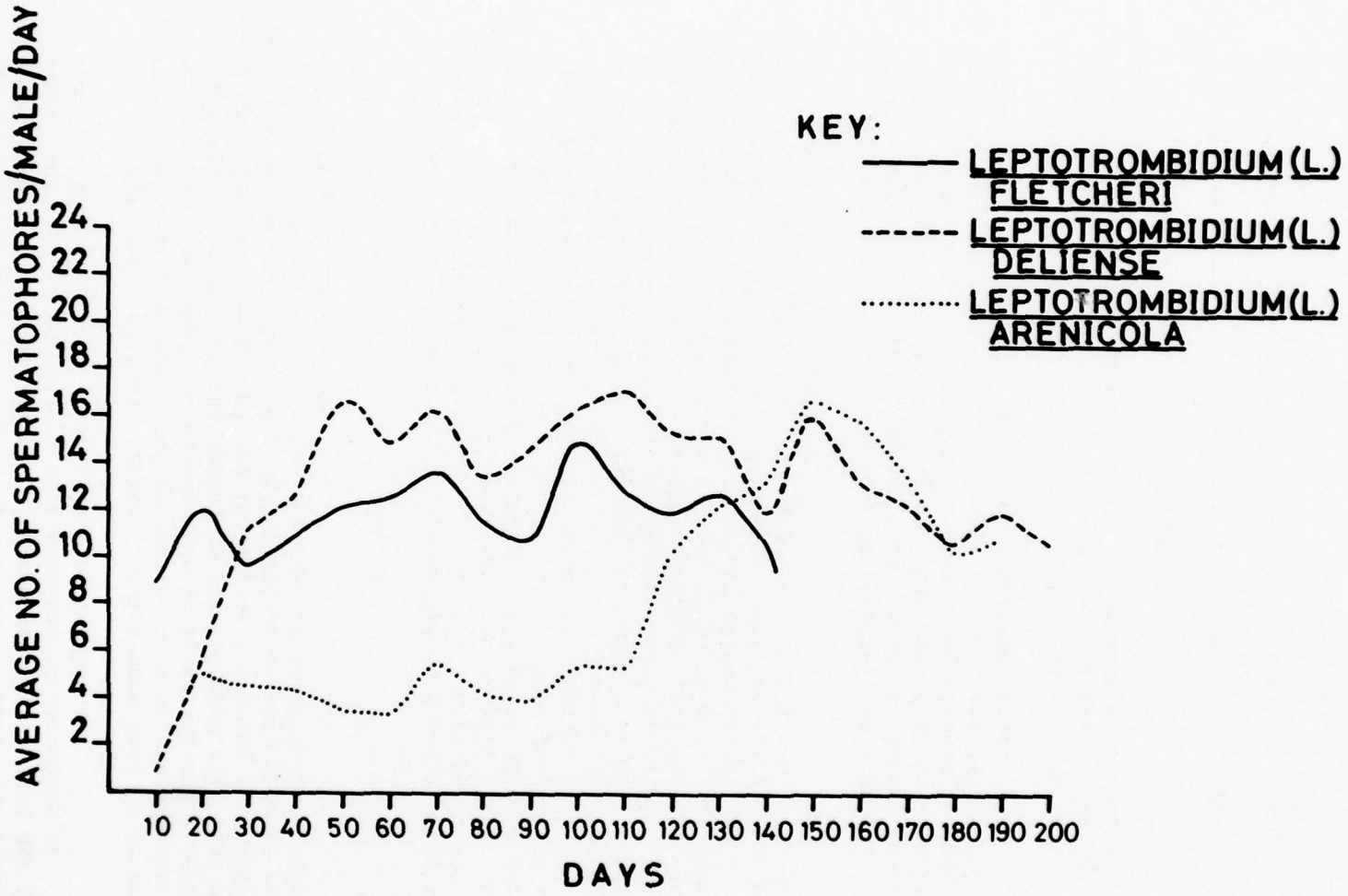


Figure 1. Rate of Spermatophore Deposition by *Leptotrombidium* Males.

The fact that *Leptotrombidium* females of one species did not utilize spermatophores from other species in the same subgenus is additional evidence that the species are distinct. Oliver (1971) indicated that the some groups, females of one species may utilize spermatophores of a different species, with subsequent parthenogenic reproduction occurring. This did not occur with the *Leptotrombidium* females tested. Since these females did readily take up spermatophoresfrom males of the same species, it seems likely that some chemical and/or morphological differences exist in the spermatophores. Without the appropriate keys a female will not take up a spermatophore.

If females influence spermatophore production in males it does not appear to be by their presence alone, i.e. there was no behavioral evidence of males responding to a pheromone. But if males deposit spermatophores at random in the absence of females it would be highly adaptive if there was some chemical that would attract the female to the spermatophore.

FUTURE WORK

Observations will be continued until all males have died or ceased psermatophore production. Additional experiments are planned to determine: (1) how long a spermatophore will remain viable after it has been deposited; (2) how long a female can continue to produce eggs after taking up a spermatophore; (3) if there are discernable morphological differences in the spermatophores of the three *Leptotrombidium* species being studied; (4) if females are attracted to spermatophores from a different species when they are coated with crushed spermatophores from males of the same species.

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Bionomic Comparisons of Infected and Noninfected Chiggers: Sex Ratios in Laboratory Colonies

by

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The infected colony of *Leptotrombidium (L.) fletcheri* described in the study by Rapmund *et al.* (1969) has been maintained through 15 laboratory generations. The transovarial transmission of rickettsial infection in this colony has been nearly 100 percent since the colony was established in 1964. The Rapmund study noted that no male offspring were produced by infected females over a period of 5 generations, and that males from the noninfected colony had to be added for the infected colony to remain viable. With one exception, individual infected females from the F_6 through F_{15} generations have only produced female progeny. Unpublished data based on 1589 progeny from our colony showed that noninfected *L. (L.) fletcheri* had an average female to male ratio of 2.45:1. Neal and Barnett (1961) reported that 9 of 13 *L. (L.) fletcheri* (=Malaysian *akamushi)* produced F_1 offspring that were either all males or all females, but sex ratios were not given for subsequent generations.

Objectives of our work were to determine: (1) if noninfected lines of chiggers can produce only female progeny over two or more generations; (2) if other colonies of infected chiggers would produce male progeny.

METHODS AND MATERIALS

Fifteen newly emerged, noninfected *L. (L.) fletcheri* females were selected at random from 7 different groups of chiggers. The females were maintained individually in clay rearing containers, and a male was added to each container. Progeny from each female were maintained in a separate group. When the progeny reached the adult stage, each was placed in an individual container to determine its sex. Since males normally begin to deposit spermatophores within 4 days after emergence (unpublished observations), any chigger not producing a spermatophore by the 10th day post emergence was considered to be a female. Microscopic examination of slide-mounted specimens was necessary to confirm the sex of presumed females that later failed to oviposit. These same methods were repeated with the progeny of F_1 females that were from exclusively female-producing lines.

With the establishment of an infected colony of *L. (L.) arenicola*, data were collected on female: male ratios as part of the normal chigger rearing procedures. These data were compared with

existing data from the *L. (L.) arenicola* colony described by Rapmund *et al.* (1972) and the infected *L. (L.) fletcheri* colony.

RESULTS

Three of the 15 individual noninfected *L. (L.) fletcheri* females produced only female progeny (Table 1). Four of the 15 produced more males than females, but none produced male offspring exclusively. The average sex ratio in the F_1 progeny was 2.22:1 (females:male). Of 8 F_1 females from exclusively female producing parents, all produced progeny that were of both sexes. The average female:male ratio was 2.93:1 for the 118 F_2 progeny examined.

Approximately 160 progeny from the infected *L. (L.) arenicola* colony have reached the adult stage, and with one exception all have been females. The exception was one infected male produced in a group of field-collected chiggers. One previous infected male, also *L. (L.) arenicola* was noted by Rapmund *et al.* (1972). To get egg production in the infected *L. (L.) arenicola* colony, males or spermatophores from a noninfected colony had to be added.

The infected colony of *L. (L.) fletcheri* has produced more than 13,000 infected progeny over 15 generations, but none have been males. In the 14th laboratory generation of the *L. (L.) fletcheri* colony, 5 parent females that had each transmitted *Rickettsia tsutsugamushi* to mice produced offspring that were noninfected (did not transmit during larval feeding). All offspring from 4 of these 5 females were noninfected and both males and females were produced. The other parent female initially produced 46 females, all of which were infected. After this female underwent a natural splitting of the integument on the postero-dorsal region (Mitchell and Nadchatram, 1969) additional progeny were produced, and 396 were reared to the adult stage. All were males, and none were infected with *R. tsutsugamushi*.

DISCUSSION

It appears that production of nearly all female progeny and the presence of *R. tsutsugamushi* in the chigger are related. Either female producers are naturally infected more frequently, if not exclusively, or the rickettsial organisms influence the sex ratio in the infected lines. The fact that both infected colonies produce nearly 100 percent females is probably not coincidental.

Spermatophores must be taken up by the infected females for oviposition to occur, but as yet we do not know if the eggs are fertilized. The production of only females from unfertilized eggs (thelytoky) is known for mites in the related family, Tetranychidae (Boudreaux, 1956, 1963). In the tetranychids, however, spermatophores were not required for oviposition. Since nearly a third of the progeny are males in our noninfected colonies of *L. (L.) fletcheri* and *L. (L.) arenicola*, production of only females probably could not continue if fertilization were occurring and female production were a heritable trait. Neither data presented in the present study nor

Table 1

Number of female and male progeny produced by individual noninfected *Leptotrombidium (L.) fletcheri* females

Parent (P_1) Chigger No.	F_1 Chiggers Female	F_1 Chiggers Male	Parent (F_1)* Chigger No.	F_2 Chiggers Female	F_2 Chiggers Male
1	39	0	1a	26	11
2	45	0	1b	10	4
3	13	0	1c	9	1
4	14	40	2a	13	3
5	8	13	2b	8	4
6	11	2	3a	12	2
7	15	2	3b	7	3
8	4	1	3c	3	2
9	8	1			
10	7	2	Total:	88	30
11	9	2			
12	5	6			
13	17	9			
14	5	3			
15	4	11			
Total:	204	92			

* Taken from P_1 lines 1-3 which produced only females.

previous rearing data show any tendency of noninfected *Leptotrombidium* chiggers to produce only females over several generations.

The *L. (L.) fletcheri* males that were produced were all noninfected, even though sibling females were infected. It is plausable that the splitting of the integument was involved in male production by the one female and that all of the males resulted from unfertilized eggs. The 2 infected males in *L. (L.) arenicola* indicate that rickettsial infections can occur in the males of this species, but these are the only 2 that have occurred. The rearing data on the infected colonies show no lower hatch rates of eggs or higher death rates in the developmental stages that could be attributed to the presence of the rickettsial infection.

Attempts are in progress to determine chromosome numbers in infected and noninfected *L. (L.) fletcheri* and *L. (L.) arenicola*. If different numbers of chromosomes are found in the eggs of both species, it seems likely that a type of parthenogenesis is occurring in the infected lines. If not, we will have additional evidence to indicate rickettsial influence on the sex ratio. An understanding of the mechanisms responsible for production of exclusively female offspring would be of great potential value in a chigger control program.

If, for whatever reason, infected chiggers produce only females, and if nearly all of these females are infected, this is in sharp contrast with observations that the proportion of infected chiggers in field collections is usually less than 1 percent (Annual Report, 1962). The need is apparent for additional comparisons of infected and noninfected lines to determine if infected chiggers are less able to survive.

FUTURE WORK

Comparisons of infected and noninfected chiggers will be continued through FY 1974. Terraria will be used to simulate field habitats, and infected and noninfected chiggers will be confined together in these habitats. Samples will be taken at intervals to determine if infected chiggers are less able to survive than noninfected ones.

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The *Rattus rajah* Group as Hosts for Scrub Typhus Vectors

by

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BACKGROUND

Harrison and Audy (1951) noted that infestations of known scrub typhus vectors and other trombiculid species were relatively light on *Rattus surifer* (*Rattus rajah* group) in Malaysia. But in later serological studies, scrub typhus was isolated from 11% of the 54 *R. rajah* and 22% of the 49 *R. surifer* captured mostly in Selangor, Malaysia (Walker *et al.*, 1973). Mites of the gamasoid group are generally abundant on *R. rajah*, *R. surifer* and other spiny-furred rats. No gamasoids have been incriminated as vectors of scrub typhus, but there has been no information published on the effect, if any, of these mites on chigger numbers on rats.

Laboratory and field tests were initiated to determine: (1) the relative affinity of the vector *L. (L.) deliense* for *R. surifer* and *R. rajah;* (2) if gamasoid mites on the *R. rajah* group affected *L. (L.) deliense* chiggers.

METHODS AND MATERIALS

Six field-collected *R. surifer* adults were used in initial chigger feeding attempts. The rats were anesthetized with Nebutal and all ectoparasites were removed. Laboratory-reared *L. (L.) deliense* larvae from a noninfected colony were fed in groups of 20 on the rats. Three groups of chiggers were fed in capsules (Baker *et al.*, 1968) on 3 of the rats, and three other groups were placed directly in the left ear of the other 3 rats. Progress of the feeding was noted daily for 5 days.

To determine relative chigger affinity for *R. surifer* rats under field conditions, 16 additional *R. surifer* were captured at Bukit Lanjan, and cleaned of ectoparasites. The approximate numbers of gamasoid and trombiculid mites present on initial capture were recorded. The rats were returned to Bukit Lanjan and released in a 20 m diam pen made of corrugated metal sheets. The pen was located in a mixed grassland habitat consisting mainly of lalang (*Imperata cylindrica*). Prior collections had established that *L. (L.) deliense* chiggers were present in the enclosure. For comparison, 15 laboratory-reared *Rattus annandalei* rats were released in the pen at the same time. *R. annandalei* do not have spiny fur and are usually infested with trombiculids when captured. The rats were recaptured 6-8 days after they were released in the pen, and counts were made of the number of *L. (L.) deliense* present on each rat.

In preliminary attempts to determine if gamasoid mites affect *L. (L.) deliense* chiggers, gamasoids collected from *R. surifer* and *L. (L.) deliense* larvae were placed together in capsules on 5 laboratory mice. The mites were observed daily for 5 days. Additionally, 50 engorged or partially engorged *L. (L.) deliense* larvae were placed in a rearing container with 100 adult gamasoid mites collected from *R. surifer*. Observations were made to determine if the gamasoids had fed on the larvae.

RESULTS

Table 1 shows the number of chiggers fed and recovered in the laboratory using *R. surifer* as hosts. Of the 120 chiggers applied, 96 attached to the rats. Only 59 (61.5%) were recovered, including 16 chiggers that were partially engorged. The remaining 37 chiggers died while attached to the hosts.

The *R. surifer* rats released in the pens had significantly fewer chiggers than did the *R. annandalei*. Upon initial capture, only 3 of the 16 *R. surifer* had been infested with chiggers, but all had been infested with gamasoid mites. Of the 16 gamasoid-free *R. surifer* released, 7 were recaptured from the pen and 3 were infested with a total of 5 chiggers. No reinfestation of gamasoids occurred. All 16 of the *R. annandalei* were recaptured from the pens, and all were infested with chiggers. The average number of chiggers per rat was 61.9, with a maximum of 320 and a minimum of 6 chiggers on individual rats. All of the chiggers infesting both species of rats were *L. (L.) deliense*.

The chiggers placed with gamasoid adults in capsules were able to feed on the mice, and no adverse affects from the gamasoids were noted. The gamasoid mites were quite active in the capsules and did not feed on the mice. There was no predation of chiggers by the gamasoids in the rearing containers or on the mice.

DISCUSSION

Although chiggers were able to feed to repletion on *R. surifer*, the number fed successfully was only about half of the total number applied. Normally, about 80 percent of the chiggers applied to *R. annandalei* or laboratory mice are successfully fed and recovered.

The low numbers of chiggers on field-caught *R. surifer* and the relatively high numbers on *R. annandalei* suggest that chiggers are less attracted to the spiny furred rats. The differences could have resulted from avoidance by *R. surifer* of the chigger microhabitats, but this seems unlikely since the rats were confined in a relatively small area and chiggers were abundant. Further investigations would be necessary to determine why *R. surifer* and other spiny furred rats are not usually infested with chiggers. More important is the question of how these rats become infected with scrub typhus. Are the few chiggers that they do pick up infected, or is another species transmitting scrub typhus to these rats? Mites of the gamasoid group

Table 1

Laboratory feeding of *Leptotrombidium (Leptotrombidium) deliense* on *Rattus surifer*

Rat No.	Site of Feeding	No. attached per No. applied	Engorged Chiggers Recovered					
			Day 1	day 2	day 3	day 4	day 5	Subtotal**
1	ear	13/20	1*	4	1	1	0	7/13
2	ear	20/20	15*	0	0	3	2	20/20
3	ear	15/20	0	12	1	0	0	13/15
4	back	8/20	0	1	6	0	0	7/8
5	back	20/20	0	0	8	0	0	8/20
6	back	20/20	0	0	1	3	0	4/20
	Total	96/120	16	17	17	7	2	59/96

* Chiggers about $\frac{1}{2}$ engorged
** Remaining chiggers dead (desiccated).

have been reported as potential vectors of other rickettsial diseases, such as endemic typhus (Dove and Shelmire, 1931; Grayson, 1961) and rickettsialpox (Huebner *et al.*, 1946; Philip and Hughes, 1948). The gamasoid mites on spiny furred rats have not been adequately investigated as possible vectors of scrub typhus.

FUTURE WORK

The species of gamasoid mites occurring on *R. surifer* will be screened to determine if they are infected with *R. tsutsugamushi*.

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REPORTS OF NEGATIVE RESULTS

Duration of Larval Feeding in Moist and Dry Air

by

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In collection attempts during dry weather, chiggers have been relatively abundant on rodents in areas where few if any chiggers could be collected with black plates. Audy and Harrison (1951) suggested that chiggers are limited to moist habitats along stream beds or similar areas during dry periods. It also seemed possible that chiggers were remaining on the host for longer periods of time during dry weather. This would appear to be adaptive, since the chigger has a constant source of moisture while attached to the host.

A preliminary experiment was designed to determine if chiggers would remain attached to a host for longer periods in dry air than in moist air.

METHODS

Ten laboratory mice were anesthetized with Nembutal, and 10 *L. (L.) deliense* chiggers were placed in the ear of each mouse. During the pretest period the chiggers had been held at an average relative humidity of 79 percent and an average temperature of 28 C. When the chiggers had attached, the anesthetized mice were put in restraining cages (Nadchatram, 1968). Five of the caged mice were placed in a desiccator jar with a drying agent (Silica gel) and five were placed in a similar jar over water. Aquarium pumps were used to provide air for the mice. The air was passed through 1000 ml flasks with water or drying agent before being pumped into the wet or dry chambers. The exact humidities of the two chambers were not measured, but the air in the wet chamber was near saturation while that in the dry chamber was less than 30 percent relative humidity. The mice were examined at 24, 48 and 72 hours after being placed in the chambers. At these intervals the number of chiggers still attached to each mouse was recorded, food was added for the mice and drying agent was replaced.

RESULTS

None of the chiggers had detached from either group of mice after 24 hours; however, 48 hours after attachment all but 8 of the 50 chiggers in dry air had dropped from the mice, while all but 10 had dropped in the wet chamber. All of the chiggers in the dry air had detached at 72 hours, while 2 remained attached to the mice in the wet chamber. These preliminary results did not demonstrate that chiggers remain attached longer in dry air than in wet air.

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Spermatophore Sterilization Attempts

by

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Oliver (1971) stated that in some species of mites the female can reproduce parthenogenetically but must take up a spermatophore, even one from another species before oviposition can occur. Parthenogenically produced offspring are all of one sex. Since our positive colonies of *L. (L.) fletcheri* and *L. (L.) arenicola* have produced almost exclusively females, it seemed possible that a type of parthenogenesis was occurring. In studies described elsewhere in this report it was determined that females of one *Leptotrombidium* species would not utilize spermatophores from another species in the same subgenus. To determine if the type of parthenogenic reproduction described by Oliver could occur in *L. (L.) fletcheri* attempts were made to obtain nonviable spermatophores.

METHODS

Twenty *L. (L.) fletcheri* males were treated with a cumulative dosage of 10 K rad of gamma irradiation over a one hour period. Spermatophores were subsequently obtained from the irradiated males, and a total of 15 individual uninfected uninseminated females were allowed to take up these spermatophores. Additionally, spermatophores from each of 15 other males were treated in attempts to render then nonviable. One of the following treatments was applied to 15 groups of 6-20 spermatophores: (1) exposure to steam for 5 minutes; (2) freezing at -4 C, thawing rapidly and refreezing; (3) exposure to UV light for 24 hours. A separate group of 5 individual females was used for evaluation of each type of treatment. The females were placed in rearing containers with the spermatophores and observations were made daily for 4 days to determine if the spermatophores were utilized by the females.

RESULTS AND DISCUSSION

Twelve of the 15 females taking up spermatophores from irradiated males produced offspring of both sexes. This shows that the males were not sterilized. The dosage should have been sufficient if properly delivered, but the hospital irradiation machine used was not designed to emit large dosages, and the chiggers may not have received the desired dosage.

Spermatophores that were frozen, thawed and refrozen appeared to be utilized by the females, since the number of spermatophores in the containers declined each day. Females were not actually observed taking up the spermatophores. No oviposition occurred in females

exposed to these spermatophores. In preliminary work we found that spermatophores frozen only once and held for up to 72 hours at -4 C remained viable. Females taking up these spermatophores produced offspring of both sexes. Spermatophores treated with UV light also remained viable as evidenced by production of offspring of both sexes. Heat treated spermatophores were not utilized by the females. It may be that some heat labile chemical attractant or "identifier" was lost by heating. Also, there may have been sufficient physical change in the spermatophores to prevent females from utilizing them.

These studies failed to demonstrate that the type of parthenogenic reproduction described by Oliver (1971) was operative in the females tested. This attempt to use ionizing radiation to inactivate spermatophores was ineffective.

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COMMENTS

GENERAL

Research on scrub typhus transmission by vector mites and on chigger bionomics is of necessity long term. To confirm that an individual chigger or one or more of a group of chiggers is infected, mouse passages and challenge are necessary. This requires a minimum of 2 months and considerable support in handling and maintenance of laboratory mice. The time from egg to adult in the infected *L. (L.) fletcheri* colony described by Rapmund *et al.* (1969) is about 50 days, and considerable technician time must be invested in maintaining chiggers. But if scrub typhus is still a disease of significance, and data from Viet Nam indicate that it is, information on scrub typhus in vectors and data on the biology of the vectors is of great potential value. The rickettsial strain in the infected *L. (L.) fletcheri* colony has remained antigenically stable through the 10 generations studied. From experimental attempts to infect rickettsia-free ("clean") chiggers by feeding them on infected hosts, it appears that vectors rarely if ever become infected from vertebrate hosts. The chigger seems to be both the reservoir and vector of scrub typhus. These findings are encouraging from a vaccine development standpoint, but they also emphasize the need for further research on *R. tsutsugamushi* in chiggers. Will other strains in other vector species remain antigenically stable?

Even if an effective scrub typhus vaccine were developed, the annoyance caused by chigger bites would justify continued research on these pests. Because of increasing restrictions on chemical control methods, other means of chigger control may be needed. If so, background data on chigger bionomics will be valuable.

FUTURE PLANS

Pools of *Leptotrombidium deliense* with one or more infected chiggers have been obtained from rodents collected at Bukit Lanjan, Selangor. An infected colony of *L. (L.) deliense* will permit us to make comparisons of infected chiggers of all three important vector species found in Malaysia. The possibility that certain gamasoid mites can vector scrub typhus will be investigated. Initial fluorescent antibody screening will be tried, using mites collected from field captured *Rattus surifer* and *Rattus rajah*.

Relatively little information has been published on the genetics of acarine vectors. Cytogenetic comparisons may explain why infected females in our colony produced only female progeny. The techniques are available for dissection of mites and preparation of slides so that chromosome numbers and configurations can be determined. Infected and noninfected vectors of each species will be compared.

It has been demonstrated that certain concentrations of CO_2 attract some chigger species and this reaction aids in host location (Sasa *et al.*, 1957). Preliminary trials are in progress to determine

if chigger collections in the field can be enhanced by the use of a controlled flow of CO_2. In initial laboratory trials, low volumes of CO_2 were passed over syracuse watch glass dishes containing larval chiggers. The chiggers became very active and appeared to move toward the source of the CO_2. Black plate collections will be made with and without CO_2 in a variety of habitats. The CO_2 will be passed through an aquarium aeration stone to achieve a difused flow over the plates.

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INVESTIGATIONS OF THE DEPARTMENT OF BACTERIAL DISEASES

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY* 30 06 73		REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY 30 06 72	4. KIND OF SUMMARY	5. SUMMARY SCTY* U	6. WORK SECURITY*	7. REGRADING* N/A	8a. DISP'N INSTR'N NL	8b. SPECIFIC DATA-CONTRACTOR ACCESS ☐ YES ☐ NO	9. LEVEL OF SUM A. WORK UNIT

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NAME:
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21. GENERAL USE

22. KEYWORDS (Precede EACH with Security Classification Code)

see continuation sheet

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

23.(U) Technical Objectives: To reappraise incidence of *Ps. pseudomallei* and *Chromo. violaceum* in flooded rice fields subject to double cropping. To relate this incidence to antibody in the human population. To determine if any relationship exists between incidence of antibody and prevalence of the buffalo leech in the environment.

To assess the silvered leaf-monkey as an animal model for melioidosis.

To study the possible relationships of *Hirudinaria spp.* to *Ps. pseudomallei* and determine if it plays a role in transmission of that organism.

24.(U) Approach: Surface water sampling survey for *Ps. pseudomallei* and *Chromo. violaceum* in four Malaysian States. Blood sampling of people of these States whose work is the cultivation of rice, to determine, by indirect hemagglutination, the incidence of antibody to *Ps. pseudomallei*.

Documentation of leech incidence in the environments concerned.

Development of a serologic test for detection of antibody to *Chromo. violaceum*.

25.(U) Progress: Reduction in incidence of *Ps. pseudomallei* from former years is shown and may relate to change in rice padi ecology. A direct relationship between the incidence of *Ps. pseudomallei* in the environment and antibody in the population exists. This also appears to relate to the incidence of *Hirudinaria spp.* Levels of antibody of 1:20 or 1:10 by IHA may be considered, in the population of an endemic area, to be evidence of past exposure to *Ps. pseudomallei*.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

DD Form 1498, Research and Technology Work Unit Summary,
Item 25 continued:

Silvered leaf-monkeys show a varied response in keeping with human experience with *Ps. pseudomallei*. They form a good model for melioidosis. Host factors alone appear to determine response to infection.

Co-existence of *Ps. pseudomallei* and *Hirudinaria spp.* is possible but there is no evidence of *Hirudinaria* being a true transmitter of infection.

Keywords: Melioidosis, *Ps. pseudomallei*, *Chromobacterium violaceum*, *Ps. pseudomallei* indirect hemagglutination, rice padi ecology, silvered leaf-monkey, *Presbytis cristatus*, buffalo leech, *Hirudinaria spp.*

COMPLETED STUDIES

Pseudomonas pseudomallei and *Chromobacterium violaceum* in Rice Growing Areas - a Reappraisal

by

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SUMMARY

The incidence of *Pseudomonas pseudomallei* and *Chromobacterium violaceum* in surface water of irrigated rice growing areas, cropped twice yearly, in four Malaysian States was investigated over a five month period. The presence of the organisms was assessed by cultural methods and by using the Golden (Syrian) Hamster *(Mesocricetus auratus)* as a biological filter.

Recovery rates for *Chromobacterium violaceum* of 77% and for *Pseudomonas pseudomallei* of 9% were shown. A significant reduction in incidence over former years, of the latter organism is evident and may relate to the increase in surface irrigation water to which the habitat is exposed.

Surface water and soil chloride ion values are low and are markedly decreased from previous years. This may be due to leaching out by long continued irrigation. Significant differences were found in the incidence of *Pseudomonas pseudomallei* between States, paralleling the results of antibody incidence in the human population of those States.

It is considered that titers of 1:20, or possibly 1:10, in the *Pseudomonas pseudomallei* indirect hemagglutination test should be taken as evidence of previous exposure to the antigen in an endemic area.

The population of *Hirudinaria spp* (the buffalo leech) is highest in the area of greatest *Pseudomonas pseudomallei* density and greatest antibody incidence.

Well and river water used in the domestic economy cannot be excluded as a major source of infection.

The status of *Pseudomonas pseudomallei* and *Chromobacterium violaceum* in causing disease is discussed and it is stressed that, in areas of high incidence of these organisms, there is need for diagnostic vigilance.

INTRODUCTION

Pseudomonas pseudomallei and *Chromobacterium violaceum* are both considered to be telluric organisms, commonly occurring in soil and surface water of Malaysia. Each of them is capable of infecting humans and animals with the production of multiple abscesses, frequently resulting in the death of the host. Both diseases are considered uncommon and frequently fail to be considered in the diagnosis of superficial sepsis, pulmonary disease or pyrexia of uncertain origin.

In view of previous work on the distribution of these organisms and the changing ecology of the habitats studied, a comparative reappraisal was undertaken.

The work of Strauss *et al* during 1964-66[15] had shown that *Pseudomonas pseudomallei* was a common organism in surface water of Malaysia, especially associated with rice fields. The highest prevalence of antibodies in the population was found in army recruits who had been resident in rice growing areas.[16] In an intensive study of Carey Island[17] a recovery rate for *Pseudomonas pseudomallei* of 4% from drainage ditches of rubber and coconut plantations was found, while only 0.4% of soil samples yielded the organism. They explained this discrepancy in terms of increased multiplication of the organism in the soil moistened by rain or by mechanical inoculation of surface water from the soil.

A soil survey of the State of Selangor, of which Carey Island is part,[22] showed a high chloride content. This feature should especially apply to Carey Island which is described as flat, most of it being below mean high tide level, effective drainage being accomplished by use of tidal gates. The soil is classified as the Kranji series, having poor drainage and high salinity but capable of improvement if bunded and allowed to leach of soluble salts. The "rice bowl" of Selangor, bounded by the rivers Bernam and Selangor, is partly made up of this type of soil. *Pseudomonas pseudomallei* has been shown to be susceptible to concentrations of sodium chloride in excess of 3%[5].

During work on leptospirosis in this unit[20] it was found that the tissues of some land leeches were capable of transmitting *Pseudomonas pseudomallei* to hamsters, causing their early death. Transmission of trypanosomes in fish[10] and, under some conditions, rinderpest virus in cattle[2] is known but, to date, bacterial transmission by leeches has not been demonstrated. *Hirudinaria spp*, the buffalo leech, is common in some rice growing areas. Leeches of the biting type are generally very sensitive even to low salt concentrations.

The rice economy of Malaysia has seen a vast improvement by the introduction of double cropping which has been made possible by the construction of canal systems to deliver either river or stored water to the rice fields. The Selangor system has been in existence for

eleven years while the Muda Agricultural Development Authority in Kedah-Perlis in the north of Malaysia and the Kemubu Agricultural Development Authority in Kelantan in the north-east, have been operating similar schemes for four and two years respectively. Continual soil wetting would be expected to have the effect of providing a milieu more suited to the growth of *Pseudomonas pseudomallei*. This condition will be attained in the rice growing areas which benefit, in one season from the monsoon rains and in the other from irrigation.

The presence, in Malaysian soil, of a mesophilic chromogenic organism, whose violet pigment is soluble in ethanol but not in water, has been documented by Sneath[11] and others. This organism is named in Bergey's Manual, 7th Ed as *Chromobacterium janthinum* but is now more usually named *Chromobacterium violaceum*. As distinct from *Pseudomonas pseudomallei* it has a much wider geographic range having been found naturally occurring in water of temperate zones. The similarity of the pathology of the disease caused by these two organisms is well known and has recently been commented on by Snowe.[14] *Chromobacterium violaceum* infections in animals have been seen by Strauss *et al*[18] and human cases documented up until 1952 are reported by Sneath *et al.*[12] At this time, no record has been found of a survey comparable to that on *Pseudomonas pseudomallei*, of any area, to determine the prevalence of *Chromobacterium violaceum* in soil or surface water.

OBJECTIVES

These are best summarized:

1. To reappraise prevalence of *Pseudomonas pseudomallei* in defined areas where it would be expected to be a commonly occurring organisms.

2. To determine if the changing ecology produced by rice cultivation methods has altered the prevalence.

3. To assess the incidence of antibody to *Pseudomonas pseudomallei* in larger numbers of rice growing people than was formerly done.

4. To assess the influence of chloride ion concentration on the organism.

5. To attempt to document the prevalence of *Hirudinaria spp* and assess whether it bears any relationship to antibody incidence.

6. To assess prevalence of *Chromobacterium violaceum* in the same habitat as *Pseudomonas pseudomallei*.

APPROACH

Four Malaysian States which form the bulk of the country's rice producing area were selected for study. Cooperation was obtained from

the State Directors of Medical Services and advice, and access to the irrigated areas was provided by the District Officer in Kuala Selangor and the responsible authorities of the Muda and Kemubu schemes. The Selangor part of the study was conducted on a day-to-day basis with USAMRU(M) laboratory as a base. In Kelantan and Kedah-Perlis, facilities were made available at the laboratories of the respective General Hospitals for all necessary laboratory investigations. Sampling was performed within areas of greatest rice density (Fig. 1) corresponding to the following:

Square Miles	Kelantan	Kedah-Perlis	Selangor
Land Area	5750	3910	3160
Sampled	980	550	95

In each area the part sampled represents the bulk of that given over to double cropping.

MATERIALS AND METHODS

The study areas were sampled by taking 20 ml of surface water from flooded rice fields into sterile screw capped universal containers which were held in an insulated container in water ice, but not frozen, until processing in the laboratory. Samples were taken at approximately 1 mile intervals where possible. The time interval between sampling and processing was never longer than 7 hours. All samples were from rice fields except for 42 in Kelantan which were taken from flowing water from positive sites of a survey conducted by this unit some years previously.

Concern had been expressed, by the director of the Muda scheme in Kedah, about the possibility of an increase in enteric disease due to the persistence of a high water table by continual flooding. To determine if this was occurring, sampling into selenite F and alkaline peptone water was also performed.

TEST ANIMALS

Two hundred and eighty and four hundred hamsters, aged 6-10 weeks were transported, caged in pairs, in the back of a Landrover, on built in racking to increase ventilation. These batches were taken respectively, to Alor Setar (300 miles) and Kota Bharu (400 miles). Losses were minimal, only 10 succumbing to the journey to Kedah. It had been established[17] that the hamster is a satisfactory test animal for the detection of *Pseudomonas pseudomallei* in natural water, an LD_{50} of 6 organisms having been shown.[7] 2 ml of the water samples was injected intraperitoneally to each of a pair of hamsters. It was felt unnecessary to use groups of five animals as had been done previously, since the infecting dose was so small. Autopsy was performed on

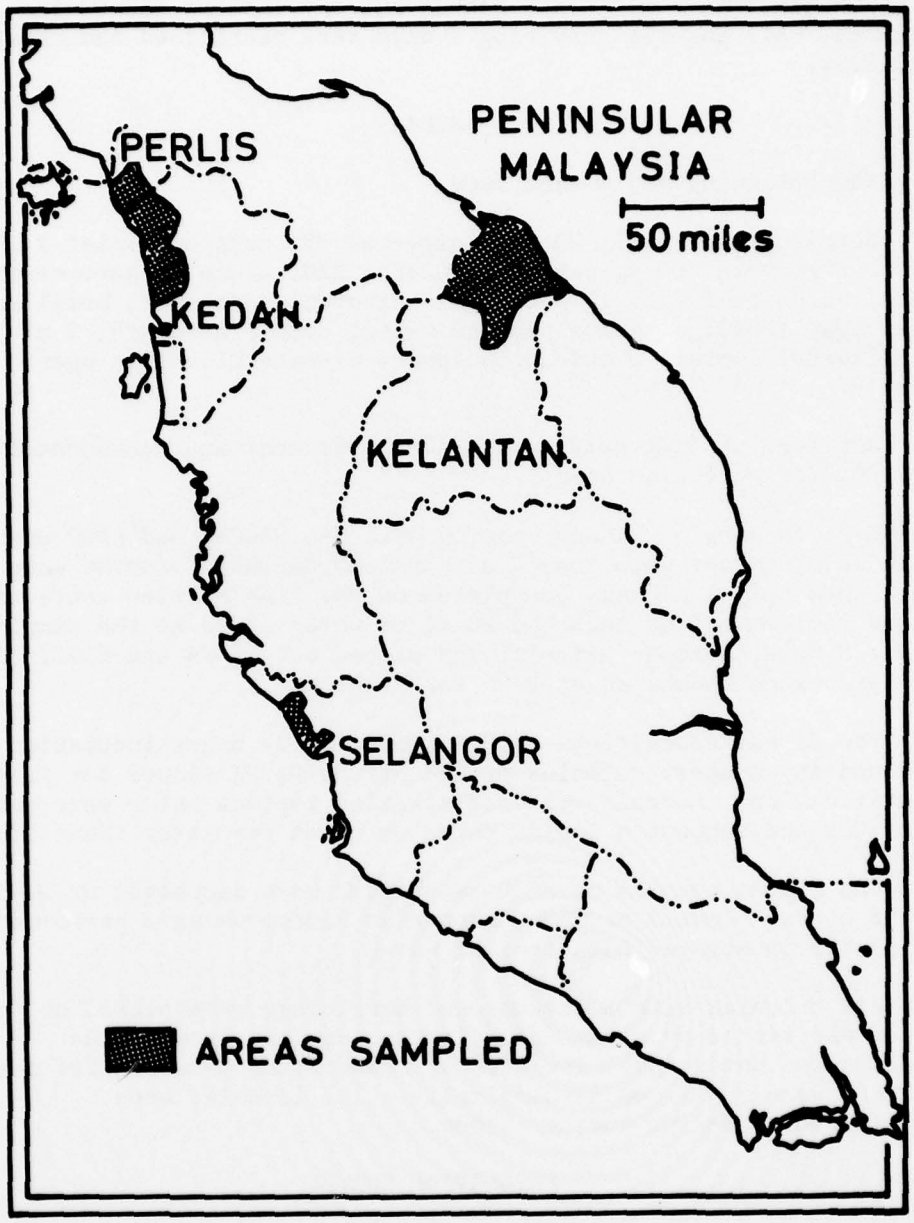


Fig.1 PENINSULAR MALAYSIA : Areas Sampled

animals dying up to seven days post-injection, observation of lesions at the injection site, in the liver, spleen, and lungs, being made at gross examination. All heart bloods and any abscess found were cultured. All animals surviving 7 days were sacrificed and treated similarly.

MEDIA

The following media were used:

Nutrient agar (NA); NA with glycerol 3%, crystal violet 1:200,000 and erythromycin 400 µg per ml (NAGC+E); NAGC alone in part of the study; Selenite F (SF) 1G per 20 ml universal container; brilliant green agar (BGA); alkaline peptone water, double strength, 2 ml per 20 ml universal container and thiosulphate citrate bile salt agar (TCBS)*.

Antisera to *Salmonella spp*, *Vibrio cholerae* and *Pseudomonas pseudomallei* were also used.*

2 ml of sample was pour-plated with NA, NAGC+E and NAGC where applicable, except when counts of *Chromobacterium violaceum* were made, when only 1 ml was pour-plated on NA. The bottles containing SF and cholera medium each had 20 ml of water added at the sampling site. Hamster autopsy material was plated out on NA and NAGC. The NA plates were incubated at 37°C for 24 hours.

The SF was subcultured to BGA following 24 hours incubation at 37°C and any suspect colonies picked off on to NA slopes for later identification. Subcultures from alkaline peptone water were made onto TCBS and suspect colonies taken on to NA for later identification.

The plates containing NAGC and NAGC+E were incubated at 37°C for 48 hours. Counts of *Chromobacterium violaceum* were performed on all plates inoculated with 1 ml of water.

All colonies with a pseudomonad morphology were picked on to NA slopes and tested by slide agglutination against *Pseudomonas pseudomallei* antiserum. Isolates of *Pseudomonas pseudomallei* from hamsters were confirmed serologically. All isolates were subcultured to NA for further study.

POPULATION SURVEY

Communities to be sampled for human blood were selected randomly within the study areas from (1 inch to the mile) Ordnance Survey maps and the Medical Directorates concerned were asked to request volunteers from each community. The criteria were that there should be an even

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* All media and antisera originated in Difco laboratories.

balance between sexes, all should be over 30 years of age, all should have been occupied in rice cultivation for their working lives so as to give a ten year-plus exposure and, if possible, all should be native to the study areas.

The volunteers were visited in their villages and 10 ml of venous blood was drawn aseptically into sterile tubes, serum being separated later at the laboratory. Controls were sought in each State but it was not possible to obtain a purely non-rice growing population of similar age range, continually resident in the same area.

Sera were frozen at -20°C and transported to USAMRU(M) for later antibody testing by *Pseudomonas pseudomallei* indirect hemagglutination.

Ten villages were visited in Kelantan, one of them being concerned in rubber cultivation only. A total of 216 males and 184 females were bled. Eleven villages in Kedah-Perlis, representing 153 males and 131 females and 5 sites in Selangor, representing 61 males and 39 females, were visited.

The Kelantan control group consisted of 76 blood bank volunteers, 13 hospital staff members and 37 rubber planters. That in Kedah-Perlis was composed of 40 hospital staff and 58 general outpatients. No controls were available from Selangor.

Water temperature recordings and pH, roughly determined by the use of nitrazine paper, were assessed at each site.

CHLORIDE ION CONCENTRATION

The method of Schales and Schales, as given in Varley[21] and slightly adapted, was used to determine Cl' concentration in water and soil samples.

Soil sampling was performed at approximately each tenth water sampling site, taking a core to a depth of 18 inches, using a 2" diameter metal borer. The top 6" and lower 6" were separated and bagged individually. Each sample was dried out, 100G taken, suspended in a similar volume of deionised water for 24 hours with occasional shaking. Aliquots of the water of suspension were tested by the method referred to.

LEECHES *(HIRUDINARIA SPP)*

An assessment of the prevalence of these was made in two ways. The local populace was asked if they saw many or few and, at each collection site, they were sought in the padi water, specimens being collected for investigation and use in other studies. It was found that, where they were present, the human foot or hand, immersed in the water for a few seconds, caused them to show themselves.

Table 1

Results of water examination in four Malaysian States

State	Period	Soil Condition	Water Samples						Soil Cl' mEq/ 100G upper/lower	Other Isolates	Estimate of *Hirudinaria* spp prevalence	Sera	
			No.	Temp°C	pH	Cl' mEq/100ml	Positive Isolates					No.	Pos 1:40 & over
							Pseudomonas pseudomallei	*Chromo. violaceum*					
Kelantan	May	Flooded: Late in cultivation: Much planting	180	30.9 (25.6-37.1)	5.0 (4.0-5.3)	0.09 (0.004-2.17)	30 (17%)	160 (89%)	0.013 0.006 (0.004-0.135) (0.002-0.06)		Abundant, widespread	489	66
Kedah-Perlis	March	Flooded: Early in cultivation: Little planting	132	31.3 (26-39.5)	5.2 (4.0-6.2)	0.04 (0.004-0.52)	5* (<4%)	63 (48%)	0.115 0.08 (0.004-0.64) (0.002-0.42)	*Salm.bovis-morbificans* (1)	Low, localized	382	20
	July	Flooded: Planted: Good growth	61				4 (6.5%)	46 (75)					
Selangor	April	Flooded: Cultivating: Some planting	62	33.5 (28.7-39.5)	5.0	0.04 (0.004-0.13)	0 (0%)	54 (87%)	0.01 0.012 (0.002-0.02) (0.002-0.02)		None	100	2
	June	Flooded: Planted: Good growth	62				5 (8%)	58 (93%)					

Ranges shown in brackets

* One of these positive results was obtained only in culture.

AD-A058 238 INSTITUTE FOR MEDICAL RESEARCH KUALA LUMPUR (MALAYSIA) F/G 6/5
TRANSMISSION, CONTROL AND TREATMENT OF INFECTIOUS DISEASES OF M--ETC(U)
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RESULTS

These are summarized in Table 1.

The conditions of cultivation represent a wide spectrum from fields of flooded stubble through fields with soil well disturbed and mixed with flood water to flooded fields with well established plants.

Water pH and temperature are materially similar to values previously recorded and are in the range of the growth capabilities of both *Pseudomonas pseudomallei* and *Chromobacterium violaceum*.

Chloride values are surprisingly low in the face of the report cited[22] and may, in Selangor, represent the result of prolonged flooding and consequent leaching over the intervening 7 years. No effect on the survival of *Pseudomonas pseudomallei* can be postulated.

Pseudomonas pseudomallei was recovered from 44 of 497 samples (8.9%). In one instance it was found on direct culture but did not cause hamster death from that sample.

If the Kelantan results are separated into samples taken from rice fields and those from running water of supply canals, small streams and the river Kelantan (Table 2), the rate of recovery is shown to vary slightly downwards in padi water, from 17% to 13.8% and upwards in running water to 26%. Thus, the recovery from running water in Kelantan is twice that from water which has been fed into and intimately mixed with, the soil. This would seem to be at variance with former concepts[5,15,17]

Table 2

Isolations in Kelantan related to type of water

Water	No. Samples	No. Positive *Ps pseudomallei*	No. Positive *Chromo. violaceum*
Rice Fields	138	19 (13.8%)	128 (93%)
Running Water	42	11 (26%)	32 (76%)

Rates of recovery in the west coast States of Kedah, Perlis and Selangor are much lower than from Kelantan. As was stated earlier, each of these States has had an irrigation facility longer than Kelantan. The lower recovery rate from areas subject to greater exposure to surface water seems also to conflict with former conclusions.[15,17]

Pseudomonas pseudomallei has been shown to have a long term survival in tap and in natural water.[15]

Fourteen samples from which *Pseudomonas pseudomallei* had been isolated and 14 matched negative samples were each held at 4°C for periods of from 15-21 days. 2 ml were injected intraperitoneally to further pairs of hamsters and the remaining water stored at a mean daily temperature of 29°C for 7 days, in the dark. At the end of this time a further 2 ml of each was injected by the same route into another batch of hamsters, singly.

Table 3

Effect of time and storage on infectivity of Kelantan water

	First Hamster Pair		Time at 4°C Days	Second Hamster Pair		Third (single) Hamster
	Days to Death	No. Infected		No. Infected		Infected
Padi Water	2	2	21	0	Samples held for 7 days at 29°C	no
	2	1	21	0		no
	1	2	20	0		no
	6	2	17	0		no
	6	1	17	0		no
Running Water	4	1	16	0		no
	3	1	16	0		no
	3	2	16	2		yes
	3	2	16	2		yes
	3	2	16	2		yes
	3	2	15	0		yes
	4	1	15	2		yes
	3	2	15	2		yes
	3	1	15	0		no

None of the negative samples, treated similarly, became positive.

Mesophilic chromogens, provisionally identified as *Chromobacterium violaceum*, showed a vastly greater incidence than *Pseudomonas pseudomallei*. Almost 77% of samples yielded the organism. There is less regional difference in incidence of *Chromobacterium violaceum*, and the comparison of Kelantan running water with padi water shows reversal of the pseudomallei trend. By extending the incubation period of the NA for more than 24 hours, the isolation rate may have been increased due to better development of chromogenesis. Furthermore, account was not taken of achromogenic variants in assessing the NA plates so that the isolation rate may well have been even greater.

Table 4

Shows the relationship of time of death to the infecting agent

State	*Ps pseudomallei* No.	Days to Death Range	Avg.	*Chromo. violaceum* No.	Days to Death Range	Avg.
Kelantan	30	2-6	4	43	1-7	3
Kedah-Perlis	8	3-6	5	10	2-6	3.5
Selangor	5	4-7	5	17	1-7	2.8
Totals	43	2-7	4.3	70	1-7	3

Coexistence of *Chromobacterium violaceum* with *Ps pseudomallei* did not reduce the time to death in those animals.

In eleven of the 43 hamsters dead of pseudomallei infection, lung abscesses from which *Ps pseudomallei* was cultured were visible on gross examination.

NUMBERS OF CHROMOBACTERIUM CAUSING DEATH

Thirty six samples, which caused death in hamsters due to *Chromobacterium violaceum* had plate counts performed on primary NA cultures. The range of counts per ml of sample water was 3-242 with an average of 46. Thus, the numbers causing death in hamsters following intraperitoneal injection of 2 ml water ranged from 6-484 with an average of 92.

OTHER ISOLATES

Salmonella bovis-morbificans was isolated from one site only, in Kedah. Neither *Shigella spp*, *Vibrio cholerae* nor *Vibrio parahaemolyticus* was recovered.

SEROLOGY

Table 5 shows the age ranges, sex ratio and results of serology.

In the padi group at the level of a titer of 1:40, the ratio of males to females is 1.2:1. The positive results are shown by State, compared to the isolation rates of *Ps pseudomallei* from water in Table 6.

Table 5

Population sampled for antibodies to *Ps pseudomallei*

	MALES							FEMALES						
	No.	No. Under 30	Age Range	Age Ave	Avg Padi Years	HA Titer 1:40 or more	HA Titer 1:20 or more	No.	No. Under 30	Age Range	Age Avg	Avg Padi Years	HA Titer 1:40 or more	HA Titer 1:20 or more
Kelantan	198	10	20-84	46	27	39(19.2)	62(31.3)	165	11	16-74	44	26	22(13.3)	35(21.2)
Kedah-Perlis	153	12	20-85	44	25	9(5.9)	18(11.8)	131	11	20-82	43	26	8(6)	13(9.9)
Selangor	61	4	21-73	43	25	1(1.6)	1(1.6)	39	3	25-60	39	19	1(2.6)	1(2.6)
Kelantan Control	92	36	18-65	33	3.8	3(3.2)	6(8.3)	34	7	19-74	37	0	3(8.8)	3(8.8)
Kedah-Perlis Control	60	12	21-65	40	0.7	2(3.3)	5(8.3)	38	8	21-63	39	0.4	1(2.6)	4(10.5)
Totals	564	74				53(9.2)	92(16.3)	407	40				35(8.6)	56(13.7)

Percentages given in brackets.

The Kelantan control group included some from a purely rubber growing village in which two of the females had titers of over 1:40 and one of the males a titer of 1:20.

Table 6

Isolation rates of *Ps pseudomallei* from water related to antibody incidence

State	% Water Samples Positive	% Sera IHA Titer 1:40 or more	% Sera IHA Titer 1:20 or more
Kelantan	17	13.5	21.7
Kedah-Perlis	4.7	5.2	10.5
Selangor	4	2	2

LEECH POPULATION

Estimates are, of necessity, crude.

In Kedah-Perlis the general consensus was that the leech population had dwindled over recent years. When they were seen, they occurred in localized areas, mostly well inland. Collections were possible in only 8 of 193 collection sites.

In Kelantan, of 138 padi sites, *Hirudinaria spp* was easily recovered from 38. This is doubtless an underestimate as many more were seen but not collected and the local people were almost unanimous in stating that buffalo leeches abounded.

In Selangor, despite assiduous searching, none was found. Long term residents of this region had never seen leeches in their area. Insecticides are used in all States to control crop pests.

DISCUSSION

Fournier and Chambon[5] state that *Pseudomonas pseudomallei* is most easily isolated from water but was not recovered from running water. Strauss *et al*[15] suggest that surface water is colonized from underlying soil. The present study has shown that isolation rates from running water are higher than from padi water but this just fails ($X^2_{(1)} = 3.577$, $P > .05$) to have significance. Sample size may be responsible for this failure. All the criteria, which are not stringent, for growth of *Pseudomonas pseudomallei* are satisfied in the varied conditions of cultivation seen during the survey. However, when Selangor is considered, the isolation rate is nil during cultivation and rises to 8% when soil/water equilibrium is reached. In the State of Perlis, there were no isolates, no matter the conditions. These facts to some extent reopen the question of the origin in nature of *Pseudomonas pseudomallei*, but answers cannnot be provided.

The question of changing ecology due to water load can be presented in the following way:

Table 7

Isolations of *Ps pseudomallei* from surface water of wet rice fields in 1964-67 (Strauss *et al*) compared to those in the present investigation

Year	Total Samples	No. Positive
1973	455	33
1964-67 ($2\frac{1}{2}$ yr study)	753	110
1967 (short term study)	45	15

The differences in isolation rates between the present study and those of the $2\frac{1}{2}$ year and short term study of Strauss are highly significant, $X^2_{(1)} = 14.7$, $P < 0.0005$ and $X^2_{(1)} = 32.09$, $P < 0.0005$, respectively. His short term study was conducted in recently established fields which did not have an irrigation facility. It may be argued that the present study was performed under different conditions to those of the earlier one but there can be little difference postulated in the seasonal variation nor in the cultivation conditions which were as varied in 1973 as they must have been in 1964-67. In any case it should be remembered that the organism in question is not fastidious in its nutritional requirements. These findings confirm that, at least in rice fields, the population of *Ps pseudomallei* is reduced from former years and suggest that increased irrigation may be responsible.

Howe *et al*[6], in a review, interpret the greater frequency of CF antibodies to *Ps pseudomallei* in Thai males which was found by Nigg[8], as "further indicating the occupational opportunities for acquiring infection from a contaminated environment". Reference to Nigg's paper does not reveal whether the males and females had had any different contact with their "contaminated environment" and it would seem illogical to draw the conclusion of Howe *et al*. There is virtually no distinction between male and female antibody incidences in the present study: at titers of 1:20 and greater, the ratio of females to males is 1:1.3 while at a level of 1:40 or greater, it is 1:1.2. If this is related to the average number of years spent in padi work by each sex, it is surprising that there is any difference. Figure 2 shows antibody incidence related to age and sex.

There is little doubt that exposure to padi work relates to antibody incidence. This is demonstrated by comparing the lengths of time spent in the environment by each group and relating this to the positive titers in the control and padi subjects. The difference in antibody incidence in the control and padi groups is highly significant

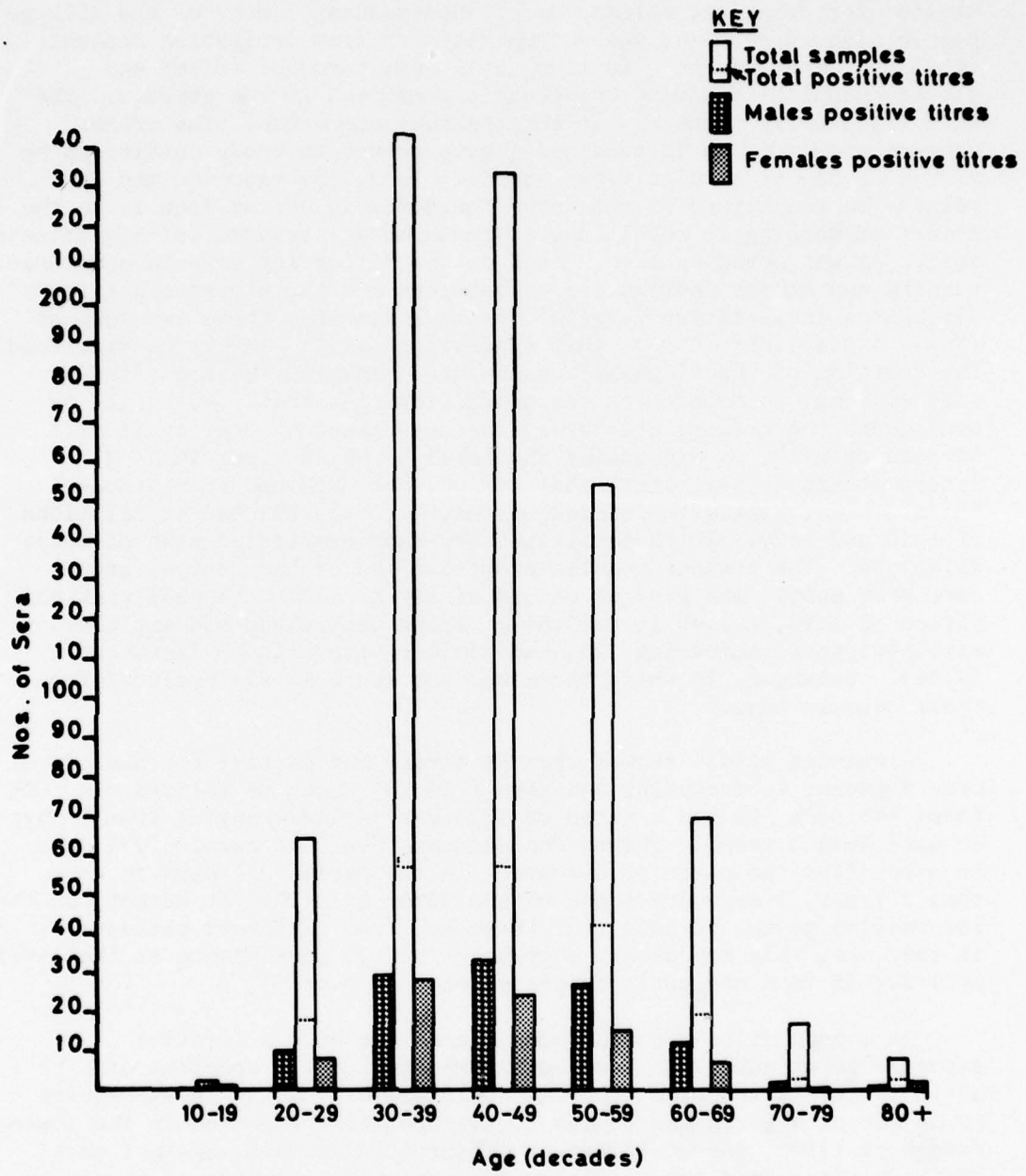


Fig. 2 Distribution of sera sampled by age and sex in rice growing population.

whether titers of 1:20 and above ($P < 0.0005$) or 1:40 and above ($P < 0.005$) are considered as evidence of infection. However, one must take into account the nature of the domestic water supply, whether for drinking, bathing or clothes-washing. Many of the village people sampled received water from wells or from irrigation channels, small and large rivers. In those villages, personal toilet and clothes-washing is almost universally performed in the streams. The water, generally speaking, is not treated before use. The urban control groups may well have had less exposure to those conditions by virtue of having a mains water supply. Thus, the exposure may be related to the nature of the water for domestic use as much as to the effect of working in conditions of *Pseudomonas pseudomallei*-contaminated soil. As was noted earlier, based on the differring effects of stored running and stored padi waters on hamsters and the almost significant difference in isolation rates of the organism from these two sources ($X^2_{(1)} = 3.577$, $P > 0.05$), this suggestion cannot lightly be dismissed. The question of the diagnostic value of a hemagglutination titer of 1:20 or more, as opposed to the prevailing view that 1:40 should be considered the breakpoint, has again been raised by Clayton *et al.*[3] Strauss *et al*[16], in discussing the level at which significant HA titers should be set, state that of 200 sera obtained from a non-endemic area, namely the United States, 7 (3.5%) reacted at dilutions of 1:10 and 1:20. It is not stated how many reacted at each of those dilutions. The present results show that 144 of the control group sera from people who give no record of any contact with padi work had titers of 1:20, < 1:40 in 8 (5.6%). Those people who had <u>any</u> contact with padi work, numbering 726, had similar titers in 53 instances (7.3%). Selangor, in which there were no controls was excluded from those calculations.

Alexander *et al*[1] showed that HA titers may persist for periods of over 2 years, in analysing 445 sera from 169 cases of melioidosis. Of these 445 sera, 23 had a titer of 1:20 for periods ranging from 2 days to more than 2 years. Thirty one patients provided samples 271 days or more after the onset of disease. In the period 271 days to more than 2 years, 3 sera are shown with a titer of 1:20. It cannot, on the information given, be said that these are from different patients: if they are, this represents a greater than 9% persistence at the level of 1:20; if from one patient, persistence is over 3%.

In a population from an endemic area who have a lifetime exposure to an antigen, it would be expected that a spectrum of antibody levels would be found. Should the diagnostic level require to be set at a given figure due to non-specific responses in the lower ranges of titer, one would expect, if plotting numbers against each antibody titer, a disproportionately high representation of those non-specific reactions. If the results for each area are treated thus, (Fig. 3) all the areas tested show a gently downward sloping curve, all the curves being parallel. There are no humps in the 1:10 - < 1:40 zone. If it is accepted that the curve represented in Figure 3 is adequate to sustain this argument, then by taking 3.5% of the total number of sera of rice-growing people and apportioning that between the titer readings 1:10 and 1:20, the integrity of the

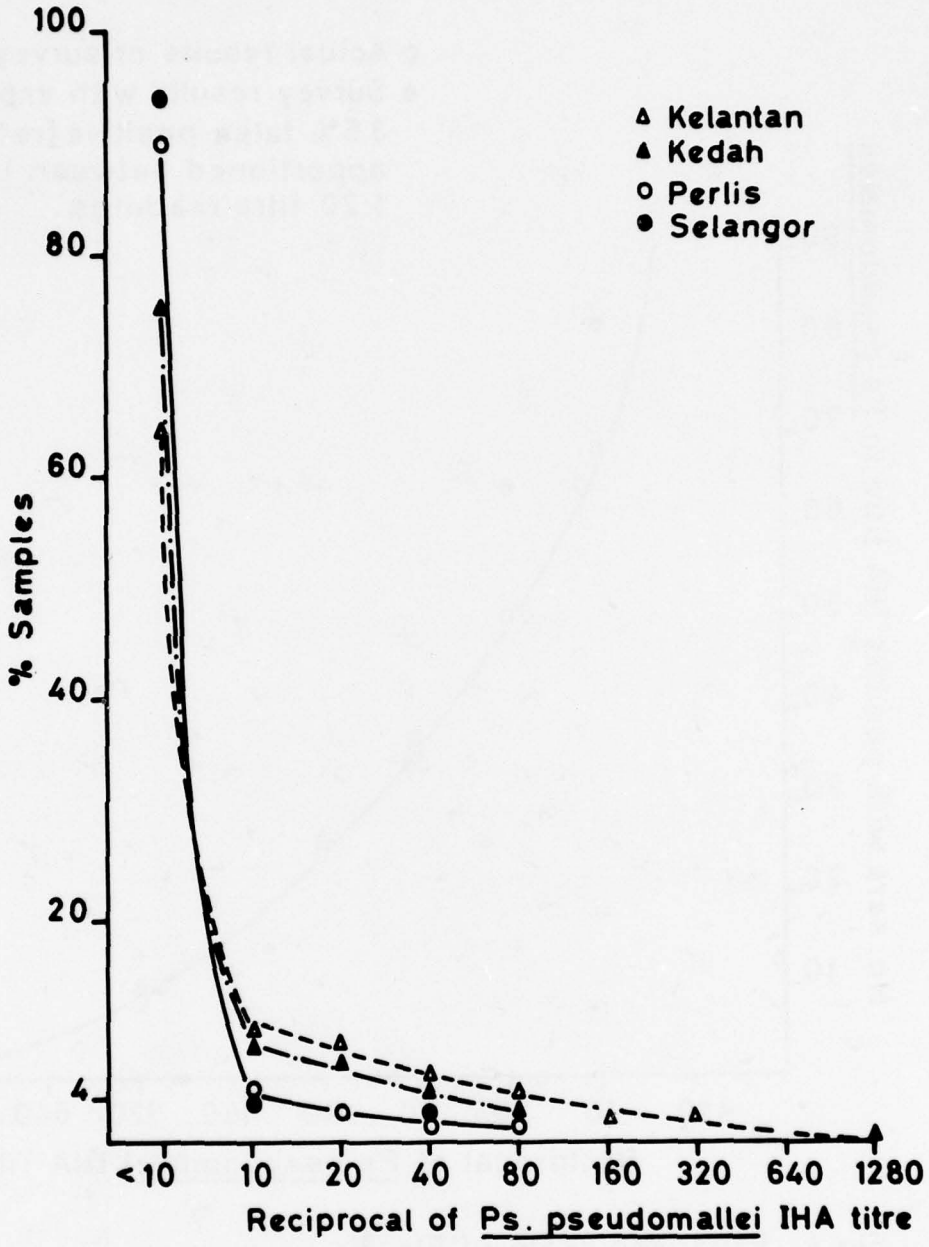


Fig.3 Percent distribution of total populations of serum samples according to Ps. pseudomallei IHA titres.

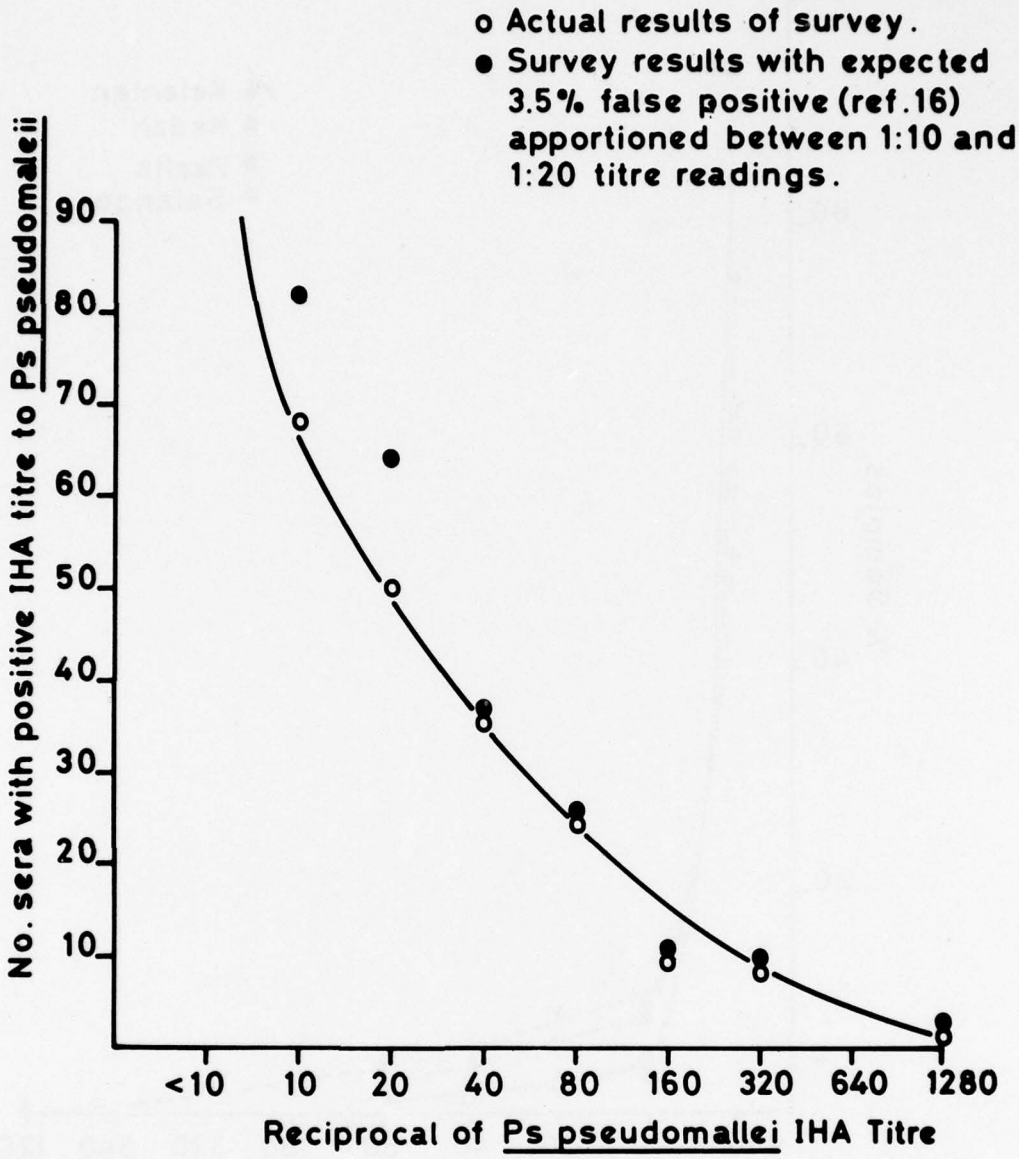


Fig.4 Expanded scale of Fig. 3
Showing distribution of positive sera to titre with and without correction for non-specific reactions.

curve should be disrupted. Figure 4 demonstrates that this is so. There is thus a very good indication that levels of 1:20, and possibly even 1:10, in an endemic area, or in people likely to have been exposed for any length of time, should be taken as indicating prior exposure.

As Sneath[12] has stated, *Chromobacterium violaceum* is commonly found in water and in soil. If not so clearly stated, it has been implied[5,15] that *Ps pseudomallei* is a widespread and common organism in South East Asia. The present survey, while challenging the ubiquitous nature of *Ps pseudomallei* demonstrates clearly that *Chromobacterium violaceum* is indeed very common and widespread. It is also clear that it is a common surface water inhabitant: the comparison between incidence in padi water and running water is significantly in favor of the former ($P < 0.005$). If run-off from fields was argued to explain the almost significantly greater presence of *Ps pseudomallei* in streams, this should equally well apply to *Chromobacterium violaceum*. It does not.

The LD_{50} of *Chromobacterium violaceum* for mice and guinea pigs has been established.[13] In the present study, hamsters were shown to be susceptible to a wide range of doses, those dying doing so more rapidly than the *Ps pseudomallei*-infected animals. Recognition of *Chromobacterium violaceum* in mixed culture is much less of a problem than with *Ps pseudomallei*, owing to the former's chromogenic characteristic. The hamster, however, has proved to be as useful a biological filter for *Chromobacterium violaceum*, whether by death or overt infection, as is the case in surveys for *Ps pseudomallei*.

Thin *et al*[19] report on 10 cases of melioidosis seen over a three year period. In a population of approximately 60,000 annually at risk, from whom these were drawn, it is evident that this disease is uncommon. In the case of infections, many of which were fatal, due to *Chromobacterium violaceum* on which information was collected by Sneath,[12] 9 were from Malaya, which, between the years 1927-1952 when the cases were seen, referred to West Malaysia and Singapore. Given that the population annually at risk approximated to 5.5 million between those years, this disease must be called rare. However, the army group in Singapore conducted their work in a sophisticated setting among a Westernized community while Malaya, in the years referred to, was less well served and the population was, and still to some extent, is, much less aware of the possibility of infection by *Chromobacterium violaceum*. Thus, the true incidence of infection by this organism may be much higher. Ognibene[9] reports two fatal infections in US soldiers in Vietnam and records the death of a third following superficial skin infection with subsequent spread to multiple abscess formation, due to *Chromobacterium violaceum*. Many of the hamsters in the present study were subject to purulent skin infection, some of which, at the time of sacrifice, had not spread. Several were kept alive and have since recovered without obvious residual infection. They will later be sacrificed and evidence of spread sought.

The widespread incidence, in appreciable numbers, of an organism which has definite potential for causing multiple abscesses and death, in like manner to *Ps pseudomallei*, must alert authorities in areas of high incidence to this diagnostic possibility in superficial skin lesions. This awareness should include knowledge of the time taken in artificial media for chromogenesis and the fact that achromogenic variants occur. Agglutination[13] has as yet not proved a satisfactory tool in sero-diagnosis but further work will be done on this aspect.

Inferences only can be drawn about the relationship of *Hirudinaria spp* to the incidence of infection. Despite the much greater leech load in rice fields in Kelantan than elsewhere, minor trauma from other causes would still appear to be the most likely route of infection. It may only be postulated that the leech adds to this risk. There is no present evidence that it is implicated as an intermediate host. Work is in progress on this subject.

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The Silvered Leaf-Monkey of Malaysia, *Presbytis cristatus*, as a Disease Model for Melioidosis

by

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SUMMARY

The use of the Silvered Leaf-Monkey of Malaysia (*Presbytis cristatus*, Raffles 1821) as an animal model for melioidosis is described.

Great variation in the effect of *Pseudomonas pseudomallei* on this animal is shown, consistent with the patterns of infection in humans. These variations are considered to be of host origin and will be further studied.

By virtue of its predominantly arboreal habitat, divorced from that of *Pseudomonas pseudomallei* and its response to infection by that organism, it forms a good animal model for the study of melioidosis.

In view of the depletion of other types of primate normally used in studies of this kind, it is recommended as an alternative.

INTRODUCTION

Melioidosis in humans is a disease showing a wide spectrum of effect varying from inapparent infection to a clinically overt, acute septicaemic process. Animal models used in experimental melioidosis have included hamsters, guinea pigs, rabbits, rats, mice, dogs, sheep, horses and monkeys. The use of the last has usually centered on macaques, either *M. mulatta*[6] or *M. fascicularis* which latter is variously termed *M. irus*[9], *M. cynomolgus*[4,12], or the crab eating monkey. Great variation in the route of infection, from conjunctival implantation[4] to intraperitoneal injection[9] and the more likely natural route of subcutaneous injection[4,6] have been used. Infecting doses have ranged from those unstated[12] to 1.5×10^6 organisms[6].

The macaque, *M. fascicularis* (Raffles 1821), typically lives near coastlines in tidal creeks and mangrove swamps, is partly terrestrial, raiding plantations and cultivated fields for food which may consist of grain, potatoes and molluscs. *M. mulatta* (Zimmermann 1780), the rhesus monkey, has similar general habits[7], while *M. nemestrina* (Linnaeus 1766), the pig-tailed macaque, is usually confined to inland forest but raids nearby grain crops for food[8]. Thus, the macaques are exposed in nature to conditions in which they

may acquire infection with *Pseudomonas pseudomallei* and cases appearing in animals confined in zoological gardens are known.[11,13] In a batch of 19 *M. speciosa*, the stump-tailed macaque, imported to the United States of America from Thailand, one developed spontaneous melioidosis.[15] Leaf monkeys, genus *Presbytis* (Escholtz 1821), the langurs, are basically arboreal, the bulk of their diet being leaves, buds, shoots and bark. Many live through a dry season without descending to drink water.[7] While they are not entirely arboreal, they are probably much less in contact with the environment of *Pseudomonas pseudomallei* than are the macaques.

The silvered leaf-monkey (*Presbytis cristatus*, Raffles 1821) is a resident of Malaysia and has been used in the study of human scrub typhus,[17] for which it forms an excellent model. Supplies of the traditionally used macaques are on the wane.[2] This, with the differing habitats of the two groups, and the variable response to pseudomallei infection shown by other animals, was considered in choosing *P. cristatus* as a model for melioidosis.

Animals captured from the wild had been tested for antibody to *Pseudomonas pseudomallei* by indirect hemagglutination, immediately following introduction to the animal colony. Of 397 macaques (95 *M. nemestrina*, 302 *M. fascicularis*) tested, 22 (5.5%) had titers of 1:40 or more, while none of the 85 silvered leaf-monkeys had significant titers.[16]

MATERIALS AND ANIMALS

Pseudomonas pseudomallei

Several "strains" from human cases which had occurred in British Army personnel during 1964-68, had been stored on nutrient agar slants, with occasional subcultures. Initially, two of these were used, one coded 603 presented a rough colonial morphology at all times, no matter the media used, while the second, called 405, has shown smooth colonial morphology throughout. Both, in minimal doses, killed hamsters within two days when given intraperitoneally.

Experimental animals

The golden hamster *(Microcricetus auratus)* was used to passage *Ps pseudomallei* 405, and as a method of detecting the organism in monkey blood drawn for the more usual blood culture technique. The monkeys used were silvered leaf-monkeys, all except one of them fully grown and some of which had previously been used in scrub typhus studies.

MEDIA

Nutrient broth, Nutrient agar (NA) and NA with glycerol 3% and crystal violet 1:200,000 (NAGC), were routinely used in preparing the organism for suspension in sterile 0.85% sodium chloride solution or in sterile distilled water, or in recovering it from animals.

METHODS

The indirect hemagglutination (IHA) test of Ileri by the tube method, in the modification described by Alexander *et al*[1], was used. All monkeys were bled from the saphenous vein to establish a baseline IHA. All were negative.

Suspensions of organisms for use were prepared by culturing *Pseudomonas pseudomallei* in nutrient broth for 24 hours at 37°C, centrifuging at 2000 rpm for 15 minutes and resuspending twice in 5 ml sterile distilled water. Following the two washes, the deposit was suspended in 3 ml sterile distilled water and standardized in the spectrophotometer. A suspension standardized to OD 0.14 at 620 nm had been shown, by plate count in triplicate, to contain 2.5 to 5.0 x 10^8 organisms per ml. Appropriate dilutions were prepared to obtain the numbers required and the counts checked retrospectively by plate count in triplicate.

One monkey, (181) was injected intraperitoneally with approximately 1.4 x 10^4 organisms of "strain" 603, suspended in sterile distilled water. All other animals were exposed to "strain" 405 in varying doses and by different routes. Number 181 and 5 of the 21 who received strain 405 had not previously been used in any experimental study. Sixteen receiving strain 405 had been exposed to *R. tsutsugamushi*, usually in the form of multiple strains several months prior to use in pseudomallei studies. Daily clinical notes were kept on each animal: IHA testing was carried out approximately two weeks post exposure and then at roughly 4-week intervals. The survivors have been followed up serologically for from 8 months to over 1 year.

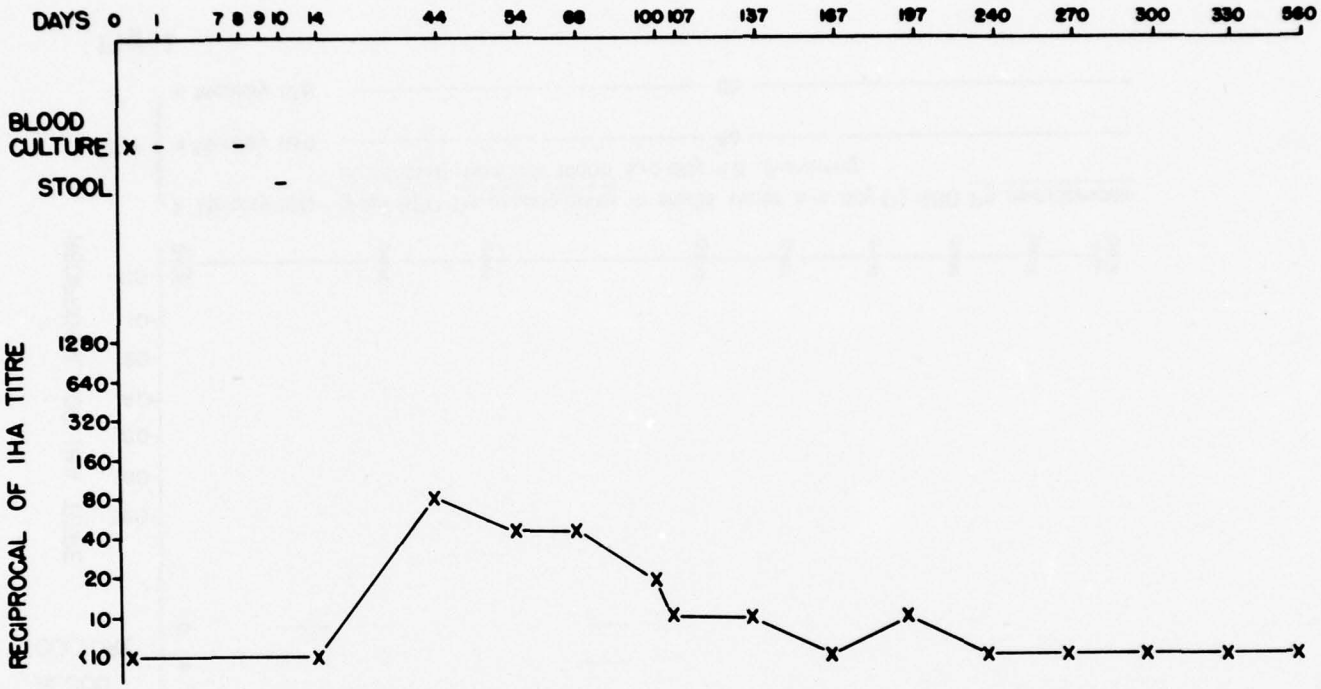
In most cases, blood cultures were performed early in the course, usually on the first, second and third days post exposure and occasionally later. Stool cultures were taken only on the first animal and temperature recordings were made only in the first few. Any superficial abscesses forming were cultured if they ruptured or aspirated and cultured if not.

All animals dying were submitted to autopsy but histology was felt unnecessary since it is described adequately elsewhere.[3,5,10,14] Cultures were made of any lesions which had formed in internal organs.

RESULTS

The accompanying figures,1-8, show the course in each animal. Where the type of exposure is similar, the animals are grouped together for convenience of presentation.

Two animals, 188 and 36, which were not injected, either intraperitoneally or subcutaneously, but housed in cages with infected animals, did not develop any changes. All animals whose scarified legs were exposed to water containing *Ps pseudomallei* or who were



KEY :- x Monkey 181 : given 1.4×10^4 Ps. pseudomallei i/p in sterile water, not passaged, day 0; Survived one year +

Fig. 1

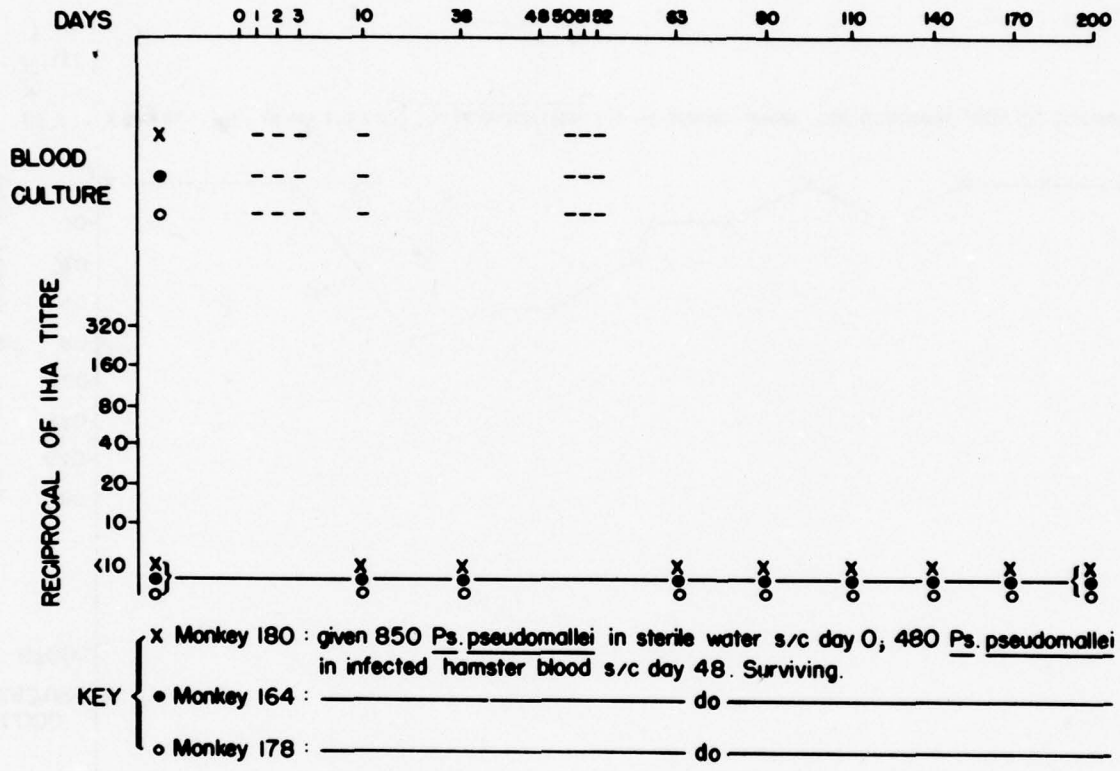
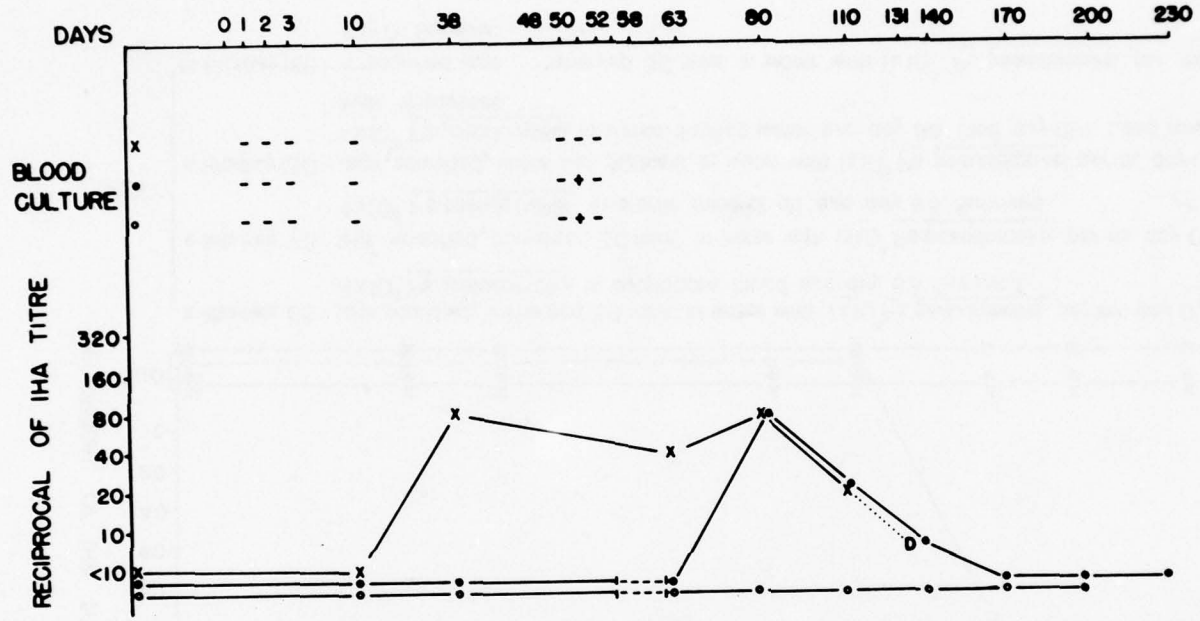

DAYS
0 1 2 3 10 38 48 50 51 52 63 80 110 140 170 200
BLOOD CULTURE
RECIPROCAL OF IHA TITRE
320
160
80
40
20
10
<10
X Monkey 180 : given 850 Ps. pseudomallei in sterile water s/c day 0; 480 Ps. pseudomallei in infected hamster blood s/c day 48. Surviving.
KEY
• Monkey 164 : do
o Monkey 178 : do

Fig. 2



KEY:

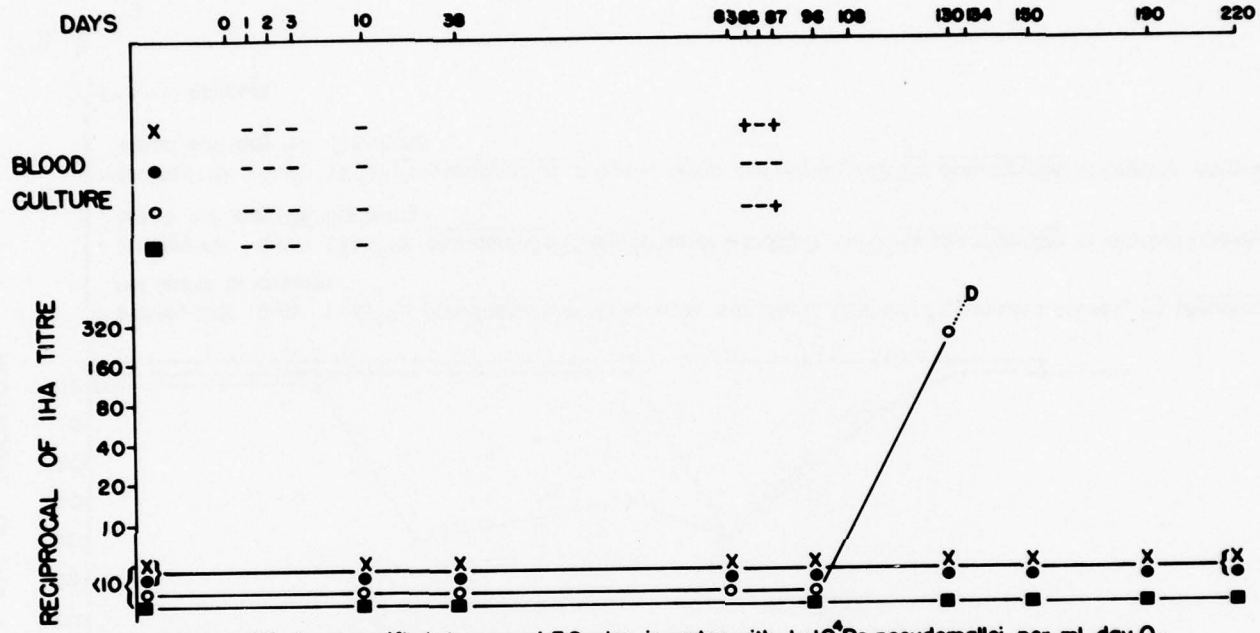
x Monkey 109 : given 1×10^4 Ps. pseudomallei in sterile water s/c day 0. Died day 151, unrelated causes; Ps. pseudomallei not found at autopsy.

• Monkey 76 : given 1×10^4 Ps. pseudomallei in sterile water s/c day 0. 1×10^4 Ps. pseudomallei in infected hamster blood s/c day 48. Surviving.

o Monkey 116 : given 1×10^4 Ps. pseudomallei in sterile water s/c day 0. 1×10^4 Ps. pseudomallei in infected hamster blood s/c day 48. Surviving.

|----| Abscess.

Fig. 3



KEY:

- x Monkey 65 : legs scarified, immersed 30 mins. in water with 1×10^4 Ps. pseudomallei per ml. day 0; 4×10^3 Ps. pseudomallei in autologous blood s/c day 83. Survived.
- ● Monkey 73 : legs scarified, immersed 30 mins. in water with 1×10^4 Ps. pseudomallei per ml. day 0; 4×10^3 Ps. pseudomallei in sterile mineral oil s/c day 83. Survived.
- o Monkey 130 : legs scarified, immersed 30 mins. in water with 1×10^4 Ps. pseudomallei per ml. day 0; 4×10^3 Ps. pseudomallei in sterile distilled water s/c day 83. Died day 134. Lung and liver abscesses.
- ■ Monkey 188 : unscarified legs immersed 30 mins. in water with 1×10^4 Ps. pseudomallei per ml. day 0. Survived.

Fig. 4

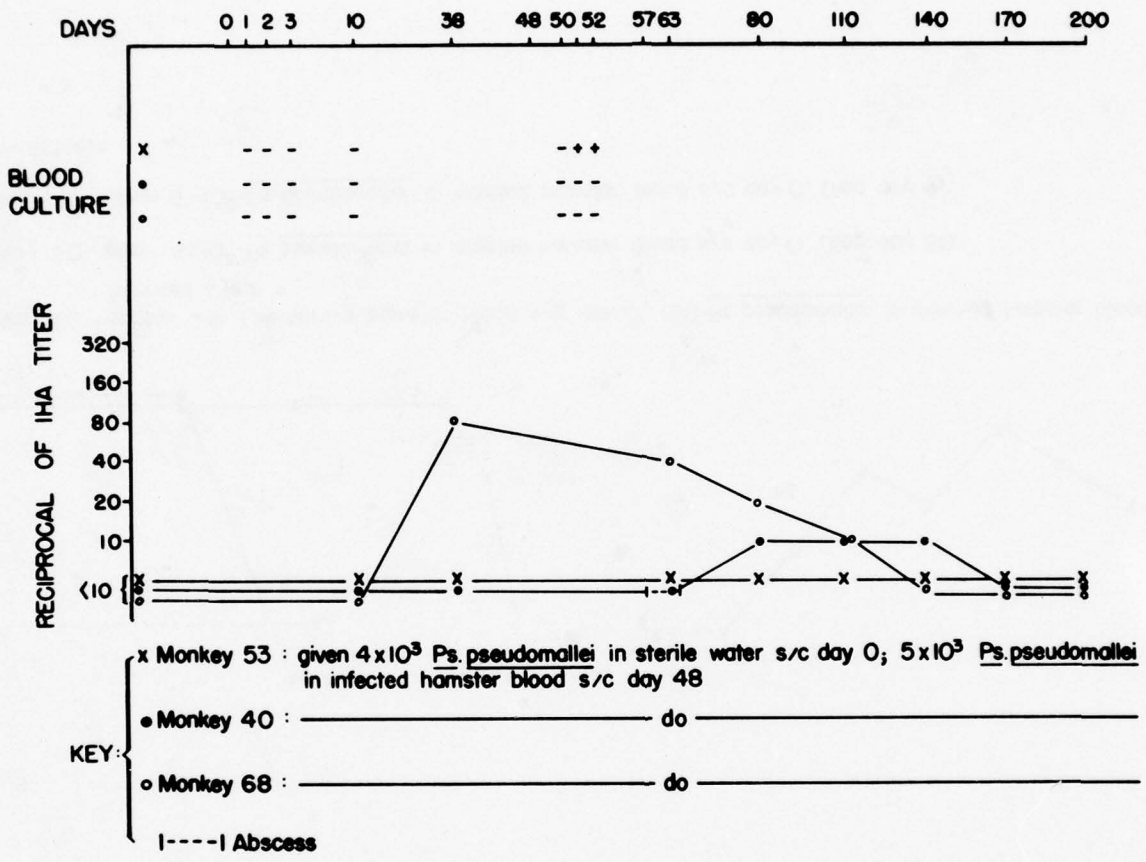
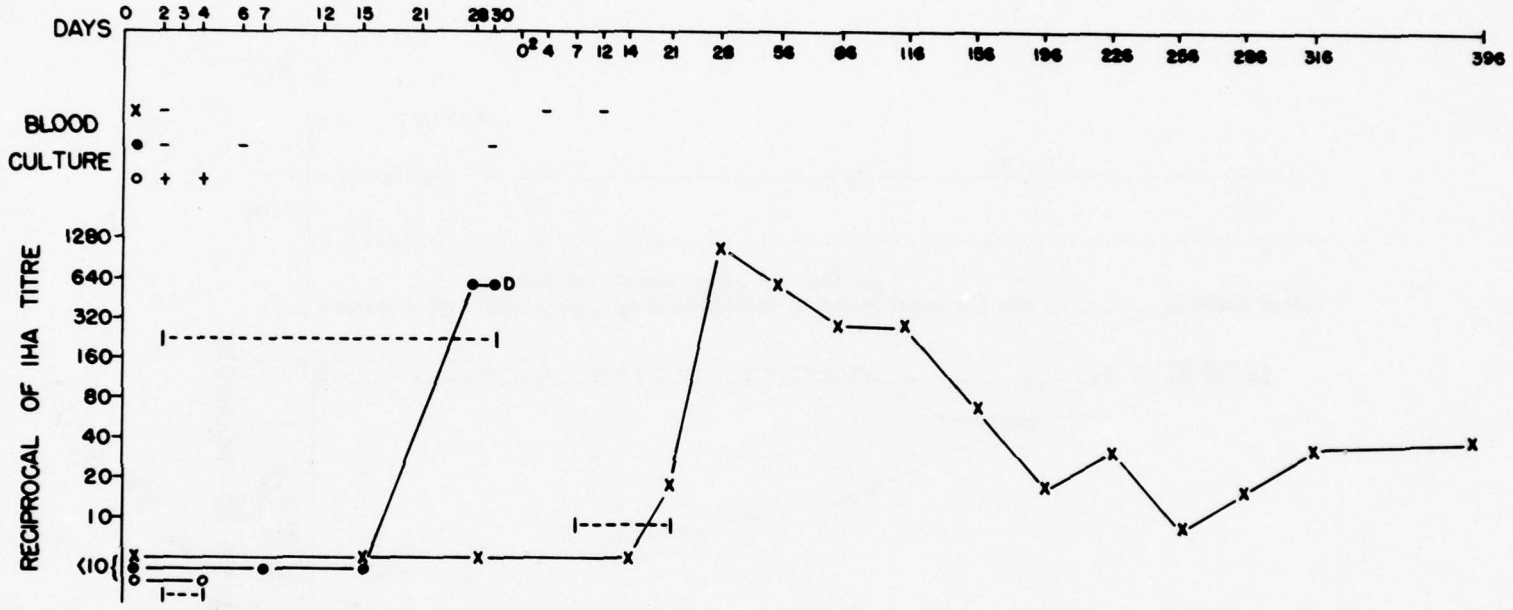
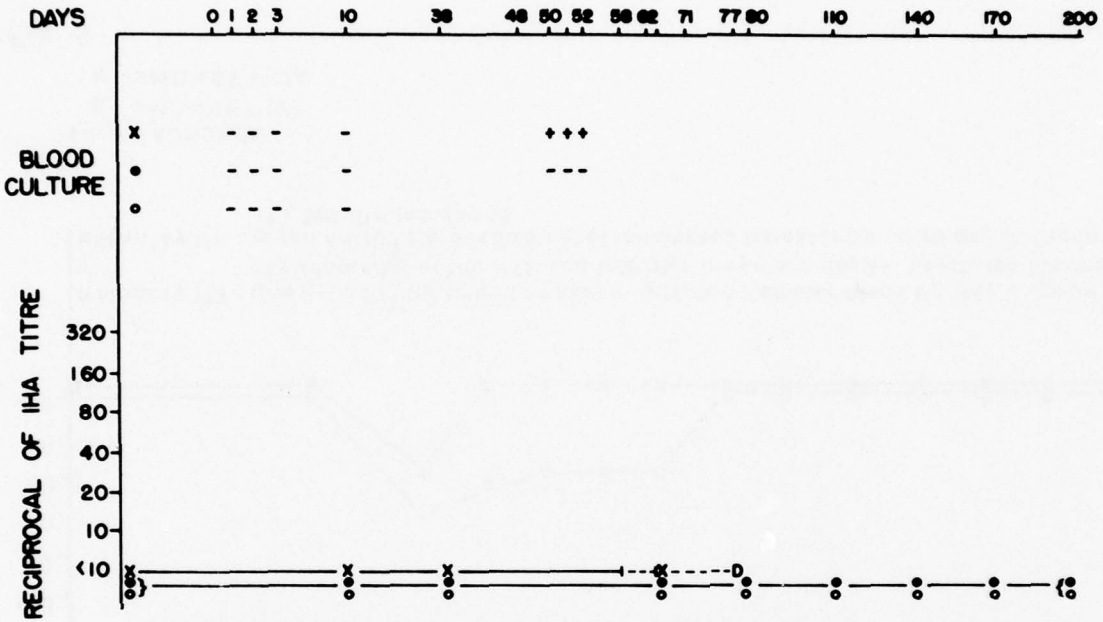

DAYS
0 1 2 3 10 38 48 50 52 57 63 80 110 140 170 200
BLOOD CULTURE
RECIPROCAL OF IHA TITER
320
160
80
40
20
10
<10
KEY:
x Monkey 53 : given 4x10³ Ps. pseudomallei in sterile water s/c day 0; 5x10³ Ps. pseudomallei in infected hamster blood s/c day 48
• Monkey 40 : do
o Monkey 68 : do
I----I Abscess

Fig. 5



KEY:
- x Monkey 172 : control with 1 ml. normal hamster blood s/c day 0; 100 Ps. pseudomallei in infected hamster blood s/c day 0²: Survived 1 year +
- • Monkey 170 : given 4×10^3 Ps. pseudomallei in infected hamster blood s/c day 0. Died day 30.
- o Monkey 171 : given 4×10^3 Ps. pseudomallei in infected hamster blood s/c day 0. Died day 4.
- I----I Abscess.

Fig. 6



KEY:

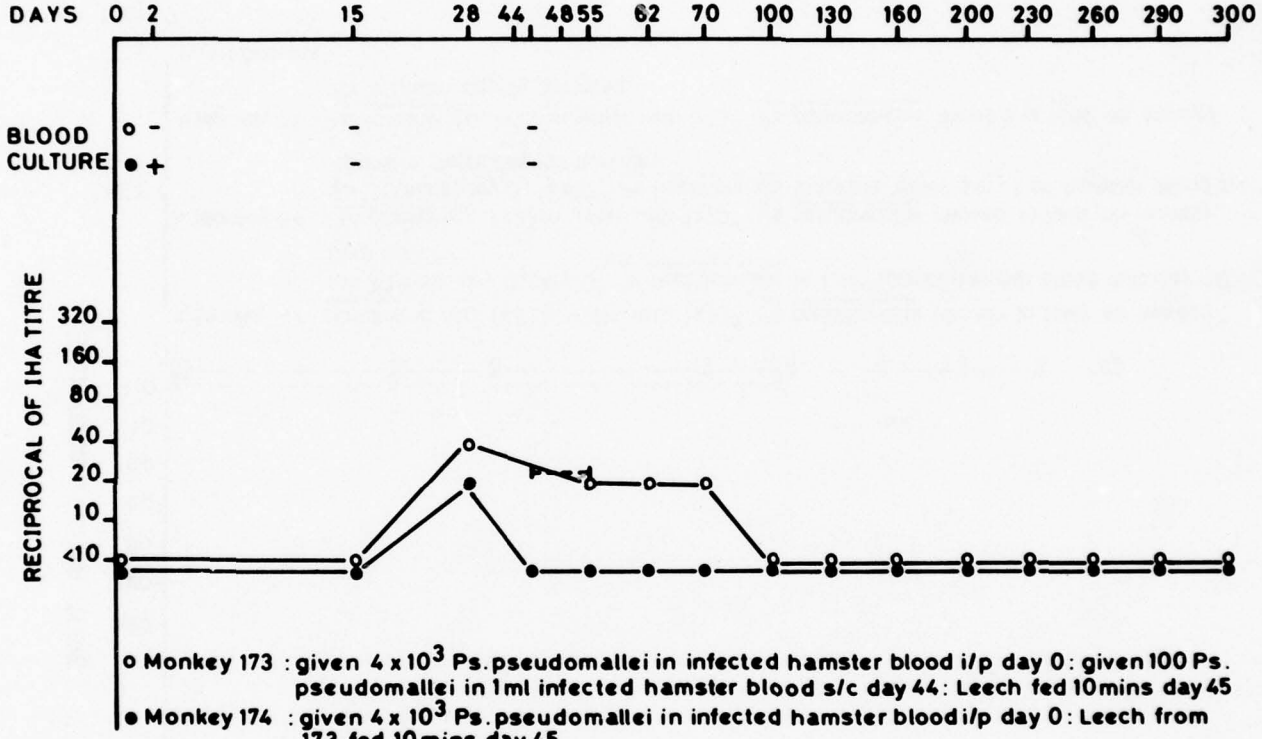
x Monkey 47: Hirudinaria spp. (x2) in water with $1x10^4$ Ps. pseudomallei allowed to feed on monkey for 30 mins. day 0; $4x10^2$ Ps. pseudomallei in 1 ml. normal hamster blood s/c day 48. Died day 77.

• Monkey 64: Hirudinaria spp. (x2) in water with $1x10^4$ Ps. pseudomallei allowed to feed on monkey for 30 mins. day 0; $4x10^3$ Ps. pseudomallei in sterile water s/c, 1 ml. hamster blood s/c opposite thigh. Day 48. Survived.

o Monkey 36: Hirudinaria spp. (x2) in water with $1x10^4$ Ps. pseudomallei allowed to feed on monkey for 30 mins. day 0; Survived.

I----I Abscess

Fig. 7



KEY:

|---| = ABSCESS

o = MONKEY 173

• = MONKEY 174

Fig. 8

subjected to leech-feeding in *Ps pseudomallei* contaminated water, did not, for these reasons, show either clinical or serological change. Of those dying due to *Ps pseudomallei* infection, two (47, 171) had no antibody response but had positive blood cultures, abscesses of 19 and 2 days duration at the site of injection and died on the 29th and 4th post-exposure days respectively, their abscesses unresolved. One (130) developed a HA titer of 1:320, 4 days before death on the 51st day, did not develop an abscess and had one of three blood cultures positive. The fourth (170) had a peak HA titer of 1:640, 2 days before and on the day of death, 30 days post exposure, an abscess at the site which lasted for 28 days and did not resolve, but negative blood cultures.

Autopsy results

The description is of gross change only.

Animal 47. A spindle-shaped abscess, 15 x 2 cms, was present over the injection site and on opening showed two large loculations of yellowish pus from which *Ps pseudomallei* was recovered. The peritoneum was studded with several small collections. The spleen was normal but in the left lobe of the liver there was a 3.5 cm collection of pus and an adjacent smaller abscess. The lungs were grossly clear but there was free pus in the bronchi from which *Ps pseudomallei* was cultured. A small fluctuant mass overlying the right frontal region on opening showed a shaggy-walled abscess containing similar material. An abscess of the right temporal lobe compressed the frontal lobe on that side. *Pseudomonas pseudomallei* was recovered from all grossly positive sites.

Animal 171, which died at 4 days, showed no gross evidence of change but blood cultures and cultures of the injection site, the spleen and liver, were positive.

Animal 130. Dissection of the injection site showed the tissue to be normal. *Ps pseudomallei* was not recovered from this site. The spleen and kidneys were normal. The liver showed multiple small abscesses average size of 2 mm diameter. There was a 3 cm diameter abscess of the lower lobe, basally, of the left lung. Almost the entire lower lobe of the right lung was occupied by a pus-filled, shaggy walled abscess with gross pleural and pericardio-pleural adhesion. *Pseudomonas pseudomallei* was recovered in culture from all the sites showing changes.

Animal 170 had a large abscess at the injection site, multiple abscesses of the liver and spleen and enlarged mesenteric lymph nodes. The lungs were clear. *Ps pseudomallei* was cultured from all grossly positive sites.

In those surviving and showing HA titers, the smallest dose of 100 organisms was associated in one (172) with abscess formation lasting 14 days, with resolution and the highest HA titer in all the

animals studied, of 1:1280 at the 28th day post-exposure. Two animals in this group (68, 109) did not show local abscess formation, neither had positive blood cultures and each developed titers of 1:80 post-exposure. Subcutaneous doses of 5×10^3 organisms (40) and 1×10^4 (76), were associated with rises in titer of 1:10 and 1:80 at 36 and 32 days, short term resolving abscesses at the site of 3 and 4 days duration, respectively. The animal with a titer of 1:80 had one of three blood cultures positive. Three animals received intraperitoneal doses: 4×10^3 in two (173, 174) and 1.4×10^4 of strain 603 in one (181). All developed antibody at titers peaking at 1:40, 1:20, and 1:80 on days 28, 28 and 44, respectively. The one with the lowest titer had one of three blood cultures positive. Animal 173 received an additional dose of 100 organisms subcutaneously, 44 days after initial exposure. This dose was associated with a resolving subcutaneous abscess of 10 days duration but with no boost in titer.

There were three animals in which there was no antibody response but which had other evidence of infection. The doses associated with infection in this group were 4×10^3 in autologous blood (65), 5×10^3 (53) and 1×10^4 (116), given subcutaneously. All had positive blood cultures and one (116), a short-term, resolving abscess of 4 days duration.

The final group are those which had been exposed but in which no evidence of any effect was seen. Three of them (164, 178 and 180) received doses of, first 850 then 480 organism subcutaneously. Two (64 and 73) received 4×10^3 organisms subcutaneously, the first in sterile water, the second in sterile mineral oil.

DISCUSSION

There is no rigid dose response in silvered leaf-monkeys to exposure to *Pseudomonas pseudomallei*, effects being seen following as few as 100 organisms given subcutaneously, to none resulting from 4×10^3 given by a similar route. No pattern can be defined except the variability of response which is thought to occur in the human disease.

Persistence of titers at varying levels up to 2-plus years in confirmed cases of human melioidosis has been shown by Alexander.[1] The results of the present study correspond in some measure with his findings. Titers in the animals which survived have persisted over periods of from 70 to 396-plus days, the longest in persistence showing a level of 1:40.

The use of a single strain, which, by colonial morphology, gave no indication of antigenic alteration throughout the study, and which has consistently retained its capacity to cause hamster death over the same period, indicates that the variability of response shown is a characteristic of the host. It is intended to pursue this aspect in attempt to determine the mechanism involved.

From all the findings it can be concluded that the silvered leaf-monkey of Malaysia (*Presbytis cristatus*, Raffles, 1821) as an animal model for melioidosis, reflects very well the disease processes as expressed in humans and emphasizes the great variability of this infection.

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STUDIES IN PROGRESS

Hirudinaria spp (the Buffalo Leech). Studies with *Pseudomonas pseudomallei*

by

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In part of the 1965 study of leptospirosis in this Unit, land leeches *(Hemadipsa spp)* were triturated and injected intraperitoneally to hamsters. One of the animals died with signs compatible with melioidosis and *Ps pseudomallei* was recovered. This led, in the following year, to several projects aimed at investigating the possible role of land leeches in the transmission of melioidosis. No conclusive evidence was adduced from these studies.

The presence of *Ps pseudomallei* in surface water has been established and the incidence of antibody to *Ps pseudomallei* has been found to be highest in people from rice-growing areas. *Hirudinaria spp* is a common inhabitant of rice fields and is known to be capable of harboring rinderpest virus from infected cattle.[2] A related species transmits trypanosomes to fish.[1] To date, bacterial transmission has not been established. It appears that leeches of most genera have a specific associated bacterial flora which, in the blood sucking species, consists of a single bacterial species.[4] In *Hirudo medicinalis* it was suggested that the gut associated flora has the ability to destroy or inhibit other bacteria.[3]

The viability of *Ps pseudomallei* in *Hirudinaria spp*, the effect of the leech on *Ps pseudomallei* with each in the same environment, and the ability of the buffalo leech to transmit *Ps pseudomallei*, were investigated.

METHODS

These were essentially similar to the methods of the 1966 group except that the host hamsters were not shaved before leech feeding in order to reduce skin trauma to that inflicted by the leech. The bacteriologic media used are described elsewhere in this report. In one procedure, that of introducing known numbers into the gut of the leech, an intubation method was employed, using a Portex intravenous catheter*. In preliminary studies a weak solution of fluorescein sodium injected transpharyngeally through the catheter was demonstrated on subsequent dissection and photography under ultraviolet light, to have reached and remained in the sacculations of the gut, thus confirming that forced feeding was feasible.

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* Portex Ltd, Hythe, England.

For convenience, the various procedures followed will be grouped together with the results of each.

RESULTS

Survival of *Ps pseudomallei* in the same environment as *Hirudinaria spp*.

Cultures of a smooth strain of *Ps pseudomallei* were grown in nutrient broth and concentrations varying from 1×10^2 to 1×10^5 per ml were prepared in sterile distilled water. Leeches of genus *Hirudinaria* which had been collected from a jungle fringe pool, were placed individually in 50 ml of sterile distilled water in a loosely capped container. Quantities of the *Ps pseudomallei* suspensions were added to give final counts of 1×10^1 to 1×10^4 organisms per ml. A control of sterile distilled water without a leech but with 1×10^4 organisms per ml was employed. Plate counts at several dilutions were performed on NAGC agar on the day of preparation and daily thereafter, for 50 days. At approximately weekly intervals, subsamples of 1 ml were also taken for intraperitoneal injection to hamsters. On each occasion, the initial volume was made up by addition of sterile distilled water. The results are given in Figure 1.

On two subsequent occasions the same procedure was followed with similar results except that the periods of occluded growth of from 10 to 25 days evident in the first experiment, did not occur.

Survival of *Ps pseudomallei* within the leech, *Hirudinaria spp*.

Sixteen leeches of a similar size were intubated transpharyngeally and each was force-fed 0.1 ml of a suspension containing 1×10^5 *Ps pseudomallei* in sterile distilled water. The leeches were distributed, in pairs, in 50 ml quantities of sterile distilled water in loosely capped jars.

Shortly following the introduction to the water, the first pair was removed and each leech of this pair sectioned at a point through the fore gut and through the hind gut. Portions of fore- and hind-gut were separately triturated in a Tenbroek tissue grinder in 1 ml sterile distilled water. 0.5 ml of the suspension was cultured on NAGC and 0.5 ml injected intraperitoneally to hamsters. The other fore- and hind-gut portions were impressed on glass slides and prepared for microscopy, using a fluorescent antibody conjugate for *Ps pseudomallei*. Finally, 1 ml of the water from the jar was cultured in several tenfold dilutions on NAGC.

This process was repeated on each of the succeeding pairs on days 1, 4, 8, 12, 16, 20 and 28. All of the waters were cultured on each of these days and on days 42 and 49. Examination of the leech by fluorescent antibody technique was erroneously ommitted on day 12.

The results, which are given in Table 1, indicate the following:

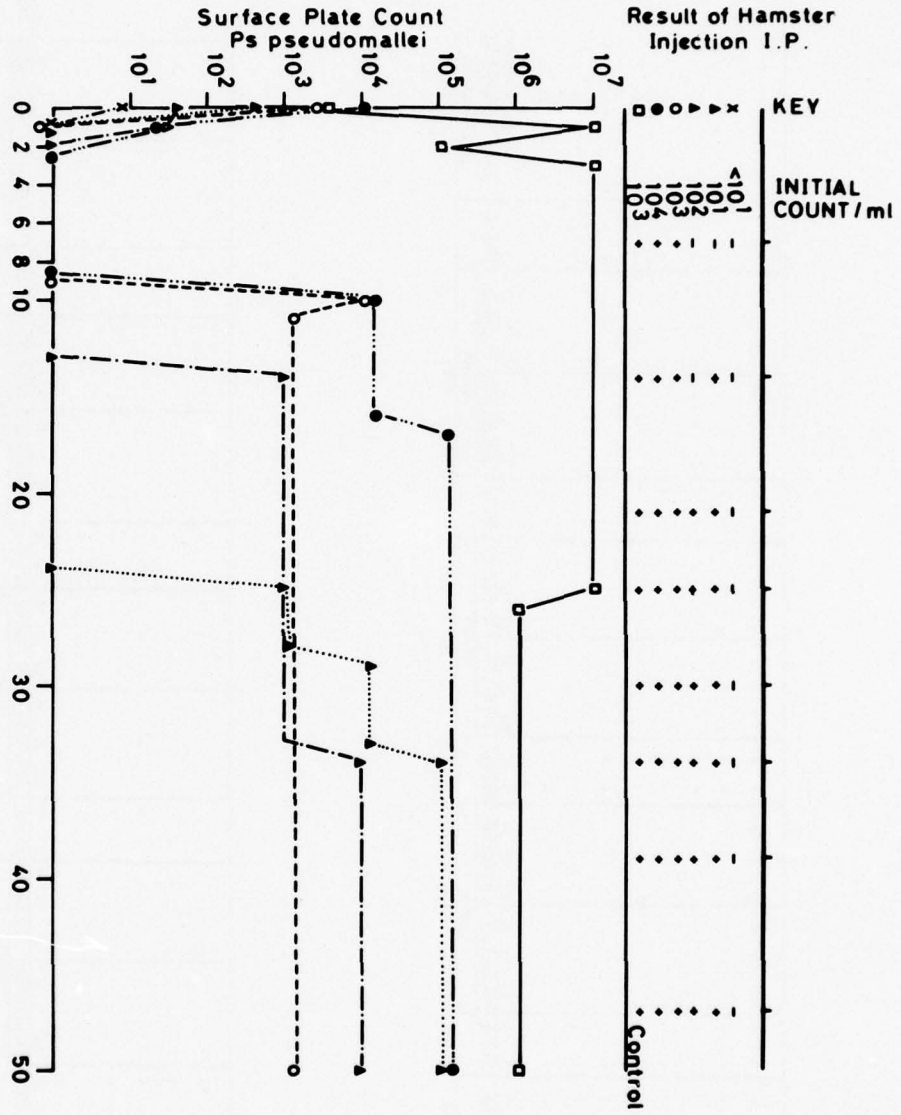


Fig.1 Surface Counts and Hamster mortality from leech containing water seeded with Ps pseudomallei

Table 1

Survival of *Ps pseudomallei* in the same environment as *Hirudinaria spp*

Day	0							1						4						8						12					
Recovery of *Ps pseudomallei*	Water		Leech					Water		Leech				Water		Leech				Water		Leech				Water		Leech			
			Anterior			Posterior				Anterior		Posterior				Anterior		Posterior				Anterior		Posterior				Anterior		Posterior	
Sample Vial No.	H	C	H	C	FA	H	C	FA	H	C	H	C	FA	H	C	H	C	FA	H	C	H	C	FA	H	C	H	C	FA	H	C	FA
1	-	-	+	+	+	+	+	+	-	-					-	-					-	-					-	-			
2									+	-	+	+	-	-	+	-					+	-					+	-			
3														+	+	-	+	+	-	-	-	+	+					+	+			
4																					+	-	-	-	+	-	-	-	+	-		
5																													+	-	-	-	ND	-	-	ND

Day	16							20						28						42		49	
Recovery of *Ps pseudomallei*	Water		Leech					Water		Leech				Water		Leech				Water		Water	
			Anterior			Posterior				Anterior		Posterior				Anterior		Posterior					
Sample Vial No.	H	C	H	C	FA	H	C	FA	H	C	H	C	FA	H	C	H	C	FA	H	C	H	C	H	C
1	-	-							-	-					-	-					-	-	-	-
2	+	-							+	-					-	-					+	-	+	-
3	+	+							+	+					+	+					+	+	+	+
4	+	-							+	+					+	+					+	-	+	-
5	+	-							+	-					-	-					-	-	-	-
6	+	+	+	+	+	-	+	+	+	+					+	+					-	-	-	-
7									+	-	-	-	±	-	-	±					-	-	-	-
															+	+	+	+	±	+	+	-	-	-	-

a. Excretion of *Ps pseudomallei* by the leech did not occur immediately following intubation.

b. While there was some variation in detection of *Ps pseudomallei* in the leech with time, it was still present in the leeches kept for 28 days (termination of the leech phase of the experiment) and had not lost its pathogenicity for hamsters.

c. Recovery of *Ps pseudomallei* from the waters tends to show a reduction following removal of the leeches from the environment.

Transmission of *Ps pseudomallei* by *Hirudinaria spp*.

Forty leeches, grouped in 14 pairs and 4 groups of three, were fed for 10 minutes on hamsters infected intraperitoneally with *Ps pseudomallei*, one group per hamster. That the hamsters were infected was confirmed by tail snip blood culture and by blood culture at necropsy following leech feeding. Counts of *Ps pseudomallei* in those animals were of the order of 5×10^3 per ml blood, at necropsy.

The leeches were kept in sterile distilled water in loosely capped jars. The leeches were re-fed on normal hamsters, each batch at intervals, subsequently sacrificing the leeches and attempting recovery of *Ps pseudomallei*. The hamsters on which they had been re-fed were followed for infection by culturing those dying or at sacrifice and necropsy. Of the original 40 leeches, 18 died before the assigned day for re-feeding on normal hamsters.

The 18 dead leeches were removed from their containers on the day of death, which varied from 2 to 61 days following the infected feed. *Ps pseudomallei* was not recovered from any of the waters in which they were kept.

Nine of these leeches had failed to feed as evidenced by lack of blood in their gut on dissection. From none of those which showed evidence of having fed, by virtue of an increase in girth and having free blood in their gut, was *Ps pseudomallei* recovered, either by direct culture of gut contents or by intraperitoneal injection of suspension into hamsters. The blood-containing leeches had died from days 19 to 41 post infected feed.

The surviving 22 leeches, in 4 batches, were fed on normal hamsters for from 5 to 15 minutes, the intervals between feeding on infected and normal hamsters varying for each batch. Re-feeding was performed on days 0, 25, 53 and 81, the leeches killed at termination of feeding, triturated in sterile saline, cultured for *Ps pseudomallei* and, with the exception of the first batch, 1 ml of leech suspension injected intraperitoneally into hamsters.

In the first batch, fed and re-fed on the same day, all of the leech suspensions contained *Ps pseudomallei* but all of the normal hamsters used in re-feeding survived until sacrifice on the 7th post

feeding day and none was infected. The second batch of 6 leeches was re-fed on normal hamsters on day 28 following the infected feed. Of those, only two gave isolates of *Ps pseudomallei* by intraperitoneal injection of suspensions into hamsters, of which one was positive on direct culture. None of the normal hamsters on which they were fed yielded isolates.

Pseudomonas pseudomallei was not recovered by any technique from groups three and four, each of six leeches, at days 53 and 81.

None of the waters in which the leeches were kept was positive for *Ps pseudomallei.*

Leech flora.

This has so far been studied in only a small number of leeches. The organism most frequently isolated conforms to the definition of family Pseudomonadaceae given in Bergey's Manual, 7th Edition. However, at present, it cannot with any certainty be placed in any one genus. As Stanier *et al*[5] stress in their taxonomic study, there are many difficulties in specifically identifying such an organism. It would be taxonomically incorrect to name it *"Pseudomonas hirudinis"* as had been done for the organism isolated from *Hirudo medicinalis* by Büsing.[3] Further examples will be sought and referred for expert opinion.

CONCLUSIONS

Ps pseudomallei appears to be able to survive in the same environment as the leech, *Hirudinaria spp.*

Survival of *Ps pseudomallei* within the environment of the leech gut is less certain, being more clearly demonstrable in the absence of blood than in its presence. Transmission by natural means, as such, has not been shown from hamster to leech to hamster.

The attraction of this work lies in the findings of transmission of trypanosomes and in the persistence of rinderpest virus in a blood sucking annelid which shares the same environment as the telluric organism *Ps pseudomallei.* Also, bacterial inhibitory processes seem to be at work against one organism when an organism of the same family not only survives in, but also may be essential to[4], the economy of the leech. This may have implications for treatment of *Ps pseudomallei* infections.

FUTURE PROPOSALS

To determine whether the effects seen to date are reproduceable. To determine if other telluric organisms, notably *Chromobacterium violaceum*, are subject to the same effects.

To define any inhibitory mechanisms involved.

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INVESTIGATIONS OF THE DEPARTMENT OF ECOLOGY

ECOLOGICAL STUDIES OF MAMMALS AND THEIR INVOLVEMENT IN TRANSMISSION OF ZOONOTIC DISEASES IN EQUATORIAL ECOSYSTEMS

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	30 06 73	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
30 06 72		U		N/A	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY		3A062110A831		
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)*

Investigations of the Department of Ecology

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

Tropical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
10 72	9 73		

17. CONTRACT/GRANT DADA17-73-G-9368

a. DATES/EFFECTIVE: 10 72 EXPIRATION: 10 73

b. NUMBER:*

c. TYPE: Y. Grant d. AMOUNT: 263

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 73	1.0	34.5
CURRENT FISCAL YEAR 74	1.0	30.4

19. RESPONSIBLE DOD ORGANIZATION

NAME:* US Army Medical Research Unit

ADDRESS:* Institute for Medical Research
Kuala Lumpur, Malaysia

RESPONSIBLE INDIVIDUAL

NAME: Dr. R. Bhagwan Singh, Director

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Lim, B.L., M. Sc.

NAME:

21. GENERAL USE

22. KEYWORDS (Precede EACH with Security Classification Code) Vertical distribution, canopy transect, *Rickettsia tsutsugamushi*, *Plasmodium*, *Hepatocystis*, Sabah, Sarawak, Peninsular

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

23.(U) Technical Objectives: To determine how the ecological niches of various species of mammals predispose their involvement in transmission of zoonotic diseases in equatorial ecosystems.

24.(U) Approach: Vertical distribution of mammals within the forest is being conducted with the aid of transect walkways through the canopy. Animals are brought to the laboratory, processed, marked and released the next day. In other areas also, capture, mark, release and recapture techniques are used to gather information, including rickettsial and viral isolations, blood parasites, etc.

25.(U) Progress: Studies of vertical distribution of mammals and their parasites and pathogens within the rainforest with the use of canopy transect walkways are in progress. In this area over 2000 animals have already been captured marked and released. Arboreal species here and elsewhere do not become infected with *Rickettsia tsutsugamushi*, however, blood parasites such as *Plasmodium* and *Hepatocystis* are more common in arboreal than in terrestrial hosts. Large differences in the rates of infections with malarial parasites occur within species of arboreal hosts which seem to be correlated with the habitat types of populations. Nests of arboreal mammals are rich with populations of mesostigmatid mites and other parasites. *Rickettsia tsutsugamushi* in terrestrial mammals appears to be most frequent in forest and lalang grass *(Imperata cylindrica)* habitats. Detailed studies using enclosures in different habitats to test sentinel animals are in progress.

In areas surveyed in Sabah, and especially Sarawak, the rates of transmission of *Rickettsia* seemed to be lower than in Peninsular Malaysia. In arbovirus studies in

*Available to contractors upon originator's approval.

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DD Form 1498, Research and Technology Work Unit Summary,
Item 25 Continued:

conjunction with the forest canopy transect walkways, 5 isolates (from 3 arboreal and 2 terrestrial host species) have been made from 823 mammals tested. Serological results are available for 201 samples, with 8 HI positives for Group B arboviruses. Studies of population dynamics have shown that some arboreal species in the primary forest have extremely slow population turnover, infrequent breeding cycles with periods of as long as 17 months with no reproduction at all, and small litters. Data for man-altered habitats is available but not yet analyzed. Several taxonomic and systematic studies remain to be written up.

COMPLETED STUDIES

Mammals and Scrub Typhus Ecology in Peninsular Malaysia

by

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Department of Ecology, USAMRU, Division of Medical Ecology, IMR
and Department of Rickettsiology, USAMRU

Abstract. 1069 blood samples and 958 kidney samples from small mammals in West Malaysia were tested to isolate *Rickettsia tsutsugamushi*, the infectious agent of "scrub typhus". Concurrently 3383 sera were tested for positive reactors against the same zoonotic agent. The results were analyzed from the standpoint of the habitat groupings and the vertical distribution of the mammals collected and tested over a two year period. The forest habitats appeared to yield higher isolation rates of *Rickettsia* than did the more transitional or edge habitats ("scrub"). Within the forest the arboreal species showed little evidence of involvement in the rickettsial transmission. No annual, seasonal pattern of infection rates could be detected, although periodically the infection rate was higher than during other times, especially in the "scrub" habitats. The nature of the "seasonality" of these fluctuations in the rate of recovery of isolates from these rodents is still not understood.

Scrub typhus derives its name from the types of habitats classically considered as the source of *Rickettsia tsutsugamushi* infections in humans in various parts of East Asia. The prevalence of reported human cases of scrub typhus may not necessarily identify the mainstream of enzootic transmission among natural hosts and vectors. Thus, it is difficult to anticipate the risk of human infections and areas of potential hazard remain unknown.

Audy[1] pointed out that the prevalence of scrub typhus in man varies according to habitats he frequents and also, in some geographic areas, according to the season of the year. Periodic fluctuations in the numbers of human cases occur in Malaysia[1] but no annual seasonality is apparent. In general, it has been reported that abandoned agricultural land grown over with "scrub" vegetation was of greatest risk to man, as well as many other transitional or preclimax habitats, such as river banks subject to periodic inundations[1]. Although stating that man seldom contracts infections in climax forests, Audy[1] alluded to a "jungle *tsutsugamushi*" transmission cycle. He postulated that in forests in Malaysia endemic foci or "...typhus islands, if present, are sparse: the endemic areas are confined to the deforested areas..." In the latter, the main vectors, as far as human infections are concerned, are thought to be ground dwelling, larval Trombiculid mites (chiggers).

Rodents and other small mammals become infected with the rickettsia[2], but it has yet to be demonstrated that they in turn infect larval Trombiculid mites in nature and that mammals thereby constitute a reservoir for the disease. Nevertheless, mammals provide a measure of rickettsial activity in Trombiculids but not necessarily those that bite man.

Cadigan *et al.* in studies of *Orang Asli* (aborigines) in Malaysia found that people in deep jungle villages had a higher prevalence of antibodies against *R. tsutsugamushi*, than those in fringe habitats ("scrub") and those in typical villages (kampongs) in rural areas[3]. Before this study, a similar situation in small mammals was already established and is now reported here. The purpose of the present paper is to analyze the results of these rickettsial isolation and serological surveys of small mammals in a broad sense on the bases of the total numbers of small mammals trapped rather than individual species and to correlate these results with ecological information. The isolation data from the host species were analyzed on the basis of the habitats and vertical distribution within forests. Trapped animals were transported to the laboratory and were etherized and bled by cardiopuncture. Some were euthanized and dissected and kidneys were used in isolation attempts. Details of the isolation and serological techniques and results are discussed in another paper[4]. In short, isolations were attempted by direct inoculation of whole blood or triturated kidneys intraperitoneally into weanling laboratory mice[5]. Serology was done by the indirect fluorescent antibody tests[6].

Small mammals included in this report came from seven areas, which can be grouped into two predominant types of habitats. These are defined below to avoid confusion that exists with terms such as "scrub". All of the areas, except Janda Baik, were in the state of Selangor in West Malaysia.

1) Edge Habitat: This type of vegetation generally exists at the interphase between forest and open areas; the latter may be agricultural lands, rivers, roads, landslides, tin mines or any other areas not grown over with forest. It consists of regenerating vegetation, usually fast-growing species which require much light. Unless the interphase is maintained by periodic clearing and cutting, by fire, or by flood, it is a transitional vegetation. If large tracts of forest are cut the entire area may pass through this phase before secondary forest develops. Other names have been given to such habitats, e.g., *belukar*, scrub, fringe habitats[3], but these often have special meanings and are not inclusive. The following areas are classified as edge habitats: A) Janda Baik: This area is located in the state of Pahang, near the Selangor border and had scattered trees, and bamboo which in few places formed a nearly complete canopy. Most of the area was intermixed with and bounded by cleared areas and cultivated land. B) Bukit Mandol: This area had a mixed, sparse growth of various aged rubber trees, often interspersed with fruit trees, differing from commercial rubber estates in that

the rubber trees were not as dense, not planted at fixed intervals or all at the same time, and did not comprise a pure stand. The area was subject to intermittent clearing of the undergrowth. C) Bukit Lagong: This was an area of shifting cultivation located at the *Orang Asli* village near Kepong, characterized by scattered relict trees of the original rainforest, small patches of secondary forest and predominated by more recent regenerating vegetation.

2) Forest Habitat: Forests with a complete or nearly complete canopy: A) Subang, B) Elmina Estate, and C) Bukit Rotan: These were collecting areas in different parts of the same large forest which had been nearly clear cut in the past, but was fairly mature. The forest was interspersed with a few relict trees from the original primary forest. D) Bukit Lanjan: Mostly original forest located near Bukit Lanjan, near Kepong, but not on the hill of that name. Tree growth reached to over 60 meters. There had been very limited selective cutting 30 to 40 years ago but the forest, although partly disturbed in the past, had all the appearances of a primary forest.

On the basis of work of Harrison[7], the present trapping data, and other broader surveys, the animals captured and tested were divided into arboreal, semi-arboreal (scansorial), and ground groups. The prevalence of isolations of *Rickettsia tsutsugamushi* in each of these groups are given in Table 1 without regard to habitat groupings. The arboreal species included squirrels: *Callosciurus notatus* (288), *C. nigrovittatus* (72), *C. caniceps* (15), *Sundasciurus tenuis* (14), *Ratufa bicolor* (1), *Iomys horsfieldii* (4), *Hylopetes spadiceus* (4), *Petinomys vordermanni* (2); and primitive primates: *Tupaia minor* (2), *Nycticebus coucang* (3), *Ptilocercus lowii* (1). *Callosciurus notatus* yielded the only two isolates of *Rickettsia tsutsugamushi*. Although considered arboreal, *C. notatus* was sometimes captured in the ground traps. In general, these data (Table 1) strongly suggest that arboreal species do not appear to be much involved in rickettsial transmission. Therefore, the arboreal species probably do not become involved in the "jungle *tsutsugamushi* transmission cycle" alluded to by Audy[1].

The data for terrestrial and scansorial (semi-arboreal) species[8] were grouped according to the two habitats in which the mammals were caught: forest and edge ("scrub"). At each trapping site constituting edge habitats each species was represented in approximately the same prevalence ratios of the total catch. Differences in infection rates in the two types of habitats can be illustrated collectively for all species or individually by single species. An example of the latter is *Rattus sabanus* which was the numerically dominant species caught on the ground in all of the trapping areas (30-50% of the catch). The blood isolates of rickettsia from *R. sabanus* in the forest habitats were 31 out of 86, and 8 out of 92 in edge habitats.

During the period of October 1969 through September 1970 the overall rickettsial isolation rate (blood) from small mammals collected in the forest habitats was 23% (N=259) while that in the

Table 1

Prevalence of *R. tsutsugamushi* isolations and sero-positives from mammals in various vertical strata in the vegetation

STRATA	NO. TESTED			PERCENT POSITIVE		
	Blood	Kidney	Serology	Blood	Kidney	Serology
Arboreal	260	198	813	0	<1	4
Semi Arboreal	478	513	1491	14	18	61
Ground	331	247	1079	14	20	55

edge habitats ("scrub") was 10% (N=336)(Fig.1A). During the same period, the serologically positive animals composed 80% of those tested from the forest (N=206), while positives among those from edge habitats composed 42% of those tested (N=298)(Fig.1C). From October 1970 through August 1971, the isolation rate (kidney) from animals collected in the forests was 21% (N=276) while the rate in those from edge habitats was 13% (N=587)(Fig.1B). The rate for serologically positive reactors was 64% (N=543) in the forest samples and 64% (N=783) in the samples from edge habitats (Fig.1D).

Collectively, these data appear to show differences in *R. tsutsugamushi* transmission in the two main habitats studied (X^2 test for isolation rates for both years, and sero-positives for period of October 1969 through September 1970, $p < 0.01$). It appears that the "jungle *tsutsugamushi*" to which Audy[1] alluded is important (but not among arboreal species) in the endemic maintenance of scrub typhus, even though man's risk in forest may be smaller than in classical scrub typhus habitats: "scrub" vegetation. The forest areas appear to be important as stable, endemic reservoirs for the disease. The edge habitats, or "scrub", are unstable, nonclimax habitats in Malaysia and are ultimately cleared again for cultivation or they regenerate into secondary forest. The probability of human infection is apparently dependent on a combination of prevalence rates in the vector chiggers, the location and the biting habits of the chiggers present and the amount of time (exposure time) spent by humans in the habitat. Relatively less time is probably spent in the forest than in "scrub" habitats which often result from extensive and intensive human activities.

In Figure 1 the rickettsial isolation and serological data for all of the small mammals (except arboreal species) in the two general habitat groups is pooled by month. In the early part of the study, November 1969 through April 1970, the samples from the forest yielded a higher isolation rate (X^2, $p < .01$) than those from the edge habitats ("scrub")(Fig.1A). By June the isolation rate in the "scrub" approximated that in the forest.

From October 1970, the source of the isolation was changed from blood to kidneys (with the exception of forest samples in April, May and June 1971). The isolation rate during that time period was initially high in both habitat groups, but decreased after December 1970 (Fig.1B). Because of the different tissues used for isolation attempts the two time periods, October 1969 through July 1970 and October 1970 through August 1971, are not strictly comparable, except for fluctuations of infection rates between habitats within the two time periods. In both time periods the isolation rates during the months of December through May were low in the "scrub" areas and this sometimes coincided with a relatively low rate in the forest (e.g., February and April 1971) and sometimes not (e.g., February through April 1970). Thus, whatever may have been responsible for these fluctuations in the isolation rate, did not always have an effect at the same time in the two habitat groups. Traub and Wisseman[9]

LEGEND

Figure 1. Rates of monthly isolations of *Rickettsia tsutsugamushi* (A and B) and serologically positive reactors (C and D) from rodents and other small mammals in two habitat groups in West Malaysia. The numbers at the top of each column indicate sample sizes. From October 1969 through July 1970 blood samples were used (A). From October 1970 through August 1971 kidneys were used as the isolation source, except the forest samples in April, May, and June 1971 which were blood (blood sample sizes in parentheses)(B). Serological samples were done concurrently with isolations (C and D).

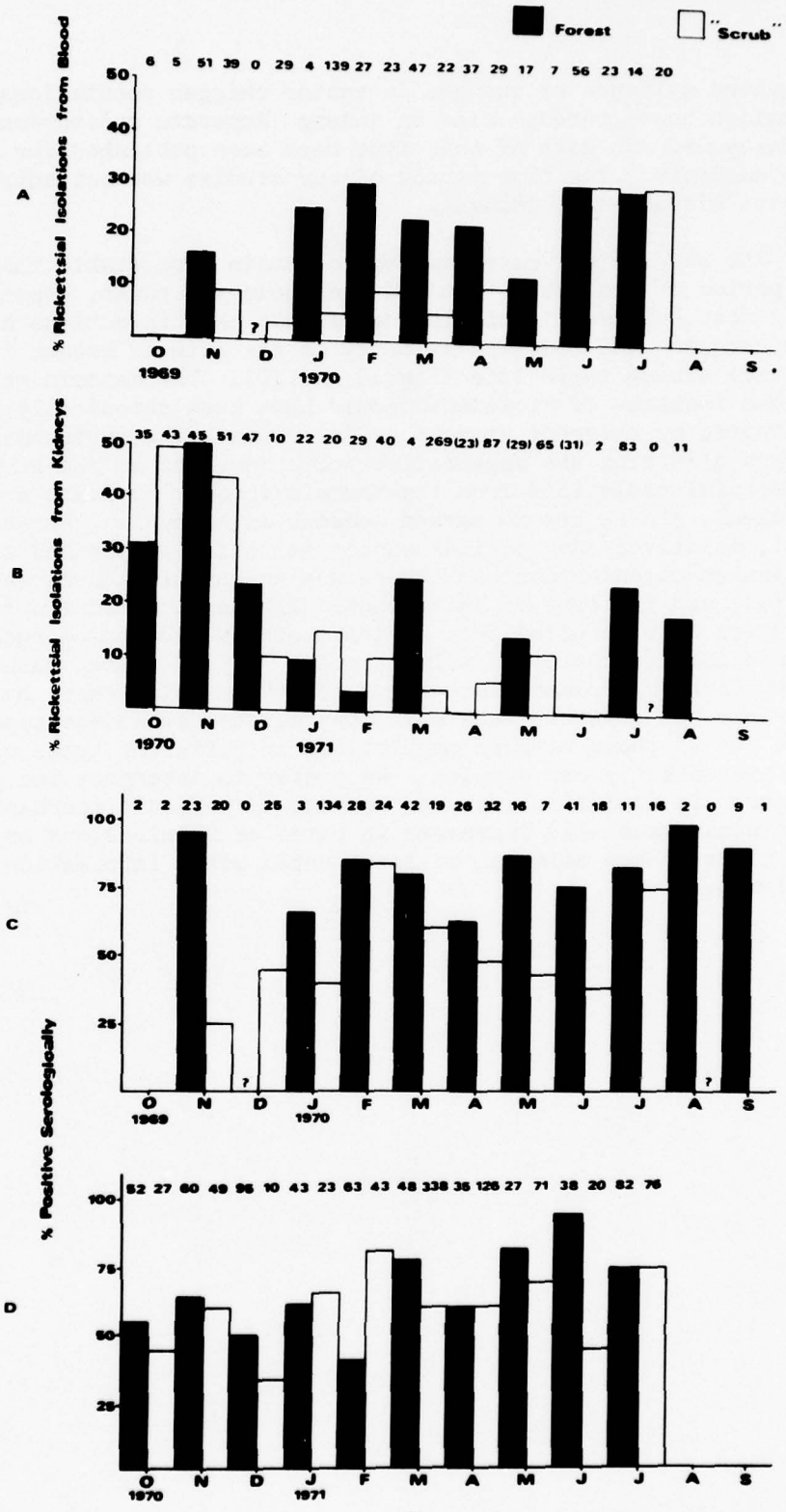

Forest
"Scrub"
A
% Rickettsial Isolations from Blood
6 5 51 39 0 29 4 139 27 23 47 22 37 29 17 7 56 23 14 20
50
40
30
20
10
?
O N D J F M A M J J A S
1969
1970
B
% Rickettsial Isolations from Kidneys
35 43 45 51 47 10 22 20 29 40 4 269 (23) 87 (29) 65 (31) 2 83 0 11
50
40
30
20
10
?
O N D J F M A M J J A S
1970
1971
C
% Positive Serologically
2 2 23 20 0 25 3 134 28 24 42 19 26 32 16 7 41 18 11 16 2 0 9 1
100
75
50
25
?
?
O N D J F M A M J J A S
1969
1970
D
82 27 60 49 96 10 43 23 63 43 48 338 36 126 27 71 38 20 82 76
100
75
50
25
O N D J F M A M J J A S
1970
1971

Figure 1

presented evidence of changes in vector chigger populations and mammalian hosts through time in lalang *(Imperata cylindrica)* habitats in Malaysia. No data of this type have been published for forest or edge habitats. The time period of our studies was not sufficiently long to discern such changes.

The serological rates seemed to remain more stable throughout the period of the study than did the isolation rates, especially in the forest habitats. This indicates that past infections had been very frequent and that the majority of the animals became infected sometime during their life (Fig.1C and 1D). The mammals tested which yielded isolates of *Rickettsia* could have been chronically infected or reinfected by chiggers at various intervals. The environmental factors effecting the apparent periodic increase in the proportion of rickettsial isolations from the mammals in some habitats are still not clear. There are no marked seasons in Malaysia. Moreover, the short, relatively dry periods cannot be reliably ascribed to any particular calendar months. There was no correlation between monthly rainfall and the rate of isolations. If the observed fluctuations in isolation rate resulted from environmentally induced recrudescence of chronic infections to a level detectable by our tests, rather than reinfections or primary infections of new animals, then the environmental inducers (whatever they may be) sometimes apparently acted out of phase between populations in different types of habitats as represented by our samples. We prefer to interpret the periodic increases in isolation rates as results of periodic (perhaps in a broad sense seasonal) increases in rates of reinfections or primary infections in new animals, at least until other information becomes available.

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Scrub Typhus Antibody in Mammals in Three Habitats in Sabah

by

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INTRODUCTION

Recent studies of scrub typhus ecology in Peninsular Malaysia showed that small mammals which restrict their activity nearly entirely to the forest canopy were not much involved in the transmission of scrub typhus *(Rickettsia tsutsugamushi)* as evidenced by blood and kidney isolation attempts and serological tests (Muul, Lim and Walker, 1973). Terrestrial and scansorial (those which climb trees at least part of the time) mammals in general yielded high rates of isolations and even higher percentages of serological positives (Walker, Gan, Chan and Muul, 1973). These studies also demonstrated that although the transmission rate of rickettsia in the terrestrial rats in "scrub" habitats (edge habitats) was high, the rate in forest rats was higher. Thus, it seems that the forest habitat may serve as the primordial source of the infection, at least for the natural mammalian hosts, and as forests are altered by man, the rickettsia "spill over" into such disturbed habitats as small animal populations establish themselves there. Further evidence for this was provided by the observation that rats not endemic to Malaysian forests, but characteristic invaders of disturbed habitats, e.g. *Rattus rattus, Rattus exulans* and *Rattus tiomanicus* (Harrison and Quah, 1962) were relatively less involved in rickettsial transmission than the forest rats (Walker, *et al.*, 1973).

The purpose of this study was to examine the distribution of serological positives among small mammals, as indicators of past infections with *Rickettsia tsutsugamushi*, in Sabah and to compare the results from that area with those obtained in Peninsular Malaysia. The data presented were collected from 14 May through 5 June 1971 at Poring, near Ranau, at the base of Mt. Kinabalu. (elevation about 1300 feet; Latitude 6° 2' N; Longitude 116° 43' E) Ecological and parasitological aspects of this study were reported elsewhere by Muul and Lim (1973).

METHODS

Traps were placed on the ground and at various heights in trees at parallel stations in three habitats: primary forest, secondary forest and edge habitats. Trapped animals were transported to the field laboratory and were etherized and bled by cardiopuncture.

Blood samples obtained from most of the specimens, were stored in filter paper (Gan, Cadigan and Walker, 1972) for later testing at the laboratories of the Institute for Medical Research, Kuala Lumpur. Serological results were obtained by the indirect fluorescent antibody test as modified by Walker *et al.* (1973). No rickettsial isolations were attempted.

RESULTS

Altogether 324 blood samples from species which are primarily ground dwelling (terrestrial and scansorial) and 167 from arboreal species were tested for antibodies against *Rickettsia tsutsugamushi*. The overall results for terrestrial and scansorial species are given in Table 1. The highest rates of sero-positives were obtained from *Rattus whiteheadi* and *R. muelleri*. The overall rate of sero-positives was 13 percent.

In Table 2 the results are grouped according to the vertical zonation of the hosts. Only two sero-positives were found among the arboreal species. The dominant arboreal species tested were *Callosciurus notatus* (91; 2 positives), *Sundasciurus lowii* (21), *Callosciurus prevostii* (18), and *Tupaia minor* (17). Also tested were *Tupaia gracilis* (8), *Pteromyscus pulverulentus* (7), *Sundasciurus brookei* (2), *Petinomys setosus* (2) and *Sundasciurus hippurus* (1).

In Table 3 the data for the terrestrial and scansorial species are grouped according to habitats in which the tested specimens were collected. The overall rate of positives in terrestrial and scansorial animals caught in the forest (primary and secondary) was 14 percent (N=266). In edge habitats the rate was 8 percent (N=49) *Rattus cremoriventer* which was caught in sufficient numbers in both habitats, had a rate of 11 percent positives (N=89) in the forest (primary and secondary) and 6 percent (N=16) in the edge habitats.

DISCUSSION

The overall proportion of animals showing antibody against *Rickettsia tsutsugamushi* in the areas studied in Sabah was lower than that observed through any of the months during our studies in similar habitats in Peninsular Malaysia. In May of 1971 in our study areas in Peninsular Malaysia, the overall rate of serological positives among terrestrial and scansorial mammals in both forest and edge habitats exceeded 60 percent (over 75 percent in the forest) (Muul *et al.*, 1973). The host species, with the exception of *Tupaia tana*, surveyed in Sabah were the same as those tested in much larger numbers in Peninsular Malaysia. However, the numerical ranking of the species differed. In Peninsular Malaysia *Rattus sabanus* was the most frequently trapped rat in the areas studied. *Rattus cremoriventer*, the dominant species in the Sabah collections, was seldom caught in Peninsular Malaysia. The absence of sero-positives in *Tupaia tana* is puzzling. But, even if the results for this species are excluded, the overall rate of sero-positives would be only 17 percent, far lower than the rate in the areas studied in Peninsular Malaysia.

We have no alternative explanation to offer to the obvious: there appears to have been less *Rickettsia tsutsugamushi* transmission occurring in the areas surveyed in Sabah than in our samples of habitats in Peninsular Malaysia. These observations would seem to warrant further investigation to determine how generally these results apply to the whole of Sabah.

Although the overall rate of sero-positives was low, there was a marked difference between the groups of mammals that spend most of their time on the ground and those which restrict most of their activity to higher vegetational zones. The paucity of sero-positives among the arboreal species is in agreement with the results of surveys in Peninsular Malaysia (Muul *et al.*, 1973). There was a statistical difference also between the scansorial and terrestrial species (Chi Square Test: $p > 0.01$).

However, unlike in the areas studied in Peninsular Malaysia there was no statistical differences (Chi Square test: $0.3 > p > 0.2$) between sero-positives among terrestrial and scansorial hosts collected in the forest (primary and secondary)(14 percent, N=226) and in edge habitats (8 percent, N=49). Unfortunately, the sample from edge habitats was small and did not contain large numbers of the species characteristic of such habitats i.e. *Rattus tiomanicus* (2), *Rattus exulans* (2), and *Rattus rattus* (5 tested). All of the latter, however were negative (Tables 1 and 3). Thus, there is a strong suggestion in the data that man-altered habitats may have somewhat less transmission than in the forest as was the case in areas studied in Peninsular Malaysia (Muul *et al.*, 1973).

Leptotrombidium chiggers were not preponderant among the total number of larval Trombiculid mites collected from the host species which yielded most of the sero-positives: *R. cremoriventer* (Trombiculids composed 27% of 104 mites), *R. muelleri* (Trombiculids composed 7% of 859 mites) *R. sabanus* (none), *R. whiteheadi* (1% of 674 mites collected). Among these sero-positive host species only *R. muelleri* (3 individuals, all serologically negative) were infested with the known vector, *Leptotrombidium deliense*. *Tupaia tana* which had four individuals infested with *L. deliense* had no sero-positives. The latter species was infrequently caught in Sabah. Even though the overall rates of sero-positives were low, it would seem difficult to attribute the infections only to *L. deliense* since so few were found to infest small mammals. The lack of sero-positives in *Tupaia tana* is remarkable in that the closely related *Tupaia glis* in Peninsular Malaysia had high rates of sero-positives (Walker *et al.*, 1973).

SUMMARY

Surveys of antibody against *Rickettsia tsutsugamushi*, the causative agent of scrub typhus in man, were conducted in various species of small mammals in three habitats in Sabah. The overall rates of sero-positives were lower than in Peninsular Malaysia. As

was the case in Peninsular Malaysia, arboreal species appeared to be little involved in rickettsial transmission. Species that spend most of their time on the ground seemed to have a somewhat higher rate of sero-positives in the primary and secondary forests than in the "scrub" (edge habitats), however, the difference was not statistically significant at $p = .05$ (Chi Square test). Infestations with the known vector, *Leptotrombidium deliense*, were few.

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Table 1

Numbers of terrestrial and scansorial* mammals captured near Ranau, Sabah, tested and found to be positive for scrub typhus *(Rickettsia tsutsugamushi)* antibodies by IFAT.

	No. Captured	No. Tested	No. Positive
*R. cremoriventer**	153	100	11 (11%)
*Tupaia tana**	100	86	0
R. muelleri	76	56	13 (23%)
R. whiteheadi	90	33	12 (36%)
*R. sabanus**	31	26	4 (15%)
R. rajah	12	11	1
*R. rattus**	8	5	0
R. tiomanicus	3	3	0
*R. exulans**	4	2	0
*Tupaia glis**	2	2	0
	479	324	41 (13%)

Table 2

Mammals classified according to their preponderant activity in the vertical vegetational zones and the overall prevalence of scrub typhus (*Rickettsia tsutsugamushi*) antibodies within these groups (collections from near Ranau, Sabah).

	No. Tested	No. Positive	% Positive
Arboreal	167	2	1
Scansorial	224	15	7
Terrestrial	100	26	26

Table 3

Habitat distribution and scrub typhus *(Rickettsia tsutsugamushi)* serological data of primarily ground dwelling mammals collected near Ranau, Sabah. (Percent species catch designates the percentage of the total specimens of the species collected within a given habitat; percent positive designates the percentage of sero-positives in the sample tested; numbers collected and numbers tested appear in parentheses). (4 *R. rattus* caught in the village not included, of these only one was tested; it was negative).

	Primary Forest		Secondary Forest		Edge Habitat ("Scrub")	
	% Species Catch	% Positive	% Species Catch	% Positive	% Species Catch	% Positive
R. cremoriventer	78 (120)	11 (72)	10 (15)	17 (12)	12 (18)	6 (16)
Tupaia tana	72 (72)	0 (62)	14 (14)	0 (10)	14 (14)	0 (14)
R. muelleri	71 (54)	28 (40)	19 (15)	11 (9)	10 (7)	14 (7)
R. whiteheadi	52 (47)	33 (18)	25 (23)	60 (10)	13 (12)	0 (4)
R. sabanus	74 (23)	17 (18)	16 (5)	0 (5)	10 (3)	33 (3)
R. rajah	55 (6)	0 (6)	0 (0)	-	45 (5)	20 (5)
Tupaia glis	50 (1)	-	50 (1)	-	0	-
R. tiomanicus	0 (0)	-	33 (1)	0 (1)	67 (2)	0 (2)
R. exulans	0 (0)	-	50 (2)	0 (2)	50 (2)	0 (2)
R. rattus	0 (0)	-	0 (0)	-	50 (4)	0 (4)
% of total catch	70 (323)	13 (216)	16 (76)	18 (49)	9 (67)	8 (49)

Scrub Typhus Transmission in Rats in Four Habitats in Peninsular Malaysia

by

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Recent surveys of *Rickettsia tsutsugamushi* transmission in small mammals in various habitats in Peninsular Malaysia showed that the rate of endemic transmission in forest habitats studied was greater than in habitats recently altered by activities of man (Muul, Lim, and Walker, 1973). These surveys drew material from several areas, some fairly distant (30 to 50 kilometers or about 20 to 30 miles) from the others. The purpose of the present study was to determine the rates of rickettsial transmission in adjacent habitats through time.

MATERIALS AND METHODS

Four trapping areas were established in the vicinity of the *Orang Asli* (aborigine) settlement at Bukit Lanjan, near Kepong, Selangor. The habitats chosen were forest, edge ("scrub"), lalang grass *(Imperata cylindrica)*, and the *Orang Asli* village. Initially, 100 traps were set in each habitat, but later the number in the forest had to be increased to obtain adequate samples (the trapping rate in the forest was lower than in other habitats). Since no single species encompassed all habitats studied, several indicator species were chosen: *Rattus sabanus* (forest), *Rattus exulans* and *Rattus rattus diardii* (village). Traps were operated five days a week. Captured animals were brought to the laboratory each day where they were weighed, measured, bled, and examined for ectoparasites. Standard toe-clipping number codes were used for later identification. The animals were released at the point of capture the next day. Recaptured animals were rebled and tested again if at least two weeks had elapsed since the previous sample had been taken. Blood samples were inoculated directly into weanling laboratory mice for rickettsial isolation attempts and serological samples were tested by the indirect fluorescent antibody technique (Walker, Gan, Chan, and Muul, 1973).

The forest area was partly disturbed primary forest. Selected logging on a very limited basis (only two species removed) were conducted in the early 1940's. The edge habitat chosen was the interphase between the forest and a swath of cleared land under some high-voltage lines. The lalang habitat was located under the high-voltage lines. This area was maintained as a fire climax by intermittent burning. Between burnings parts of the area had grown up with vinaceous vegetation, wild ginger, wild banana, and fast-growing trees. The site chosen for the trapping grid, however, was nearly pure lalang grass. This lalang grass habitat, although not

wider than 100 meters at any point (and usually less), extended for kilometers under the high-voltage wires. The village was set in a mixture of vegetation consisting of fruit trees, palms which were intermixed with open spaces and houses. All four habitats in which trapping was conducted were adjoining.

The trapping was conducted from August 1971 through January 1973, but the sample sizes in December 1972 and January 1973 were small. In January 1972 the forest grid area was timbered and the traps were removed farther into the forest where a new grid was established.

RESULTS

Altogether 880 specimens of the indicator species were captured and tested in the four habitats. Some of these were recaptured and retested a total of 788 times. In the forest all but one specimen of the indicator species were *Rattus sabanus*. In the edge habitat *R. tiomanicus* comprized 56 percent of the collections, *R. sabanus* 14 percent. In lalang *R. tiomanicus* was 61 percent, *R. sabanus* less than 1 percent, *R. argentiventer* 31 percent, and *R. exulans* 7.6 percent. In the village *R. tiomanicus* was 52 percent, there were no *R. sabanus*, *R. argentiventer* was 17 percent, and *R. exulans* 31 percent. The results for *R. rattus* are not included as only 4 were captured in the edge habitat and twelve in the village (3 were positive). Since there was no statistical difference between the isolation results from initial captures (238 positives in 852 samples, 28 percent, collected from August 1971 through November 1972) and repeat captures (201 positives in 760 samples, 26 percent, collected at the same time), these data were combined. The combined monthly samples from all four habitats varied from 19 percent to 34 percent positives. There were no statistically significant differences between consecutive monthly samples nor between the lowest (July 1972, 19 percent, N=78) and the highest (November 1971, 34 percent, N=108) monthly isolation rates.

Habitats. The lowest rate of isolations was obtained from the village, 20 percent of 490 samples positive. The overall rate in the forest was 33 percent (N=340 samples). In the portion of the forest which was cut over in January 1972, the samples collected before (3/23 positive) and the samples collected after the timbering operations (4/25 positive) were not statistically different.

In lalang the isolation rate was 31 percent (N=480) and in the edge habitat, 26 percent (N=333).

Hosts. The highest isolation rate was obtained from *R. argentiventer* (34 percent of 295 samples), followed by *R. sabanus* (32 percent of 379 samples). There was no statistical difference between the isolation rates in these two rats. In *R. tiomanicus* the overall isolation rate was 26 percent (N=736 samples).

R. tiomanicus was trapped in the village (52 percent of total catch), lalang (61 percent) and scrub (56 percent). The lowest isolation rate for this species was obtained in the village (20 percent of 255 samples), the highest rate in the lalang (32 percent

of 297 samples). The isolation rate in *R. sabanus* was significantly higher (X^2, $p > .02$) in the forest (34 percent of 339 samples) than in the edge habitat (16 percent, 38 samples). The isolation rate in *R. argentiventer* was high in all habitats in which it was caught (34 percent in the village, N=83; 31 percent in lalang, N=150; 45 percent in the edge, N=62). *R. exulans* had the lowest isolation rate in all habitats in which it was caught (14 percent in the village, N=152; 16 percent in lalang, N=37; 17 percent in the edge, N=48).

DISCUSSION

Unlike in our earlier studies (Muul *et al.*, 1973) no marked fluctuations in the overall transmission rate could be discerned in the area within the 16 month period under study. However, our earlier results were based on pooled samples from several areas to obtain sufficiently large sample sizes. The present data are more reliable since they were obtained from adjacent habitats, the extremes about one kilometer apart. Also, the differences in isolation rates in these adjacent habitats were not as great as those obtained from those earlier studies. Nevertheless, the endemic transmission rate among rats still appears to have been somewhat higher in the forest than in the forest edge ("scrub").

The overall rate of isolations from the forest (115 positives of 340 samples) was significantly higher (X^2, $.05 > p > .02$) than that in the adjacent edge habitat (88 positives of 333 samples). Also the overall isolation rate in *R. sabanus*, the base of the activity of which seems to be the forest, was significantly higher (X^2, $.05 > p > .02$) than in *R. tiomanicus*, which seems to be based in the "scrub" and other man-altered habitats. Those few *R. sabanus* which were caught in the edge habitat had a significantly lower (X^2, $.05 > p > .02$) rate than those caught in the forest. The isolation rates in *R. sabanus* and *R. tiomanicus* were not significantly different in the edge habitat, but the rate in *R. sabanus* in the forest was significantly higher (X^2 $.05 > p > .02$) than the rate in *R. tiomanicus* in the edge habitat.

The lalang habitat also had a high transmission rate although the rates in lalang and in the edge habitat were not significantly different (X^2 $p > .20$). According to Harrison & Quah (1962) *R. argentiventer* is a rat primarily based in lalang grass habitats, whereas, *R. tiomanicus* is a rat of scrub habitats (edge) and plantations. Although *R. argentiventer* was caught in lalang more often than anywhere else, it was also caught regularly in the adjoining village and in the adjacent edge habitat. In the latter two habitats its isolation rate was higher than that of the rats characteristic of those habitats: in village *R. argentiventer* (28/83) vs. *R. exulans* (22/152), X^2, $p < .01$; in the edge habitat *R. argentiventer* (28/62) vs. *R. tiomanicus* (46/185), X^2, $p < .01$. This seems to indicate that lalang, where *R. argentiventer* seems to be based, has a high rate of transmission and the rates for the adjoining village and edge habitats are embellished by lalang rats wandering

through these areas and rats characteristic of the edge and village perhaps wandering through the lalang on occasion. Further evidence of this is provided by *R. tiomanicus* which were 32 percent (N=297) infected in lalang and 25 percent (N=185) infected in the edge habitat. The difference in this case is not statistically significant X^2, $.10 > p > .05$, but the numerical difference in the results is very suggestive. The overall blood isolation rate obtained in our previous study, in more extensive edge habitats that bordered neither mature forest nor lalang, was 10 percent (N=336).

If the houses were removed from the village area, it would be most similar to the edge habitat. The isolation rates are certainly similar (20 percent for village, N=490, 26 percent for edge, N=333).

The rat most often caught in the lalang was *R. tiomanicus*. Perhaps this happened because the lalang strip although extensive, was narrow throughout its length. Also, in most areas it did not consist of only lalang, but was mixed with other vegetation. Thus, it appears that *R. tiomanicus* spent quite a lot of time in this area, including the nearly pure stand of lalang in which the trapping grid was located. Their activity in lalang may have boosted the overall isolation rate for that species. *R. argentiventer* probably spent most of its itme in lalang, but wandered out occasionally to the adjoining areas.

From these results it appears that the endemic transmission is greatest in the forest and in lalang. Since in the area studied the edge and village habitats were adjacent to the forest and lalang, especially the edge which was situated between them, the transmission rates may have been enhanced from "spillower" from these adjacent habitats. This may account for the relatively higher transmission rate found in the edge habitat as compared with other man-altered environments of larger size (Muul *et al.*, 1973).

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Table 1

Isolation rates of *Rickettsia tsutsugamushi* in four habitats

	R. tiom		*R. sabanus*		*R. argent*		*R. exulans*		Overall	
	Pos	Tested	Pos	Tested	Pos	Tested	Pos	Tested	Pos	Tested
Village	50	255			28	83	22	152	100	490
Lalang	96	297	0	2	46	150	6	37	148	485
Scrub	46	185	6	38	28	62	8	48	88	333
Forest	0	1	118	362					118	363
Overall	192	738	124	402	102	295	36	237		

Medical Ecological Considerations of a Collection of Mammals from East Malaysia

by

Illar Muul and Lim Boo Liat

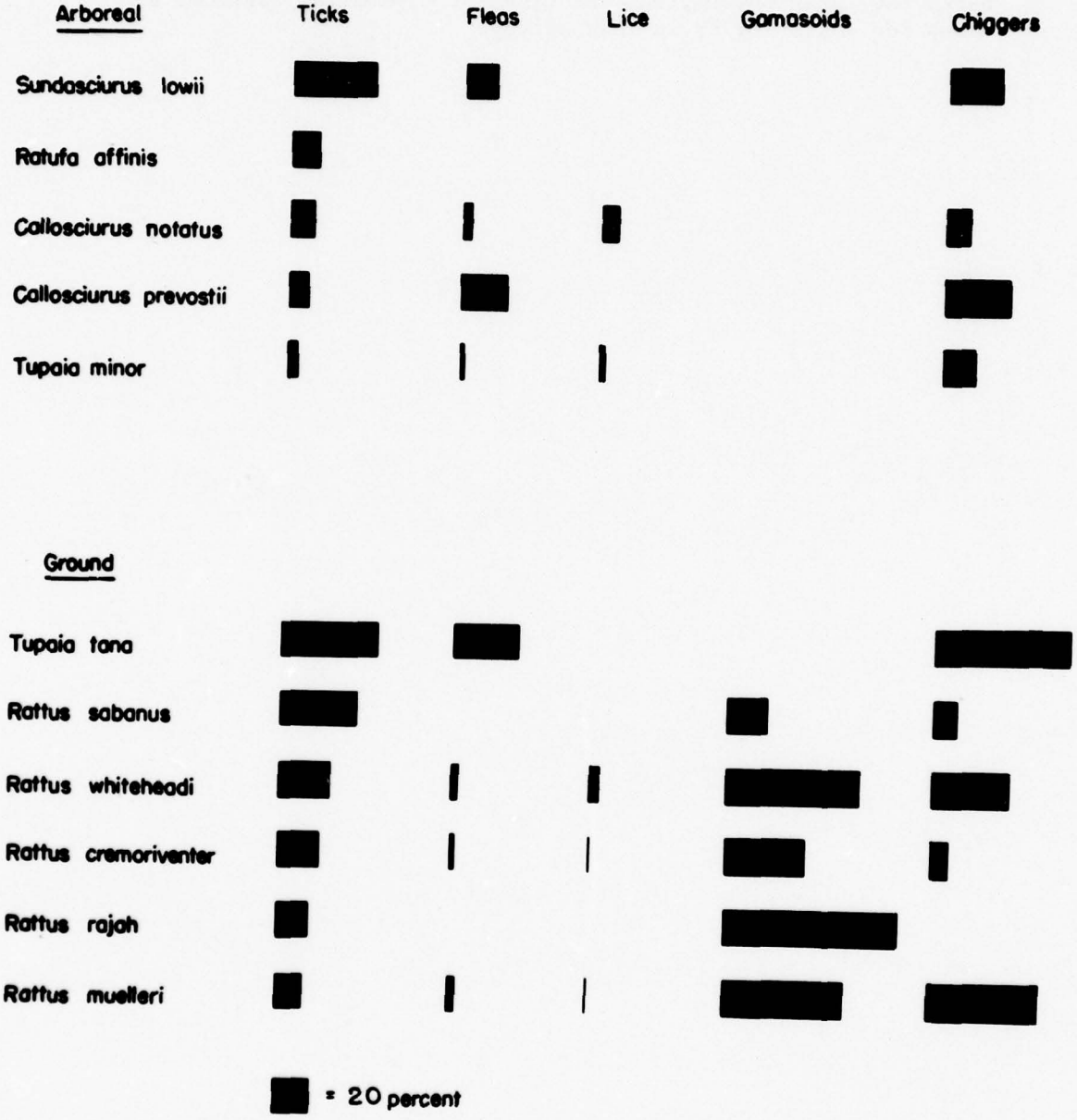
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From 14th May through 5th June 1971, 860 mammals were collected by a team from the Division of Medical Ecology, Institute for Medical Research, Kuala Lumpur, Malaysia, in the vicinity of Poring (6° 2' N, 116° 43' E), East Malaysia (Sabah). Collections were made mainly in the primary forest, and also in secondary forests and edge habitats ("scrub", *belukar*). The mammalian species associations as measured by trapping were about the same in both primary and secondary forests. The secondary forest samples did not include several of the species collected in the primary forest, but the numerical proportions of the arboreal species were about the same. Among the terrestrial species, the numerical proportion of certain species, such as *Rattus whiteheadi* was greater in the secondary than in the primary forests. Several species appeared in the collections from the secondary forest which were absent from among those collected in the primary forest, e.g. *Rattus rajah, R. exulans, R. tiomanicus.*

Rattus rattus diardii appeared in collections from houses and edge habitats. In other respects the terrestrial species associations collected in edge habitats did not differ markedly from those from secondary forest. The arboreal collection in edge habitats consisted of only two species, *Callosciurus notatus* and *Tupaia minor.* This indicates that the arboreal fauna in secondary forest and edge habitats is mainly an impoverished primary forest fauna, whereas the ground mammals include species which seem to be specialized for disturbed habitats. The ectoparasite patterns of the mammals collected are shown in Figure 1. Ticks appear to be more preponderant on terrestrial species than among arboreal species. The infestations of fleas and lice do not appear to follow any marked arboreal/terrestrial patterns. Gamasoids (hard mites) were virtually absent from arboreal species, but quite frequent among terrestrial species, except *Tupaia tana.* Larval Trombiculid mites (chiggers) were more preponderant among ground species than among arboreal ones. It is remarkable in this regard, however, that *Tupaia tana* which had large numbers of chiggers, including known vectors such as *Leptotrombidium deliense* (4 individuals infested with this species among 78 that had infestations with chiggers), had no sero-positives among 86 blood samples tested for scrub typhus *(Rickettsia tsutsugamushi)* antibodies. *Rattus rajah* was not found to be infested with chiggers. It did not have any scrub typhus sero-positives either (10 tested). The overall rate for scrub typhus sero-positives was 2 percent in arboreal species and 13 percent in semi-arboreal and ground species.

We are very grateful to members of the team: Chai Koh Shin and Phang Ong Wah (U.S. Army Medical Research Unit, USAMRU) and P. Ramachandran, Sharif bin Mansor. The team also included Ulang bin Sipang. The chiggers were identified by the Department of Acarology (USAMRU). The blood samples were tested by Miss Elsie Gan, Department of Serology (USAMRU). We are grateful also to the Agriculture Department, the Director of the Mt. Kinabalu National Park, the Sabah Museum, and the Director of Medical Services of Sabah for assisting us in this survey.

Fig. 1. Ectoparasite Patterns in Mammals Collected in East Malaysia
Percent Individuals Infested (10 or more)



Ecological Distribution of Blood Parasites in Some Arboreal Rodents

by

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Infections with *Plasmodium* and other blood parasites have been reported in several species of mammals in Malaysia (Sandosham, 1967; Dunn, Lim and Yap, 1968). Some of these, although not human parasites, are morphologically very similar to forms parasitic to humans (Green, 1933; Sandosham, Eyles and Yap, 1962). With the second record of a human infection with *Plasmodium knowlesi* (Yap, Cadigan, and Coatney, 1971), it is worthwhile to examine the ecological patterns of distribution of the various red cell protozoa and other blood parasites in mammals of West Malaysia. This gives us some information on the ecological requirements for the enzootic transmission of various blood parasites. The purpose of this report is to present data on various blood parasites of five species of giant squirrels. The ecological niches of these species differ as well as their specific habitats. Two of these hosts locomote like most squirrels, *Ratufa bicolor* and *R. affinis*, and three are gliders ("flying squirrels"), *Petaurista petaurista*, *P. elegans*, and *Aeromys tephromelas*. All five can be observed in the same general areas in the canopy of rainforests, although they seem to have specific ecological affinities to specific habitats depending on the degree of alteration of the forests. *Petaurista petaurista* in some areas that have sustained heavy logging have been found to be very common, but neither of the two *Ratufa spp.* are common in such areas. *Ratufa bicolor* seems to be an inhabitant of deeper forest than *R. affinis* and *Petaurista elegans* seems to occur more commonly in deep forest, or recently cut forest, than do *P. petaurista* and *Aeromys*.

Another major ecological difference between the two species of *Ratufa* and the "flying squirrels" is that both *Ratufa* are diurnal and the "flying squirrels" are nocturnal. All of these giant squirrels feed on fruit and the flying squirrels also feed on leaves. None of them usually descends to the ground and they are usually observed at least 30 feet (about 10 meters) from the ground, and often up to 150 feet (about 46 meters) or higher in the emergent trees.

Sandosham (1967) and Dunn *et al.* (1968) reported prevalence rates for *Hepatocystis* (probably *H. vassali*) in *Ratufa affinis* and *R. bicolor*. Sandosham, Yap and Omar (1965) described a new species of *Plasmodium* from *Petaurista petaurista*: *Plasmodium booliati*. Another, not yet named, new species of *Plasmodium* from *Petaurista elegans* has been collected and preliminarily described by Yap, Muul and Lim (1970).

METHODS AND MATERIALS

A total of 380 blood specimens from giant squirrels have been examined and reported here. These were collected from 1 February 1968 through 30 March 1973. Giant squirrels are very difficult to trap, thus all but three specimens were collected by shooting. Thick and thin blood films were made on microscope glass slides and stained with Giemsa. Other samples and data were collected for related studies. Specimens were prepared as standard research reference skins and skulls.

RESULTS

The results of most of the examinations of blood samples are given in Table 1. In addition to *Plasmodium* and *Hepatocystis*, prevalence rates for microfilariae are given, but it is not clear at this point what species the latter represent. Not included in Table 1 are results from examination of 42 *Petaurista petaurista* from Kuala Langat area of Selangor. These results are given in Table 2. No *Plasmodium* was found in these samples, although 24 percent had infections with microfilariae. These squirrels from the Kuala Langat area were not collected in forest but in groves of rather isolated trees adjacent to cultivated areas, particularly oil palm. These trees were primarily cultivated durian (*Durio*) and remnants of the original forest. Neither species of *Ratufa* was observed in those highly disturbed habitats.

Another sample left out of Table 1 were eight *P. petaurista* collected from Pulau Tioman, an island off the east coast of the peninsula opposite Kuala Rompin, Pahang. Five of these "flying squirrels" had patent *Plasmodium* infections.

The rest of the samples of "flying squirrels" came from forest habitats in various parts of West Malaysia. The specimens of *Aeromys* came primarily from Bukit Lagong, Selangor and Tamok, Johore where many *P. petaurista* were also collected. *P. elegans* were mostly collected in the vicinity of Tamok, Johore, but not around the village, as was the case with the *Aeromys* and *P. petaurista*. Four *P. elegans* were collected in forest at elevations above 5000 feet at Cameron Highlands; none of these had *Plasmodium*, but are included in the totals in Table 1.

All of the samples of *R. bicolor* are included in Table 1. This species was usually collected in mature forests. Most of the samples (91/129) came from Bukit Lagong, where the rate of positive *Hepatocystis* samples was 50%, or about the same as that for the total of the samples.

The samples from *Ratufa affinis* collected from Subang Forest Reserve, Selangor a secondary forest, are included in Table 2 but not in Table 1. Of 26 samples examined from Subang only one was positive for *Hepatocystis*. In another series (included in Table 1) from

Trengganu, collected from a partly timbered primary forest, 26 out of 28 samples were positive for *Hepatocystis*. At Bukit Lagong, a forest intermediate in the degree of disturbance, the rate of *Hepatocystis* infections in *R. affinis* was 12 out of 22 samples, or about the same rate as in *R. bicolor* collected in the same area.

DISCUSSION

Several ecological factors seem to be important regarding the distribution of blood parasites in these giant squirrels. The presence of either *Plasmodium* or *Hepatocystis* is correlated with the periodicity of activity of the hosts. *Plasmodium* infections occurred in the nocturnal species, *Hepatocystis* in the diurnal ones. However, one nocturnal species, *Aeromys* had neither. How much of this correlation is based on the susceptibility of hosts is not known. *Hepatocystis* is known to occur in nocturnal bats and mousedeer (Sandosham, 1967). Yet, nearly all of the diurnal squirrels in West Malaysia have been reported to have *Hepatocystis*, but not *Plasmodium*, infections (Sandosham, 1967; Dunn *et al.*, 1968; Muul, Lim and Yap, 1970). On the other hand, we have found neither *Plasmodium* nor *Hepatocystis* infections among hundreds of blood specimens examined of the smaller flying squirrels, all nocturnal, which occur in West Malaysia (*Hylopetes lepidus (=spadiceus), H. platyurus (=lepidus), Pteromyscus pulverulentus, Petinomys setosus, P. vordermanni, Iomys horsfieldii*). It seems that *Plasmodium* infections are fairly species specific, but so far no taxonomic distinction has been made between the *Hepatocystis* occurring in the various diurnal squirrels.

The genera *Ratufa* and *Petaurista* are not closely related systematically. It is therefore uncertain whether the periodicity of activity is the cause for the dearth of *Hepatocystis* infections in the *Petauristinae*. The absence of *Plasmodium* in *Ratufa* may be explained by the host species specificity of this parasite. There are other arboreal, nocturnal mammals in Malaysia that have *Plasmodium* infections, e.g., the colugo or flying lemur, harboring a different species (Dunn, Eyles, and Yap, 1963). In Taiwan (Formosa) *Plasmodium watteni* infects *Petaurista* (Lien and Cross, 1968).

Table 2 gives the combined infection rates of *Hepatocystis* for the two species of *Ratufa* and of *Plasmodium* for the two species of *Petaurista*. Only localities from which sufficient samples were collected are included and these are arranged according to the degree to which they have sustained alteration of the original forest, with the most disturbed areas shown at the top and the least at the bottom. Although only a few habitats are available for such a comparison, it seems that the least disturbed areas favor a higher rate of transmission of *Hepatocystis*. This was found to be the case for other species of squirrels also (Muul *et al.*, 1970).

We are dealing with two species of *Plasmodium* in the two *Petaurista* hosts, but again it seems that even within the same host species *P. petaurista*, the infection rate is higher in less disturbed areas. It was highest in *P. elegans* collected in primary and recently logged primary forests in Johore.

The infection rates with microfilariae seemed to be lowest in the less disturbed areas, but variable in the others.

A possible further complication of the distribution patterns of some of these infections is temporal periodicity. This is suggested by the data on *Hepatocystis* infections in *Ratufa bicolor*. *R. bicolor* was the only species collected in sufficiently large numbers over a period of years in one locality (Bukit Lagong, Selangor) for such a comparison. A total of 91 specimens have been collected and examined from 1968 through 1972. During one six month period, January through June 1970, 23 of 28 specimens examined were positive. During the remainder of the time the rate was less than 50%. In addition to demonstrating ecological differences between hosts of different types of blood parasites, these data indicate that surveys of parasite prevalence must take into consideration the type of habitats in which the potential hosts are collected and the possible influence of seasonal periodicity on the rate of infections.

SUMMARY AND CONCLUSIONS

The overall rates of infections of *Hepatocystis* in two species of *Ratufa* and *Plasmodium* in two species of *Petaurista* were high (54 to 72% and 34 to 46%, respectively). *Aeromys* was not found to be infected with blood protozoa. *Plasmodium* infections seemed to be host specific and correlated with (but not necessarily dependent on) nocturnal habits of the hosts. The two hosts of *Hepactocystis* reported here are diurnal. In habitats greatly altered by man the infection rates with both *Hepatocystis* and *Plasmodium* seemed to be lower than in deeper forest.

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Table 1

Prevalence of blood parasites in some forest canopy rodents

Species	No. Exam.	*Plasmodium*	*Hepatocystis*	*Microfilariae*	*Trypanosoma*
Petaurista petaurista	61	21 (34%)	0	21 (34%)	3
Petaurista elegans	24	11 (46%)	0	2 (8%)	0
Aeromys tephromelas	22	0	0	3 (14%)	0
Ratufa affinis	68	0	49 (72%)	8 (15%)	0
Ratufa bicolor	129	0	70 (54%)	23 (18%)	0

Table 2

Prevalence of blood parasites in different types of forest

Ratufa bicolor and *R. affinis*

Type of Forest	No. Examined	*Hepatocystis*	Microfilariae
Secondary (Selangor)	32	5 (16%)	3 (10%)
Secondary/Primary (Selangor)	113	58 (51%)	21 (19%)
Partly Timbered Primary (Trengganu)	34	32 (94%)	1 (3%)

Petaurista petaurista and *P. elegans*

Type of Forest	No. Examined	*Plasmodium*	Microfilariae
Scattered Large Trees (Selangor)	42	0	11 (26%)
Scattered Large Trees/ Forest Edge (Johore)	13	2 (15%)	4 (31%)
Secondary/Primary (Selangor)	19	8 (42%)	9 (47%)
Recently Timbered (Partly) Primary/Primary (Johore)	18	9 (50%)	0

Ecological and Parasitological Considerations of a Collection of Mammals from East Malaysia

by

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Several publications since 1962 have presented new locality records and reviews of older surveys of mammals of East Malaysia (Sabah, formerly North Borneo)(Davis, 1962, for the lowland forests; Lim and Heyneman, 1968, for the high elevations on Mt. Kinabalu). Medway (1965) published a taxonomic review. This report presents data on a collection of mammals obtained from 14 May 1971 through 5 June 1971 at Poring, near Ranau, at the base (SE) of Mt. Kinabalu. A total of 860 mammals were collected from various habitats, but mostly from the primary forest. The elevation in the vicinity of Poring (6° 2' N, 116° 43' E) is around 1300 feet (400 meters). All collections were made within about 12 miles of Poring.

The purpose of this study was to determine the species associations of small mammals in the primary forest and to some extent in adjacent secondary forests and edge habitats ("scrub", *belukar*) and around villages. Within the forest we attempted to ascertain also the vertical zonal associations of arboreal mammals. Various ecological data, such as reproductive status of populations, were gathered. We attempted also, to determine the habitat differences in distribution patterns of ectoparasites and pathogens (*Rickettsia tsutsugamushi*, zoonotic pathogen responsible for "scrub typhus"). Detailed accounts of ectoparasites and *Rickettsia* are not included in this report, but we expect to publish them elsewhere.

METHODS

Animals were collected by wire-basket traps (Harrison, 1966), by hand, and by shotgun. Traps were distributed among interested villagers who were directed to place the traps in the habitats desired. Approximately half of the traps were placed at various heights in trees, the remainder on the ground. It was presumed that the various species had equal access to the traps and that those which spend more time in trees were more likely to be caught in traps placed in trees than those species which were primarily terrestrial. Thus, the percentage of the species catch in trees is taken as a relative measure of the degree of arboreal activity. It was accepted that certain species would not enter traps and that the predisposition to enter traps varies among species, so that the trapping results alone would not necessarily be indicative of relative abundance or the total species composition. The other collecting methods were used to partially alleviate this deficiency.

Under each species account, the number of specimens collected are given. For the purpose of this report measurements and Institute for Medical Research (IMR) catalog numbers are given for only up to 20 individuals (10 male, 10 female) per species to save space. The mean and range (in parentheses) of body measurements are given in millimeters; the hind foot length excluded the claw, and the tail was measured to the end of the vertebrae. All weights are in grams. The following abbreviations are used: H&B, head and body length; T, tail length; HF, hind foot length; E ear length; Wt, weight.

Along with the binomial nomenclature (mostly following Medway, 1965), common names are given. These were either taken from Harrison (1964) or Medway (1965), or invented as descriptive of the species in cases where these authorities have merely given a binomial derivative (e.g. *Sundasciurus brookei*, Brooke's Squirrel).

Blood parasites were searched for in thick and thin smears of blood. Each specimen was euthenized with chloroform and the ectoparasites (fleas, lice, gamasoids (or Mesostigmatid mites)) were brushed from the fur and chiggers were dislodged from the ears and elsewhere with a sharpened wooden applicator stick. Females were examined for signs of lactation and their uteri were examined for embryos.

This report includes all mammals collected except bats.

RESULTS

SPECIES ACCOUNTS

Order Dermoptera
Family Cynocephalidae

Cynocephalus variegatus (Audebert) Flying Lemur
One specimen, subadult male (IMR 91220)
Measurements: H&B, 245; T, 170; HF, 62; E, 23; Wt, 309.
Habitat: Primary forest.
Observations: The specimen was caught 12 feet upon a tree by hand. No blood parasites were found.
Ectoparasites: None was found.

Order Primates
Family Tupaiidae

Tupaia glis (Diard) Common Treeshrew
Two specimens, adult females (IMR 90857, 91135).
Measurements: H&B, 194, 204; T, 180, 185; HF, 47, 48; E, 15, 18; Wt, 169, 202.
Habitats: One in primary forest, one secondary forest.
Observations: One specimen was trapped on the ground, the other 3 feet up on a tree. Both specimens had swollen mammae. The female from the primary forest contained two embryos. One specimen had a blood infection with Microfilaria.

Ectoparasites: Both specimens were found to have chiggers only (*Helenicula signata* (21), *Walchiella impar* (17), *W. oudemansi* (3), *Leptotrombidium spp.* (2), *Eutrombicula wichmanni* (2)).

Tupaia minor Gunther — Lesser Treeshrew

Thirty seven specimens collected, 20 examined, 10 males (R 90560, -618, -639, -651, -738, -885, 91002, -074, -152, -208), 10 females (R 90547, -554, -607, -625, -766, -774, -984, 91121, -196, -209).
Measurements: H&B, males 129 (125-136), females 129 (125-133); T, males 161 (155-169), females 151 (142-160); HF, males 31 (29-33), females 30 (28-32); E, males 13 (11-14), females 13 (12-15); Wt, males 54 (49-58), females 56 (45-71).
Habitats: This species was caught in all three habitats at about the same ratio in terms of the total catch.
Observations: Only two specimens were caught on the ground, the rest were trapped or shot in trees. The heights at which they were trapped ranged from 2 to 10 feet, the average was 6 feet. They seem to range quite high into the canopy, however, since they were shot as high as 70 feet; the average height was 39 feet. Most of the females examined had swollen mammae indicating lactation. One female was pregnant with 2 embryos. Apparently a high percentage of females had parturitions just prior to this period. No blood parasites were found.
Ectoparasites: 4% had ticks, none had fleas, 2% had lice (*Haplopleura*, probably new species), and 10% chiggers (*Ascoschoengastia lorius* (74), *Walchiella oudemansi* (22), *W. impar* (13), *Leptotrombidium spp.* (10), *Eutrombicula wichmanni* (1)); none had Gamasoid (Mesostigmatic) mites.

Tupaia gracilis Thomas — Slender Treeshrew

Ten specimens, seven males (adults) (R 90505, -574, -670, 91075, -078, -116, -262), three females (two adults)(R 90504, -546, -567).
Measurements: H&B, males 139 (135-144), females 142, 143; T, males 170 (162-178), females 167, 174; HF, males 38 (36-41), females 38, 41; E, males 14 (13-17), females 13, 14; Wt, males 62 (61-69), females 84, 98.

In addition to hind foot measurements, this species may be distinguished from *T. minor* by the gray underfur on the inner flanks; in *T. minor* the fur is buff also at the bases.

Habitats: This species was caught in forest habitats, both primary and secondary.
Observations: Only one out of the ten specimens was collected on the ground. The average height was six feet (2-15 feet). The single adult female examined was lactating. One specimen had a Microfilaria blood infection.
Ectoparasites: One specimen had fleas and four had chiggers (*Leptotrombidium spp.* (74), *Helenicula signata* (32), *Walchiella oudemansi* (1)). No lice or Gamasoids (Mesostigmatic mites) were found.

Tupaia tana Raffles — Large Treeshrew

Ninety eight specimens collected, 20 adults examined, 10 males (R 90507, -542, -545, -575, -610, -614, -626, -650, -662, -692), 10 females (R 90501, -555, -611, -612, -633, -664, -665, -682, -683, -684).

Measurements: H&B, males 208 (197-217), females 204 (187-218); T, males 176 (161-186), females 172 (161-183); HF, males 46 (44-49), females 45 (43-49); E, males 18 (15-20), females 16 (15-18); Wt, males 212 (150-255), females 218 (173-237).
Habitats: This species was caught in all three habitats at about the same ratio in terms of the total catch.
Observations: Although this species has also been called the "Terrestrial Treeshrew" (Medway, 1965) and Davis (1962) notes that it was mostly observed on the ground, nearly 80% of the individuals were caught in trees 2 to 10 feet from the ground, average 5 feet from the ground. Thus, this species is not so confined to a terrestrial existence as has been suggested. One out of 45 females examined was pregnant, 10 had enlarged mammae, indicating recent parturitions. More than 1/3 of the individuals tested had Microfilaria infections. Five individuals had *Trypanosoma* blood infections.
Ectoparasites: 56% of the individuals had ticks; 37% had fleas; 80% had chiggers (of those identified (N=2346) the following were found: *Walchiella oudemansi* (42%), *Leptotrombidium spp.* (26%), *Helenicula signata* (21%), *Walchiella impar* (6%), *Eutrombicula wichmanni* (5%). None was found to have lice or Gamasoid mites. This species yielded the highest infestation rates with ticks and fleas.

Family Lorisidae

Nycticebus coucang (Boddaert) Slow Loris
One specimen, female (R 91354)
Measurements: H&B, 275; T, 25; HF, 53; E, 17; Wt, 429.
Habitat: This specimen was collected in a fruit plantation.
Observations: This female was caught 15 feet up in a tree. She was not pregnant and did not appear to be lactating. No blood parasites were found.
Ectoparasites: Not examined.

Family Tarsiidae

Tarsius bancanus Horsfield Tarsier
Nine specimens collected, 6 males (R 90881, 91145, -146, -195, -269, -334), 3 females (R 91008, -296, -333).
Measurements: H&B, males 132 (116-147), females 137 (133-144); T, males 194 (181-212), females 208 (202-216);HF, males 68 (63-70), females 68 (65-71); E, males 30 (29-34), females 31 (29-33); Wt, males 106 (73-138), females 109 (101-119).
Habitat: Six were caught in primary forest, three in secondary forest.
Observations: They were captured 5-15 feet from the ground in trees. One female was pregnant with one embryo. The other females were not lactating. No blood parasites were found.
Ectoparasites: Two individuals were found to have ticks, six had chiggers (*Leptotrombidium spp.* (462), *Walchiella oudemansi* (1)). None was found to have fleas or lice.

Order Pholidota
Family Manidae

Manis javanica Desmarest — Pangolin
One specimen, adult female (R 90993)
Measurements: H&B, 397; T, 351; HF, 64; E, 17; Wt, 2874.
Habitat: Primary forest
Observations: Specimen was captured by hand on the forest floor. It showed no signs of reproductive activity. No blood parasites were found.
Ectoparasites: Only ticks were found on this specimen.

Order Carnivora
Family Mustelidae

Martes flavigula — Yellow-throated Marten
One specimen, adult female (R 90954)
Measurements: H&B, 463; T, 345; HF, 91; E, 28; Wt, 1373.
Habitat: Deep primary forest.
Observations: Specimen was shot in a tree. She was neither pregnant nor lactating. No blood parasites were found.
Ectoparasites: Only ticks were found on this specimen.

Family Viverridae

Prionodon linsang (Hardwicke) — Banded Linsang
Two specimens, adult females (R 90715, 91270)
Measurements: H&B, 355-411; T, 295-362; HF, 62-66; E, 27-28; Wt, 533-795.
Habitat: Both were shot in trees 5 to 15 feet from the ground. The larger female was lactating. No embryos were found. No blood parasites were found.
Ectoparasites: None was found.

Paguma larvata (Hamilton-Smith) — Masked Musang
Two specimens, females (R 90986, 90989)
Measurements: H&B, 580-605; T, 561-599; HF, 95-101; E, 47-49; Wt, -.
Habitat: Deep primary forest
Observations: Shot at about 40 and 60 feet up in trees. Neither female was lactating nor pregnant. No blood parasites were found.
Ectoparasites: One specimen had ticks. No other ectoparasites were found.

Arctogalidia trivirgata Gray — Small-toothed Palm Civet
Two specimens, males, adult (R 90786), subadult (R 91197)
Measurements: H&B, 440, 363; T, 480, 425; HF, 78, 69; E, 40, 40; Wt, 1218, 795.
Habitats: The adult was caught near the village and the subadult in primary forest.
Observations: The adult was caught on the ground by a dog; the subadult was shot 20 feet up in a tree. One individual had a blood infection with Microfilaria.
Ectoparasites: No ectoparasites were found.

Hemigalus derbyanus (Gray) Banded Palm Civet
Two specimens, adult females (R 90716, 91185).
Measurements: H&B, 525, 535; T, 337, 296; HF, 76, 72; E, 36, 32; Wt, 1909, 1687.
Habitat: Primary forest
Observations: Specimens shot 15 to 45 feet up in trees. One of the females had swollen mammae. Neither female contained embryos. The blood of one was infected with Microfilaria.
Ectoparasites: One of the specimens had ticks.

Order Rodentia
Family Sciuridae

Ratufa affinis (Raffles) Giant Squirrel
Seventeen specimens collected and examined, nine males (R 90551, -821, -822, -904, -990, 91005, -006, -134, -299), eight females (R 90961, 91007, -025, -039, -040, -194, -271, -285).
Measurements: H&B, males 361 (336-375), females 363 (349-395); T, males 430 (421-444), females 439 (419-471); HF, males 76 (67-82), females 77 (63-86); E, males 37 (23-29), females 26 (23-28); Wt, males 1153 (961-1238), females 1277 (1112-1485).
Habitat: Primary forest.
Observations: Shot 30-100 feet up, average 52 feet. This species seems to occupy mostly the upper canopy zone. One of the six females examined had swollen mammae. None contained embryos. Some had *Hepatocystis* parasites in their blood.
Ectoparasites: Three specimens had ticks. No other ectoparasites were found.

Callosciurus prevostii (Desmarest) White-striped Squirrel (also Black Squirrel in some areas)
Fifty three specimens collected, 20 examined, 10 males (R. 90513, -548, -552, -553, -581, -584, -606, -615-17), 10 females (R 90576, -580, -585, -591-95, -601-02).
Measurements: H&B, males 237 (222-247), females 234 (222-251); T, males 226 (190-245), females 231 (212-250); HF, males 50 (44-56), females 50 (45-52); E, males 20 (17-23), females 20 (17-22); Wt, males 403 (364-454), females 420 (377-480).
Habitats: All but seven specimens were collected in primary forest; five were shot in secondary forest, two in edge habitats.
Observations: Five were trapped at heights from 7 to 25 feet. The rest were shot from 15 to 120 feet from the ground, average height 50 feet. Thus, it appears this is primarily an arboreal species which does not readily enter traps at heights at which they were set. Seven females out of 28 examined had swollen mammae. Six were found to be pregnant, with 2 embryos each. Many individuals had blood infections with *Hepatocystis* and some had Microfilaria.
Ectoparasites: Six specimens were found to be infested with ticks, 13 with fleas, and twenty with chiggers. (Mostly *Ascoschoengastia audyi*, some *A. lorius*, *Helenicula signata*, *Leptotrombidium* spp., and *Walchiella oudemansi*.) No lice or Gamasoid mites were found.

Callosciurus notatus (Boddaert) Red-bellied Squirrel

One hundred and thirty seven specimens collected, 20 examined, 10 males (R 90514, -16, -49, -50, -57, -87, 90620, -23, -30, -31), 10 females (R 90515, -17, -19, -37, -38, -56, -58, -59, -63, -77).
Measurements: H&B, males 202 (175-222), females 196 (190-210); T, males 180 (150-210), females 191 (172-205); HF, males 45 (41-46), females 45 (41-47); E, males 17 (15-21), females 18 (17-19); Wt, males 222 (174-248), females 238 (180-297).
Habitats: Collected in about equal proportions of the total catch in each of the habitats: Primary forest, secondary forest, edge habitats.
Observations: Trapped in trees at heights from 2 to 30 feet, average 8 feet. Five were caught in traps on the ground. They were also shot in trees at heights from 20 to over 60 feet, average 39 feet. Fourteen of 70 females examined showed signs of lactation. Twelve females were pregnant,with 1-3 embryos each. Many individuals (30% of those examined) had blood infections with *Hepatocystis*, and some had Microfilaria.
Ectoparasites: Fourteen percent had ticks; 4% had fleas; 8% had lice *(Neohaematopinus callosciuri)*; 13% had chiggers (mostly *Ascoschoengastia audyi*, some *Walchiella oudemansi, Leptotrombidium spp., Eutrombicula wichmanni, Helenicula signata.*); no Gamasoid mites were found.

Sundasciurus hippurus (Geoffroy) Horse-tailed Squirrel

One specimen collected, adult female (R 90666).
Measurements: H&B, 237; T, 246; HF, 46; E, 13; Wt, 329.
Habitat: Secondary forest.
Observations: Trapped 7 feet from the ground in a tree. Since only one was trapped and none were shot it appears that this squirrel was relatively rare in the localities collected. This female was not lactating or pregnant. The blood contained no protozoan parasites.
Ectoparasites: Fleas and chiggers were present *(Ascoschoengastia lorius, A. audyi)*. No ticks or Gamasoids were found.

Sundasciurus lowii (Thomas) White-bellied Squirrel

Thirty specimens collected, twenty examined, 10 males (R 90571, 90628, -34, 90736, -41, 90970, -77, -79, -85, -99), 10 females (R 90740, -95, -96, 90817, -27, -68, -71, 90929, -80, -94).
Measurements: H&B, males 152 (146-157), females 143 (137-148); T, males 89 (85-98); females 94 (77-106); HF, males 34 (33-37), females 34 (32-37); E, males 14 (13-17), females 14 (13-15); Wt, males 91 (81-102), females 88 (74-103).
Habitats: Collected in about equal proportions in primary and secondary forests. None was collected in edge habitats.
Observations: Collected mostly in trees at heights from 2 to 10 feet, average 6 feet. One was shot 50 feet up in a tree. Only four were caught on the ground. Davis (1962) considered this to be a terrestrial species. Four females out of twelve appeared to be lactating, one was pregnant with 2 embryos. Only one was infected with blood parasites, *Hepatocystis*; none were found infected with Microfilaria.
Ectoparasites: Out of 29 examined 14 had ticks; 5 (all males) had fleas; 8 had chiggers (mostly *Walchiella oudemansi*, some *Leptotrombidium spp.*); none was found to have Gamasoid mites.

Sundasciurus brookei (Thomas) Grey-bellied Squirrel

Three specimens collected, one male (R 90788), two females (R 90905, 91147).

Measurements: H&B, male 164, females 161, 164; T, male 132, females 117, 142; HF, male 32, (?), females 34, 38; E, male 13, females 14, 14.5; Wt, male 124, females 128, 103.

Habitat: All were collected in primary forest.

Observations: Two were trapped at 8 and 20 feet from the ground: one was shot at a height of 50 feet. Medway (1965) describes this species as "characteristic of hills and submontane forest." Apparently it descends to elevations as low as 1300 to 1500 feet. Neither female was pregnant nor lactating. Two of the three were infected with *Hepatocystis* and one of these had a concurrent infection with Microfilaria.

Ectoparasites: Two specimens were infested with ticks. No other ectoparasites were found.

Lariscus hosei (Thomas) Four-striped Ground Squirrel

Two specimens collected, adult females (R 90608, 90874)

Measurements: H&B, 172, 189; T, 142, -; HF, 42, 43; E, 14, 14; Wt, 145, 175.

Habitat: Collected in primary forest.

Observations: One was trapped 3 feet from the ground on vegetation (no information recorded for the other). This species is considered rare (Medway, 1965, Davis, 1962). Both specimens showed signs of lactation; one contained a single embryo. No blood parasites were found.

Ectoparasites: One specimen had fleas. No other ectoparasites were found.

Exilisciurus exilis (Muller) Pigmy Squirrel

Seven specimens collected, two males (R 90649, 91337), five females (R 90643, 90718, 91329, 91335, -36).

Measurements: H&B, males 80, 64, females 74 (62-83); T, males 52, 52, females 47 (30-54); HF, males 21.5, 20, females 20 (18-21); E, males 10, 9, females 10 (9-10.5); Wt, males 18.5, 12, females 15.5 (14-17).

Habitat: Primary forest.

Observations: None entered traps. Specimens were shot from 10 to 160 feet from the ground in trees. They move around slowly on the bark of large branches and trunks of trees. Because of their small size and manner of movements they could be mistaken for large insects. One female appeared to be lactating; none were pregnant. No blood parasites were found.

Ectoparasites: No ticks; one specimen had fleas; no lice; one had Gamasoids; two had chiggers (*Leptotrombidium spp.* (20) and *Ascoschoengastia audyi* (8)). The presence of *Leptotrombidium* indicates that either this squirrel spends some time on the ground or, less likely, that this *Leptotrombidium* occurs in the canopy.

Rheithrosciurus macrotis Tufted Ground Squirrel

Three specimens collected, all females (R 90991, 91193, 91297).

Measurements: H&B, 338, 339, 352; T, 302, 342, 337; HF, 82, 88, 84; E, 46, 42, 47; Wt, 1170, 1227, 1281.

Habitat: Primary forest.
Observations: Specimens were shot on the ground and 10 feet and 30 feet up in trees. Thus, this species does not appear to be strictly a ground squirrel. Two of the females showed signs of lactating and one was pregnant with two embryos. None had blood parasites.
Ectoparasites: None was found.

Petinomys setosus (Temminck) Small Grey Flying Squirrel
Two specimens collected, one male (R 91036), one female (R 91133).
Measurements: H&B, male 115, female 127; T, male 107, female 105; HF, male 22, female 21; E, male 14, female 14; Wt, male 40, female 45.
Habitat: Primary forest.
Observations: These specimens were caught by hand in cavities in live trees 2 and 8 feet from the ground. The female was neither lactating nor pregnant. No blood parasites were found.
Ectoparasites: Both specimens had chiggers *(Ascoschoengastia indica* and *Leptotrombidium spp.)*.

Hylopetes lepidus (Horsfield) Red-cheeked Flying Squirrel
(formerly *H. spadiceus*, see Muul & Lim, 1971)
Three specimens collected, one male (R 90710), two females (R 90711, 91298).
Measurements: H&B, male 119, females 119, 129; T, male 109, females 88, -; HF, male 23, females 23, 22; E, male 18, females 20, 18; Wt, male 46, females 49, 53.
Habitat: Primary forest.
Observations: These specimens were caught by hand in nest cavities in live trees. R 90711 and R 90712 occupied the same nest. R 91298 was in a nest 5 feet from the ground. One of the females was pregnant with two embryos. The other was neither pregnant or lactating. No blood parasites were found.
Ectoparasites: Only chiggers *(Leptotrombidium spp., Walchiella impar)* were found on two of the specimens. No other ectoparasites were found.

Pteromyscus pulverulentus (Gunther) Smoky Flying Squirrel
Ten specimens collected, six adult males (R 90787, -987, 91132, -36, -90, 91324), one subadult male (91192), two adult females (R 91037, 91191), and one subadult female (91038).
Measurements: H&B, males (adult) 230 (221-240), females (adult) 251, 250, T, males (adult) 224 (215-235), females (adult) 221, 229; HF, males (adult) 43 (42-44), females (adult) 42, 44; E, males (adult) 23 (21-24), females (adult) 21, 23; Wt, males (adult) 275 (232-298), females (adult) 305, 287.
Habitat: Primary forest.
Observations: All the specimens were collected by hand in nests in tree cavities, ranging from 10 to 12 feet from the ground. One of the nests contained an adult female (R 91037) and a subadult (R 91038); another nest had an adult male (R 91190), an adult female (R 91191) and a subadult male (R 91192). Recent reproduction in both adult females is indicated by the presence of the subadults in the same nests. This is a new species for Sabah.

No blood parasites were found.
Ectoparasites: None was found, except for ticks on one individual. On the whole this species is remarkably free of parasites.

Family Muridae

Rattus rattus diardii (Jentink) House Rat
Nine specimens collected, three adult males (R 90564, -779, 91242), one subadult male (R 90764), three adult females (R 90853, 91115, -246), and one subadult female (R 90765).
Measurements: H&B, males (adult) 182, 174, 165, females (adult) 171, 161, 158; T, males 171, 161, 158, females 164, 212, 171; HF, males 35, 35, 37, females 35, 39, 35; E, males 21, 20, 20, females 20, 21, 16; Wt, males 152, 121, 94, females 93, 170, 126.
Habitats: Four were caught in houses, three in edge habitats, one in a rice-field.
Observations: All were caught in ground traps. One female was pregnant, with six embryos. The others were not lactating. No blood parasites were found.
Ectoparasites: Three specimens had chiggers *(Walchiella oudemansi, Gahrliepia disparunguis, Leptotrombidium spp., Eutrombicula wichmanni).* No other ectoparasites were found.

Rattus tiomanicus Field Rat
One specimen collected, male (R 90751).
Measurements: H&B, 131; T, 156; HF, 33; E, 19; Wt, 72.
Habitat: Primary forest
Observations: Specimen was caught on the ground. No blood parasites were found.
Ectoparasites: No ectoparasites were found except chiggers *(Walchiella oudemansi).*

Rattus exulans (Peale) Little Rat
Four specimens collected, one male (R 90927) and three females (R 90679, -849, 91283).
Measurements: H&B, male 126, females 125, 112, 110; T, male 129, females 132, 118, 115; HF, male 24, females 24, 23, 20; E, male 17, females 16, 17, 16; Wt, male 45, females 47, 40, 49.
Habitats: Two caught in secondary forest, two in edge habitats.
Observations: All were caught on the ground. None of the females showed signs of reproductive activity. No blood parasites were found.
Ectoparasites: None was found.

Rattus muelleri (Jentink) Grizzled Giant Rat
Seventy nine specimens collected, 43 males, 36 females, 20 adults examined, ten males (R 90561, -632, -641, -646; -654, -658, 90737, -768, -773, -777), ten females (R 90626, -653, -656, -685, -776, -781, -842, -865, -886, -889).
Measurements: H&B, males 209 (196-229), females 196 (181-210); T, males 237 (215-258), females 242 (222-257); HF, males 42 (40-45), females 40 (37-43); E, males 22 (20-25) females 22 (21-25); Wt, males 241 (191-283), females 188 (153-228).
Habitats: Collected in about equal proportions of the total catch in each of the three habitats.

Observations: This species was caught in equal proportions on the ground and in low vegetation (1 to 20 feet, average 6 feet). Six out of 34 females were pregnant, with 4 to 8 embryos (average 5.2). Three females showed signs of lactation. One subadult had a *Trypanosoma* blood infection.
Ectoparasites: Eleven individuals had ticks, two had fleas, one had lice *(Haplopleura dissicula)*, 54 had Gamasoids, and 52 had chiggers (mostly *Walchiella oudemansi*, (75% of infested individuals), also *Gahrliepia disparunguis* (about 40%), *Leptotrombidium spp.* (about 40%), *Ascoschoengastia lorius*, (about 7%), *A. audyi, Helenicula signata, Eutrombicula wichmanni, Chelodenta reidi*, and *Blankaartia acuscutellaris*).

Rattus sabanus (Thomas) Long-tailed Giant Rat
Thirty one specimens collected, 16 males, 15 females; 20 adult specimens examined, 10 males (R 90503, -597, -696, -784, -785, -813, -838, -888, -894, 91020), 10 females (R 90598, -640, -739, -879, -909, -910, -969, 91032, 91054, 91068).
Measurements: H&B, males 252 (240-273), females 248 (226-256); T, males 363 (320-398), females 373 (320-400); HF, males 47 (44-50), females 46 (44-48); E, males 26 (23-29), females 27 (27-29); Wt, males 388 (338-453), females 351 (278-407).
Habitats: Collected in about equal proportions of the total catch in both primary and secondary forests. Two were caught in edge habitats.
Observations: This species was caught in arboreal traps about half of the time, ranging from 2 to 12 feet from the ground, average 6 feet. Five out of 14 females examined were lactating; none was pregnant. No blood parasites were found.
Ectoparasites: Fourteen individuals had ticks, seven had Gamasoids, three had chiggers *(Gahrliepia morrowae)*. No fleas or lice were found.

Rattus cremoriventer (Miller) Dark-tailed Tree Rat
One hundred-fifty specimens collected, 20 adults examined, 10 males (R 90540, -569, -599, -635, -642, -674, -681, -695, -705, -731), 10 females (R 90609, -693, -699, -713, -733, -752, -772, -782, -792, -797).
Measurements: H&B, males 142 (134-151), females 133 (125-141); T, males 192 (175-221), females 180 (160-210); HF, males 26 (24-28), females 25 (22-27); E, males 17 (16-19), females 17 (16-19); Wt, males 72 (63-85), females 60 (48-66).
Habitats: This was the most frequently trapped species in the primary forest. It ranked second in the collections of terrestrial species from secondary forest, and was predominant in edge habitats.
Observations: It was caught in arboreal traps (from 1 to 15 feet from the ground, average 6 feet) about as frequently as in those on the ground. Thus, unlike its name implies, it is not strictly an arboreal rat. Six of 62 females examined showed signs of lactation; six of 71 were pregnant with 2 to 3 embryos. No blood parasites were found.
Ectoparasites: Twenty one percent were infested with ticks, one individual each had lice *(Haplopleura sicata)* and fleas. Forty six percent had Gamasoids and nine percent had chiggers (mostly *Walchiella oudemansi* and *Leptotrombidium spp.* also *Gahrliepia disparunguis, Walchiella nadchatrami, W. lucanosa, W. impar*, and *Eutrombicula wichmanni)*.

Rattus rajah (Thomas) Brown Spiny Rat

Eleven specimens collected and examined, seven males (R 90543, -655, -661, -667, -702, -771, -850), four females (R 90565, -735, -744, -769).

Measurements: H&B, males 191 (178-218), females 191 (166-202); T, males 201 (193-210), females 193 (171-204); HF, males 40 (38-43), females 39 (35-41); E, males 23 (23), females 22 (21-23); Wt, males 142 (124-171), females 168 (152-197).

Habitats: Six were caught in primary forest, five in edge habitats.

Observations: All but one, which was caught in vegetation 3 feet from the ground, were caught on the ground. One of three females examined showed signs of lactation. None was pregnant. One individual had a blood infection with Microfilaria.

Ectoparasites: Two individuals had ticks, all had Gamasoids. No other ectoparasites were found.

Rattus baeodon (Thomas) Small Spiny Rat

Two specimens collected, one male (R 90697), one female (R 90761).

Measurements: H&B, male 133, female 139; T, male 125, female -; HF, male 26, female 27; E, male 18, female 19; Wt, male 54, female 75.

Habitat: Edge habitat.

Observations: Both were caught on the ground. The female showed signs of lactation, but was not pregnant. No blood parasites were found.

Ectoparasites: Only Gamasoids were found on both specimens.

Rattus whiteheadi (Thomas) Lesser Spiny Rat

Eighty eight specimens collected, 20 adults examined, 10 males (R 90588, -657, -671, -721, -732, -760, -762, -816, -820, -837), 10 females (R 90566, -589, -600, -613, -648, -707, -753, -755, -756, -758).

Measurements: H&B, males 127 (120-138), females 126 (117-139); T, males 114 (103-127), females 105 (70-117); HF, males 27 (25-28), females 26 (23-29); E, males 17.5 (16-18), females 18 (16-20); Wt, males 54 (47-61.5), females 56 (44-68).

Habitat: This species was collected in all three habitats, however, in the secondary forest it was the predominant species collected. It ranked fourth in edge habitats (third among terrestrial species) and fifth in primary forest (fourth among terrestrial species).

Observations: All but five specimens were caught on the ground. Six females out of 39 examined were pregnant, with 2 to 6 embryos. Seven were lactating. No blood parasites were found.

Ectoparasites: Ten of 75 specimens examined had ticks, two had fleas, three had lice *(Hoplopleura pectinata)*, 58 had Gamasoids, and 29 had chiggers (mostly *Gahrliepia disparunguis* (642), few *Walchiella oudemansi* (25), *Leptotrombidium spp.* (3), *Chelodenta reidi* (3), and one *Helenicula signata.* Despite the paucity of known vectors on this species, it had the highest rate of seropositives (12 of 33 tested, 36%) in antibody surveys for scrub typhus *(Rickettsia tsutsugamushi).*

Chiropodomys major Thomas Large Pencil-tailed Tree-mouse

Five specimens collected, two males (R 91187, -88), three females (R 91081, -186, -189).

Measurements: H&B, males 114, 109, females 106, 112, 102; T, males 117, 116, females 116, 123, 118; HF, males 22, 21, females 23, 22, 20; E, males 17, 17, females 17, 18, 16; Wt, males 40, 36, females 32, 43, 35.
Habitats: One was trapped in primary forest, four caught by hand in secondary forest.
Observations: One specimen trapped 10 feet above ground in a tree, four were collected from bamboo internodes at a height of about 8 feet. None showed signs of reproductive activity. No blood parasites were found. Because of the paucity of published information on measurements, it is difficult to determine whether this species is correctly identified. Harrison (1964) gives a range for *C. gliroides*; H&B 50-100; T 110-150% of H&B, HF 14-21; Wt 15. Ten *Chiropodomys glioroides* from Selangor, W. Malaysia measured: H&B 85-96; T 104-126; HF 18-20; E 15-20; Wt 20-33.5.
Ectoparasites: Only chiggers were found (83 *Ascoschoengastia lorius*, one *A. lacunosa*).

Family Tragulidae

Tragulus javanicus (Osbeck) Lesser Mouse-deer
Three specimens collected, two males (R 90903, 91030), one female (R 90958).
Measurements: H&B, males 382, 335, female 413; T, males 60, 51, female 70, HF, male 111, female 125; E, males 35, 34, female 35; Wt, males 1159, 750, female 1393.
Habitats: Primary and secondary forests.
Observations: The female showed no signs of reproduction. No blood parasites were collected. This species was found in the same habitats as *T. napu*. No blood parasites were found.
Ectoparasites: Two individuals had ticks. No other ectoparasites were found.

Tragulus napu (Cuvier) Large Mouse-deer
Eight specimens collected, five males (R 90956, -957, -959, 91028-29), three females (R 90746, -902, -955).
Measurements: H&B, males 483 (446-514), females 462, 561, 460; T, males 76 (65-88), females 84, 94, 74; HF, males 132 (125-137), females 140, 157, 134; E, males 40 (35-43), females 40, 42, 39; Wt, males 1821 (1190-2369), females 2245, 3535, 2486.
Habitat: Primary forest.
Observations: None of the females showed signs of reproductive activity. Two individuals had blood infections with *Trypanosoma sp.*
Ectoparasites: Three individuals had ticks. No other ectoparasites were found.

DISCUSSION

Measurements: Measurements are provided for all or samples (10 of each sex of each species collected in large numbers) of the specimens collected since there is a paucity of such information published. Measurements provide a valuable tool for identifications. Moreover,

published descriptions of many species are still based on very small series. However, taxonomic revisions where necessary will be treated elsewhere.

Habitats: The general associations of species in each of the habitats, as measured by trapping, is illustrated in Figs.1, 2. Although fewer specimens were collected in secondary forests and edge habitats than in the primary forest, the samples taken are large enough for general comparisons. The primary forest species diversity is considerably enhanced if species are included which were collected by means other than trapping. This indicates that many forest species do not enter traps readily, and would be missed in surveys that depended solely on trapping.

The numerically dominant terrestrial species in both primary forest and edge habitats was *Rattus cremoriventer*. In a similar survey in West Malaysia this species has been found to be a minor component of the trappable fauna (Bukit Lagong, Selangor and elsewhere) or even absent (Bukit Lanjan, Selangor)(Records of the Division of Medical Ecology, IMR). In secondary forests the dominant terrestrial species was *Rattus whiteheadi*, with *R. cremoriventer* the second. With this and the following exceptions, the numerical ranking of the terrestrial, mammalian species associations were about the same in all habitats. *Rattus rajah* appeared only in collections from secondary forests and edge habitats. Several species, too few in numbers collected to be included in Figure 1, appeared in the collections from secondary forests and edge habitats, but were absent from those from the primary forest, *i.e. Rattus exulans, R. tiomanicus, R. diardii.* Therefore, it appears that the latter three species are mostly confined to and perhaps specialized for utilization of disturbed habitats. The arboreal fauna in secondary forests and edge habitats seems to be mainly an impoverished primary forest fauna.

Arboreal vs. Terrestrial: The following data are presented which seem to indicate that few species are exclusively either arboreal or terrestrial, although broad categories in this regard can be drawn. The evidence comes from several different lines.

Trapping: Traps were set in pairs at each site, one on the ground and one in the vegetation above. The heights of the arboreal traps varied, but the animals presumably had equal opportunity to reach them if all species made equal use of the various vertical zones in the vegetation. Differences in average heights would indicate differences in the utilization of vertical zonations.

Most of the species classified as terrestrial species (Harrison, 1957, Davis, 1962) were caught about half of the time, or more, in the vegetation above ground, *Tupaia tana* (79% caught in arboreal traps), *Rattus sabanus* (53%), *R. cremoriventer* (47%), *R. muelleri* (46%). Others seemed to be more confined to the ground *R. rajah* (9% arboreal) and *R. whiteheadi* (6%). The average height of collection in the vegetation was about the same: *Tupaia tana*, 5 feet (range 2-10), *R. sabanus* 6 feet (2-12), *R. cremoriventer* 6 feet (1-10), *R. muelleri* 6 feet (1-20), *R. rajah* 3 feet, *R. whiteheadi* 5 feet (3-8).

Several of the arboreal species were occasionally caught on the ground *i.e. Callosciurus notatus* (5 times), *Tupaia minor* (once), *Sundasciurus lowii* (4 times), *T. gracilis* (once).

Ectoparasites: Infestations with certain species of chiggers provide evidence that some species considered to be strongly arboreal actually appear to spend some time on the ground. *Leptotrombidium spp.* are considered to be terrestrial chiggers(Audy, 1952). Yet, several of the arboreal species had infestations with these species (the numbers in parentheses following the species show the average numbers of identified *Leptotrombidium spp.* per infested animal and the ratio of specimens infested with these species): *Tarsius bancanus* (77, 6/9), *Tupaia gracilis* (19, 3/10), *Exilisciurus exilis* (10, 1/7), *Sundasciurus lowii* (3, 3/29), *Tupaia minor* (2, 2/39), *Petinomys setosus* (1, 1/2), *Hylopetes lepidus* (1, 1/3), *Callosciurus notatus* (0.3, 3/134), *C. prevostii* (0.24, 2/53). It would appear from this that *Tupaia gracilis* is more terrestrial than *T. minor* which in appearance is very similar. Such evidence provides valuable clues to the ecology of little known species such as *T. gracilis*.

Reproduction: Among the diurnal squirrels the rate of pregnancy was 21 of 132 or 16 percent of those females examined. 26 of 116 (22 percent) showed signs of lactation. Among the nocturnal squirrels, one female out of four examined was pregnant, two others were accompanied by subadults in the same nest. Among the forest rats the rate of pregnancy was 17 of 163 or about 10 percent.

The treeshrews also showed signs of reproductive activity. One female of two *Tupaia glis* was pregnant the other showed signs of lactation, but only one was pregnant. Only one *T. tana* female of 45 examined was pregnant. The overall pregnancy rate among the Tupaiids was 3/70 or 4 percent, including a single non-reproductive female, *T. gracilis*. Since 24/58 or 41 percent of those examined showed signs of lactation, this seems to indicate that there were some synchronous parturitions just prior to the period of collections and that breeding among the Tupaiidae may be seasonal.

One of the female Tarsiers was pregnant. None of the carnivores showed signs of reproductive activity nor did the Tragulids. These larger mammals probably breed less frequently than do the rodents and Tupaiids.

Blood Parasites: As in West Malaysia (Dunn, *et al.*, 1968), the occurrence of blood parasites, particularly *Hepatocystis* and Microfilaria, was most frequent among the diurnal squirrels. Microfilarial blood infections were particularly common among *Tupaia tana* (39 percent). *Trypanosoma sp.* infections were found in *Tragulus napu* and *Rattus muelleri*.

Ectoparasites: The distribution of ectoparasites in the mammals collected is given by general categories in Fig. 3. The specific identifications of ticks, fleas, and Gamasoids have not been completed yet by the authorities to whom the material has been sent. The Trombiculid mites (chiggers) will be discussed in detail in another paper.

In general, ticks were found more frequently on terrestrial species than on those that are mainly arboreal. *Sundasciurus lowii*, which had the highest infestation rate with ticks among the arboreal mammals, spends quite a large portion of its time on the ground; indeed, it has been considered to be a ground squirrel (Davis, 1962).

The infestation rates with fleas and lice appear to be species specific and do not appear to show any marked distributional patterns correlated with the vertical zonation of the hosts. Fleas seemed to be most preponderant, however, on certain arboreal *(Sundasciurus lowii, Callosciurus prevostii)* and semi-arboreal species *(Tupaia tana)*.

The prevalence of Gamasoid infestations correlated well with terrestrial existence of the host. Since *T. tana* lacked Gamasoids it would seem that nesting habits may be important. Tupaiids, unlike the rats, generally do not inhabit burrows in the ground, but usually nest in the vegetation, particularly in crotches of trees. However, little is known of the nesting habits of *T. tana*, specifically, and it is only presumed that they are similar to those of other Tupaiids.

Various forms of chiggers infest both arboreal and terrestrial mammals. In terms of numbers, the infestations among terrestrial species seem to be more frequent than among arboreal species.

Some of the gaps in chigger infestations are puzzling, e.g. *R. rajah* which had none. It is interesting to note that this species was 100% infested with Gamasoids. Also the species most frequently infested with chiggers, *Tupaia tana*, had no Gamasoids. However, *Rattus muelleri* had plenty of both. A species considered strictly arboreal, *Tarsius bancanus*, was infested with large numbers of *Leptotrombidium spp.* which are thought to be ground inhabiting chiggers (Audy, 1952). This apparently indicates that the Tarsier spends quite a lot of time roaming about on the ground. One of the authors (LBL) has frequently seen them on the ground in pursuit of insects during the night. Another little-known species, *Tupaia gracilis*, apparently spends quite a large part of its time on the ground as evidenced by the *Leptotrombidium spp.* found on them. Even the flying squirrels, *Hylopetes lepidus* and *Petinomys setosus* had *Leptotrombidium spp.* infestations.

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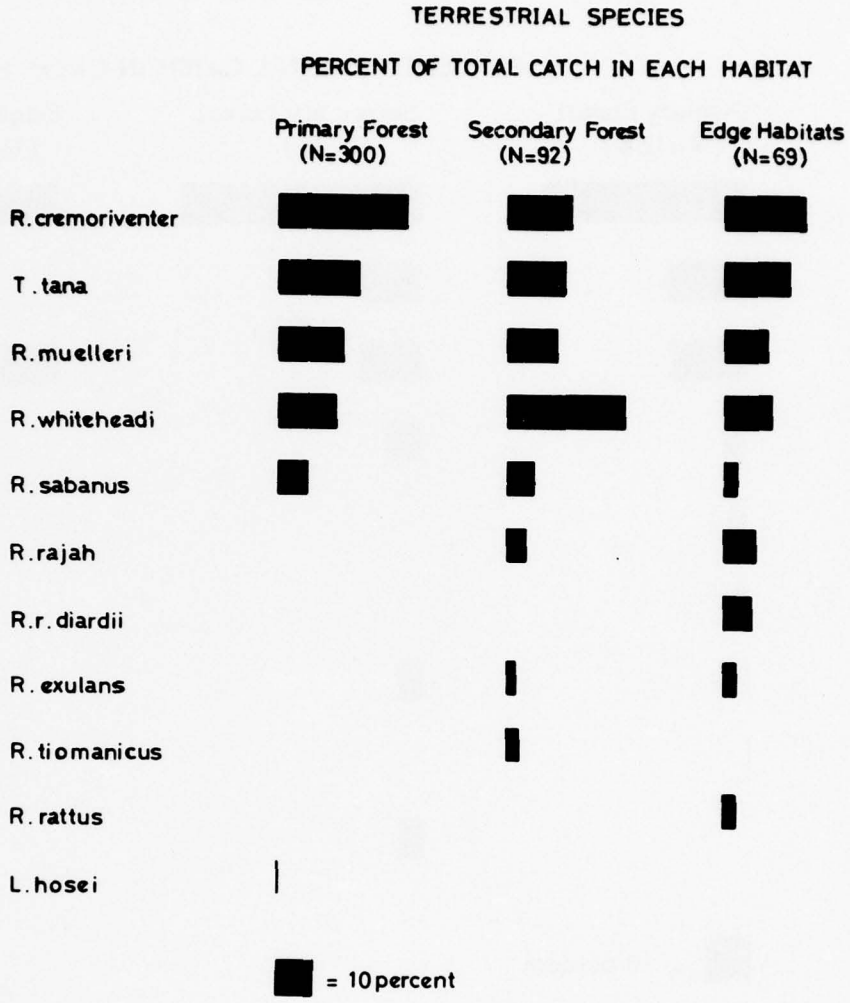


Figure 1. Habitat distribution of terrestrial mammals and their numerical rank in each habitat as indicated by trapping. Percentages indicate the ratio of each species in the total collection from each of the habitats.

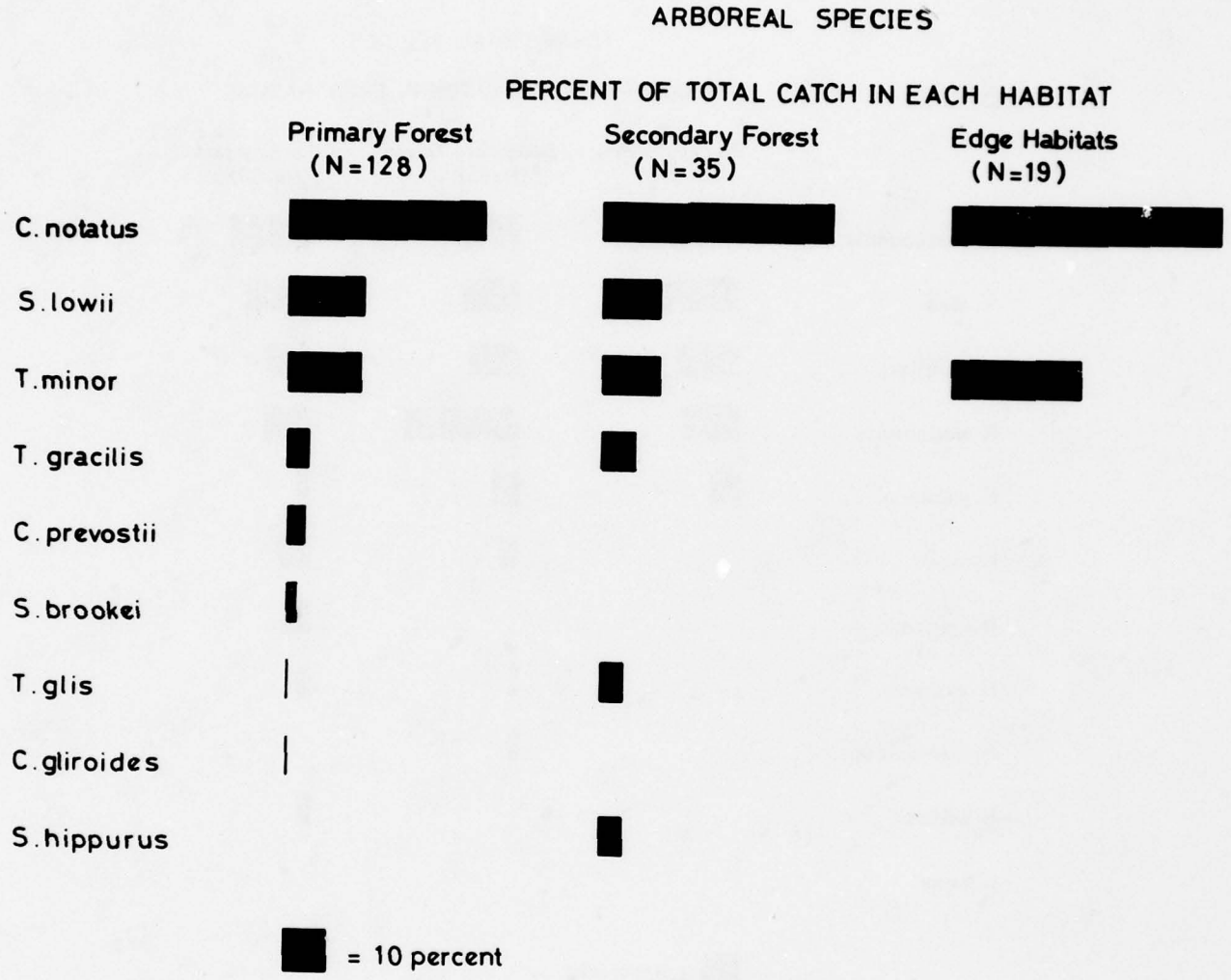


Figure 2. Habitat distribution of arboreal mammals and their numerical rank in each habitat as indicated by trapping. Other arboreal species not trapped are listed in the text. Percentages as in Figure 1.

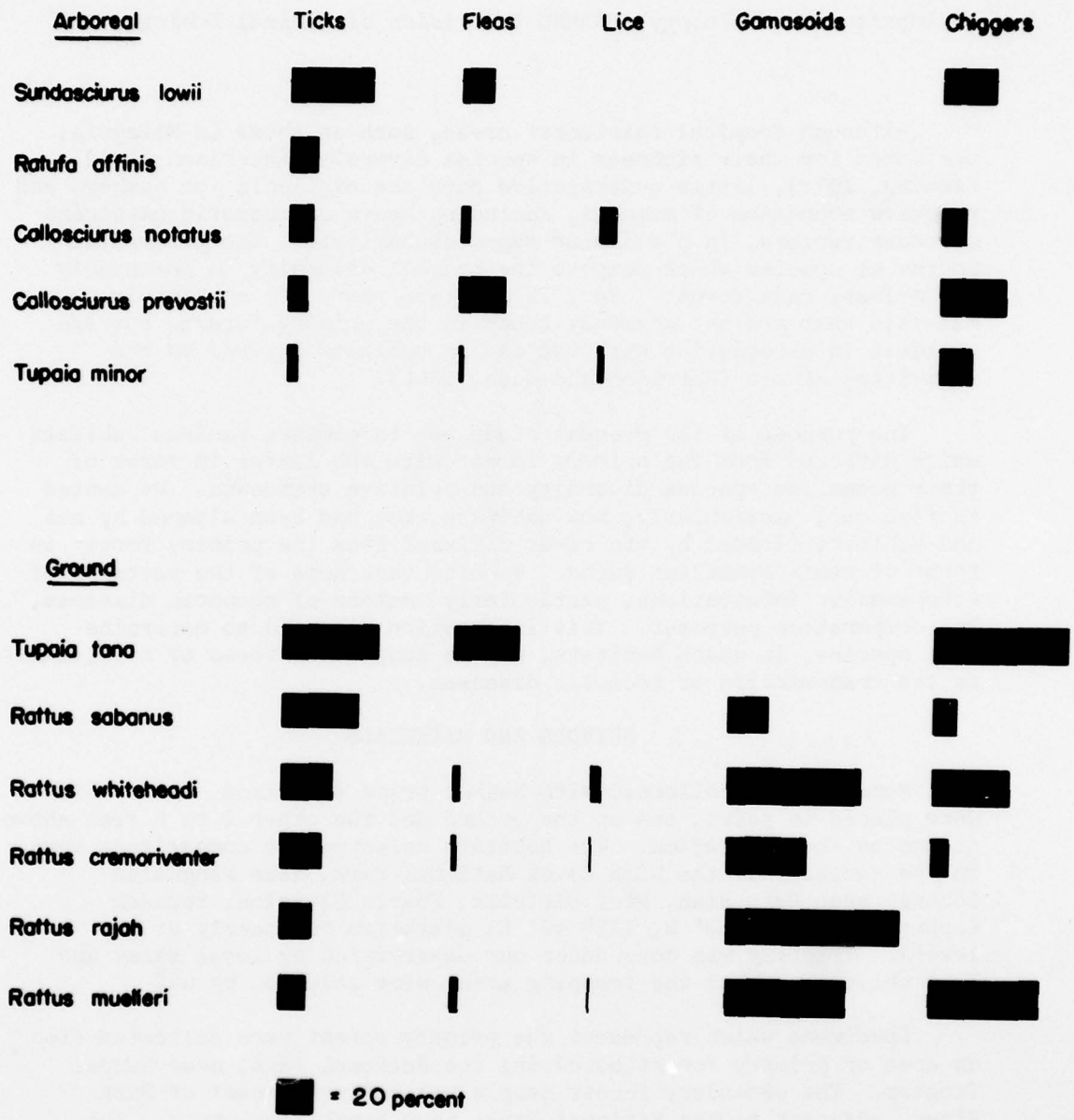


Figure 3. Ectoparasite distribution from arboreal and terrestrial (ground) mammals. Percentages indicate ratio of individuals collected infested with given ectoparasites.

Habitat Distribution and Ectoparasites of Small Mammals in Sarawak

by

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Although tropical rainforest areas, such as those in Malaysia, are noted for their richness in species diversity (Harrison, 1962, Fleming, 1973), little quantitative data are available for numbers and relative abundance of mammals, including hosts of zoonotic pathogens or their vectors, in particular types of habitats. The primordial source of species which compose the overall diversity is presumably the primary rain forest. Yet, in addition there are species in Malaysia that are not commonly found in the primary forest, but are abundant in association with man and in habitats altered by the activities of man (Harrison and Quah, 1962).

The purpose of the present study was to compare various habitats which differed from the primary forest with the latter in terms of their mammalian species diversity and relative abundance. We wanted to find out, particularly, how habitats that had been altered by man and habitats flooded by the river differed from the primary forest in terms of their mammalian fauna. We also took note of the patterns of ectoparasite infestations, particularly vectors of zoonotic diseases, for comparative purposes. This information will aid to determine what species, in which habitats, may be suspect in terms of involvement in the transmission of zoonotic diseases.

METHODS AND MATERIALS

Mammals were collected with basket traps (Harrison, 1966) which were placed in pairs, one on the ground and the other 2 to 6 feet above ground on the vegetation. The habitats selected for comparisons were in the vicinity of the Niah Caves National Park, near Pangkalan Lobang, near Batu Niah, Miri District, Fourth Division, Sarawak (approximately 3° 53' N, 113° 42' E; elevation was nearly at sea level). Trapping was done under our supervision by local Malay and Iban collectors, but the trapping areas were selected by us.

Specimens which represent the primary forest were collected from an area of primary forest bordering the National Park, near Sungei Tangkap. The secondary forest sample was collected east of Niah River, adjacent to the National Park, near Pangkalan Lobang. The swamp forest sample was collected on the west side of Niah River, north of Pangkalan Lobang. The edge habitat sample came mostly from the west side of Niah River near Pangkalan Lobang.

Animals were removed from the trap into cloth bags in which they were euthenized with chloroform. Thick and thin blood smears were prepared for examination of blood parasites (to be reported elsewhere). Serological samples were obtained for studies of scrub typhus *(Rickettsia tsutsugamushi)* prevalence (to be reported elsewhere). Fleas, lice, and mesostigmatid mites were brushed from the host. Ticks were removed from the ears and other parts of the body. Chiggers (larval Trombiculid mites) were dislodged from the ears and elsewhere on the body with a sharpened wooden applicator stick. All ectoparasites were placed in 70% alcohol for later identification and the cloth bags were washed between examinations. Although each specimen was examined for ectoparasites by the same person and equal time and effort was devoted to each, it is doubtful that every ectoparasite was recovered.

Females were examined for signs of lactation and their uteri were examined under a binocular dissecting microscope for the occurrence of embryos and placental scars (to be reported elsewhere).

This report includes all mammals trapped and those captured in nest cavities in trees in the habitats listed above, with the exception of bats (reported elsewhere: Lim, Chai and Muul, 1973).

DESCRIPTION OF TRAPPING AREAS

Primary forest:- According to the District Forestry Officer, this forest had not been opened for commercial timbering in the past. There were no signs of timbering on any scale in the area chosen for trapping. The canopy was from 80 to over 100 feet. Ground cover was sparse, consisting of palms and small trees.

Secondary forest:- According to the District Forestry Officer, this area has sustained some commercial timbering. However, tall trees were frequently encountered. The ground cover was denser than in the primary forest, with an abundance of small trees forming a lower canopy than in the primary forest.

Swamp forest:- This area had a high water table with exposed water abundant among the trees. The trees were not as tall as in the primary forest, but the canopy was complete.

Edge habitats:- This stage in regeneration of vegetation is known in Malaysia as *belukar*. It results from clear cutting. With the exception of occasional taller trees, the vegetation was up to about 30 feet tall, consisting mostly of fast growing species of trees which are characteristically primary invaders of such cut-over areas. These trees were intermixed with wild banana, wild ginger, vines, and herbaceous vegetation, forming a dense cover which was difficult to penetrate.

RESULTS

A total of 270 arboreal and 232 scansorial and terrestrial mammals were trapped. Additional arboreal species were captured in

their nests in tree holes. Most of the terrestrial species (Figure 1) appear to be partly scansorial as they were trapped sometimes in vegetation above ground.

Callosciurus notatus was the predominantly caught arboreal species in all habitats and *Tupaia tana* was the terrestrial species most abundantly caught in all habitats except the swamp forest; where its dominance was replaced by *Rattus muelleri*. *Rattus rajah, R. exulans, R. whiteheadi, R. tiomanicus,* and *R. rattus* were captured only in the man-altered habitats and *R. rattus* were captured only in the man-altered habitats and the swamp forest which was subject to periodic inundations of water from the river. Most of the species in the catch in each habitat were approximately in the same numerical proportion as those caught in the primary forest, although these species (primary forest component) comprised a smaller percentage of the total collection than in primary forest.

Figure 2 demonstrates the patterns of infestation of the mammalian hosts with ectoparasites. Only species which had 10 or more individuals examined are included. The canopy species tended to have a lower infestation rate with ticks than did the terrestrial species. The observed tick load varied from 1 to 4, with averages of 1 to 2 per individual among the canopy species. Terrestrial species varied from 5% to nearly 40% infested and tick loads varied from 1 to 40. The highest mean tick loads were in *Tupaia glis* 3.5, *Tupaia tana* 4, and *Rattus muelleri* 16.

Fleas were scarce among most of the canopy and terrestrial species (Fig. 2). The highest infestation rates were among the squirrels and terrestrial tree shrews. The mean flea loads were highest among the terrestrial tree shrews: *Tupaia tana* 6 (one specimen with 65 fleas), *Tupaia glis* 4 (range 1-8); in the squirrels the mean loads were less: *Callosciurus notatus* 1.6 (range 1-5), *C. prevostii* 2.5 (range 1-4).

No lice were found in three of the tree shrews and *Rattus tiomanicus* (Fig. 2). In the other species infestation rates varied from 18% *(R. sabanus)* to over 50% *(C. prevostii)*. Mean louse loads varied from 6 (range 1-18) in *R. cremoriventer* to 17 (range 1-130) in *Callosciurus notatus*.

Most species had fairly high infestation rates with gamasoid (mesostigmatid) mites, with the exception of the tree shrews. Rates varied from 10 percent *(Hylopetes lepidus)* to nearly 100 percent *(R. muelleri)* in mammals other than tree shrews. Gamasoid loads varied from 10 to 20 in *C. prevostii* to over 300 in *R. cremoriventer*; the highest mean loads were on *R. muelleri* (42) and *R. cremoriventer* (41). The mean loads in the squirrels were lower: *C. notatus* 10 (range 1-100), *C. prevostii* 17 (range 10-20).

Chigger infestation rates were highest among the squirrels, tree shrews and in *R. muelleri*. Individual chiggers were not counted so chigger loads were not determined.

DISCUSSION

Although the known mammalian fauna of Borneo is very diverse, only a fraction of the species known to occur there were obtained in the sample of over 500 mammals trapped in the few habitats under study. The trapped species were supplemented by those caught in arboreal nest cavities, but still many species were not caught. Of the 10 species of the lowland terrestrial forest rats known from Borneo (Harrison, 1964; Medway, 1965) only six were caught. None of the five tree rats were caught. Four of the six lowland commensal rats were trapped. Of the 7 lowland tree shrews, four were captured in addition to the related *Ptilocercus lowii*.

The squirrels were even less represented: one of the four species of lowland ground squirrels, five of the 11 species of lowland tree squirrels, and five of the 13 species of flying squirrels. Altogether, 44% of the rodent species within these groups known from the lowlands of Borneo were represented in our sample.

The absence of certain terrestrial forest rats and terrestrial squirrels may be owed to the limited number of habitats sampled. The commensal rats not caught were *Rattus norvegicus* and *Mus musculus* which seem to be restricted to coastal cities and *R. argentiventer* which seems to be confined to ricefields and grasslands (Harrison and Quah, 1962). The tree rats, tree squirrels, flying squirrels, and tree shrews may also be restricted to habitats other than the ones sampled by us.

In general, the arboreal animals in the man-altered habitats and the swamp forest which is periodically distributed by floods were about the same as those in the primary forest. This seems to indicate that no specialized arboreal forms exist in these disturbed areas. *Callosciurus notatus* which was numerically dominant in the primary forest was even more prevalent in the edge habitats. For the first few days in the edge habitats it comprized nearly the entire catch and their capture had to be discontinued (the trappers were asked to release them after the 4th day of collections) in order to include sufficient numbers of other species in the sample which was originally to be limited to 200 specimens per habitat. In Peninsular Malaysia, *C. nigrovittatus* is the numerically dominant species in most primary forests and *C. notatus* is more abundant in secondary forests and edge habitats (Division of Medical Ecology, IMR, unpublished records).

The assortment of terrestrial species found in the primary forest seems to have been represented in approximately the same proportions in the disturbed habitats, with the exception of *R. muelleri* in swamp forest. This rat seems to be also more common in wet habitats, such as edges of streams, than in more dry areas in Peninsular Malaysia (Lim, 1970). In addition, the disturbed areas had an abundance of commensal rats, not represented in the primary forest sample (Fig. 1). This indicates that in this instance, the disturbed areas had a more

diverse fauna, at least in terms of species which responded to our trapping efforts. The remaining 66% of the species not obtained by us probably do not enter traps readily or may be confined to specialized habitats, such as the taller growing rainforests found in the interior. In another study in Sabah (Muul and Lim, 1973), a much more diverse assortment of species was obtained in a forest with a canopy over 150 feet, with taller emergent trees, than in the forest near Pangkalan Lobang which had a canopy about 100 feet high.

In terms of ectoparasites, the arboreal species tended to have lower infestation rates with ticks and smaller tick loads than did the terrestrial species (Fig. 2). This was also the case in Sabah (Muul and Lim, 1973). It is interesting to note that fleas were not found on species which construct sub-terranean nests (infrequent or absent on such hosts also in Sabah). The elaborate grooming behavior of the tree shrews probably is responsible for the absence of lice in three of the four species examined and low numbers found on the fourth (three infested specimens with 1, 3 and 25 lice recovered from each). Gamasoid mites were few or absent in the tree shrews, again perhaps because of their grooming behavior. Larval Trombiculid mites (chiggers), on the other hand, were abundant on tree shrews (in the ears) and squirrels. With the exception of *R. muelleri*, the rats were not frequently infested with chiggers.

These patterns of distribution of hosts and their parasites should be helpful in determining which species may be candidates for investigation in epidemiological studies of zoonotic pathogens in nature.

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Figure 1

PATTERNS OF DISTRIBUTION OF MAMMALS TRAPPED IN FOUR HABITATS IN SARAWAK

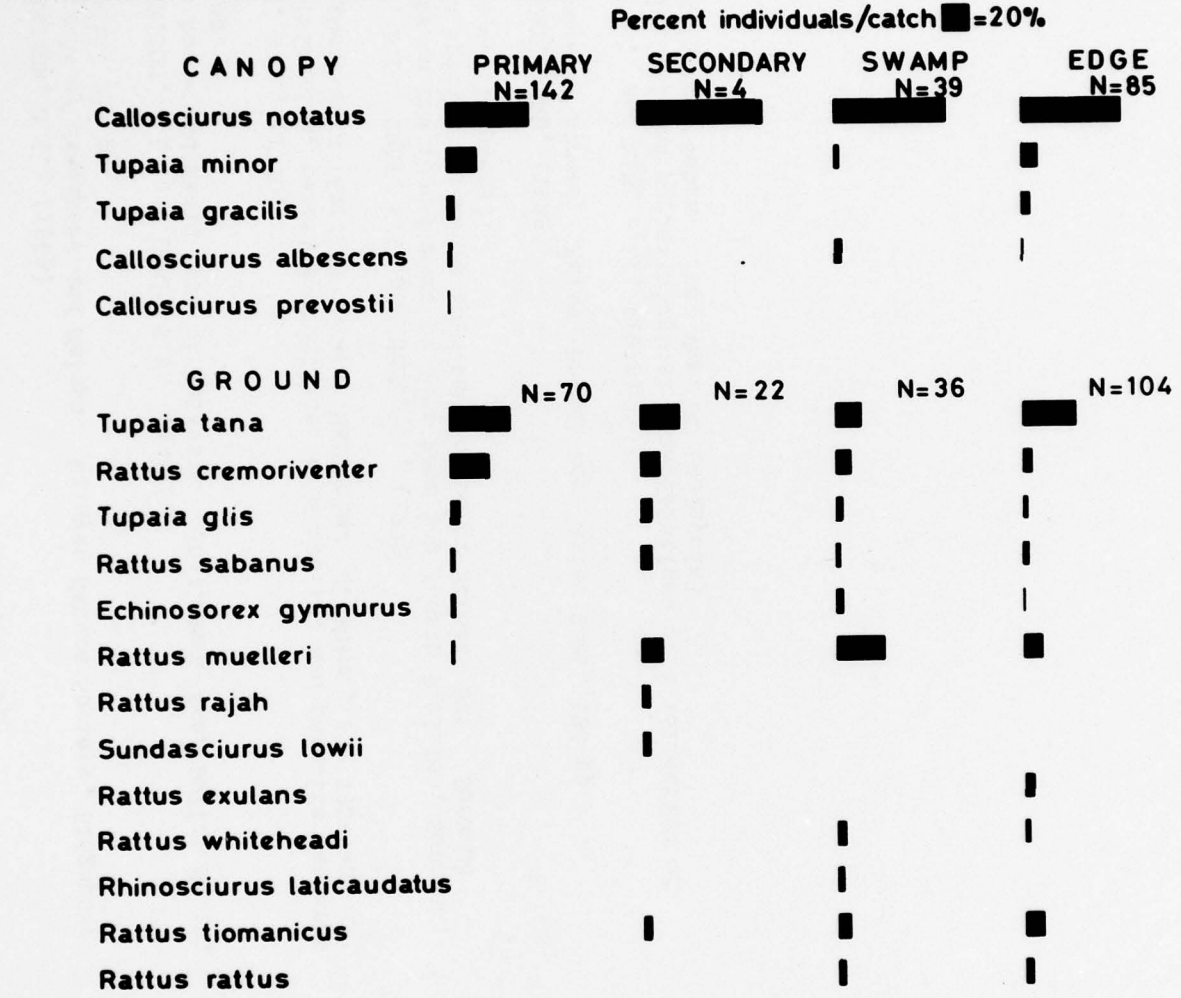
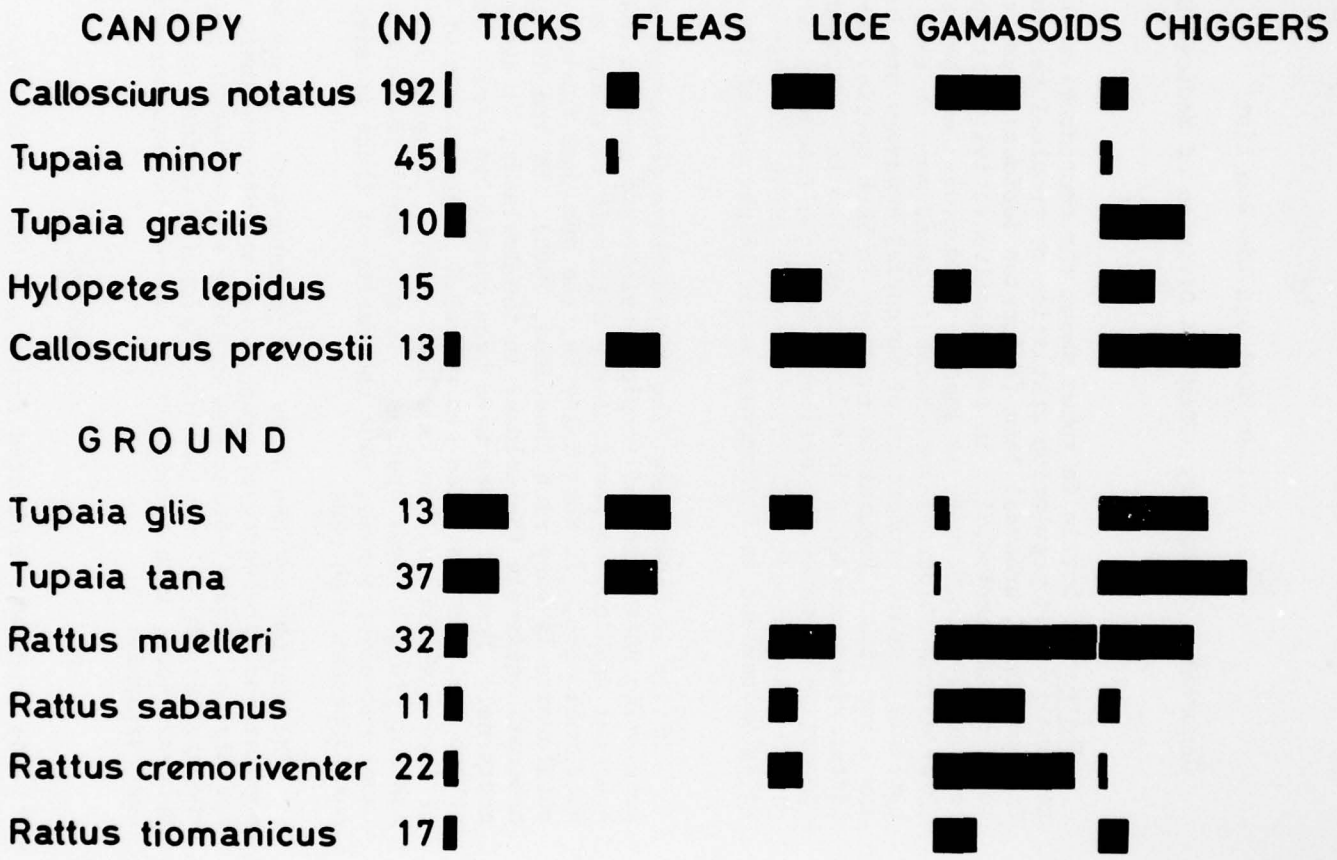


Figure 2

ECTOPARASITE PATTERNS IN MAMMALS COLLECTED IN SARAWAK

Percent individual infested ■ = 20%



Reproductive Frequency in Some Rainforest Mammals in Malaysia

by

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Abstract. Little is known about the reproductive frequency, seasonality and population statistics of tropical rainforest mammals. One nocturnal, arboreal form *(Hylopetes lepidus)* seems to have a super-annual periodicity in reproductive activity with periods of reproductive inactivity as long as 17 months. Another species *(Pteromyscus pulverulentus)* seems to breed more or less continuously, but only a small proportion of potential breeders are involved at any given time. Population turnover in both species seems to be slow. Litter sizes average 2 in *Hylopetes* and 1.3 in *Pteromyscus*. Population turnover, as estimated from the frequency rate, gestation period (estimated), and litter size, is about 1/3 that of forest rats in *Hylopetes* and in *Pteromyscus* about 15 percent of the rate in rats.

Little is known about the reproductive frequency, seasonality and population statistics of tropical rainforest mammals (Harrison, 1952). Arboreal species are particularly difficult to study, especially the nocturnal ones. In West Malaysia, the arboreal fauna in the rainforests is very rich (Harrison, 1966). Of the 20 species of arboreal squirrels (Sciuridae) in the lowlands, 11 are gliders and nocturnal. Most of these have been considered rare. However, search of arboreal nest cavities has revealed large numbers of some species of flying squirrels (Muul and Lim, 1971). Trapping in the same areas has not yielded these species. In most areas trapping has been the usual method of survey, thus the rarity of these species seems to be more apparent than real.

The purpose of this study was to determine the age structure of populations, frequency of pregnancies and parturitions, and the monthly and annual variations in these statistics in populations of two species of flying squirrels, *Hylopetes lepidus* and *Pteromyscus pulverulentus*, in a rainforest in Johore, the southernmost state in West Malaysia.

METHODS

The study was conducted from July 1968 through April 1972. At first samples were taken each month, less frequently later. Nests of the animals were located in the daytime by *Orang Asli* (aborigines). Animals were removed from nest cavities in trees, together with nesting materials. The advantage of nest collections over trapping is that the whole population is represented including the very

young, availing statistics on populations that otherwise can be arrived at only indirectly. The animals were placed into cloth sacks, euthenized with chloroform and for related studies they and the sacks were examined for ectoparasites; blood samples were taken and on autopsy, endoparasites were collected. The reproductive tracts were removed and these and all parasite samples were preserved in 70% alcohol. Uteri were examined under a dissecting microscope for the presence of embryos. Neonates were preserved in alcohol. Each juvenile and adult specimen was prepared as a standard reference study skin and skull. Subadults were distinguished by size and pelage coloration, which in *Hylopetes* is rufous in adults and gray or brown in subadults. In *Pteromyscus* subadult pelage is shorter than in adults, and the tail is narrower. Subadult pelage was observed to be retained in captivity for 3 to 4 months in *Hylopetes* and longer, up to 6 months, in *Pteromyscus*. We were not reluctant to collect large samples of these species because the forest in this area was being timbered and the area was destined to become agricultural land.

RESULTS

Monthly statistics on populations of these species appear in Figures 1 and 2. During the 46 month period, a total of 1166 (596 ♂♂, 470 ♀♀) adult *Hylopetes* were collected, in addition to young of various ages. At six month intervals, the immatures/adult females ratios, beginning in July 1968 through April 1972, (last period 4 months only) were 1.0, 0.1, 0, 0.74, 1.7, 0.37, 1.12, 0.16, or an overall ratio of 0.6. Average litter size at birth was 2.0 (N=39 females examined for embryos; range 1 to 4).

During the same period 440 (211 ♂♂, 229 ♀♀) adult *Pteromyscus* were collected, in addition to young of various ages. For each year the immatures/adult females ratios were 1.0 (N=51 ♀♀) (5 months only), 1968; 3.30 (N=58 ♀♀), 1969; 0.52, 1970; 0.73, 1971. During the entire period there were 144 immatures collected or an overall immatures/adult females ratio of 0.63. Average litter size at birth was 1.3 (N=14 females examined for embryos; range 1 to 2).

In *Hylopetes* in the first part of the collections young were present in the nests and pregnancies among females were observed (July and August, 1968). From September 1968 through January 1969, no more pregnancies were noted. After January 1969, *Hylopetes* populations collected composed of only adults, until February 1970, when pregnancies were once again observed. Thus, the time interval between the successive observed pregnancies was 17 months. In February and March 1970, the majority of the females examined were pregnant. There were no subadults to indicate any previous reproduction within the population. Reproduction apparently continued through July. After this no pregnancies or young litters were observed. About half the subadults observed in September and October were adult size (over 130 mm head and body length) but still bearing the juvenile pelage. In April 1971, some of the females were pregnant again (one also in March), this peak in prevalence being 14 months since the beginning of the previous period of

prevalent pregnancy rates (February, 1970) and 9 months after the end of the 1970 period of reproductive activity. Breeding appeared to continue through September, with less in October, 1971. No samples were collected in November and December. Reproductive activity was low in January 1972 and subadults were few. The last sample, in April 1972 included one litter. In *Pteromyscus* recent litters or pregnancies were seen in nearly every month, with the exception of November 1968 through February 1969, and September and October, 1970.

DISCUSSION

In *Hylopetes* the pattern of reproduction from year to year seems to have been highly variable. No particular part of the calendar year seemed to be devoted specifically to reproduction. Yet, the reproductive activity seemed to be highly synchronized within the population. Once begun, reproductive activity seemed to continue for about 6 or 7 months. Periodicity did not seem to follow an annual pattern, and indeed, in 1969 there seemed to be no reproduction at all. The onset of breeding in 1968 was not determined, but perhaps it occurred in February or March (assuming the length of the reproductive period to last 6 or 7 months as was the case in 1970, 1971). In 1969 there was none and in 1970 the onset was in February or about 19 months since the calculated beginning of the last one (or July 1968 at the latest). In 1971 the onset was in April, or 14 months after the beginning of the previous one in February 1970. In 1972, the peak in reproductive activity was not determined, but apparently it had not yet occurred in April when the last sample was taken. Thus, the interval since the beginning of the last reproductive period in April 1971 was at least 13 months.

It is not clear whether these peaks in reproductive activity represent some sort of a super-annual reproductive periodicity based on erratic climatic conditions with at least 13 months, and perhaps, up to 19 months, between onsets, or whether this unusual reproductive periodicity follows fruiting cycles of some forest trees that form a food source for the species. Our unpublished data indicate that both species are largely frugivorous (fruit eating). Some trees, such as *Saniria, Myristica, Sterculia,* and *Shorea,* appear to bear fruit infrequently, at some sort of a super-annual cycle (McClure, 1966; Medway, 1972). McClure (1966) noted squirrels fed on fruit of members of these genera. In 1969, no *Shorea* or *Myristica* were observed to fruit in Gombak in the State of Selangor by Medway (1972), although there was synchronous fruiting the year before. But, this is only circumstantial evidence and we have no records of fruiting in Johore. No lunar rhythm, as described by Harrison (1952), was evident.

There are no marked seasonal fluctuations in the weather in most of West Malaysia, including Johore. The range of daily fluctuations in temperatures exceeds that of the annual mean daily temperatures (Dale, 1963). There are no dry months, but some months tend to be wetter than others, particularly November through January (Dale, 1959, 1960). Therefore, it is very difficult to correlate the

peculiar reproductive periodicity of *Hylopetes* with any particular environmental factor or cue. If there is an irregular, but usually super-annual reproductive periodicity in *Hylopetes*, this observation would shed new light on reports of annual reproduction patterns reported in the literature especially in cases in which data have been lumped over a period of years under calendar months (Medway, 1969). In such cases, given samples over a sufficient number of years, the results would appear to show that breeding occurs in nearly every calendar month. If annual samples are small, the lumped data would give the impression that breeding is more or less continuous all year round. For this reason one must be cautious in trying to fit ecological phenomena in the tropics into calendar years. That this sometimes (we don't really know how often since so few species have been studied) does not work out has been dramatically illustrated by McClure (1966) and Medway (1972) with flowering and fruiting cycles of many trees in Malaysia.

In *Pteromyscus*, on the other hand, breeding did not seem to be as synchronized or as restricted seasonally as in *Hylopetes*. There was evidence of recent reproductive activity through most of the time, excluding only November 1968 through February 1969, and September and October 1970. Something of peaks seemed to have occurred in May-June 1970 and in April-June 1971. On the whole, *Pteromyscus* seems to be a continuous breeder, mostly at a low level.

In terms of population turnover, *Hylopetes* seems to be highly variable. In the last part of 1968 the ratio of immatures/adult females was 1.0; in 1969, .04; in 1970, .92; in 1971, .67; in the first 4 months of 1972, .16. In *Pteromyscus* the immatures/adult females ratios were 1.0 in 1968, 0.3 in 1969, 0.52 in 1970, and 0.73 in 1971. Thus, since the average litter size at birth in *Hylopetes* is 2 it appears that usually less than half the adult females were accompanied by litters and less than one quarter were observed with litters in 4 out of 8 of the six-month periods.

These ratios, which reflect population turnover, seem very low. Population turnover can be calculated in another way, i.e., from pregnancy rates. These have been reported for other small mammals studied in West Malaysia by Harrison (1952). The pregnancy rate in 72 female tree squirrels *(Callosciurus notatus* and *C. nigrovittatus)* was found to be as high as 20 percent during four months of collection in 1949 (Harrison, 1952). During a period of over 3 years, ground rats were found to have an overall pregnancy rate of 16 percent (N=968) tree rats 16 percent (N=111), and diurnal tree squirrels, including the two species named above, nearly 13 percent (N=474)(Ibid.). In *Hylopetes* the overall pregnancy rate during the 46 months of study was 13 percent (N=470 ♀♀). In *Pteromyscus* the pregnancy rate over the same time span was 9 percent (N=229 ♀♀). These overall pregnancy rates, however, are not directly related to population turnover since not all species have the same period of gestation and litter sizes are different. In Malaysian flying squirrels the gestation period is not known, but we assume it to be similar to that of North American flying squirrels, i.e., 40 days (Sollberger, 1943). If

forest rats have a gestation period around 20 days (estimate based on laboratory rats, *Rattus norvegicus)* their rates of observed pregnancies would represent twice the number of litters as the same rate in flying squirrels. The median litter size among 9 species of forest rats was about 3 (Harrison, 1956). Since litter size in *Hylopetes* is also smaller (2) than in rats, the overall pregnancy rate of 13 percent would represent 26 young per 100 females over a period of time, whereas the same pregnancy rate over time in rats would represent 78 young per 100 females. In *Pteromyscus*, since the litter size is still smaller (average 1.3) and the pregnancy rate is lower (9 percent) than in *Hylopetes*, even fewer or about 12 young would be produced per 100 females over an equivalent period of time. Another factor needed to get a more accurate picture of population turnover is the period of reproductive activity, related to longevity. Unfortunately we do not have such data.

ACKNOWLEDGEMENTS

We are grateful to the Director of the Institute for Medical Research for facilitating this study. We thank the following persons in the Division of Medical Ecology for their efforts in field and laboratory work: Chai Koh Shin, Abdul Rahman bin Omar, Kamalludin bin Sagap, Lee Eng Kee, M. Krishnasamy, P. Ramachandran, Ramdzan bin Hamjan, Shariff bin Mansor, R.D. Soosai. We are also grateful to the *Orang Asli* (aborigines) collectors in the villages of Tamok and Kudong, Johore. This study was supported mainly by a Research Grant (DADA17-73-G-9368) from the U.S. Army Medical Research and Development Command.

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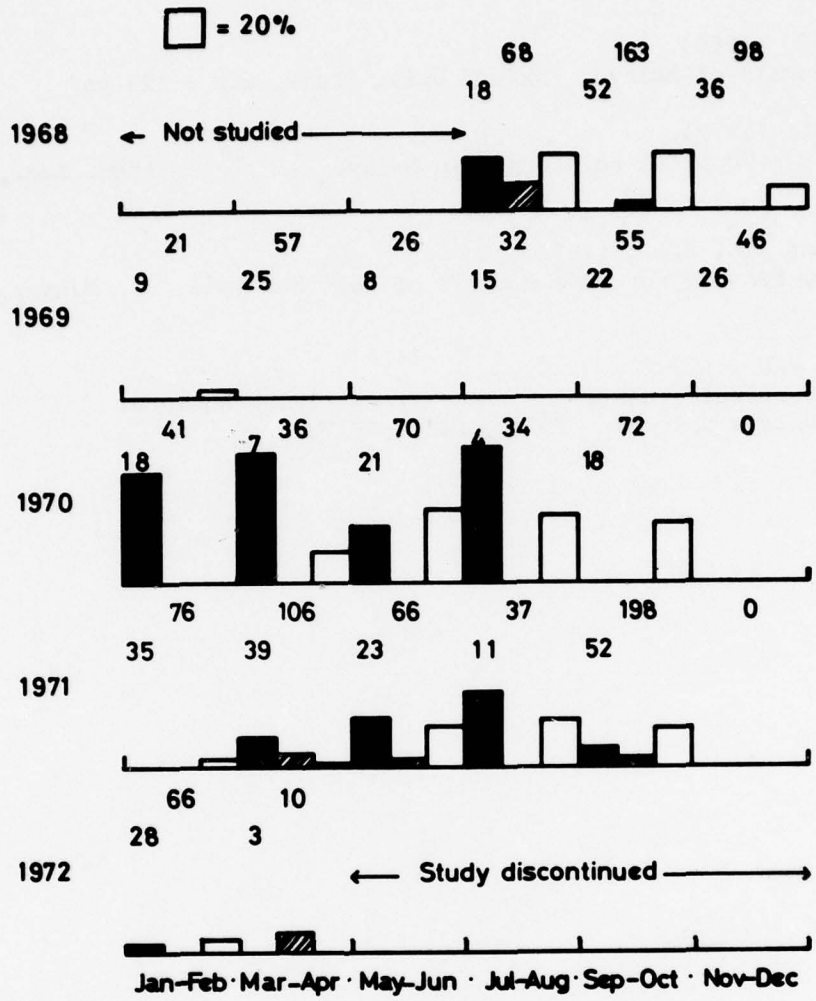


Figure 1. Population statistics at two month intervals of *Hylopetes lepidus* collected from a primary rainforest in Johore, W. Malaysia. Numbers at top represent total specimens (adults and sub-adults) collected in each of the two month periods; below, to the left, the number of adult females in the sample; dark bars represent percentage of females pregnant, hatched bars represent percentage of females with young litters, open bars represent the percentage of sub-adults and immatures in each sample.

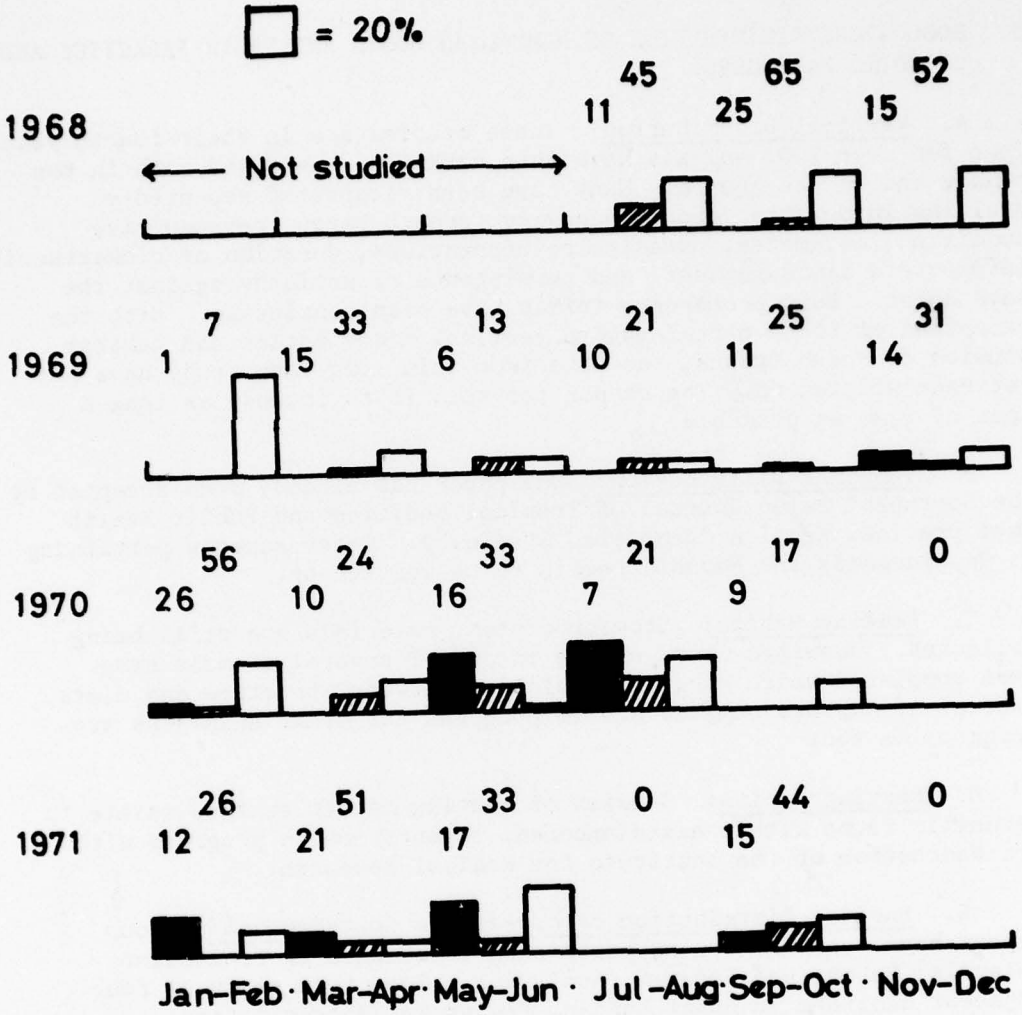


Figure 2. Population statistics at two month intervals of *Pteromyscus pulverulentus* collected from a primary rainforest in Johore, W. Malaysia. Legend as in Figure 1.

STUDIES IN PROGRESS

I. ECOLOGICAL DISTRIBUTION OF MAMMALIAN HOSTS AND THEIR PARASITES AND ZOONOTIC PATHOGENS.

A. Vertical Distribution: These studies are in their fourth year. Thus far over 2000 animals have been marked and released both in the canopy and on the ground. Many have been recaptured repeatedly yielding information about longevity, growth rates, reproductive condition, movements, parasite reinfestations, duration of rickettsemia *(Rickettsia tsutsugamushi)* and persistence of antibody against the same agent. Bait preference trials have been continuing. With the exception of those pertaining to vertical distribution and habitat studies of scrub typhus, the data from this long term study have not yet been written up. The reason for this is to include as long a span of time as possible.

B. Temporal Distribution: One paper has already been accepted by the Southeast Asian Journal of Tropical Medicine and Public Health (see previous section "Completed Studies"). Other aspects pertaining to *Hepatocystis* and *Babesia* remain to be written up.

C. Feeding Habits: Stomach content materials are still being collected. Drawings of digestive tracts of several mammals have been completed which show a correlation between structure and diets. Studies of *Angiostrongylus* are in progress and other helminths are being collected.

D. Nesting Habits: Studies of nesting habits as they relate to parasitic fauna within nests (arboreal rodents) are in progress with Mr. Nadchatram of the Institute for Medical Research.

E. Habitat Distribution of *Rickettsia tsutsugamushi*: Four papers have been written up pertaining to studies in Peninsular Malaysia, Sabah, and Sarawak, including a long term study of four adjacent habitats in Bukit Lanjan, Peninsular Malaysia (see "Completed Studies"). One additional study is in progress at Bukit Lanjan involving 20 meter diameter enclosures in which sentinel rats are placed once each month.

F. Arbovirus Studies: To date 823 isolation attempts have been made by Dr. Lim Teong Wah's laboratory of the Institute for Medical Research. Five isolates have been made which have not yet been characterized: 3 from arboreal animals, *Callosciurus notatus* (2) and *Nycticebus coucang* and two from terrestrial animals, *Tupaia glis* and *Rattus surifer.*

Serological tests (H.I.) have been completed for 201 specimens which yielded 8 positives for Group B arboviruses.

G. *Angiostrongylus*: These studies are in progress. Data are ready for writing up.

II. POPULATION DYNAMICS OF HOSTS AND IMPORTANCE IN TRANSMISSION OF ZOONOTIC PATHOGENS.

A. Population Dynamics: Data are ready for analysis in relation to scrub typhus and *Angiostrongylus*.

B. Seasonality of Reproduction: One paper written up and accepted by the Journal of Mammalogy (see previous section "Completed Studies"). Data for a different habitat, a "kampong rubber plantation" are ready to be written up.

C. Reproductive Frequency and Litter Size: Additional data are being collected. Some data already incorporated in manuscript listed under I,B.

D. Longevity: Data are being accumulated at the canopy transect study area and in other study areas where we have mark and release programs.

III. SEASONAL PHENOMENA CORRELATED WITH FLUCTUATIONS IN ZOONOTIC INFECTIONS.

A. Phenological Studies: Studies at the Bukit Lanjan canopy transect area are being continued with the cooperation of Dr. Ng, Forest Research Institute.

B. Seasonal Patterns of Parasitization: Some of the data are presented and discussed in the papers on *Plasmodium* and *Rickettsia* infections (see "Completed Studies").

IV. SPECIES ASSOCIATION INDICES.

Relevant data have been presented and discussed in: "New locality records for some mammals of West Malaysia", Journal of Mammalogy, 52: 430-437; "Habitat distribution and ectoparasites of small mammals in Sarawak" (accepted by the Sarawak Mus. J.); "Land use and small mammal ecology in Cameron Highlands, Malaysia" (accepted by the Federation Mus. J.); and "Ecological considerations of a collection of mammals from Sabah" (accepted by the Federation Mus. J.) (see "Completed Studies"). The overall analysis for derivation of actual indices will be done at the termination of the study in order to include as much data as possible.

V. ZOOGEOGRAPHIC BOUNDARIES. (see "Plans for Future").

VI. DISEASE ENDEMICITY IN SUNDA REGION.

Three papers have already been written up in reference to *Rickettsia tsutsugamushi* in Peninsular Malaysia and Sabah (see "Completed Studies"). We now have the serological results for four habitats studied in Sarawak. In each of these habitats: primary forest, secondary forest, swamp forest, and edge habitats, the rates of serological positives *(Rickettsia tsutsugamushi)* are very much

lower than those obtained in Sabah, and particularly Peninsular Malaysia. These results are now being verified. Comparisons of rates of infections with other pathogens have not yet been analyzed.

VII. SYSTEMATICS, TAXONOMY, AND ZOOGEOGRAPHY OF MAMMALS.

One study was published in 1971, "Taxonomic status of *Petinomys morrisi* Carter (Sciuridae) and its relationship to *Petinomys setosus* Temminck", Journal of Mammalogy, 52: 362-369. Other studies await being written up including: "Neotype of *Petaurillus kinlochii* (Robinson and Kloss)", "Mongoose, *Herpestes urva*, new to Peninsular Malaysia", "New species of arboreal rat, *Pithechir parvus* Kloss", "Taxonomic revision of *Hylopetes*".

ABANDONED INVESTIGATORY AVENUES

None

PLANS FOR THE FUTURE

With the vast amounts of data gathered and through use and comparisons of specimens collected by other investigators in the past which are now available at the Smithsonian, British Museum, and other research institutes, it should be possible to get a better idea of various zoogeographic boundaries which seem to limit the distributions of hosts and probably their parasites and pathogens. Once identified, these boundaries would be useful for epidemiologists for checking the spread of zoonotic diseases in that control measures would be augmented by the environmental barriers. These types of collations of new data with previously accepted entities would be best done at the termination of the study.

INVESTIGATIONS OF THE DEPARTMENT OF MEDICAL ENTOMOLOGY

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	30 06 73	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
30 06 72		U		N/A	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY		3A062110A831		
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)*
Investigations of the Department of Medical Entomology

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
Tropical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
10 72	9 73		

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: DADA17-73-G-9368 10 72 EXPIRATION: 10 73
b. NUMBER:*
c. TYPE: Y. Grant d. AMOUNT: 263
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	1.0	20.1
	CURRENT 74	1.0	25.6

19. RESPONSIBLE DOD ORGANIZATION
NAME:* US Army Medical Research Unit
ADDRESS:* Institute for Medical Research
Kuala Lumpur, Malaysia

RESPONSIBLE INDIVIDUAL
NAME: Dr. R. Bhagwan Singh, Director
TELEPHONE: Institute for Medical Research

20. PERFORMING ORGANIZATION
NAME:* Institute for Medical Research
ADDRESS:* Kuala Lumpur, Malaysia

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Parsons, R.E., MAJ, MSC
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Dondero, T.J., Jr., MAJ, MC
NAME: Robinson, D.M., MAJ, VC
NAME: Cheong, W.H.

21. GENERAL USE

22. KEYWORDS (Procede EACH with Security Classification Code) Malaria vectors, mosquito biology, ULV equipment, *Anopheles balabacensis*, arboviruses, *Anopheles maculatus*

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

23.(U) Technical Objectives: To determine the biology of malaria vectors and associated mosquitoes and relate it to such variables as transmission rate, chloroquine resistance and plasmodial species. To determine the human ecology aspects to malaria vectors. To isolate and identify arboviruses in mosquitoes from West Malaysia. To relate the species of mosquitoes and viruses to various ecological manifestations. To determine if *Anopheles balabacensis* is a species or species complex.

24.(U) Approach: Surveys for malaria vectors are made in conjunction with each chloroquine resistance study. Mosquitoes are collected by human biting collections, human bait traps, Communicable Disease Center (CDC) light traps with CO_2 (dry ice), larval collections, and resting collections. Anophelines are dissected for malaria parasites. Culicines are screened for arboviruses.

25.(U) Progress: Mosquito surveys have been made at five rubber estates. *Anopheles maculatus* was the predominant anopheline at all estates. One oocyst-positive *An. maculatus* was found during the surveys. *An. aconitus* and *An. kochi* were the predominant anophelines collected during the Trengganu surveys. No positive stomachs or glands were found in this area. The malaria vectors in this area remain unknown. One *An. balabacensis* was collected during the Trengganu surveys. A total of 30551 mosquitoes were identified, pooled, and screened for arboviruses. Eleven positive isolates were found in 323 mosquito pools. Definite identifications are not available at this time. Plans for ordering ULV equipment and its application in the field are being made. It is anticipated that initial tests will begin in January 1974.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

COMPLETED STUDIES

Comparison of CDC Miniature Light Traps and Human Biting Collections for Mosquito Catches during Malaria Vector Surveys in Peninsular Malaysia

(To be submitted to Mosquito News for Publication)

by

Ray E. Parsons, Timothy J. Dondero, Jr. & Cheong Weng Hooi

Departments of Medical Entomology and Parasitology, USAMRU &
Division of Medical Entomology, IMR

Malaria surveys in Malaysia in past years have relied primarily on human bait as an attractant for adult anopheline mosquitoes. The most common collection methods utilizing humans are bare leg collections and human bait traps. Both are effective methods of collecting anopheline mosquitoes. They do however, require the physical presence of collectors during night-time hours and are subject to the usual biases associated with such collections.

A number of studies have been done since Sudia and Chamberlain introduced their battery operated light trap (CDC) in 1962. Newhouse *et al.* (1966) showed that light traps plus CO_2 (dry ice) collected more species and greater total numbers than those with light alone. In Thailand, Miller *et al.* (1969), compared light alone, CO_2 alone, and light plus CO_2, and found that the latter significantly increased the mosquito catch. Herbert *et al.*(1972), working in the Republic of Vietnam with a similar evaluation of the CDC light trap came to approximately the same conclusions. In addition to their basic evaluations, both experiments showed that anophelines could be collected routinely with the CDC light trap if CO_2 were added. However, none of the anophelines collected during these surveys are considered vectors in the areas studied.

This present experiment had a two-fold objective. To determine if known malaria vectors could be routinely collected in CDC light traps and to compare light trap catches with human bait catches.

MATERIALS AND METHODS

The tests were conducted in three villages in a rural area near the eastern coast of Peninsular Malaysia. A malaria survey of the village children was made concurrently with this experiment to determine the malaria rate in the area.

The standard CDC miniature light trap was utilized for the tests. The regular collecting bag was replaced with a rigid cloth bag 12 inches long and 7 inches in diameter. The distal end of the bag was cloth screen so the mosquitoes inside could be seen. The fans and lights of the traps were powered by 6 volt DC flashlight batteries. Fresh batteries were used each night.

A total of nine light traps, three in each village, were set out nightly from 6:00 PM to 6:00 AM. Each village had one light trap with CO_2 plus light, one CO_2 only and one light only. Four human collectors doing bare leg collections were set out in each village. They collected from 6:00 PM to 6:00 AM. The collectors were placed out of sight of the light traps so they would not act as an attracting source for the traps.

The human biting collections were made by placing a small, glass vial over the mosquito as it landed on the collectors leg. The vial was quickly closed by inserting a wad of cotton in one end. Collections were timed hourly and each hour's collection placed in a separate white, cloth bag.

The following morning specimens were collected and taken to a field laboratory alive. Here they were killed with chloroform, counted and identified. The anophelines were further examined for malaria parasites.

The light traps were run for 8 nights and the human biting collections for 5 nights. The data is presented on a unit per hour basis, i.e., the number of mosquitoes per hour per total light trap collections versus the number collected per total man hours.

RESULTS AND DISCUSSION

Table 1 represents the results of the data by species or genera collected. A total of 9458 mosquitoes were collected during the study. Of these, 1248, or 13 percent, were anophelines. The human biting collections accounted for 0.5 anopheline and 3.5 culicines per man hour. The traps with light only collected 1.1 anophelines and 2.2 culicines per trap hour. With CO_2 only the catch was 0.3 anophelines and 4.5 culicines per trap hour. The trap with CO_2 plus light collected 1.8 anophelines and 13.1 culicines per trap hour.

Culex species were the most common mosquitoes collected. *Culex annulus*, a potential arbovirus vector, was the most frequently caught *Culex*.

Ten species of anophelines were collected during the survey. Of these, only *Anopheles barbirostris* is considered a vector in Peninsular Malaysia. *An. aconitus*, an important vector in Java but not considered a vector in Malaysia was collected in small numbers. *An. kochi* was the predominant anopheline collected by all methods, and was more attracted to light plus CO_2 than human bait. This species, along with *An. aconitus*, are not considered vectors in Malaysia, however, they both have been listed by Sandosham (1969) as efficient transmitters of human malaria under laboratory conditions.

Previous collections, in the same villages, using light traps plus CO_2 and bare leg collections have yielded small numbers of *An. maculatus* and *An. balabacensis*, both considered vectors of malaria in parts of Malaysia.

Table 1

Comparison of mosquito catches of various CDC miniature light trap combinations and human biting collections from three villages in Peninsular Malaysia

	Human Biting Collections[a]	Light Only[b]	CO_2 Only[b]	Light plus[b] CO_2
Anopheles aconitus	10	1	2	7
Anopheles barbirostris	11	5	4	1
Anopheles crawfordi	26	14	2	2
Anopheles indiensis	33	20		59
Anopheles karwari	11	2		16
Anopheles kochi	187	246	82	335
Anopheles philippinensis	2			5
Anopheles separatus	11	2		2
Anopheles tessellatus	31	30	10	75
Anopheles vagus	2			2
Aedes albopictus	14	2	2	8
Aedes caecus	1			
Aedes chrysolineatus	1			
Aedes lineatopennis	88	7		19
Aedes poicelus	3			
Aedes vexans	66		2	9
Aedes sp.	5	1		1
Armigeres subalbatus	4	1	1	3
Coquillettidia nigrosignata	1		2	1
Culex annulus	809	462	830	3201
Culex bitaeniorhynchus	23	4	16	19
Culex fuscocephalus	16	10	4	25
Culex gelidus	117	23	50	106
Culex nigropunctatus		6	35	51
Culex sinensis	2			
Culex tritaeniorhynchus	733	27	272	148
Culex whitmorei	25		1	
Culex (Lopho) sp.	1	6	7	4
Mansonia annulata	1			
Mansonia dives	564	59	73	165
Mansonia uniformis	35			4
Uranotaenia sp.		22	1	11
Total Anophelines	324	320	100	504
per unit hour	0.5	1.1	0.3	1.8
Total Culicines	2509	630	1296	3775
per unit hour	3.5	2.2	4.5	13.1
Total Mosquitoes	2833	950	1396	4279
per unit hour	4.0	3.3	4.8	14.9

a - Twelve collectors for 5 nights, 12 hours per night (720 man biting hours)

b - Three light traps for 8 nights, 12 hours per night (288 light trap hours)

The results of the tests show that CDC light traps plus CO_2 was the most efficient method of collecting anophelines and culicines in the area studied. Human biting collections, the method considered best for collecting anopheline vectors in Malaysia was not as productive as light alone or light plus CO_2. Considerable bias entered into these human biting collections. The collectors were local villages and even though they had collected during previous surveys they were not as experienced or motivated as regular technicians. The collections do however, represent the usual method of human biting collections during routine malaria surveys and therefore is considered a valid comparison to light traps. For specific information such as biting rates or biting times trained technicians would have to be used. The biting collections using local collectors are still considered valuable for they provide information on vector preference that cannot be obtained from light traps. In addition, in remote villages such as the study area, considerable cooperation can be obtained from the local people if collectors are hired from the village surveyed.

SUMMARY

The total number of mosquitoes per unit hour was greater with CDC light traps plus CO_2 than any other method. The light traps with light alone or CO_2 alone were less effective than a combination of the two and are not considered as valuable for collecting. If CO_2 were not available light alone could be used but previous collections indicate this is usually not very effective for most anopheline species. Human biting collections, though not as successful in total numbers, are considered necessary when collectors are available.

It is apparent from these and studies by other workers that new and improved methods are needed to evaluate malaria vector populations. Evaluation of new collection devices are being made by the authors with the hope of developing techniques that will make it possible to determine present anopheline populations and predict future populations.

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A Potential New Method of Identifying Animal Species and Mosquito Blood Meals by the Hemoglobin Crystallization Technique

(Lab. demonstration 1972 Southeast Asian J. Trop. Med. Pub. Hlth. 2: 286)

by
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The hemoglobin crystallization method of identifying blood meals of hematophagous arthropods was developed by Dr. Robert K. Washino, University of California at Davis.

This technique involves crystallizing hemoglobin from blood samples taken from arthropod midguts, and comparing crystal structure with that of known materials.

The technique is still in the experimental stage even though initial results have been very encouraging. This laboratory is attempting to use this technique not only as a tool for identifying mosquito blood meals but as a potential method of separating closely related animal species.

The slides demonstrated are blood crystals from the hemoglobin of a guinea pig and a white, laboratory reared rat. To date six species of rats have been tested by this technique.

Preliminary Studies of the Vectors of Malaria in West Malaysia

(Paper presented at the 1972 Annual Meeting of the American Mosquito Control Association 25-28 March)

by
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Mosquito surveys were made in conjunction with chloroquine resistant *Plasmodium falciparum* malaria studies in two locations in West Malaysia. The first area consisted of three kampungs (villages) in Trengganu State on the East Coast of West Malaysia. The second site, Tuan Rubber Estate is approximately 50 miles east of Kuala Lumpur in Pahang State.

The areas vary considerably both ecologically and culturally. The kampungs typify rural Malaysia of the past i.e. the residents are primarily "tillers of the soil", and have little movement out of their home area. The people usually own their own small acreage where they raise basic crops and keep a few animals, usually cows and chickens. The topography varies from flat to rolling hills but mountains to 4000 feet can be seen in the distance (approximately 10 miles). During the rainy season this area is usually cut off from the outside by the flooding Trengganu river. Many of the people work on cooperative FLDA (Federal Land Development Authority) Schemes where individuals get a basic allotment from the Government and share profits of the crops involved. The major crops on the Schemes in this area are rice, palm oil and peanuts.

Tuan Estate is an entirely different situation. It is a privately owned, efficiently organized and managed, commercial organization. It is one of numerous estates owned by a British company and managed locally by a resident manager. This estate consists of approximately 2000 acres of planted rubber trees varying in size from seedlings to old rubber trees ready to be cleared and replanted. All the workers (tappers) on the estate are residents on the estate. They live in neat, well kept rows of houses called "labor lines". The topography of the estate consists primarily of rolling hills and intermittent streams. Most valleys in the area form swampy areas where little or no rubber is planted. These areas create ideal breeding areas for anophelines when they collect with rainwater.

The labor lines mentioned above are all located in one small section of the estate about $\frac{1}{4}$ square mile in total area. None of the houses are screened but they had been sprayed within the previous two months with benzene hexachloride (BHC) or "Gamma Hexane", as it is marketed locally. No background data is available on the effectiveness of this material in this area.

MATERIALS AND METHODS

Trengganu: Three kampungs, Kuala Dura, Kuala Jeneris and Payang Kayu, were surveyed for malaria vectors. Malaria prevalence and chloroquine sensitivity studies were done concurrently with vector surveys. Mosquitoes were collected by Communicable Disease Center (CDC) light trap plus CO_2 (dry ice), light trap alone, or CO_2 with no light source. Biting collections were made with human bait using their bare legs or back.

Three light traps, were placed in each village and operated from 6:00 PM to 6:00 AM. Each morning, mosquitoes were taken alive to a field processing laboratory, the anophelines were identified and dissected immediately. Culicines were identified and certain specimens were saved for reference; and the others were discarded.

Local residents were hired nightly to assist trained technicians with the bare leg and bare back collections (BLC). The collectors, using flashlights, trapped mosquitoes individually off their legs as soon as the mosquito came to rest. Specimens were collected in small glass vials and a cotton wad then placed in the end to prevent the mosquito from escaping. The following morning they were taken to the field laboratory and processed the same as light trap collections.

Larval surveys were made in all three villages and adjacent areas. Larvae were dipped from their breeding habitat, placed in plastic food containers fitted with lids and taken to the laboratory for subsequent rearing. Specimens that died were placed in vials with 70 percent alcohol and later identified. The larval and pupal skins and adults were saved for further taxonomic study.

Concurrent malaria and chloroquine resistant *falciparum* studies were done in this area. Malaria infection rates were determined initially and all infections were treated and followed for 28 days for chloroquine resistance.

Tuan Estate: This site has been surveyed more extensively than Trengganu, and will be a long-term malaria vector study site. Collections have been made at least weekly since November 1972 and will continue at least one year.

Ten light traps with dry ice (CO_2) were run nightly during collection periods. Various habitats such as tappers living area, seedlings, young and mature rubber were sampled with the light traps. Anophelines were processed as in Trengganu. Culicines were pooled by Genus, frozen in dry ice, and saved for arbovirus screening.

Bare leg collections were made nightly from 6:00 PM to midnight. Specimens were processed the same as with light traps.

Human bait traps of the Magoon-type were run nightly. These traps use two men as bait. The men were protected by an inner

mosquito net. Every two hours the men would lower an outer net and collect the mosquitoes trapped inside. Collections were made from 6:00 PM to 6:00 AM. The anophelines were identified and dissected. Culicines were pooled by Genus, frozen in dry ice and saved for arbovirus screening.

Larval surveys were made throughout the estate. Larvae were collected, separated individually and reared. Larval and pupal skins and adults were saved for taxonomic study. Larvae that died were placed in 70 percent alcohol and held for identification.

During the first phase of this long-term study everyone on the estate was checked for malaria parasites. Persons found with *P. falciparum* infections were treated with chloroquine to access the prevalence of chloroquine sensitivity. The estate has a resident hospital attendant who checks daily for new cases of malaria and makes a thick smear for future reading. Each new case is recorded by name, house and date. All malaria cases are treated with chloroquine if the patient does not clear of parasites they are then treated with other drugs. In the future mosquito surveys will be concentrated in areas of the estate where transmission is known to occur.

RESULTS AND DISCUSSION

Trengganu: *Anopheles kochi* was the predominant anopheline collected during the survey. This species is not thought to be a vector of human malaria but is susceptible to experimental infection in the laboratory (Sandosham 1959). As shown by Table 1, it appears to be relatively more attracted to light than to human collectors. *An. aconitus*, an important malaria vector in Java was the second most common anopheline collected. This species is readily infected in the laboratory but is very rarely found naturally infected and is not considered an important vector in West Malaysia (Sandosham 1959). One specimen of *An. b. balabacensis* was collected during the survey. Past collections in this area have yielded small numbers of adults of this species but the larvae have not yet been collected (Andre 1971).

Table 1 gives the number of anopheles collected according to method of collection and vector information. All anophelines were identified; live specimens were examined for gonotrophic aging. Parous specimens were dissected to determine infection rates. No mosquitoes were found positive for oocysts or sporozoites. Table 2 summarizes dissection results. In spite of these and previous surveys into this area the vectors of malaria have not been identified. The remoteness of the area makes it impractical for continuous surveillance but periodic surveys of the area are feasible. Future mosquito work in the area will be done to try to identify the malaria vectors in the area and to search for *An. b. balabacensis* larvae. If successful, attempts will be made to colonize a *balabacensis* strain from this locality.

Table 1

Anopheles captured by CDC light traps and bare leg catches in Trengganu and Tuan Estate

Trengganu

9 nights, August and September 1972

Species	Method of Collection		Total
	Light Trap	Bare Leg	
Anopheles aconitus	115	200	315
Anopheles balabacensis	-	1	1
Anopheles barbirostris	6	11	17
Anopheles crawfordi	18	41	59
Anopheles indiensis	71	34	105
Anopheles karwari	25	25	50
Anopheles kochi	692	193	885
Anopheles philippinensis	11	6	17
Anopheles separatus	5	13	18
Anopheles tessellatus	115	29	144
Anopheles vagus	2	2	4

Tuan Estate

12 nights, November 1972 - February 1973

Species	Method of Collection			Total
	Light Trap	Bare Leg	Human Bait Trap	
Anopheles barbirostris	2	0	0	2
Anopheles crawfordi	0	1	0	1
Anopheles kochi	0	0	1	1
Anopheles leucosphyrus	0	1	0	1
Anopheles maculatus	9	4	22	35
Anopheles tessellatus	2	-	1	3
Anopheles philippinensis	2	0	0	2

Very little malaria transmission was occurring during the study period. The three kampungs were screened for malaria prevalence. There were 602 persons, mostly school children, examined. Of these, 26 percent were found infected with malaria, 14 percent *Plasmodium falciparum*, 9 percent *P. vivax*, and 6 percent *P. malariae* including mixed infections. A chloroquine resistance survey was made on 445 members of this group. Only 6 percent of the 75 *P. falciparum* infections exhibited chloroquine resistance. Details of the study will be published elsewhere.

Tuan Estate: *Anopheles maculatus*, reported to be the principal vector of malaria in the hill regions of West Malaysia was the predominant anopheline collected at Tuan Estate, located in central Pahang. Table 1 also summarizes the adult collections from Tuan Estate.

Dissection and parity data are given in Table 2. This study was initiated in November 1972. Collections have been made during at least one week per month. Data presented is summarized through February 1973. Long term malaria and vector studies are planned for this area. The site presents a unique opportunity for such studies for a number of reasons. All the workers live in a rather small area and can be followed individually if they contract malaria. The manager of the estate is very cooperative and assists in every way possible with the project. Future work will include host preference studies, quantitatively relating malaria cases to mosquito vectors, testing of new collection equipment and techniques, and (near the end of the study) evaluation of mosquito control procedures.

The malaria study done in conjunction with the mosquito work surveyed everyone on the estate for malaria parasites. There have been 557 workers and dependents examined. Of these 34.5 percent were infected with malaria. *P. falciparum* accounted for 33.6 percent the remaining were *P. vivax* or mixed infections.

A chloroquine resistance test was done on 175 *P. falciparum* carriers and 50 controls. More than 50 percent of the study group demonstrated resistance to chloroquine. It was noted by Scanlon and Sandhinand (1965) that chloroquine resistance in Southeast Asia appeared to be associated with the presence of *An. b. balabacensis*. There have been no *An. b. balabacensis* collected in this area thus far. The data collected demonstrates that chloroquine resistance can occur in areas in Southeast Asia where *An. b. balabacensis* apparently is not present. Current and future studies should reveal the distribution and prevalence of chloroquine resistance in West Malaysia.

Summary: The short study made in Trengganu did not reveal the malaria vectors present in the area but collections did show that potential vectors were present. CDC light traps were demonstrated to be useful tool in malaria surveys.

Data from Tuan Estate in Central Pahang have been limited by small numbers of anophelines, but the estate appears to be an

Table 2

Results of dissections of *Anopheles* collected at Trengganu and Tuan Estate

Trengganu

Species	Number Dissected	Parous	Positive
Anopheles aconitus	229	69	0
Anopheles barbirostris	5	1	0
Anopheles crawfordi	26	5	0
Anopheles indiensis	38	13	0
Anopheles karwari	23	5	0
Anopheles kochi	231	82	0
Anopheles philippinensis	11	5	0
Anopheles separatus	11	0	0
Anopheles tessellatus	26	6	0

Tuan Estate

Species	Number Dissected	Parous	Positive
Anopheles barbirostris	2	1	0
Anopheles crawfordi	3	0	0
Anopheles kochi	1	0	0
Anopheles maculatus	35	0	0
Anopheles philippinensis	1	1	0
Anopheles tessellatus	2	0	0

excellent location for a detailed malaria vector study. *An. maculatus* has been taken only in small numbers. Dry conditions have limited its breeding capability. It is anticipated that the mosquito population should build-up to a peak in May or June. At this time maximum effort to get the data described above will be made. The occurrence of high levels of chloroquine resistant *P. falciparum* in the absence of *An. b. balabacensis* demonstrated that resistance can occur when this vector apparently is not present. More work is needed to determine the geographical distribution of *An. b. balabacensis* in Southeast Asia. Possibly, when the range and taxonomic status of this important vector is worked out, its relationship to chloroquine resistant malaria if any can be firmly established.

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Plague in Southeast Asia

(Presented at the January 1973 Seminar of the Malaysian Society of Parasitology and Tropical Medicine held in Kuala Lumpur)

by
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INTRODUCTION

According to Wu Lien-teh *et al.* (1936) plague *(Yersinia pestis)*, had its origin within or near the central Asiatic plateau. He maintains that the first plague epidemic on actual record was the outbreak among the Philistines in 1320 B.C., which, as described in the Bible (1 Samuel, V and VI), was characterized by the appearance of "emerods in their secret parts."

Probably the best known epidemic as far as modern man's historical record is concerned was during the Middle Ages, when the "Black Death" as it was then called, killed 25 million people in Europe (Herms. 1961).

In spite of many years work by large numbers of dedicated workers plague remains an important problem in many parts of the world. Thousands of human cases have been recorded even in recent years. In the temperate regions of the world, where human populations are usually low, this disease exists primarily in sylvatic form and creates minimal human health problems. Most of the classical work has been done in these areas where seasons are distinct and the ecological status of the rodents and flea vectors are reasonably well known. The tropical zones of the world, such as Southeast Asia present quite a different picture. Almost every phase of tropical ecology is behind work in temperate zones. This lag is very conspicuous in plague research. Ecological work and some components of the infectious cycle are not well known. The population density in many areas of Southeast Asia creates a continual potential plague problem.

South Vietnam is the area of major concern in Southeast Asia today. Plague cases have occurred by the thousands since the middle 1960's. Even though a peace treaty has just been reached, plague will most likely remain a problem until health conditions have improved in this country. The remainder of this report deals primarily with the plague situation in South Vietnam and a study that took place there.

Plague first appeared in the Republic of Vietnam in 1906. It was imported by rats, transported in bundles, from Canton and Hong Kong to the port of Saigon. Plague spread outward from the Saigon area, appearing in the lower Mekong River Delta at Soc Trang in 1907. In 1908, it was imported from the south, by junk, to the port of Phu Bai and hence to Phan Thiet and through much of Binh Thuan Province.

From 1911 on, the disease moved northward, appearing in Phan Rang. Since the onset of the major fighting in Vietnam in 1965, plague has rapidly spread throughout most of South Vietnam. It is suspected this rapid dissemination was due to supplies airlifted from known plague areas such as Saigon or Nha Trang to remote locations such as the study area (Dak To).

Dak To, at the time of this study was a U.S. Special Forces Camp. The Camp contained approximately 20 American soldiers and 500 Vietnamese and Montagyard soldier and families. The altitude at the Camp was 600 meters, surrounding mountains had a maximum elevation of 1200 meters. The Dak Poko River is directly in back of the Camp and during the dry season, December through April, this river represents the only significant water source in the area. Vegetation in the area consists primarily of low scrub brush and trees, lallang grass and bamboo. Dense jungle begins 5 to 10 kilometers out from the Camp.

An epidemic of bubonic plague began at Dak To on February 21, 1967. The distribution of plague cases was bimodal, with a three-week interval between the two models, during which no cases occurred. This lapse was contributed to the application of insecticide dust. Trapping and the collection of ecologic data began on 29 March 1967. Trapping of rodent populations was not accomplished prior to, or during the first portion of the epidemic.

OBJECTIVES

To study the following known relationships.

1. The occurrence of plague cases exclusively among the local soldiers and their dependents living along the east and west sides of the Camp.

2. Why attack rates were selectively higher among dependent women and children than among the soldiers themselves.

3. The relationship of spraying and dusting to the initial cessation of the occurrence of plague cases, and their subsequent reappearance. In addition we set as our objectives to determine:

(1) The rodent and flea species in the Camp and adjacent areas.

(2) The flea index, by rodent species.

(3) The prevalence of plague in infected rodents.

(4) The relationships between these and other environmental variables, and the occurrence of human plague cases.

METHODS AND MATERIALS

1. The animals were live trapped with Tomahawk collapsible traps. A variety of local food stuffs were tried as baits. Eventually dried fish was found to be the bait of choice for the rodents. Traps were placed in bunkers and storerooms throughout the Camp. In addition, both transect and grid trapping were attempted in scrub and lallang areas adjacent to the Camp. These areas were shortly ruined for trapping purposes because a U.S. Army Unit deployed into the area and destroyed the trap sites.

2. All rodents and their ectoparasites were processed as follows:

a. The animal was anesthetized with ether.

b. Heart blood was taken for serum.

c. The animal was combed and examined for ectoparasites. Fleas were placed in 70 percent ethyl alcohol.

d. The animal was autopsied and examined grossly for evidence of plague infection. The spleen was removed, placed in normal saline and frozen.

3. Sentinel mice.

(1) Approximately one-hundred Swiss albino mice were placed in cages at predetermined locations throughout the Camp and in immediately adjacent areas.

(2) A few mice fitted with leashes were released into what appeared to be deserted rodent burrows, in an attempt to attract fleas.

(3) After exposure, mice were examined for ectoparasites, and held for 21 days, or until they died. Spleens were removed; placed in normal saline, and frozen for subsequent laboratory tests.

RESULTS

1. A total of 110 rodents were collected. Of these, 54 were *Rattus rattus*, 28 *R. nitidus*, 16 *R. exulans*, 2 *R. argentiventer* and one *Rhyzomys pruinosus*.

2. Two specimens of *R. nitidus* found dead, had *Yersinia pestis* positive spleens. Both were collected on 1 April 1967, one day prior to the onset of the last confirmed case of plague.

3. One of the 14 *R. rattus* tested serologically demonstrated a positive hemagglutination titer.

4. Only 84 fleas were found on the 110 animal specimens collected. Flea indices during the study period were 1.22 for April, .65 for May, .30 for June and .12 for July. Flea indices showed a sharp reduction in May which was the beginning of the rainy season. All fleas were identified as *Xenopsylla cheopis*.

5. Of the 110 sentinel mice exposed, none were found positive for *Y. pestis*. The only ectoparasite collected from the mice was one *Dermacenter sp.* tick.

DISCUSSION

1. Seasonal factors and plaque transmissions:

a. In the Republic of Vietnam, maximum plaque transmission occurs beginning in the dry season, which varies in the months of its occurrence from location to location. Flea indices in Vietnam have been noted repeatedly to increase in ambient temperatures associated with the dry season, and to decrease rapidly with the advent of the rains.

b. An eight year study done by Cavanaugh and Marshall (1972) demonstrated that climatic conditions were found to influence the course of plague epidemics in two ways:

(1) by regulating the density of a flea population; and

(2) by regulating the efficiency of *Xenopsylla cheopis* in transmitting the plague bacillus. Slight variations in temperature, relative humidity, and vapor pressure deficits either permit an epidemic to flourish or cause a decline in its intensity.

2. Attack rates among local soldiers and dependents:

a. At Camp Dak To, most of the soldiers and their dependents are housed in underground bunkers. The relative darkness within the bunkers during daylight hours, where dependent wives and children unquestionably spend more time than the soldiers, offers a means of accounting for a higher plague attack rates observed among dependents.

3. Localization of cases:

a. Plague cases occurred only among the soldiers and their dependents housed along the east and west sides (walls) of the camp, and virtually simultaneously on both sides. Along the east and west walls, lallang grass, scrub brush and short grasses extend up to the outermost barbed-wire perimeter, while to the north was an asphalt aircraft runway and an aircraft parking area and to the south, was the Dak Poko river, sparse grass and scrub brush were found between the camp and the river.

b. A burn-off in the lallang and brush on the east and west sides during the weeks just preceding the outbreak suggested that plague-infected rodents might have been driven into the camp and harbored a pre-existing sylvatic focus. However, sentinel mice failed to confirm infection in these areas, and trapping failed to produce animals for study.

c. The plague cases at Dak To were the first bacteriological confirmed cases in Kontum Provinces, Republic of Vietnam. At the time of the Dak To outbreak, major plague epidemics were in progress in Nha Trang, from which supplies were frequently delivered by air. It is possible that the source of infection (i.e., infected rats and/or fleas) was introduced via food shipments aboard cargo aircraft.

4. Persistence of plague:

a. Dusting with 0.5 percent diazinon dust was apparently effective in temporarily interrupting flea-borne transmission. The occurrence of a second outbreak of human cases three weeks later, and the recovery of *Y. pestis* from a *R. nitidus* suggest that the transmission continued among rodents. This was believed due to inadequate insecticide coverage and/or plague persisting in chronic form in rodents and new flea generations, which in the pupal stage were protected from the insecticide.

SUMMARY

1. Rodent trapping at Dak To resulted in the collection of several rodent species. The following rodents were collected: *Rattus rattus*, *R. nitidus*, *R. exulans*, *R. argentiventer* and *Rhyzomys pruinosus*.

2. The Oriental rat flea, *Xenopsylla cheopis*, was the only flea species found on rodents at Dak To. As in other parts of Vietnam, rodent flea indices were observed to decrease with the advent of the rains, which at Dak To, began in May.

3. The environmental conditions in bunkers, which serve as housing for soldiers and their dependents, favor plague transmission. In particular, the relative darkness inside, known to encourage biting activities of *Xenopsylla cheopis*, was probably a significant factor in accounting for observed higher attack rates among the dependents than among soldiers themselves.

4. The localization of human plague cases exclusively along only two of the sides of the camp could have been due to a sylvatic focus. This was not confirmed, either by trapping or the use of sentinel mice. The plague bacillus probably was introduced by infected rats and/or fleas in air cargo from Nha Trang.

5. Termination of the first portion of the outbreak was related to dispersal of 0.5 diazinon dust. The second outbreak three weeks later, and the finding of a *R. nitidus* positive for plague, indicates

that transmission among rodents continued in areas not reached by the insecticide; chronic plague infections in rodents were responsible for the persistence during the interval between the two outbreaks.

This paper, though primarily a report on a single outbreak of plague in the Highlands of the Republic of Vietnam, includes information of interest to all health workers in Southeast Asia. Plague is a major health problem in South Vietnam, it does occur sporadically in Thailand, Laos, Kymer, Burma and Indonesia. Malaysia has never had a recorded case of plague. *Xenopsylla cheopis*, the principle plague vector throughout the world is present in Malaysia, as are a number of the rodent reservoirs. The absence of plague from the plague-free areas had not been explained. The possibilities for its absence could be: ecological variations that prevent development of plague organisms, higher standards of living in some Southeast Asian countries, and less movement of people and goods into these plague-free areas.

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STUDIES IN PROGRESS

Malaria Vector and Anopheline Biology Investigations in Conjunction with Chloroquine Resistance Studies in West Malaysia

by

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BACKGROUND

Joint studies with the Department of Parasitology are being conducted at various locations in West Malaysia to determine the chloroquine susceptibility levels in the population and associated anopheline vectors found in each area.

Five rubber estates, Tuan, Renjok, Sungai Kawang, Paroi, and Terentang, the first three in Pahang State the latter two in Negri Sembilan (Figure 1) have been surveyed. In addition, a study was done in the Kuala Brang District of Trengganu State. The latter survey was a follow-up of work done by Andre *et al.* (See USAMRU Annual Reports 1972-73).

Preliminary surveys have shown that *Anopheles maculatus*, the presumptive vector of malaria in the foothills region of West Malaysia, is the predominant anopheline at all the rubber estates. In Trengganu, *An. maculatus* has been collected but more data would be required to incriminate this species as the principal vector in this area. *An. balabacensis* an important vector in some areas of Southeast Asia has also been collected in very small numbers near Kuala Brang. A long range study, involving malaria and malaria vectors was initiated at Paroi Estate. The other estates will be surveyed periodically for comparison purposes.

OBJECTIVES

1. To determine the biology of malaria vectors and associated mosquitoes, and relate it to such variables as transmission rate, chloroquine resistance and malaria species.

2. To relate the human ecology aspects to malaria vectors.

MATERIALS AND METHODS

Evaluation of collection techniques: One important factor has evolved in these studies to date. The present methods of collecting mosquitoes do not give a valid random sample of the populations present, or allow suitable means to predict future fluctuations in populations. Various new or modified techniques of accessing mosquito populations are being evaluated during the course of these


Fig. 1
N
7.
TRENGGANU
PERAK
WEST MALAYSIA
6.
PAHANG
8.
5.
2.
1.
NEGRI
SEMBILAN
3.4.
1 TUAN ESTATE
2 RENJOK ESTATE
3 PAROI ESTATE
4 TERENTANG ESTATE
5 SUNGEI KAWANG ESTATE
6 SUNGEI WANGI ESTATE
7 KUALA BRANG
8 KAMPONG SERTIK

studies. Remarks and progress, negative or positive are discussed later in this section.

COLLECTION OF ADULT MOSQUITOES

Light traps: Communicable Disease Center (CDC) light traps with CO_2 (dry ice) as an added attractant, were set nightly from 1700-1800 hours and picked up the following morning from 0600-0700 hours. After the morning pick-up the mosquitoes were returned to the field laboratory and killed in dry ice. The anophelines were quickly separated, identified and dissected. During this manipulation, parity was determined, and parous specimens further examined for oocysts or sporozoites. Culicines were identified, pooled by species and saved for virus screening by the Department of Viral and Rickettsial Diseases. (See Arbovirus section)

Biting collections: Man-biting collections are made nightly during each survey. These collections were made by USAMRU technicians and local resident collectors. Mosquitoes are collected individually off the legs or back of the collectors by placing a small plastic vial over the specimen as it rests or feeds on the individual. A cotton pad is then quickly inserted into the vial, thus trapping the mosquito. Specimens were separated hourly and placed in white animal bags until processed.

Bait-trap collections: In some of the areas a man-bait trap similar to a Magoon trap has been utilized to collect mosquitoes. The trap consists of inner and outer nettings. Two collectors place themselves on cots within the inner net, and at hourly or two hourly intervals they go out, lower the outer net and collect the mosquitoes trapped inside. This trap gives little data except to provide mosquitoes for dissection. Specimens are continually lost during collecting and very little comparable data on biting times, or populations is collected. A new trap, used with or without a human for bait, that may give a random representative sample of mosquitoes present in an area has been designed but is not yet fabricated. When complete, this trap will be evaluated for its potential as a mosquito survey devise.

Larval collections: Larvae were collected by the standard method of dipping. They were pipetted from the dipper into plastic cups or directly into vials with 70 percent alcohol. Larvae placed in the cups were later individually reared and larval and pupal skins saved for taxonomic study. The specimens placed in alcohol were returned to the laboratory where they were cleared and mounted in Canada balsam.

Resting collections: These collections were made in houses, sheds, etc., with a mouth aspirator and in vegetation with a sweep net. After collecting the mosquitoes were placed in a holding cage and held for identification and subsequent dissection for malaria parasites or blood meal determination.

RESULTS AND DISCUSSION

Rubber estates: Mosquito surveys were conducted at the following rubber estates, Tuan, Renjok, Paroi, Sungai Kawang, and Terentang (Figure 1). Tuan and Paroi were studied in more detail and Paroi will be followed for at least one year. The other three estates were surveyed but will be dropped from future studies.

Anopheles maculatus was the predominant anopheline collected at all the estates. This species was collected principally during man-biting collections though small numbers were captured in human bait traps and CDC light traps plus CO_2 (dry ice). Other potential vectors collected included *An. barbirostris* and *An. letifer.* These latter two species were taken in such small numbers it is very unlikely they could represent an important vector population.

Larval collections were made at all rubber estates. Attempts were made to set up permanent larval sites at Tuan and Paroi, however, either dry weather or rapid flooding eliminated each new site established. Anophelines, usually *An. maculatus* were collected in small numbers at every estate.

Dissection of anophelines was made at all estates. Only one oocyst positive *An. maculatus* was found. This was during a presurvey at Paroi estate. Specimens were examined for parity at each location. A summary of the parity rate is given in Table 1.

The objectives of the malaria vector program at the rubber estates have only been partially met. Routine collections will continue at Paroi and spot checks will be made at Tuan. It is very unlikely that anopheline populations will be suitable to evaluate ULV spraying or form population models to predict future mosquito fluctuations.

Trengganu: Three surveys were made in the Kuala Brang area of Trengganu (Figure 1) in August and September 1972. No anophelines were found positive for malaria parasites during these surveys. In 1971, Andre found one *An. maculatus* with oocysts (USAMRU Annual Report 1972). A total of 8898 mosquitoes were taken during the surveys. Of these, 1227 or approximately 14 percent were anophelines. *An. aconitus* and *An. kochi* were the most commonly collected anophelines. Potential vectors such as *An. barbirostris* were taken in very small numbers. No *An. maculatus* were collected during these surveys. The anopheline vector in this area is still unknown. Malaria rates as high as 40 percent have been found in some of the kampungs. The past data thus far, incriminates *An. maculatus* but the extremely low numbers in earlier collections makes it an unlikely candidate as the only, or principal vector in this area. *An. aconitus* a vector in Java is usually considered a zoophilic species in Malaysia, this may be true in most areas but it exhibits a strong anthropophic nature in the Kuala Brang area. This species should be examined closely in future surveys.

Table 1

Parous rates of *Anopheles maculatus* collected in West Malaysia 1972-1973

Location	No. Examined	Parous	Percent Parous
Paroi	28	9	32.1
Tuan	193	45	23.3
Renjok	7	2	28.6
Terentang	37	14	37.8
Sungai Kawang	31	11	35.8

Summary: Malaria vector surveys in conjunction with chloroquine resistance studies have been made in various areas of West Malaysia. *An. maculatus* is the predominant anopheline at all the rubber estates surveyed and probably the principal vector in these areas. In Trengganu the vector situation is very vague and it would be difficult to incriminate any one species at this time.

A total list of mosquitoes collected and numbers of each would be rudundant of past USAMRU reports. However, to give future workers an idea of species encountered, and location, a summary of collections of all mosquitoes is given in Table 2.

Table 7

Species and collection location of mosquitoes collected during malaria vector surveys by USAMRU in West Malaysia from August 1972 to September 1973

Species	Location by Number[a]							
	1	2	3	4	5	6	7	8
Aedes albopictus	+	+	+	+	+	+	+	+
Ae. butleri						+		
Ae. caecus							+	+
Ae. chrysolineatus							+	
Ae. desmotes	+							
Ae. laniger	+							
Ae. lineatopennis	+		+			+	+	+
Ae. niveus	+							+
Ae. poicelus							+	
Ae. prominens	+							
Ae. vexans	+	+	+	+	+	+	+	+
Aedomyia catasticta	+	+	+	+			+	+
Anopheles aconitus			+				+	+
An. argyropus					+			
An. balabacensis							+	
An. barbirostris	+	+	+				+	+
An. brevirostris							+	
An. crawfordi	+	+			+		+	+
An. donaldi							+	+
An. indiensis			+	+	+		+	+
An. karwari			+		+		+	
An. kochi	+		+	+			+	+
An. letifer					+	+		+
An. leucosphyrus	+							
An. maculatus	+	+	+	+	+			+
An. peditaeniatus								+
An. philippinensis	+		+		+	+	+	+
An. separatus						+	+	+
An. tessellatus	+		+		+		+	+
An. vagus				+				
Armigeres flavus	+							
Ar. subalbatus	+	+	+	+	+	+	+	+
Culex annulus	+	+	+	+	+	+	+	+
C. bitaeniorhynchus	+	+	+	+	+	+	+	+
C. fatigans	+	+	+	+	+			+
C. fuscanus								+
C. fuscocephalus	+	+	+	+			+	+
C. gelidus	+	+	+	+	+	+	+	+
C. halifaxi								+
C. mimulus					+	+	+	+
C. nigropunctatus	+	+	+	+	+	+	+	+
C. pseudovishnui			+	+			+	+
C. sinensis			+		+		+	+
C. tritaeniorhynchus	+	+	+	+	+	+	+	+
C. whitmorei							+	
Malaya genurostris								+
Mansonia annulata	+					+	+	
M. annulifera	+		+					+
M. crassipes[b]	+	+	+	+	+	+	+	+
M. dives	+	+	+	+	+	+	+	+
M. hodgkini[b]						+		
M. nigrosignata[b]					+	+	+	
M. ochracea	+	+	+	+	+	+	+	+
M. uniformis	+	+	+	+	+	+	+	+
Uranotaenia campestris								+
U. macfarlanei								+
U. nivipleura								+
U. obscura								+
U. testacea								+
U. trilineata					+			+

a. See Figure 1 for locations.
   1 - Tuan Estate
   2 - Renjok Estate
   3 - Paroi Estate
   4 - Terentang Estate
   5 - Sungei Kawang Estate
   6 - Sungei Wangi Estate
   7 - Kuala Brang
   8 - Kampung Sertik, FLDA Scheme

b. Now considered under Genus Coquillettidia.

Isolations of Arboviruses from Wild-Caught Mosquitoes in West Malaysia

by

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BACKGROUND

In late 1972, investigations were initiated to screen mosquitoes for arbovirus isolations. During recent years at USAMRU culicine mosquitoes had been identified to Genus, or in some case to species and either saved for reference collections or discarded. Earlier work by Traub *et al.* (1956-1960 USAMRU Annual Reports) resulted in isolating arboviruses from various mosquito species. Very little detailed work has been done by USAMRU since that time.

The interest and expertise is available at USAMRU again, thus it was agreed that the data previously being lost when mosquitoes were being discarded could provide valuable information on various aspects of arbovirus transmission and ecology. The work being done is a cooperative effort between the Departments of Medical Entomology and Viral and Rickettsial Diseases.

OBJECTIVES

1. To isolate and identify arboviruses in mosquitoes from West Malaysia.

2. To relate the species of mosquitoes and viruses to various ecological manifestations.

MATERIALS AND METHODS

Collection methods are described elsewhere in this section. With few exceptions all mosquitoes utilized for virus screening were collected during malaria vector surveys.

Specimens were processed differently than the anophelines used in malaria vector investigations. The morning after collection, specimens were killed in dry ice, separated, and pooled to Genus in the field, while they were still frozen. The culicines remained on dry ice and were returned to Medical Entomology USAMRU in Kuala Lumpur. Here, specimens were identified and pooled by species. Rare specimens were not pooled but saved for reference collections. The identification was accomplished by placing a petri dish holding the mosquitoes over a piece of dry ice. The dry ice was contained in a small, white enamel pan. The mosquitoes were identified with the aid of a dissecting microscope

and quickly transferred to plastic vials, also retained in dry ice. The specimens were then turned over to the Department of Viral and Rickettsial Diseases for screening.

RESULTS AND DISCUSSION

A total of 30551 mosquitoes were pooled and screened for arboviruses. Table 1 summarizes the results of these screenings. From 323 pools, 11 were found positive. Five of these positives were taken at Tuan Estate. Definitive identifications are not available at this time. In addition to the positive mosquito pools, one human isolate was found from a 12 year old boy at Paroi Estate. The positive isolates will be sent to the U.S. Army Medical Component, SEATO Laboratory, Bangkok, for further typing.

Future arbovirus investigations will consider data related to the human, mosquito and animal reservoir aspects of the diseases. A new location, Ulu Lui, in the Ulu Langat region of Selangor State, approximately 20 miles from Kuala Lumpur, will be investigated for the above considerations.

Table 1

Numbers of isolations of virus from wild-caught mosquitoes in West Malaysia 1972-1973

	Location and Numbers of Mosquitoes Collected[a]									Results	
Species	1	2	3	4	5	6	7	8	9	Total Inoculated	Number Pools Positive / Negative
Aedes albopictus						355+	6	75	2	438	1 / 4
Ae. laniger						2				2	/ 1
Ae. lineotopennis						9		11		20	/ 1
Ae. vexans						7		5		12	/ 1
Aedomyia catasticta						15		2		17	/ 1
Armigeres subalbatus						9	4	18	15	46	/ 1
Culex annulus						736+	11	83	6	836	1 / 8
C. bitaeniorhynchus						74	13	10	4	101	/ 1
C. fatigans						192+	20	3	18	233	1 / 2
C. fuscocephalus							1	7	12	20	/ 1
C. gelidus						1077+	151	249	39	1516	1 / 15
C. Lophoceraomyia sp.						44		43	1	88	/ 1
C. nigropunctatus						27	12	79	2	120	/ 2
C. tritaeniorhychus						329	15	114	54	512	/ 6
C. pseudovishnui								2		2	/ 1
C. sinensis								1		1	/ 1
Mansonia annulifera								2		2	/ 1
M. crassipes						146	1	24	5	176	/ 2
M. dives						498+	84	15	6	603	1 / 5
M. ochracea						36	1	1	1	39	/ 1
M. uniformis						972	6	22	18	1018	/ 11
Anopheles sp.	487		7	8	48					550	/ 6
Aedes sp.	1	17	74	894	276	1425				2687	/ 27
Armigeres sp.		226	42	201	240	135				844	/ 9
Culex sp.	2761+	5	1912+	6992++	1414+	1758				14842	5 / 144
Mansonia sp.	929		381	1170+	3053	292				5825	1 / 58
Orthopodomyia sp.							1			1	/ 1
									Total:	30551	11 / 312

a - Represents Location of Collection

1 - Ampang Forest Reservoir, Primary Forest, near Kuala Lumpur.

2 - Gombak Road, Primary Forest, 22 miles from Kuala Lumpur.

3 - Sungai Wangi Estate, Rubber Estate, approximately 100 miles North of Kuala Lumpur.

4 - Arcadia Estate, Rubber Estate, same as 3.

5 - Kampung Sertik, FLDA Scheme, 50 miles east of Kuala Lumpur.

6 - Tuan Estate, Rubber Estate, 60 miles southeast of Kuala Lumpur.

7 - Renjok Estate, same as 6.

8 - Paroi Estate, Rubber Estate, 5 miles south of Seremban in Negri Sembilan.

9 - Terentang, Rubber Estate same as 8.

+ - Represents a positive arbovirus isolate.

Mosquito Studies in a Federal Land Development Authority (FLDA) Scheme (Kampung Sertik) in West Malaysia

by

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BACKGROUND

In 1967 a cooperative project between IMR and USAMRU to study malaria incidence, vectors and associated mosquitoes species was undertaken. The site was a newly opened FLDA scheme in western Pahang State named Kampung Sertik. It was surmised the study would give background malaria data for other FLDA schemes in West Malaysia. Very little malaria was present in the residents and with minimum treatment it fell below 1 percent. However, the experiment did run from 1967-1971 and considerable data was collected on mosquito bionomics.

Future studies will be primarily concerned with changes in mosquito species from jungle fauna to the urban or kampung fauna. Of particular interest is the time sequence involved in the introduction of *Aedes aegypti* into such an area.

OBJECTIVES

1. To follow the changes, if any, in the mosquito fauna of an FLDA scheme.

2. To screen the mosquitoes collected for arboviruses.

MATERIALS AND METHODS

Collecting: The same methods will be used as described previously. Two surveys are planned per year and collection locations and types will be the same as in previous years.

When sufficient data is collected (at least one year) the species spectrum will be compared with earlier collections and the changes noted.

PROGRESS

The species collected during two surveys in 1972 and 1973 are given in Table 2 of Malaria Vector's section. No changes were noted in the mosquito fauna from previous years. *Ae. aegypti* has not yet been introduced into the scheme. *Culex fatigans* has been present since the opening of the scheme in 1967. Malaria vectors such as *Anopheles maculatus, An. barbirostris* and *An. letifer* were collected but no positive specimens have been found. One positive virus isolation from a *Culex* pool has been made (see Table 1 of Arbovirus section).

The Study of *Anopheles balabacensis* in Southeast Asia and its Relation to Chloroquine Resistant Malaria Transmission

by

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BACKGROUND

An excellent review of *An. balabacensis* and its relation to malaria through 1971 is given by Slooff and Verdrger (WHO/MAL/72.765).

This mosquito, because of its exophilic and exophagic nature has created many problems for the WHO Eradication Programs. The routine residual spraying usually done by WHO is virtually ineffective against this vector in most areas. In Sabah, however, control of this species seems to be possible with residual spraying.

Variations from the normally accepted taxonomic patterns in wing scales have been noted in the process of identifying *An. balabacensis* and other members of the Leucosphyrus Group. To adequately identify, not only *An. balabacensis* but closely related anophelines a study of so called "strains" needs to be made. Collections from Thailand, Indonesia, East and West Malaysia need to be examined if this problem is to be resolved.

Another important reason to study *An. balabacensis* is that the geographical extent of this species seems to coincide with the presence of chloroquine resistant malaria. This is only supposition to date and may be proven invalid when more areas are surveyed for chloroquine.

OBJECTIVES

1. To taxonomically define *An. balabacensis* and related anophelines are species, subspecies, strains, etc.

2. To collect, rear and cross specimens of *An. balabacensis* from geographically distinct regions, e.g. West and East Malaysia, Thailand, and Indonesia.

MATERIALS AND METHODS

Proposed locations, available specimens, and rearing status:

THAILAND: Museum specimens are available at SEATO Laboratory in Bangkok. Specimens from a Thailand strain being reared at SEATO will be requested for rearing studies at USAMRU.

WEST MALAYSIA: Museum specimens are available at USAMRU, IMR, and at the University of Malaya (Dr. Ramalingam). A Perlis strain is available at IMR. In addition SEAMP, Washington, D.C. has specimens from this area.

EAST MALAYSIA: Specimens from East Malaysia are available at SEAMP, Washington, D.C., University of Malaya, and Institute for Medical Research (IMR) Kuala Lumpur.

INDONESIA: *An. balabacensis* has been recorded from Borneo and Java. North Sumatra has not reported this species, a trip to this area to determine if it present and collect specimens for rearing is planned.

Collecting techniques: Conventional methods of collection, e.g. biting, resting larval, light traps, etc. will be used but they will be concentrated in areas likely to have *An. balabacensis*. Associated ecological data will be taken with all collections.

Larval specimens will be reared and associated skins saved. Adult rearing will be attempted when it is feasible to bring back specimens alive; otherwise adults will be pinned for taxonomic study.

Rearing and crossing: An attempt will be made to establish colonies of *An. balabacensis* from each of the above areas. Once sufficient numbers are present crossings will be made and the progeny examined for taxonomic variation.

PROGRESS

A trip to Trengganu (Figure 1) was made to August 1973 to attempt to collect enough *An. balabacensis* for rearing. No specimens of this species were collected during the trip. Eggs of *An. balabacensis* are being sent to IMR from Sabah, East Malaysia. These will provide specimens from that area. A recent contact in Sarawak, East Malaysia has provided an opportunity to obtain *An. balabacensis* and *An. leucosphyrus* from this area.

Collections in mountain regions of West Malaysia are also desirable. Past collectors have described *An. balabacensis introlatus* from various mountainous areas such as Frazers Hill and Cameron Highlands. If collections can be made, crosses with *An. b. balabacensis* are planned. These should test the validity of both sub-species.

Systematics of the Flea Fauna of West Malaysia

by

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BACKGROUND

Little data is available on the flea fauna of West Malaysia. Dr. Traub of the University of Maryland has identified a few species from this area but has not collected extensively within most animal groups. The oriental rat flea, *Xenopsylla cheopis* is taken routinely in rats collected in Kuala Lumpur. The cat flea *Ctenocenphalides felis* is common in Malaysia. Squirrel fleas, collected by Ecology, USAMRU, and identified by Traub include: *Stevalius robinsoni*, *Thaumapsylla breviceps orientalis* and *Tagacopsylla mera*.

No systematic collecting of animals with the primary purpose of collecting fleas has been undertaken. Ecology has proposed collections in the higher elevations of Malaysia (Cameron Highlands, etc.) to screen animals for plague *Yersinia pestis* (USAMRU OBJECTIVES, 1973). These cool, highland areas are the most productive flea habitats in the tropics. These collections could be very valuable to flea systematic studies if proper attention is given to the collection of fleas.

In addition to the above source, trapping will be done during malaria studies. One man can set and collect 100 traps and process the animals collected in about four hours per day. Blood will be taken from all animals and used for arbovirus screening and possibly rickettsia isolations.

OBJECTIVES

1. To classify the flea fauna of West Malaysia.

2. To determine the host-flea relationship of the animals collected.

MATERIALS AND METHODS

Ecology collections: Fleas collected by Ecology will be passed to Medical Entomology for screening and then forwarded to Dr. Traub, University of Maryland for taxonomic study.

Medical Entomology:

Trapping - Approximately 100 animals traps will be set in the late afternoon (usually in conjunction with the setting of light traps.) The traps will be set in distinct habitat types, e.g. lallang,

rubber, primary forest, etc. Animals will be picked up the following morning, placed in white, cloth bags and taken to the field lab for processing.

Processing - The animal will be anesthetized with chloroform while it is still in the cloth bag. It will be removed and held over a white, enamel tray (to catch any ectoparasites) and heart's blood taken. This blood will be allowed to set until the sera separates. The sera will then be taken off and frozen. Next the animals will be combed for fleas and lice. These ectoparasites will be placed in 70 percent alcohol for storage and subsequent identification. The animal will then be observed under dissecting microscope for mites or other ectoparasites. These will also be placed in 70 percent alcohol. The animal will then be frozen on dry ice and returned to the IMR for final identification (Ecology) and disposition.

PROGRESS

Fleas collected by Ecology in Sarawak and by the Acarology Department in North Sumatra have been screened and sent to Dr. Traub at the University of Maryland. No fleas have been collected during routine trapping at Rubber Estates.

REPORT OF NEGATIVE RESULTS

Hemoglobin Crystallization Technique for Identifying Mosquito Blood Meals

by

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This technique, described by Washino and Else (*Amer. J. Trop. Med. Hyg., 21(1):* 120-122) involved crystallizing hemoglobin from blood samples taken from the mosquito midgut and comparing crystal structure with that of known materials.

We were able to crystallize hemoglobins from the following animals: guinea pig, white rat, *Rattus bowersi, R. annandalei, R. muelleri, R. cremoriventer* and the mouse deer, *Tragulus spp.* It was not possible to get crystal formation in any of the primates. Washino and Else also found this to be the case. Because primates are critical for blood meal studies in malaria investigations it was felt that further investigation would be fruitless. The technique readily separates rat species and may have some value in mammal taxonomic studies. It is however, considered undesirable for mosquito blood meal studies.

FUTURE PLANS AND COMMENTS

I. Malaria Vector Studies

Results from these studies have been disappointing. Future investigations will be done only at Tuan and Paroi Estates. Paroi will be surveyed every two weeks. At Tuan, surveys will be made once every 3 months. Data relating malaria incidence to anopheline vectors will still be collected and possibly some patterns might emerge. When the Department of Parasitology finishes their malaria epidemiology study (one year hence) the areas will be treated by an ULV apparatus using malathion for mosquito control. In addition the malaria rate will be checked to see if this method of control might reduce malaria incidence.

II. Mosquito Control and Repellent Studies

In the previous paragraph the use of ULV (Ultra Low Volume) ground aerosols for malaria vector control was mentioned. Upon recommendation of the Armed Forces Pest Control Board (AFPCB) two ULV rigs, one hand carried, the other hand-pushed, will be tested under varying conditions for mosquito control. These light weight, highly versatile pieces of equipment seem ideal for small area control problems. Areas such as rubber estates and small, isolated village would be comparable to military bivouac areas in size. Data from such studies would be invaluable to future military operations. No doubt, after the Vietnam experience, much practical research is needed in mosquito control techniques.

Repellent studies under laboratory and field conditions will be made on various species of mosquitoes. Standardized techniques developed by the U.S. Department of Agriculture Laboratory, Insect Affecting Man Branch, Gainesville, Florida, will be used for these tests. The standard military repellent, DEET (Di-ethyl-toluamide) plus repellents suggested by the AFPCB will be tested.

The USDA Laboratory at Gainesville, Florida will be a collaborator either indirectly by recommendations, or directly by utilization of their personnel here in Malaysia. Details of these projects are yet to be worked out but preliminary tests should be started by January 1974.

III. Mosquito Collection Techniques

Several new devices (gadgets) are, or will be evaluated for collecting mosquitoes. A large, 8 inch wide dipper with a flat bottom is being tested for collecting larvae in very shallow ditches. A trap for adult collections used with or without human bait has been designed and is being fabricated. Modifications of the CDC light trap using an animal instead of CO_2 as an attractant is being made. There is no doubt that new collection techniques, especially for malaria surveys, are needed to allow better assessment of vector populations.

IV. Mosquito Blood Meal Studies

A new technique of mosquito blood meal identification by disc electrophoresis using polyacrylamide gel is being tested by the Departments of Viral and Rickettsial Diseases and Medical Entomology. This technique which has considerable application to malaria vector or other arthropod-borne disease studies is discussed in detail in the Viral and Rickettsial Diseases section.

INVESTIGATIONS OF LABORATORY ANIMAL RESOURCES

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	30 06 73	DD-DR&E(AR)636

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
30 06 72		U		N/A	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY		3A062110A831		
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)*

Investigations of Laboratory Animal Resources

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

Tropical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
10 72	9 73		

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: DADA17-73-G-9368 10 72 EXPIRATION: 10 73
b. NUMBER:*
c. TYPE: Y. Grant
d. AMOUNT: 263
e. KIND OF AWARD:
f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	1.0	33.3
FISCAL YEAR CURRENT 74	1.0	60.0

19. RESPONSIBLE DOD ORGANIZATION
NAME:*
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RESPONSIBLE INDIVIDUAL
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20. PERFORMING ORGANIZATION
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Roberts, C.R., MAJ, VC
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Sirimanne, R.A., B.V.Sc & A.H.
NAME:

21. GENERAL USE

22. KEYWORDS (Procede EACH with Security Classification Code)
Animal model, laboratory animal, cost accounting, guinea pigs, nutrition, new building.

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

23.(U) Technical Objectives: To continue to modernize and improve the facilities, methods of operation, nutrition, and caging of the laboratory animal colonies. To improve procedures that can be used for maintaining the silvered leaf monkey. To improve the production colony of *Rattus annandalei* and other natural animal models for human disease.

24.(U) Approach: Studies will be done to evaluate the need for supplementation of commercially available animal chow with additional vitamin sources. Growth curves will be determined for all species bred here. New caging will be purchased for rodents and simians. Consultation with the architect on the new animal facility will be continued.

25.(U) Progress: All breeding stock in the guinea pig colony is now Hartley albino. All of the suckling mouse, rat, hamster, and 25% of the weaned mouse production colonies have been housed in new caging. The design of the new animal facility has been approved by the Malaysian government and construction is now scheduled to begin in January 1973.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

STUDIES IN PROGRESS

An assessment of laboratory animal production and per diem costs is now in progress. The following paper outlining the method used was presented at the July, 1973 meeting of the Malaysian Society of Parasitology and Tropical Medicine.

Cost Accounting for Laboratory Animals in Malaysia

by

Clifford R. Roberts

Department of Laboratory Animal Resources
USAMRU

The cost of production for one laboratory animal is important data for both the manager of the animal production facility and the manager of the research facility. The animal production facility management uses the cost per animal to measure the efficiency of production and to help predict his budgetary requirements. The research manager uses the cost per animal to help predict his own budgetary requirements and to help assess the cost-benefit ratio of research projects.

METHOD OF COMPUTATION

The components of the cost per animal (CPA) are per diem (daily) costs and initial costs. The per diem costs are those for:

1. Direct labor
2. Indirect labor
3. Food
4. Bedding
5. Caging system
6. Utilities
7. Building maintenance

Initial costs are those for:

1. Initial cost of parents (pro rated)
2. Per diem of parents (pro rated)
3. Per diem from birth to date of issue.

The method by which the components of the CPA are given a dollar value should be as simple as possible while including pertinent cost factors, since a recomputation will be necessary each time one of the components changes significantly in value. Unfortunately, changes in component values commonly occurs several times each year and so recomputations of the CPA are usually necessary at quarterly intervals.

DEFINITION AND METHOD OF COMPUTATION FOR PER DIEM COST FACTORS

Direct labor is defined as including the A) Animal Caretaker - a pro-rata share of his cost. The most accurate method in theory is to actually measure the time the caretaker needs to perform each task, such as cleaning one cage, weaning one litter of mice, or filling 100

water bottles. However, the process of measuring may influence the rate of work enough to invalidate data acquired. A simpler, and probably as accurate, method is to divide the total number of working hours per day by the total number of animals cared for during that day. It must be emphasized here that working hours only are used in the calculation; tea breaks and lunch period are excluded. B) Cage Washer - A pro-rata share of his cost. Again, careful timing of each task is theoretically most accurate, but total number of cages processed per working day is a close approximation. C) Supervisor - A pro-rata share of his cost. This is the direct supervisor of the animal caretaker and the cage washer. His duties usually include record-keeping for the colony, scheduling of leaves and overtime, supervision of animal breeding procedures, and preparation of reports for the superintendent or chief of the department. The simplest way to allocate his cost is to assign a pro-rata share for each mouse produced during the period of computation, usually 3 months to a year.

Indirect labor is defined as including the A) Superintendent of the laboratory animal facility - a pro-rata share of his cost. This individual is usually a university graduate, probably a veterinarian, and will have other duties besides the supervision of animal production. In his case, an assessment must be made of the average number of hours devoted each week to the animal production unit. Since the superintendent's salary is usually much higher than the animal caretaker's, it is important to measure the distribution of his time accurately, or the calculated CPA will not be realistic. B) Purchasing office - a pro-rata share of costs. The proportional amount of time spent by this office ordering supplies for the animal production unit should be calculated and given a dollar value. A simple method is to use the ratio of money spent for animal supplies to the total amount spent through this office. Special consideration may have to be given if especially difficult supply contracts or tenders are completed during the period of computation. C) Personnel office - A pro-rata share of costs. The proportional costs of this office may be figured as a ratio of the number of animal facility personnel to the total number of employees.

Although the relationship of direct labor expenses to animal cost is easily understood, the relationship of indirect labor expenses may not be so clear, especially to the departments who use, and should pay, for the animals. Therefore, in some institutions, the indirect labor costs are paid through the administrative budget of the research center and not added to the cost of the individual animal. In some unusual cases, even direct labor costs are borne by the central administrative budget. In such cases, the animal facility manager and the research manager should be aware that the administrative budget of the institution conceals part of the true cost of animals utilized. The problem with this method of cost-accounting is that it forces every department of an institution to pay animal costs, even though many departments may never utilize animals in their work. Heads of departments not using animals may object to contributing to the support of the animal facility - from which their departments get no direct benefit.

Food costs may be simply calculated, but oversimplification must be guarded against. The various sizes of animals of the same species and different amounts of waste occurring with different feeders must be taken into account and preclude the use of standardized tables giving the amount of food consumed per day per animal.

Laboratory animal bedding in Malaysia is usually sawdust or wood shavings and is inexpensive. Its cost may be calculated by dividing the total cost of bedding for each class of animals by the number in that class.

The cage system is defined as including the cage itself, the rack which holds the cage, the food dispenser, and the water dispenser. The complexity can vary from a wire fence around a plot of grass for sheep to an isolator system for germ-free animals. For any case, the projected lifetime of each component must be known. The maximum lifetime or period of use may be limited by ordinary wear and tear, by the time required to become out-of-date, or for certain special purpose caging, by termination of a project.

The method of cost computation for caging is then defined as complete, constant rate depreciation over the projected lifetime. The daily amount of depreciation is, then, the per diem rate.

Utilities are defined as including electricity, water, gas, telephone, and trash collection. The usual method of computation is to divide the total utility costs for a year by the total number of animals produced. If different species are involved in the calculation, the complexity of the computation can become very great. In such cases, a simple method is to divide the utility fees by the area in square feet of occupied animal rooms to give a utilities cost per square foot. It is then easy to assign costs to animals on the basis of the number of square feet occupied per animal.

Building maintenance is defined as including repair of equipment other than caging, repair of the building, replacement of light bulbs, minor remodeling, and may include charges for fire and police protection, insurance, or taxes. It may be difficult in some institutions to accurately assess these costs, and in such cases, they are usually borne by the administrative budget. Where they can be accurately assessed they are assigned to each animal on the same basis as utility charges.

DEFINITION AND METHODS OF COMPUTATIONS FOR INITIAL COST FACTORS

Initial cost of parents is defined as either their purchase price or the cost of rearing them until they reach producing age, pro-rated over the total number of weaned young produced during the parents lifetime.

The per diem cost of parents is defined as the total per diem charges for the parents from mating age to the time of weaning of the last litter produced. This cost is pro-rated over the total number of weaned young produced.

Calculation of the costs of parents requires knowledge of the per diem cost factors described above, the average age of the parents at birth of their first litter, the average time between litters, and the average number of weaned young produced per pair of parent animals. With this information the cycle length from birth of first litter to replacement of the parents and birth of the first litter of the replacement parents can be computed. A simple, yet equitable, method of figuring both initial cost of parents and per diem cost of parents is to apply the per diem charges as defined above to both parents for the length of this reproduction cycle, and to divide this figure by the total number of weaned young produced per pair.

The per diem cost weaning to issue is defined as above, using the cost factors peculiar to weanling animals.

PRACTICAL CONSIDERATIONS

Before beginning computation of the CPA, the director of the institution should be consulted for policy decisions defining which factors of the per diem costs will be borne by the animal user and which costs will be borne by the institutional administrative budget.

Calculations of dollar amounts should initially be made to 4 or 5 decimal places since the amount per day per item can be very small. When the final total is reached, it is usually rounded off to the nearest one cent per day except in the case of mice, which may be rounded to the nearest one-half cent per day.

Finally, graphs of each cost factor should be prepared showing cost of the factor per unit purchased on one axis against CPA or per diem cost on the other axis. The use of such graphs can ease the necessary task of recalculating the costs whenever expenses for contributory factors change.

CONCLUSION

Although the initial computation of the cost per animal is both difficult and time-consuming, accurate assessment of the CPA can be a valuable tool in animal resources management. While research administrators may not easily understand the value of laboratory animals if it is stated only in numbers of animals, they will certainly appreciate a dollars and cents valuation.

Growth Curves for Laboratory Animals Produced at the IMR

by

Clifford R. Roberts

Department of Laboratory Animal Resources
USAMRU

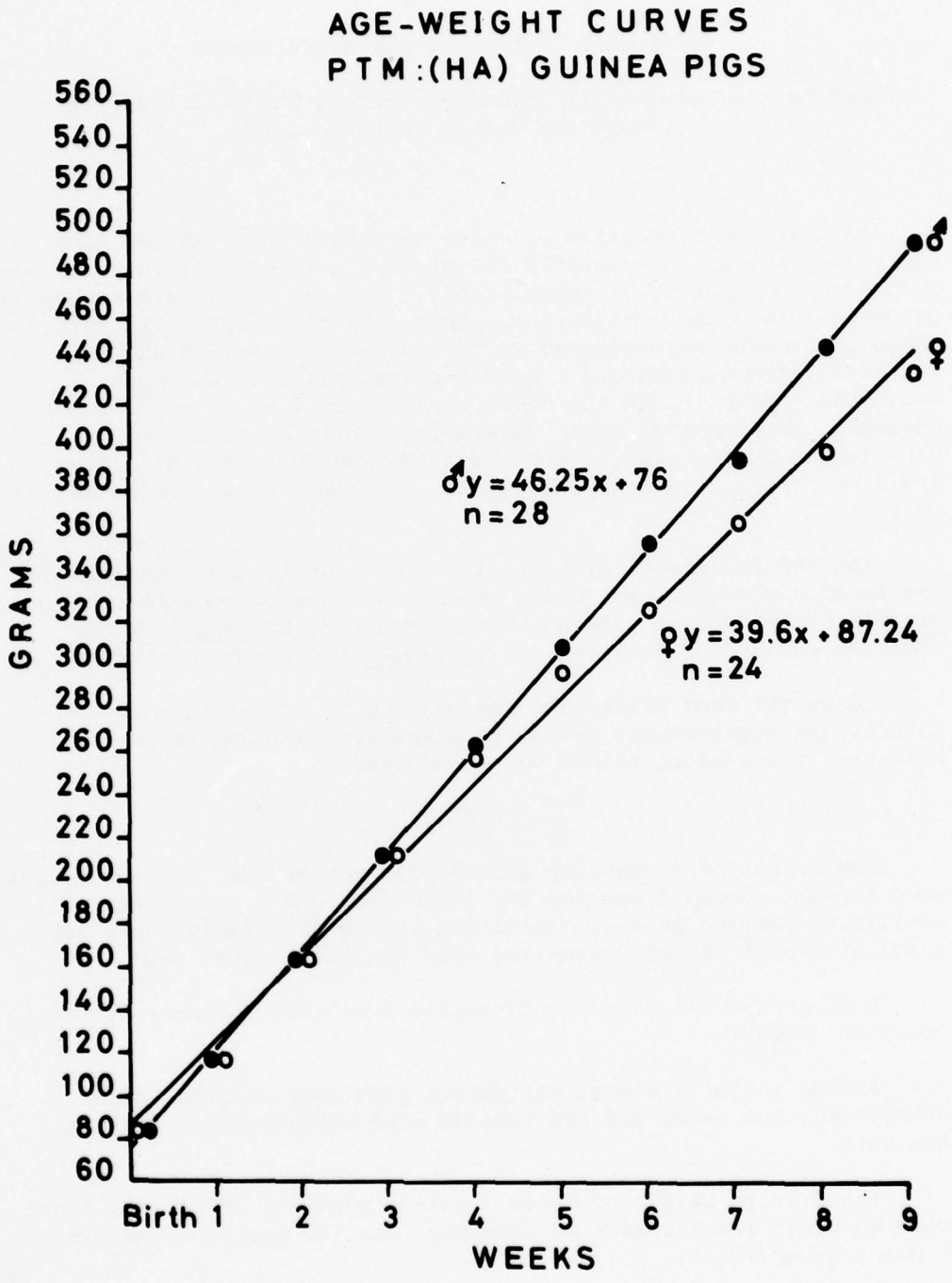
Although laboratory animals have long been produced at the IMR for issue to USAMRU-M investigators, no age versus weight data for these animals has been available. This data should be useful to predict the time necessary to produce animals of a desired weight range, to give investigators an expected weight range for experimental groups, and to serve as a "quality control" check on the production colonies.

Curves have been completed for outbred Hartley strain guinea pigs from birth through 9 weeks of age (Fig. 1). The average weight of these guinea pigs at the ages measured is slightly greater than a strain of outbred American shorthair guinea pigs raised in the U.S.A.*

Growth curves for rabbits, hamsters, mice and gerbils are in progress.

* Poiley, Samuel M. Growth tables for 66 strains and stocks of laboratory animals. *Lab Anim Sci, 22:* 758-779, 1972.

Figure 1


AGE-WEIGHT CURVES
PTM:(HA) GUINEA PIGS
GRAMS
560
540
520
500
480
460
440
420
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
♂ y = 46.25x + 76
n = 28
♀ y = 39.6x + 87.24
n = 24
Birth 1 2 3 4 5 6 7 8 9
WEEKS

Nutrition of Laboratory Animals in Malaysia using Locally Available Feeds

by

Clifford R. Roberts & Roy A. Sirimanne

Department of Laboratory Animal Resources, USAMRU & Division of Laboratory Animal Resources, IMR

Because the production colonies supplying the USAMRU-M with laboratory animals are jointly operated with the IMR, it has not been practical to import U.S. animal feeds. A mouse chow and a monkey chow procured from a local miller have been used since 1961. In 1970, these chows were reformulated at the request of the IMR and the USAMRU-M veterinarian, and a rabbit-guinea pig chow was made available. Even though the chows were supposed to be nutritionally adequate, supplemental green vegetables continued to be fed to rabbits and guinea pigs, and monkeys *(Macaca sp.)* were also given fruit and sweet potatoes. Mice, rats and hamsters were fed the chow only.

The IMR Department of Nutrition did a nutritional analysis of the locally produced chow which showed the chow to be deficient in vitamin C, for guinea pigs and Rhesus monkeys, in vitamin A for hamsters and Rhesus monkeys, and in niacin for Rhesus monkeys.[1]

Since the feed miller was not willing to accept data on nutritional requirements generated elsewhere as being valid in Malaysia, two feeding trials were conducted.

TRIAL 1

Two groups of 5 weanling guinea pigs matched for age and weight were formed. Group 1 was fed the local chow and a local vegetable similar to mustard greens, containing 102 mg of vitamin C per 100 grams of leaf.[2] Group 2 received only the local chow.

Both groups had water continuously available and were housed in identical cages.

At the end of 4 weeks, all guinea pigs were weighed, killed, necropsied, and serum and fed samples were submitted for vitamin C analysis.

The average weight of Group 1 guinea pigs was 350 grams, there were no gross lesions seen at necropsy, and the pooled serum vitamin C was 1.35 mg/100 ml.

The average weight of Group 2 guinea pigs was 205 grams, they were very thin, soiled with feces, and appeared reluctant to move

about in the cage. The only lesions seen at necropsy were petecchial hemorrhages of the urinary bladder mucosa. The pooled serum vitamin C was 0.15 mg/100 ml. The level of vitamin C in the chow was 1.8 mg/100 gm.

Although these young guinea pigs did not develop all of the classical signs of scurvy, their serum vitamin C levels were well below the 0.4 mg/100 ml reported as necessary[3] and their clinical signs were consistent with vitamin C deficiency. Vitamin C levels in the chow were also much lower than the recommended levels of 222 mg/kg.[1]

TRIAL 2

Supplementary vitamins were added to the drinking water of the hamster production colony. Approximately 100 I.U. of vitamin A were added to 60 ml of water daily in the water bottles; no other water was provided. The local animal chow was constantly available. The number of live young born in the first 45 litters conceived after the vitamin supplement was started was compared to the number of live young in the previous 142 litters (control litters) whose dams had received no vitamin supplement. The size of an additional 30 litters was noted after 6 months of continuous vitamin supplementation. The t test for two independent sample means was used to compare the mean litter size.

The mean size of the control litters was 6.7, the mean size of the first litters conceived after the start of vitamin supplementation was 7.6, and the mean size after 6 months of supplementation was 8.2. The t test showed that the change in litter size after supplementation was significantly larger in the first litters, but there was no significant difference between the size of those litters born one month and those born six months after supplementation was begun.

REFERENCES

1. Nutrient requirements of laboratory animals. 2nd Ed., 1972, *Nat. Acad. Sci.*, Wash, D.C.

2. Department of social medicine and public health tables of representative values of foods commonly used in Singapore. Department of Social Medicine and Public Health, University of Malaya in Singapore, Singapore, 1960.

3. Nungester, W.J. and Amers, A.M. The relationship between ascorbic acid and phagocytic activity. *J. Infect. Dis.*, *83*: 50, 1948.

PLANS FOR THE FUTURE AND COMMENTS

New Animal Facility

Construction of the new animal facility for the IMR was initially to begin in October, 1972. However, by that time only preliminary floor plans had been completed. The present USAMRU veterinarian felt that the proposed design could be improved by simplification of the flow of personnel and material through the building, and by centralizing services, such as cage washing, common to several activities in the building. Rather than proposing a new building design, a list of performance criteria was given to the architect with suggestions on solutions to some of the problems. This approach is generally more successful than that of attempting to relegate the architect to draftsman's status while a health professional dictates building design and construction. Excellent cooperation has been achieved between the architect and the veterinary staff, and the new animal facility should be both practical to build and operate in Malaysia and suitable for accreditation by AAALAC.

Funds for the facility were approved by the Malaysian government in July 1973, final drawings will be completed in October, 1973, and start of construction is now scheduled for January, 1974.

The building will contain 17,600 net square feet of animal rooms, of which 2700 square feet will be air conditioned and designed to provide a quarantine and an isolation suite. The remaining animal rooms have natural ventilation with the two outside walls enclosed by wire screening. A laboratory is provided for each 9 experimental animal rooms to avoid the undesirable movement of animals to laboratories outside the building. Facilities are also provided for surgery, clinical and gross pathology, and radiology. Because of cost and difficulty of proper maintenance in Malaysia, automatic cage washing equipment and an autoclave for the food and bedding supply have been excluded from the initial equipment inventory. However, provision has been made in the design for the future utilization of such equipment.

If construction and occupation of the new animal facility occur as now planned, it will serve not only as a national center for laboratory animal production and housing, but as a training center for Malaysian animal technicians. The completed facility will be able to provide the USAMRU-M with much-needed additional animal housing and rodent production capability.

Comments

A. Supply of Feeds

Several meetings have been held with the local feed miller's representative and improvement of the nutritional quality of the feed is possible. The physical qualities of the local feed are not satisfactory in that the mouse pellets are too small and too soft. However, two factors are operating to prevent a speedy rectification of the feed problems. One, laboratory animal chows are only a small fraction of the miller's total production, so that he is not too concerned with our problems. Two, the price of animal chow has increased 40% in 1973, and the other users of the chows are extremely reluctant to have any changes made in the chow formulation if such changes would result in even a small further increase in the price per bag. It has been difficult to convince the other users that the savings in waste and supplementary feeds would pay for the needed improvements in formulation and pelleting. A new, fully-automated, Malaysian feed mill is due to begin operation in January, 1974, and the management has expressed interest in producing laboratory chows to our specifications. It is probable that this creation of some competition in the feed industry will improve our bargaining position. Due to the lack of military space-available air freight to Kuala Lumpur, and to the sharing of feed costs with the IMR, it is still not considered feasible to import animal feeds from the USA.

B. Caging

Approximately 950 new, US-made rodent cages were introduced into the rodent production colony this year. Another 2000 cages are needed to completely replace the old cages in the production colony and of USAMRU-M experimental rodents. Funds have been requested in the FY 74 budget for this purpose. It appears at this time that the cages can be manufactured in Malaysia at less cost than importing them from the U.S.

The training program for local employees projected for this year has begun, with first priorities being given to the training of a technician for the departmental clinical laboratory. The individual selected is making very satisfactory progress.

New cages for the *Rattus annandeli* colony have been designed and are now under construction. The availability of these cages should allow an increase in the size of the colony to permit both research into the reproductive cycle of these rats and provision of sufficient weanling animals for scrub typhus investigations.

Reproduction in the Lesser Mouse Deer *(Tragulus javanicus)* colony is satisfactory, but more investigation is necessary to arrive at a optimum diet and caging system.

INVESTIGATIONS OF THE DEPARTMENT OF PARASITOLOGY

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	30 06 73	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
30 06 72		U		N/A	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY		3A062110A831		
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)*

Investigations of the Department of Parasitology

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

Tropical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
10 72	9 73		

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: 10 72 EXPIRATION: 10 73
b. NUMBER:* DADA17-73-G-9368
c. TYPE: Y. Grant d. AMOUNT: 263
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	1.0	15.4
	CURRENT 74	1.0	22.3

19. RESPONSIBLE DOD ORGANIZATION
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RESPONSIBLE INDIVIDUAL
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21. GENERAL USE

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NAME: J.T. Ponnampalam, MBBS
NAME: M. Mariappan

22. KEYWORDS (Procede EACH with Security Classification Code) Human malaria, chloroquine-resistance, epidemiology, *in vitro* testing, Malaysia, *P. falciparum*, *P. vivax*, urine testing

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

23.(U) Technical Objective: To locate areas in Peninsular Malaysia, Sabah (North Borneo), and Sumatra, Indonesia, which have chloroquine resistant falciparum malaria; to assess the proportion of resistant infections; to determine the degree of resistance, e.g. R-1, R-2, etc., to seek chloroquine resistant *P. vivax*; to attempt to improve *in vitro* testing for drug resistance; to compare and improve on existing field methods for demonstration of urinary chloroquine; to determine seasonality of malaria in a part of Peninsular Malaysia; to determine in a population with an approximately 15% malaria rate whether nearly everyone is infected during the course of a year or whether sub-groups of "malaria-prone" repeaters exist; to compare such sub-groups demographically with the others.

24.(U) Approach: Conduct in many areas 28-day follow-up *in vivo* chloroquine resistance studies with provisions to assess the rate of possible reinfection; modify the culture method portion of the Rieckmann *in vitro* drug testing technique by employing the method of Diggs *et al.*; modify and field test several described urine analytical methods for chloroquine; conduct over a 2 year period 4-weekly malaria surveillance on a stable population, treating the infections found; compare the malaria-prone sub-group, if one exists, in terms of age, sex, occupation, use of mechanical or chemical protection against mosquitoes, sleeping habits, social activities (e.g. travelling, etc.), and if possible individual attractiveness to mosquitoes.

25.(U) Progress: 4 *in vivo* chloroquine resistance studies completed and analysed showing considerably more resistance than previously realized, mostly of the R-1 type; 4 additional studies underway or completed but not yet analysed; surveys conducted for

*Available to contractors upon originator's approval.

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DD Form 1498, Research and Technology Work Unit Summary,
Item 25 Continued:

further prospective study sites; preliminary study (7-day) conducted in Sumatra, Indonesia; preliminary visit and feasibility assessment made in Sabah; thus far no chloroquine resistance found in *P. vivax* or *P. malariae; in vitro* method still in developmental stages - parasites kept alive but not yet growing; Dill-Glazko urine test found less satisfactory under field conditions in Malaysia than other methods; 6 months worth of malaria surveillance completed thus far - existence or non-existence of a malaria prone sub-group not yet apparent; infections are wide-spread but do seem somewhat more frequent in certain households.

COMPLETED STUDIES

Chloroquine Resistant Falciparum Malaria in West Malaysia

by

Timothy J. Dondero,Jr., Ray E. Parsons & J.T. Ponnampalam

Departments of Parasitology and Medical Entomology,USAMRU
& Malaria Research Division, IMR

Resistance of falciparum malaria to chloroquine hinders the treatment, prevention, and control of malaria in many parts of South East Asia.

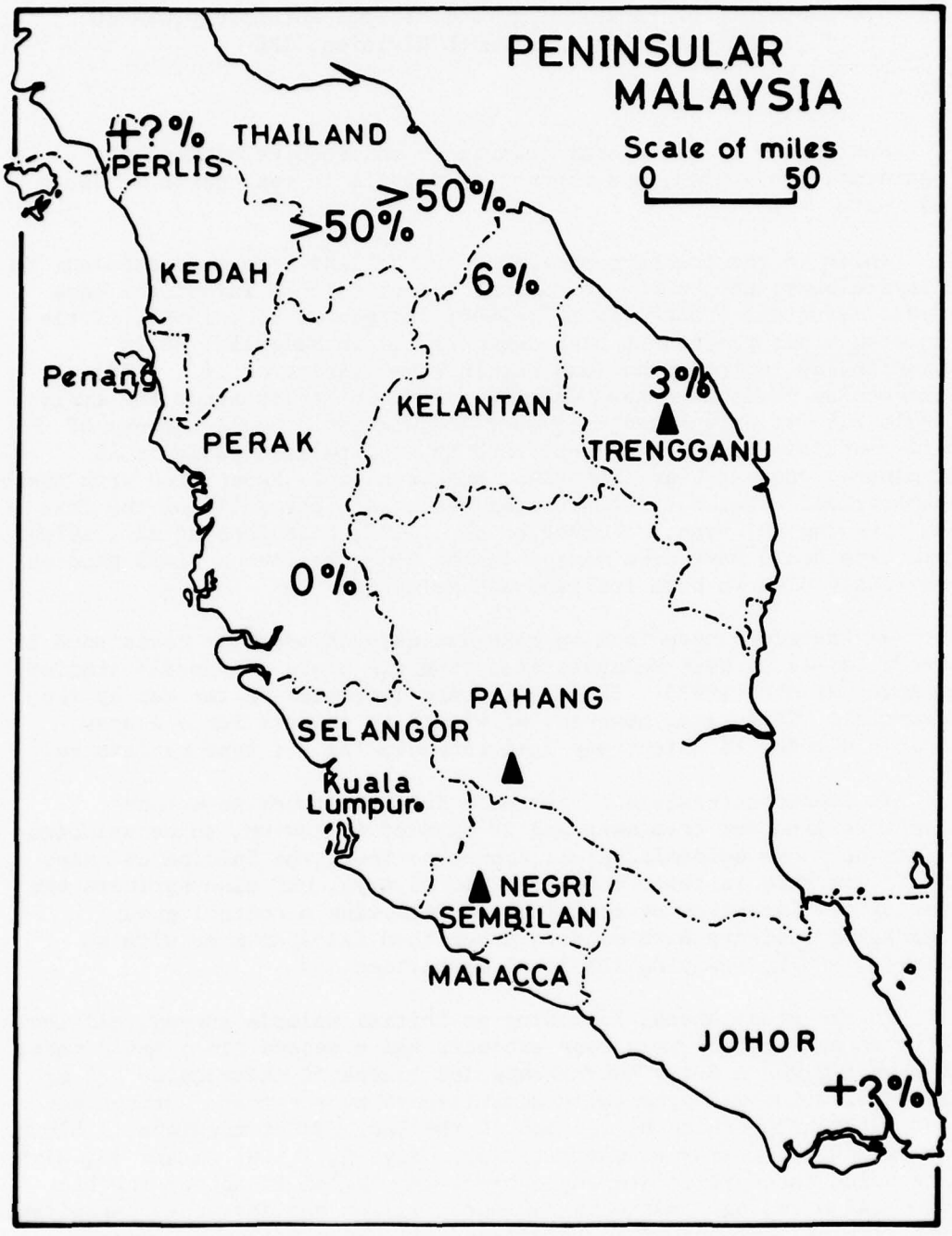
While in the southern provinces of Thailand (see map) adjacent to Malaysia over half of the tested falciparum malaria infections have proved resistant (Bourke *et al.*, 1966; Cadigan *et al.*, 1968), little resistance has previously been demonstrated in Malaysia: 6% in Kelantan, 3% in Trengganu, and nil in Perak (Andre *et al.*, 1972). Chloroquine resistance has been recognized in Perlis since the early 1960's but its extent and frequency are unknown. Small numbers of resistant infections have been found in hospitalized patients in Singapore, Johore, Negri Sembilan, and Selangor. Experience with these hospitalized cases has demonstrated resistance primarily of the late recrudescing R I type (McKelvey *et al.*, 1971; Colbourne *et al.*, 1970). This type would have been missed in the 7-day follow-up field studies previously used in both Thailand and Malaysia.

We therefore undertook to reinvestigate chloroquine resistance in several areas of West Malaysia including one place previously studied by Andre *et al.* (1972). These sites are indicated on the map by the triangles. This time, however, we wished to observe for a longer period in order to detect any late recrudescing R I type resistance.

It seemed unfeasible to evacuate infected cases to a non-malarious area for treatment and 28 days of follow-up, so we selected a somewhat more epidemiological approach: treat the falciparum cases and follow them in their home area for 28 days, but also estimate the risk of new infection by treating and following a control group comprising subjects with malaria other than falciparum or with no malaria, and by studying the local mosquitoes.

In the study areas, following an initial malaria survey, all the infected and control volunteer subjects had a second blood smear made, then were given a 3-day WHO-recommended course of chloroquine (25 mg base/kg), which was personally administered by our team. Urines were tested for chloroquine before and on the last day of treatment. Blood was examined for asexual parasitemia on days 5, 7, 14, 21 and 28, with care being taken to locate any subject who failed to appear for his scheduled bleeding. Subjects seriously ill or who developed parasitemia with clinical disease after treatment were given alternate therapy.

MAP OF THE LOWER MALAY PENINSULA SHOWING THE PUBLISHED RATES OF CHLOROQUINE RESISTANCE AMONG *P. FALCIPARUM* INFECTIONS. THE TRIANGLES INDICATE THE SITES OF CURRENT STUDIES COMPLETED TO DATE.



In each study we estimated the attack rate for new falciparum infection by determining the rate of post-treatment falciparum parasitemia among those with only *P. vivax* or *P. malariae* before treatment and among those without malaria on two examinations when the study began. These negatives were usually sibling or spouses of infected subjects. Thus we arrived at several figures: first a resistance rate based on parasitemia on the 7th day after instituting therapy - this conforms to WHO standards and corresponds with the method for determining rates in the earlier studies. The second figure is an estimated overall resistance rate based on the full 28-day post-treatment observations and adjusted downward to account for the estimated attack rate for new infections.

The first study area comprised 6 villages in a rice growing region in Trengganu, about 100 miles south of the Thai border. The subjects were Malay primary school children, and in two places, villagers of all ages. The initial prevalence rates of malaria are shown in Table 1. We did not determine spleen rates there.

Table 2 shows the results of treatment and our estimate of a 5% overall chloroquine resistance rate. The 7-day estimate of 1.4% is consistent with the 3% found by Andre *et al* (1972) in the same schools and villages in 1970.

The second study area comprised two adjacent rubber plantations in a hilly area of Western Pahang about 80 miles east of Kuala Lumpur. The subjects were the resident laborers and their dependents of all ages and were primarily of South Indian origin born in Malaysia but also included Malays and Chinese. An initial and a subsequent confirmatory study was performed there. The pre-treatment malaria prevalences are shown in Table 3.

Chloroquine had been extensively used for treatment of fevers on the larger plantation prior to the first study. Interestingly, very little *P. vivax* was found at that time. The spleen rate was 30%.

The initial study (Table 4) resulted in an estimated overall resistance rate was 54%, with 21% positive on day 7. Over half of those 36 cases positive on day 7 were negative on day 5, meaning early recrudescing R I resistance.

In the repeat study (Table 5) the overall rate of resistance was 50% while the 7-day figure is a low 5.5%. This time there were more controls and also a reduced *P. falciparum* attack rate.

The third study area thus far completed was a rubber plantation near Seremban, about 50 miles south of Kuala Lumpur. The group there were about 80% Indians, 20% Malays.

In late February 1973 the *P. falciparum* prevalence rate was 12.6%, *vivax* 1.2%, spleen rate 5%. During the study period no *P. falciparum* appeared among the 44 control subjects. As shown

TABLE 1

RESULTS OF INITIAL BLOOD EXAMINATION IN ULU TRENGGANU, AUGUST 1972

Place	No. Examined	No. positive					Prevalence Rate
		falcip.	vivax	malariae	(mixed)	Total	
Kampungs (Not including school children)							
Kuala Dura	102	4	2	1	-	7	7%
Kuala Jeneris	68	10	2	1	-	13	18%
Schools							
Kuala Dura	99	14	12	6	(2)	30	30%
Kuala Jeneris	33	8	3	1	(1)	11	33%
Sungei Buloh	49	15	9	4	(4)	24	47%
Tengkawang	104	11	16	13	(6)	34	33%
Matang	106	18	8	5	(2)	29	27%
Bukit Tadok	38	2	2	5	(2)	7	18%
All places combined	602	82	54	36	(17)	155	26%

TABLE 2

ASEXUAL *P. FALCIPARUM* PARASITEMIA FOUND AFTER FULL CHLOROQUINE TREATMENT IN SUBJECTS FROM ULU TRENGGANU, AUGUST-OCTOBER 1972

Infection Prior to Treatment	No. of Subjects Fully Studied	No. of Cases of Parasitemia Listed by Date of First Appearance				Total Cases of Post-treatment Parasitemia	Percent Post-treatment Parasitemia
		day 7	day 14	day 21	day 28		
P. falciparum	70	1	1	1	1	4	5.7%
Species other than *P. falciparum*, or no infection	370	1	-	2	-	3	0.8%

ESTIMATED *P. FALCIPARUM* ATTACK RATE 0.8%

7-DAY RESISTANCE RATE 1.4%

ESTIMATED OVERALL (28 DAY) RESISTANCE RATE 5%

TABLE 3

RESULTS OF INITIAL BLOOD EXAMINATION ON TUAN AND RENJOK ESTATES, TELEMONG, PAHANG, NOVEMBER 1972 AND MARCH 1973

	No. Examined	No. Positive				Prevalence Rate
		falcip.	vivax	(mixed)	Total	
1st study - Tuan (all ages)	557	188	5	(1)	192	34.5%
2nd study - Tuan (children 0-10 yr)	172	39	20	(2)	57	33.2%
Renjok (all ages)	206	18	8	(1)	25	12.2%

SPLEEN RATE (TUAN ESTATE) 30%

TABLE 4

ASEXUAL P. PALCIPARUM PARASITEMIA FOUND AFTER FULL CHLOROQUINE TREATMENT IN SUBJECTS FROM TUAN ESTATE, TELEMONG, PAHANG, (1st STUDY), NOVEMBER - DECEMBER 1972

Infection Prior to Treatment	No. of Subjects Fully Studied	No. of Cases of Parasitemia Listed by Date of First Appearance					Total Cases of Post-Treatment Parasitemia	Percent Post-Treatment Parasitemia
		day 5	day 7	day 14	day 21	day 28		
P. falciparum (asexual)	172	17	19	40	27	16	119	69
P. falciparum (gametocytes*)	16	-	2	4	2	2	10	62
P. vivax or no infection	33	-	-	4	1	-	5	15

ESTIMATED P. FALCIPARUM ATTACK RATE 15%

7-DAY RESISTANCE RATE 21%

ESTIMATED OVERALL (28 DAY) RESISTANCE RATE 54%

*Many previously treated

TABLE 5

ASEXUAL P. FALCIPARUM PARASITEMIA FOUND AFTER FULL CHLOROQUINE TREATMENT IN SUBJECTS FROM TUAN AND RENJOK ESTATES (2ND STUDY), MARCH - APRIL 1973

Infection Prior to Treatment	No. of Subjects Fully Studied	No. of Cases of Parasitemia Listed by Date of First Appearance					Total Cases of Post-Treatment Parasitemia	Percent Post-Treatment Parasitemia
		day 5	day 7	day 14	day 21	day 28		
P. falciparum	55	1	2	12	10	4	29	53%
P. vivax or no infection	84	-	-	-	1	1	1	2.4%

ESTIMATED P. FALCIPARUM ATTACK RATE 2.4%

7-DAY RESISTANCE RATE 5.5%

ESTIMATED OVERALL (28-DAY) RESISTANCE RATE 50%

In Table 6, among the 35 *P. falciparum* cases treated and fully followed up the recurrence rate was 14%, which we estimate to be the resistance rate, virtually all due to late recrudescing RI resistance.

An interesting feature in all our study areas is that the great majority of resistant infections are of the R I type, primarily late recrudescing.

In all, 94 *P. vivax* infections, pure or mixed, and 35 *P. malariae* infections were treated and fully followed up. There was no evidence of resistance, although there was one *P. vivax* "relapse" after 14 days.

The roughly 50% chloroquine resistance rate found on two studies in Pahang is by far the highest yet found in Malaysia. However it is much lower than that found in southern Thailand where over 50% were positive on the 7th day after treatment compared with our maximum of our 21% on day 7.

The results of these and previous field studies in Peninsular Malaysia (map) suggest great variations in the frequency of resistance, with as yet no apparent geographical pattern. However, appreciable chloroquine resistance has been seen thus far only in areas such as rubber estates, where chloroquine has been long and heavily used. The low frequencies have appeared in rural areas and in populations little exposed to chloroquine treatment or prophylaxis. Our present and very preliminary guess is that the falciparum malaria in much of Peninsular Malaysia is potentially resistant to chloroquine and that the past local use of the drug has had a strong influence on the present local frequency of chloroquine resistance.

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TABLE 6

ASEXUAL P. FALCIPARUM PARASITEMIA FOUND AFTER FULL CHLOROQUINE TREATMENT IN SUBJECTS FROM PAROI ESTATE, AMPANGAN, NEGERI SEMBILAN, MARCH-APRIL 1973

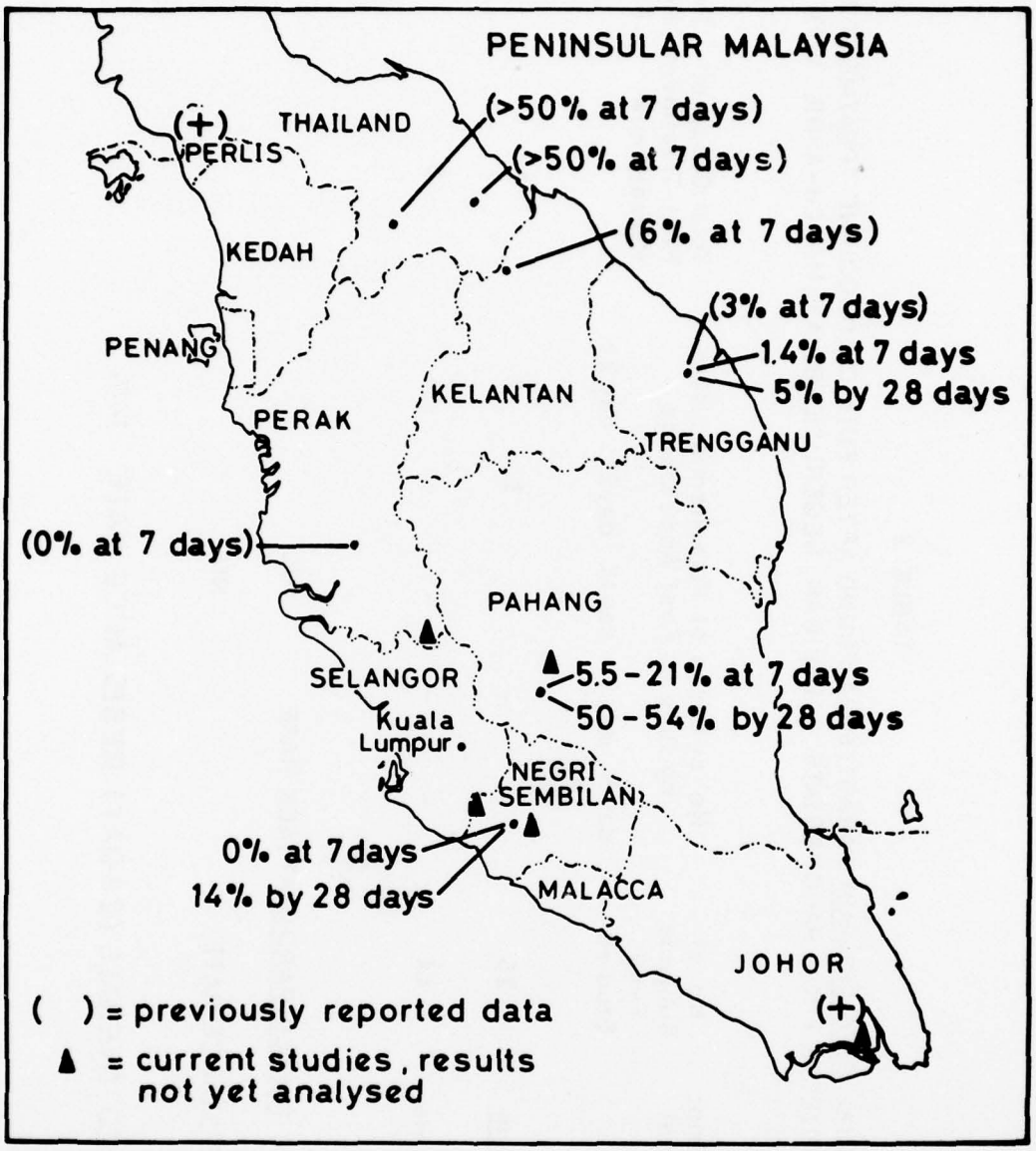
Infection Prior to Treatment	No. of Subjects Fully Studied	No. of Cases of Parasitemia Listed by Date of First Appearance					Total Cases of Post-Treatment Parasitemia	Percent Post-Treatment Parasitemia
		day 5	day 7	day 14	day 21	day 28		
P. falciparum	35	-	-	-	3	2	5	14%
P. vivax or no infection	44	-	-	-	-	-	0	NIL

ESTIMATED P. FALCIPARUM ATTACK RATE NIL

7-DAY RESISTANCE RATE NIL

ESTIMATED OVERALL (28-DAY) RESISTANCE RATE 14%

MAP SHOWING COMPILED RESULTS OF COMPLETED STUDIES PLUS SITES OF CURRENT STUDIES



Falciparum Malaria Resistant to Chloroquine Suppression but Sensitive to Chloroquine Treatment in West Malaysia

by

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In the course of a study on prophylaxis by a combination of sulfadoxine and pyrimethamine (Lewis and Ponnampalam, in preparation) on a rubber estate in Negri Sembilan, West Malaysia, we observed in one of the control groups an interesting phenomenon: falciparum malaria which could be treated by chloroquine but not prevented by it. Two comparable control groups of approximately 100 persons each were seen at 4-weekly intervals from February through November 1972. One group was on weekly chloroquine (5 mg. base/kg. Avlochor, I.C.I.) the other on monthly vitamin C placebo.

Blood from all subjects was examined prior to and during the study at 4-weekly intervals and all positive cases thus detected were treated with a 3 day, 25 mg./kg. course of chloroquine. Throughout we counted as new infections only those positive cases which had been negative the previous months.

The initial survey on the estate revealed 11% *P. vivax* and 12% *P. falciparum*. During the nine 4-week periods of the study, the group on weekly chloroquine was protected from *P. vivax* infection. Only one infection occurred and this was in a 1-year old baby whose retention of the drug may be questioned. On the other hand in the placebo group there were 50 new infections giving an average monthly attack rate of 5.6%

However chloroquine apparently did not protect against *P. falciparum*. In the placebo group there were 56 new *falciparum* infections with an average monthly attack rate of 6.3% while in the chloroquine group there were 45 new infections (in 35 different people)for an average attack rate of 5.7%. During the 44 man months where chloroquine taking was incomplete (over half during the Muslim fasting month) 2 new infections with *P. falciparum* (4.5%) appeared. There is no statistical difference between the overall incidences of falciparum in the placebo and the full chloroquine groups ($X^2 = 3.303$, $P > 0.1$). Moreover the monthly incidences for this species were generally comparable for both groups. No difference was apparent between the densities of parasitemia in the two groups when measured by the semi-quantitative method of Field *et al.*(1963).

We are reasonably assured that most of the chloroquine group actually received their drug since all doses were personally administered by either the Estate hospital assistant or one of the

authors. During month 5 when all drug taking was personally supervised by one of the authors, 5 people still developed *P. falciparum* infections. When urines of the chloroquine subjects were surprise tested by the Haskins method (1958) 7 days after their previous 5 mg./kg. suppressive dose (approaching the limits of sensitivity of the test) among those 35 people who developed malaria 75% were strongly positive, 18% were weakly positive, while 7% registered negative. Finally no *P. vivax* appeared in the chloroquine group except in the baby.

Although chloroquine appeared to provide little protection against infection with *P. falciparum* in these "semi-immune" subjects it was none the less reasonably effective therapeutically. When treated with the full 3 day 1500 mg. equivalent course, 40 of the 45 cases which broke through chloroquine suppression cleared and were negative on blood examination 3 weeks later. Experience with 82 other treated cases of *P. falciparum* was similar. In all, 86% of treated cases were still blood negative 3 weeks post-treatment. Some of those who were positive by 3 weeks may represent reinfection: There was an average monthly attack rate of about 6% for the study group as a whole. The actual rate of drug resistance (therapeutically judged) is probably less than 14%. (Previously, resistance to chloroquine has been only rarely noted in this area of the country (Field *et al.*, 1952; Wilson and Edeson 1954, 1957.)

Thus the seeming paradox: while "chloroquine resistance" appeared well under 15% - based on treatment as are most published rates - chloroquine's value in suppression appeared to be nil at the standard dosage.

We are unaware of published reports of this nature, although several studies in Southeast Asia have found falciparum malaria somewhat less responsive to lower therapeutic doses such as 10 mg./kg. than to the higher doses in the range of 25 mg./kg. over 3 days. (Sandosham *et al.*, 1966; Bourke *et al.*, 1966; I.M.R., 1967; Andre *et al.*, 1972).

These earlier investigations implied that chloroquine suppression would not be fully adequate in areas such as Malaysia where there occurs some reduced sensitivity to chloroquine therapy. However a false sense of security might result if one inferred that the great majority of falciparum "strains" would still be suppressed by chloroquine simply because they could be successfully treated. Our findings suggest this is not necessarily so. We wonder if resistance to the lower suppressive doses might be a more sensitive *in vivo* indicator of chloroquine resistance.

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Urine Tests for Chloroquine

by

T.J. Dondero, Jr. & M. Mariappan

Department of Parasitology, USAMRU

Attempts were made at improving the simple qualitative field test for chloroquine in urine described by Lelijveld and Kortmann (*Bull. Wld Hlth. Org., 42:* 477-479, 1970). We found that extraction of a 2 cc specimen of urine with n-hexane or n-heptane before adding the indicator eosin solution resulted in a bright pink precipitate when there was as little as 2 ug/ml of chloroquine present, a 2 to 3-fold increase in sensitivity over the parent procedure, and comparable in sensitivity to the more laborious but standard Haskins test (*Am. J. Trop. Med. Hyg., 7:* 199-200, 1958). Although the modified test worked very well in chloroquine-containing urine in the laboratory and on post-treatment urine in the field, unfortunately 30-40% of the urine specimens from previously untreated Malay and Indian subjects in the field gave false positive reactions with this test but not with the Haskins test. Probably some urinary substances, possibly alkeloids of dietary origin, were causing the false positives.

It occurred to us that possibly the parent test might also give false reactions under similar field conditions. (The method was developed and field-tested in Africa where the dietary by-products might be different.) To date we have conducted 3 comparative studies of the Lelijveld-Kortmann method (L-K) and Meyers reagent (Wilson & Edeson, *Med. J. Malaya, 9:* 115-131, 1954), an older, more standard test which has similar reported sensitivity. Any specimen on which there was a difference in result between the two tests was subjected to the more sensitive Haskins test. On 124 urines collected from employees at the Institute for Medical Research, primarily urban dwellers not taking malaria prophylaxis, none registered positive for either test except for one trace positive (±) for the L.K. In two field studies a total of 770 urine samples were tested, about half collected before our chloroquine treatment (many people had taken some chloroquine on their own) and half after. Among the post-treatment samples there were no differences in results between the two methods: all except one sample were positive. However the L.K. method was disappointing with the pre-treatment urines. In the first study among 116 such urines L.K. gave false positives relative to the Meyers (and verified as negative by Haskins) in 6% of the cases (plus about 20% additional false positives if the trace positives are counted), and false negatives in 3.5%. One urine, positive by Haskins, was negative by Meyers and trace positive by L.K. In the second study among 276 pre-treatment urines L.K. gave 4.2% false positives and 5.3% false negatives. Meyers reagent gave only 1 false negative where the L.K. was positive.

Thus under field conditions in Peninsular Malaysia the Lelijveld and Kortmann method in non chloroquine-containing urine gave 4-6% false positive results and in urine apparently containing small amounts of chloroquine (from treatment some time before the test or from recent partial treatment) gave 3-5% false negatives. It is therefore both less specific and less sensitive than its chief rival, the Meyers reagent of Wilson and Edeson. Moreover the L.K. is slightly more tedious than the Meyers on the average urine, requiring several extra steps, and is, in our hands, more difficult to read. Its one possible advantage is that virtually all urines can be tested. Albumin containing urine must be heated and filtered before testing with Meyers and (reportedly) on rare occasions a urine cannot be sufficiently clarified to test.

STUDIES IN PROGRESS

Malaria Epidemiology

by

T.J. Dondero, Jr., R.E. Parsons & D.R. O'Holohan

Departments of Parasitology, and Medical Entomology, USAMRU
& Kelinik O'Holohan, Seremban.

In collaboration with Dr. R.E. Parsons, Department of Medical Entomology, USAMRU-M, and with Dr. D.R. O'Holohan, Kelinik O'Holohan, Seremban, we are conducting long term 4-weekly malaria surveillance on a stable rubber estate population. Parallel entomological surveillance is being performed by Dr. Parsons.

The purposes of this work are: 1) to determine the seasonal incidences of infection with *P. falciparum* and *P. vivax* (no recent information is available). 2) To determine the meaning of, for example, a 15% malaria rate (point prevalence) - are most people actually infected during the course of a year? 3) To determine whether there is a sub-group of "malaria prone" people and if so to compare them demographically with the non-malaria group. 4) To attempt to relate seasonality of malaria with rainfall and anopheline dynamics. 5) To obtain cooperative volunteer subjects with known recent malaria histories for chloroquine resistance studies in vivo (one study already completed) and in vitro. 6) To look for evidence of increasing rates of chloroquine-resistant *P. falciparum* after various periods of "chloroquine pressure", i.e., regular chloroquine treatment of all parasitemias.

The study site is a reasonably isolated rubber estate near Seremban about 50 miles south of Kuala Lumpur. The population of about 170, mostly of South Indian origin, live in virtually identical dwellings on one small central area of the estate, some 2 miles from the nearest living area. Monthly blood smears are collected on all who wish to cooperate (usually > 90%) by USAMRU technicians; within 7-10 days all blood positive subjects are treated with a 3-day, 25 mg/kg course of chloroquine by the estate Hospital Assistant, under the supervision of Dr. O'Holohan. Most fever cases other than those detected in the monthly examination seek medical assistance either from the Hospital Assistant or from Dr. O'Holohan. Smears are made on these cases for our records prior to anti-malaria therapy. Medical problems other than those related to malaria remain the responsibility of the estate's medical service.

A baseline chloroquine resistance test was conducted at the beginning of the study period in February-March 1973. As reported above the resistance was estimated at 14 percent.

MONTHLY INCIDENCE (OF NEW INFECTIONS) OF *P. FALCIPARUM* AND *P. VIVAX* PLUS OVERALL MALARIA PREVALENCE* BY MONTH ON PAROI ESTATE, SUNGEI GADUT, NEGRI SEMBILAN

Date	Incidence of *P. falciparum*	Incidence of *P. vivax*	Overall Prevalence
13 Feb 73	-	-	13.7%
13 Mar 73	5.3%	2.0%	9.9%
10 Apr 73	5.8%	7.1%	14.8%
8 May 73	1.9%	5.0%	8.2%
5 Jun 73	7.1%	12.3%	18.8%
2 Jul 73	5.8%	5.1%	13.1%
30 Jul 73	11.3%	12.0%	24.8%

* Includes all current positive asexual infections - new, old, treatment failures etc.

The initial prevalence rates were 12.6% for *P. falciparum* and 1.2% for *P. vivax* with a spleen rate of 5%. The monthly incidences for new infection (attack rates) plus the overall prevalence each month are listed for the first 6 months of observation in the table and illustrated in the figure. For purposes of the study a "new" infection is defined as asexual parasitemia appearing after at least one negative monthly blood smear. Also considered as "new" infections are a few cases of *P. falciparum* gametocytemia which appeared at least 4 months after the last known *P. falciparum* infection. (We cannot completely exclude the possibility of self medication with chloroquine although the avenues for free diagnosis and treatment are readily available through the study.) Since radical treatment has not yet been instituted for *P. vivax*, a few of these "new" infections may actually be relapses following treatment (6 possible relapses so far). The overall prevalence figure includes all persons who are positive whether with a new infection or are carried over from the previous month.

Including the infections present on the first survey and new infections in the first 6 subsequent 4-weekly observations, there have been 128 malaria infections affecting 95 different people (56% of the total observed population): 74 *P. falciparum* and 54 *P. vivax* (of whom 6 had known *P. vivax* 2 or more months previously).

AD-A058 238 INSTITUTE FOR MEDICAL RESEARCH KUALA LUMPUR (MALAYSIA) F/G 6/5
TRANSMISSION, CONTROL AND TREATMENT OF INFECTIOUS DISEASES OF M--ETC(U)
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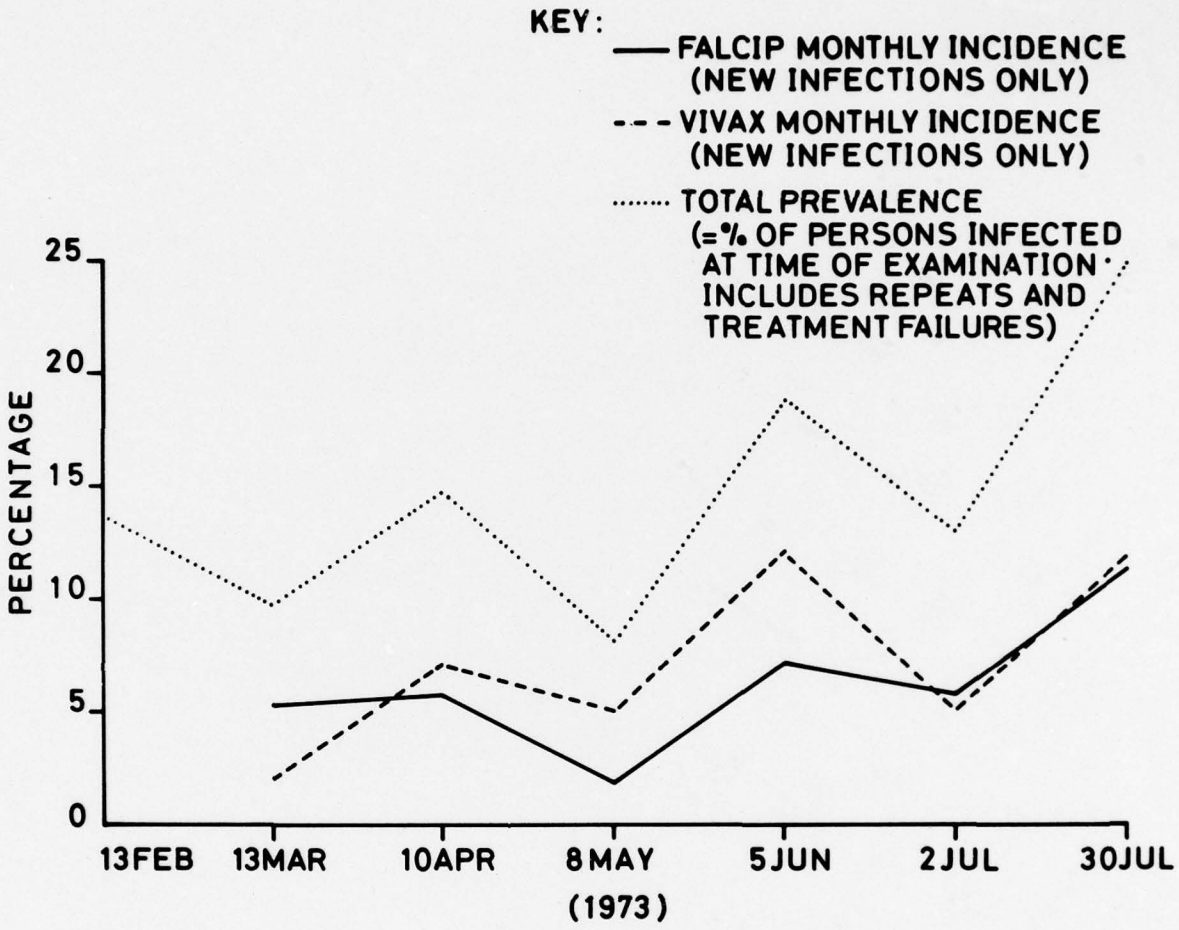


Figure 1. Monthly incidence and prevalence of malaria, Paroi Estate, Sungei Gadut, Negri Sembilan, Malaysia.

Results of the mosquito studies are found in the Department of Medical Entomology's report.

No conclusions are yet justified on the existence of a "malaria prone" sub-group or on the other questions asked.

Chloroquine Resistance Studies in Sumatra, Indonesia

by

T.J. Dondero, Jr., Kwo Eh Hoa & Gino Tan

Department of Parasitology, USAMRU, University of North Sumatra, Medan & General Hospital, Medan.

During the reporting year a two-week preliminary survey and chloroquine resistance study was conducted in North Sumatra, Indonesia, in collaboration with Dr. Kwo Eh Hoa, University of North Sumatra, Medan, with Professor (Dr.) J. Sulianti Saroso, National Institute for Medical Research, Jakarta, and with Captain P.F.D. Van Peenan, NAMRU-2 Jakarta. Chloroquine resistance has been alleged by Dr. H. Wofensberger in reports to Prof. Sulianti to occur in *P. vivax* in various parts of Indonesia including Pulau Nias in North Sumatra.

Surveys were conducted in two kampungs (villages) and two rubber estates. The only site with sufficient malaria for the resistance studies was Seruwai Plantation, near Belawan some 25 miles northeast of Medan. A short (7-day) *in vivo* study among 10 *P. vivax* and 29 *P. falciparum* cases failed to detect any chloroquine resistance. A 28 day follow-up study on more subjects is scheduled for late 1973, since late-recrudescing R I resistance could not be detected under the short follow-up conditions used.

In vitro Chloroquine Resistance Testing

by

T.J. Dondero, Jr. & M. Mariappan

Department of Parasitology, USAMRU

Work is still in progress attempting to improve the culture technique for the *in vitro* chloroquine resistance detection method of Rieckmann *et al.* (*Am. J. Trop. Med. Hyg., 17:* 661-671, 1968). We are trying to employ the more supportive technique of Diggs *et al.* (*J. Parasit., 57:* 187-188, 1971) to circumvent some of the technical limitations of the Rieckmann method. However should the cultures prove successful we plan to maintain Rieckmann's criterion of inhibition of parasite maturation in the presence of chloroquine as evidence of drug resistance.

INVESTIGATIONS OF THE DEPARTMENT OF VIRAL AND RICKETTSIAL DISEASES

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	30 06 73	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
30 06 72		U		N/A	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY		3A062110A831		
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

Investigations of the Department of Viral and Rickettsial Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

Tropical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
10 72	9 73		

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: 10 72 EXPIRATION: 10 73
b. NUMBER: DADA17-73-G-9368
c. TYPE: Y. Grant d. AMOUNT: 263
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	1.0	30.7
FISCAL YEAR CURRENT 74	1.0	43.0

19. RESPONSIBLE DOD ORGANIZATION
NAME:
ADDRESS: US Army Medical Research Unit
Institute for Medical Research
Kuala Lumpur, Malaysia

RESPONSIBLE INDIVIDUAL
NAME: Dr. R. Bhagwan Singh, Director
TELEPHONE: Institute for Medical Research

21. GENERAL USE

20. PERFORMING ORGANIZATION
NAME:
ADDRESS: Institute for Medical Research
Kuala Lumpur, Malaysia

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Robinson, D.M., MAJ, VC
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Gan, E., B.A.
NAME:

22. KEYWORDS (Procede EACH with Security Classification Code) *Rickettsia tsutsugamushi*, Silvered leaf-monkey, *Pseudomonas pseudomallei*, Antigens, Fluorescent Antibody

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

23.(U) Technical Objectives: Define the incidence of disease in specific tropical habitats. Relate the incidence/risk of human infection to the occurrence of the organism in potential vectors and amplifying hosts. Relate strain differences to degree of virulence and host response.

24.(U) Approach: Isolation and antibody rates are determined for vectors, small mammals, and humans in specific tropical habitats. A spectrum of modern biological techniques are employed to study the antigens present in *R. tsutsugamushi* and *Ps. pseudomallei* and to compare the host's response to these antigens.

25.(U) Progress: The response of Silvered leaf-monkeys to several strains of *R. tsutsugamushi* has been studied. Identification of the source of mosquito blood meals by means of polyacrylamide gel electrophoresis has proven feasible. Several antigenic fractions have been prepared from *Ps. pseudomallei*. The laboratory is producing all the necessary reagents for the FA identification of *R. tsutsugamushi* strains and antibody. Preliminary attempts to purify *R. tsutsugamushi* have not been successful.

*Available to contractors upon originator's approval.

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STUDIES IN PROGRESS

Response of the Silvered Leaf-monkey to Challenge with Single Strains of *Rickettsia tsutsugamushi*

by

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Previous studies from this laboratory (Annual Reports 1970, 1971 and 1972) have investigated the utility of the Silvered leaf-monkey *(Presbytis cristatus)* (SLM) as an animal model for scrub typhus. However, almost all the data was derived from animals which had been given multiple strain inoculations. Early work had shown that the strains of *R. tsutsugamushi* were distantly related in that natural infection with one strain would not confer lasting protection against subsequent infection with all heterologous strains. Under these circumstances we felt the multiple inoculations did not reflect the natural challenge delivered by the chigger, and probably caused much greater clinical signs than occur in natural infections.

Based on these premises the response of SLM to the inoculation of graded doses of selected single strains of *R. tsutsugamushi* was studied.

MATERIALS AND METHODS

Rickettsia: The Karp, Gilliam, Kato, and TA 678 strains of *R. tsutsugamushi* were obtained from the Department of Rickettsial Diseases, WRAIR. All seed stocks were prepared as 20% infected yolk sac pools in sucrose - phosphate - glutamine (SPG).

Inoculation: Locally procured SLM were inoculated subcutaneously (SC) on the left thigh with preselected dilutions of rickettsia. A concurrent titration was conducted in white mice to determine the actually dose inoculated. Control monkeys were not inoculated.

Clinical Laboratory Studies: At specified intervals pre- and postinoculation the experimental and control SLM were temperatured and bloods were drawn for packed cell volumes (PCV) and total white blood cell counts (WBC).

Serological Tests: Antibody titers were determined by indirect fluorescent antibody techniques which have been described (USAMRU-M Annual Report 1971).

Rickettsial Isolations: Mice were inoculated with freshly drawn blood (prior to clotting) by the IP route. When sick or at 14 days, these mice were killed and a suspension of spleen-kidney tissue prepared in SPG with a Ten Broeck grinder. This suspension was

inoculated into additional mice. At 14 days postinoculation or when sick, tissues from these mice were passed into a third group. The third set of mice were given chloramphenicol in the drinking water for 21 days, rested for 5 to 7 days, and subsequently challenged with 100-1000 LD_{50} of the Karp strain.

RESULTS

Strain Parameters: Table 1 lists the strains of rickettsia with their passage histories and the dose delivered to the SLM. Because of decreases in titer during -70C storage the delivered doses varied from the anticipated doses (10^2, 10^4, 10^6 $MIPLD_{50}$). However, only in the case of the Kato strain was the titer greater than one log_{10} different than the anticipated titer.

Clinical Laboratory Results: Figures 1, 2 and 3 illustrate the clinical laboratory results from the monkeys inoculated with the three levels of the Karp strain. Monkey number 194 which was given the largest dose of Karp strain died on day 27 postinoculation. (The only other SLM which died was given an intermediate dose of the Gilliam strain.) The majority of the animals (4 of 6) had significantly depressed PCV, but no relationship could be detected between the severity of the anemia and the dose of rickettsia inoculated. Monkeys numbered 192, 193, and 194 were all febrile for two or more consecutive days during the observation period (these same animals developed eschars). Since these monkeys had been given the largest doses of organism the febrile response was dose dependent. The WBC varied widely and followed no pattern with dose inoculated.

Figures 4, 5, and 6 present the data from the 6 monkeys inoculated with the dilutions of the Kato strain. Again PCV were depressed in 4 of 6 of the animals, and the depression was not related to dose inoculated. Five of the 6 monkeys were febrile at some time during the 30 day observation period, but these febrile periods were of 1 to 3 days duration rather than being sustained over a longer period of time as were those produced by the Karp strain. Significant leucopenia occurred in the two animals given the smallest dose of rickettsia. While 4 of the 6 had swollen lymph nodes only two of the animals had eschars.

Figures 7, 8, and 9 present the clinical laboratory data from the SLM given the Gilliam strain. Monkey number 203, who was given $10^{4.3}$ $MIPLD_{50}$ of this strain, died on day 21 postinoculation. These monkeys appeared to be the sickest of any inoculated, and 5 of the 6 were febrile for four or more consecutive days during the observation period. In contrast to the other groups 4 of 6 exhibited a significant leucocytosis during the experimental period. PCV paralleled those of the previous groups in that 5 of 6 had significant decreases. The same five had eschars.

Figures 10, 11, and 12 present the data from SLM inoculated with the TA 678 strain. Monkeys 211 and 212 (Figure 12) had

Table 1

Parameters of the experimental *Rickettsia tsutsugamushi* Strains

Strain	Passages	Titer[1]	Inoculum dose[2]
Karp	YS[3]-45	$10^{7.4}$	$10^{1.4}$, $10^{3.4}$, $10^{5.4}$
Kato	YS-139	$10^{7.9}$	$10^{0.9}$, $10^{2.9}$, $10^{4.9}$
Gilliam	YS-163	$10^{7.3}$	$10^{2.3}$, $10^{4.3}$, $10^{6.3}$
TA-678	A[4]9/TC[5]1/YS-120	$10^{6.5}$	$10^{1.5}$, $10^{3.5}$, $10^{5.5}$

1. Mouse intraperitoneal median lethal doses per ml in seed stock.
2. Dose of rickettsia delivered in 0.1 ml
3. YS = yolk sac passage
4. A = animal passage
5. TC = tissue culture passage

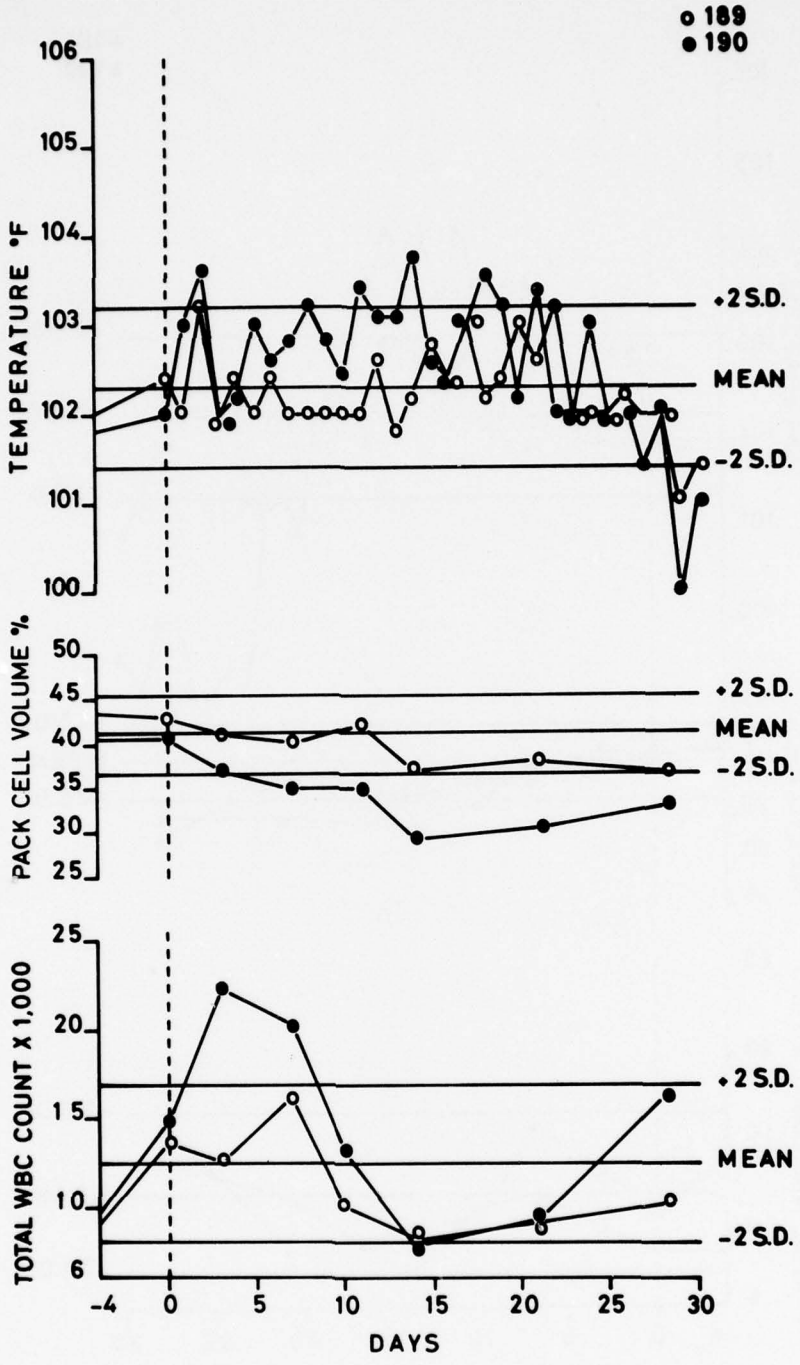


Figure 1. Response of Silvered leaf-monkeys to $10^{1.4}$ $MIPID_{50}$ of the Karp strain of *R. tsutsugamushi*.

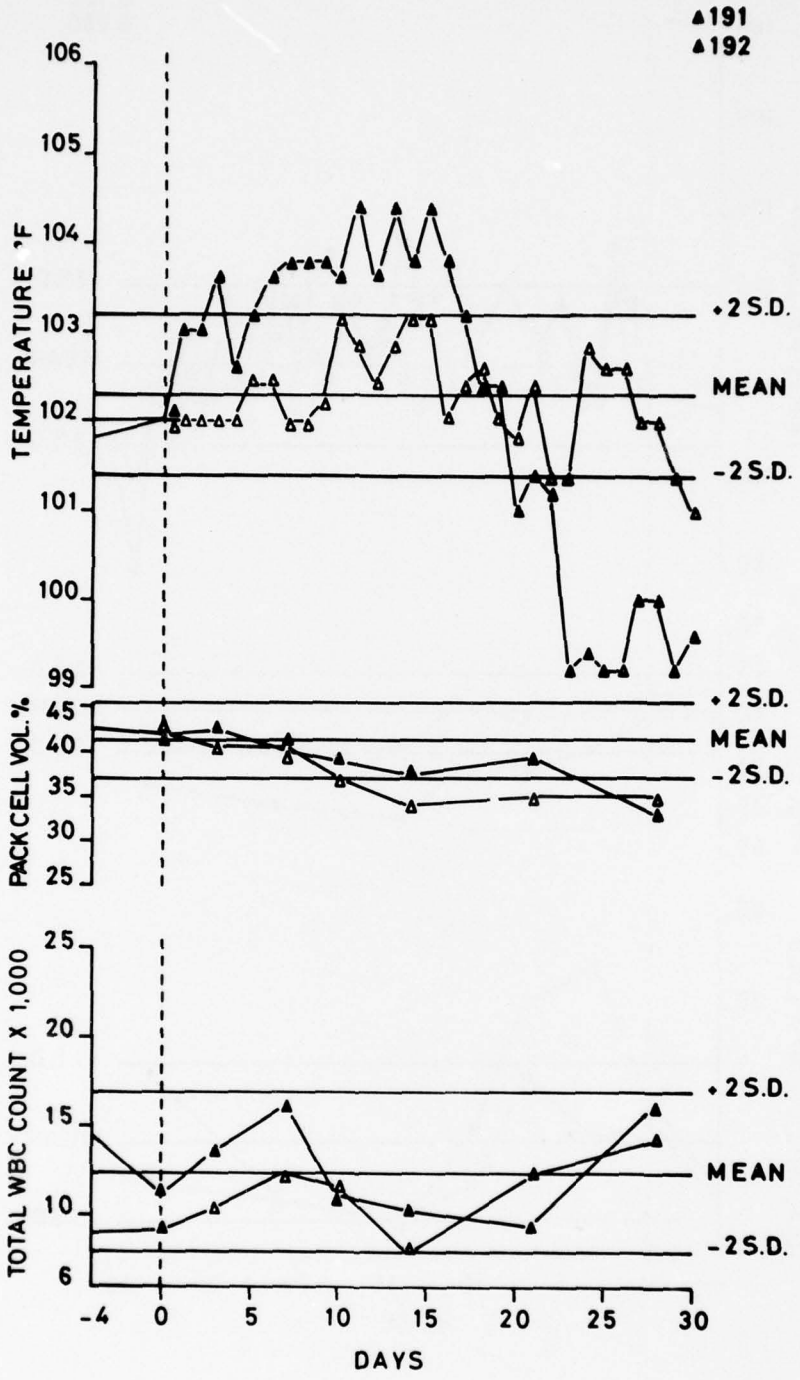


Figure 2. Response of Silvered leaf-monkeys to $10^{3.4}$ $MIPID_{50}$ of the Karp strain of *R. tsutsugamushi*.

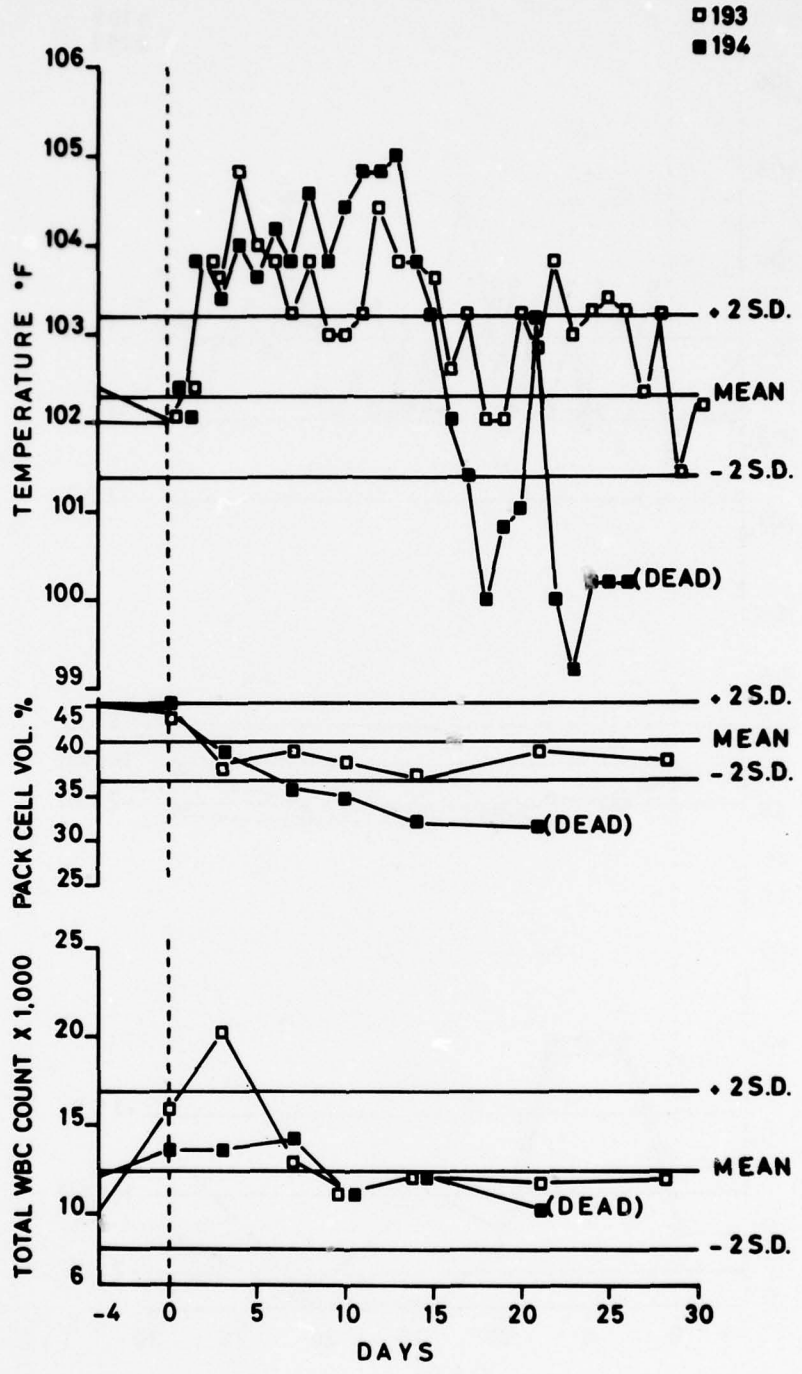


Figure 3. Response of Silvered leaf-monkeys to $10^{5.4}$ $MIPID_{50}$ of the Karp strain of *R. tsutsugamushi*.

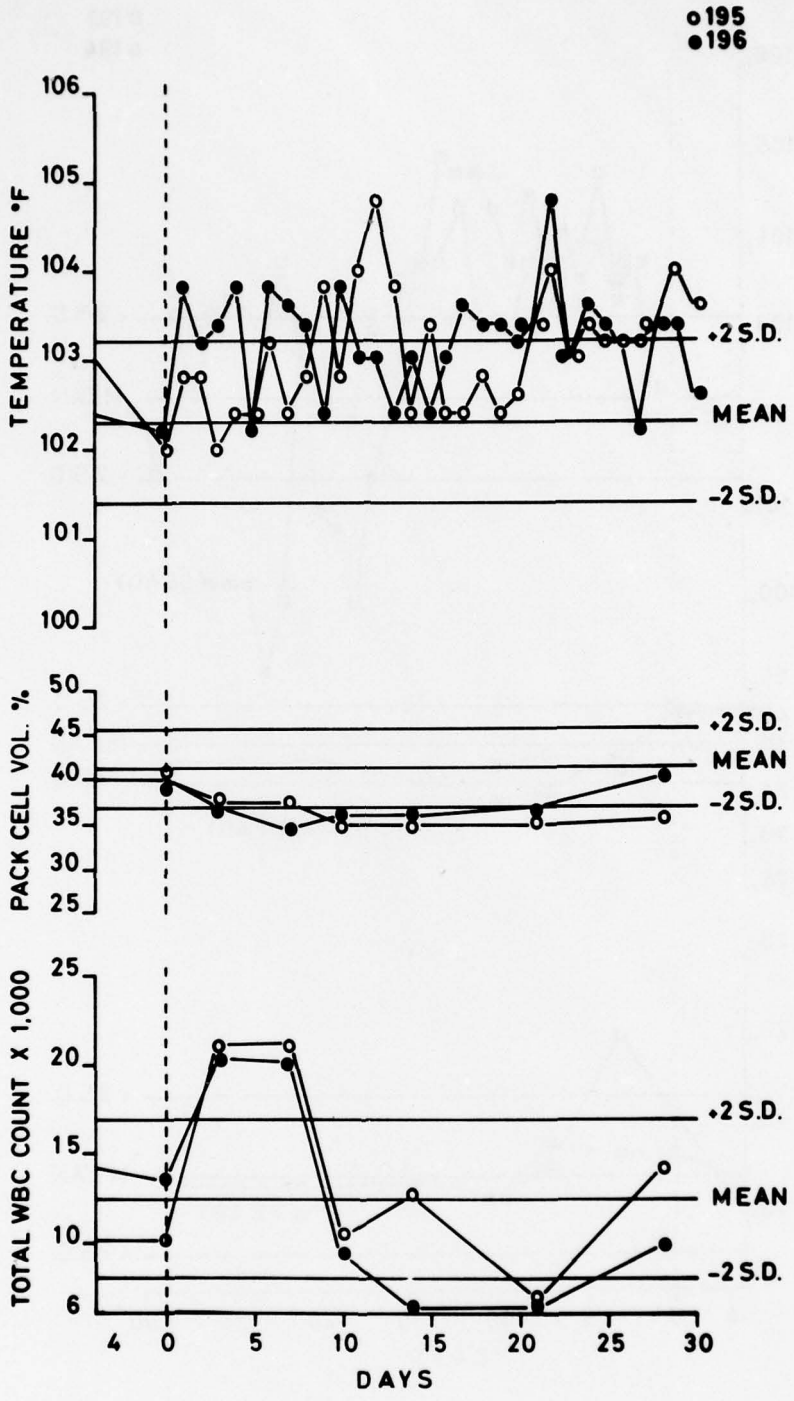


Figure 4. Response of Silvered leaf-monkeys to $10^{0.9}$ $MIPID_{50}$ of the Kato strain of *R. tsutsugamushi*.

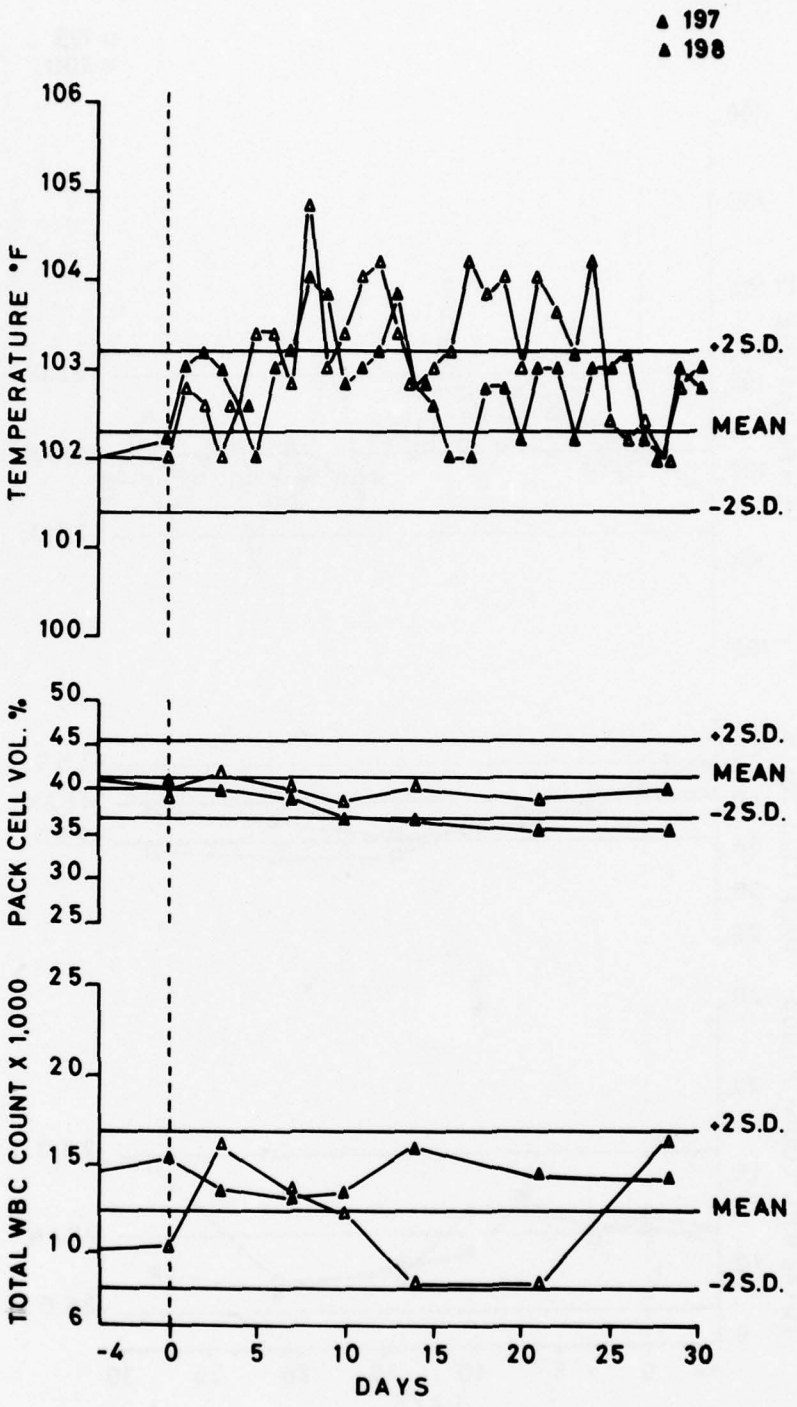


Figure 5. Response of Silvered leaf-monkeys to $10^{2.9}$ $MIPID_{50}$ of the Kato strain of *R. tsutsugamushi*.

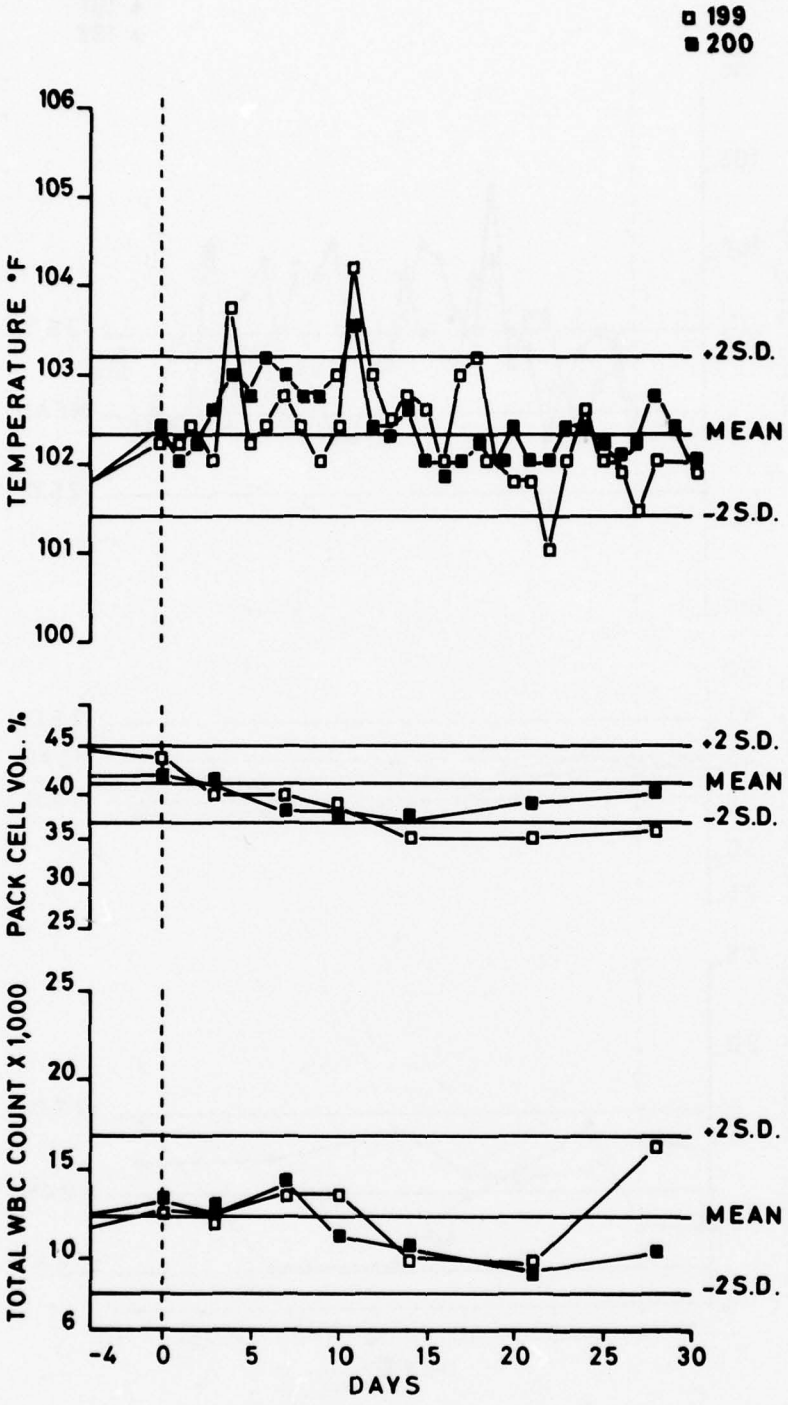


Figure 6. Response of Silvered leaf-monkeys to $10^{4.9}$ $MIPID_{50}$ of the Kato strain of *R. tsutsugamushi*.

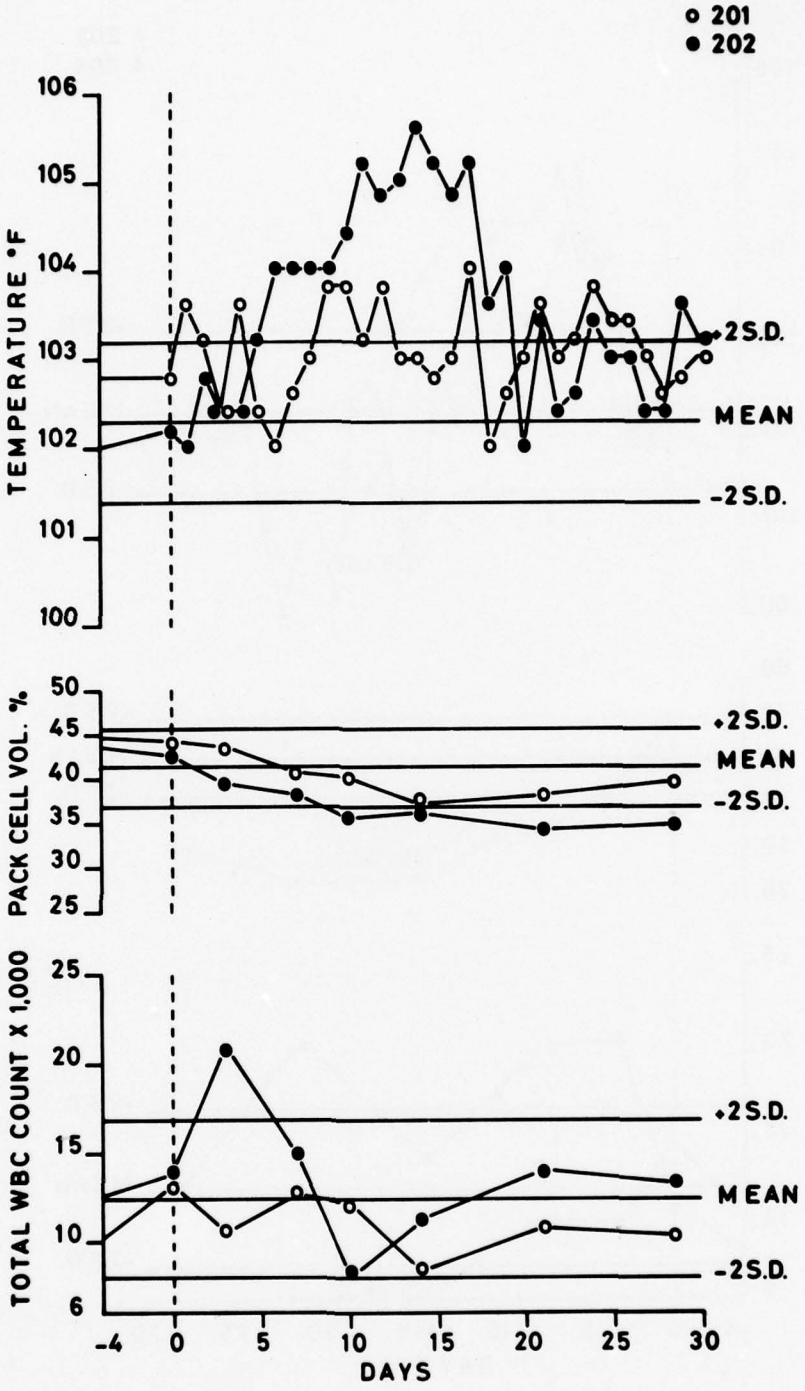


Figure 7. Response of Silvered leaf-monkeys to $10^{2.3}$ $MIPID_{50}$ of the Gilliam strain of *R. tsutsugamushi*.

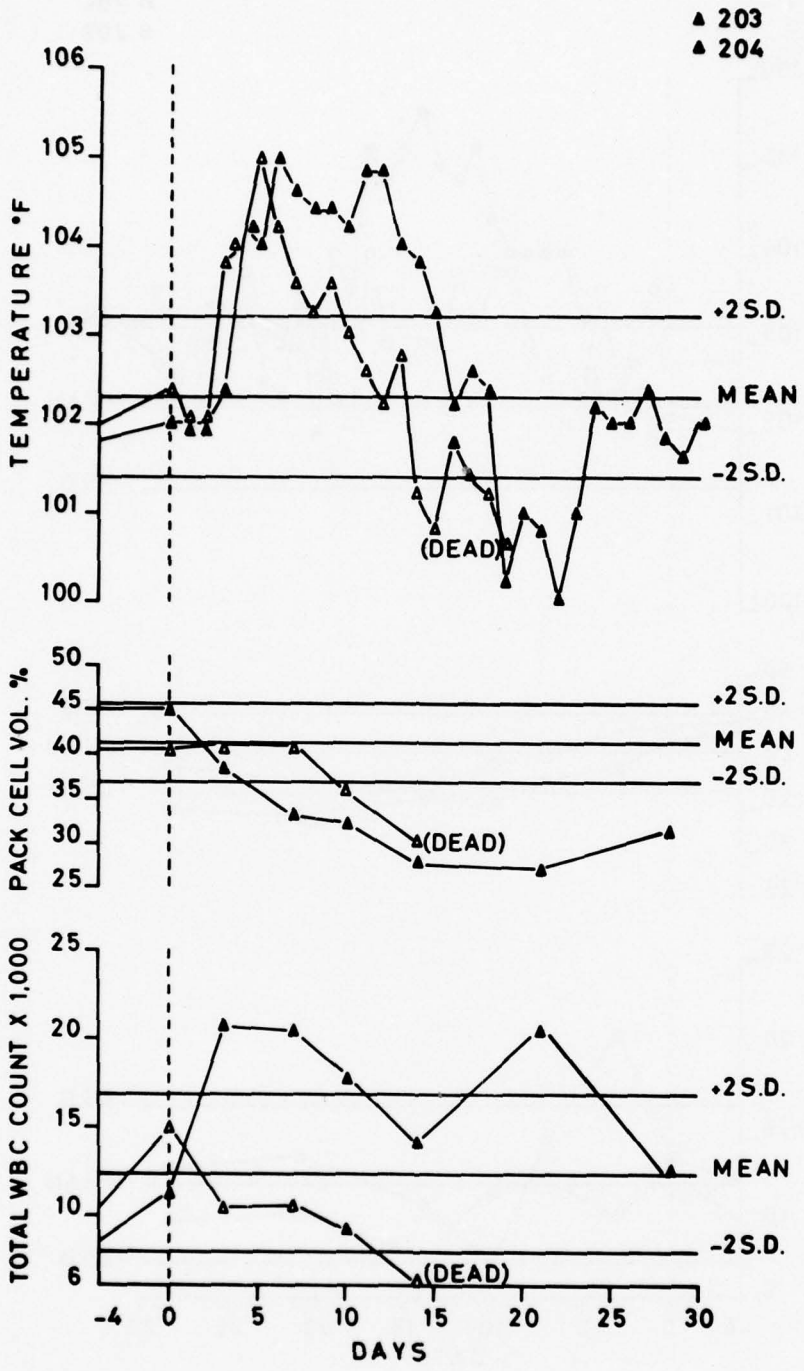


Figure 8. Response of Silvered leaf-monkeys to $10^{4.3}$ $MIPID_{50}$ of the Gilliam strain of *R. tsutsugamushi*.

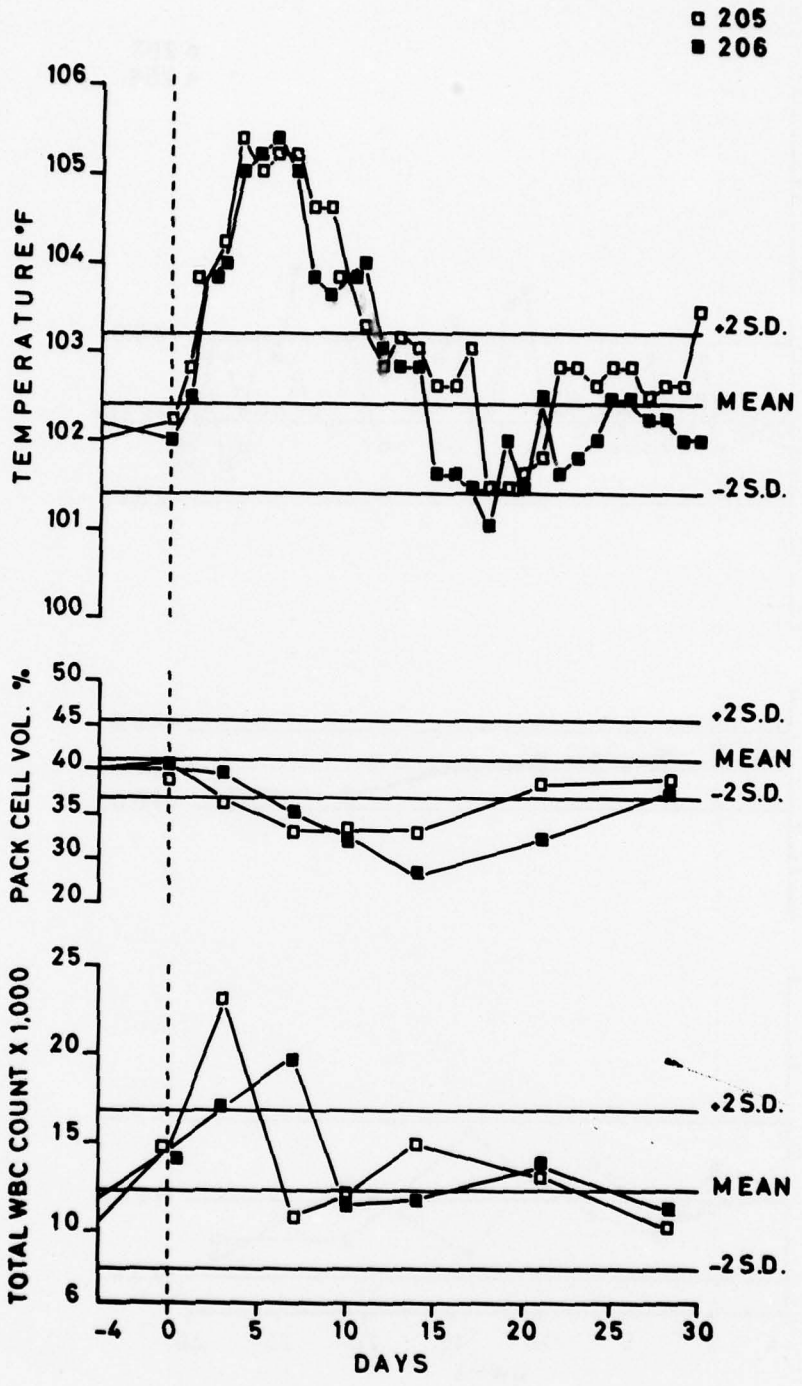


Figure 9. Response of Silvered leaf-monkeys to $10^{6.3}$ $MIPID_{50}$ of the Gilliam strain of *R. tsutsugamushi*.

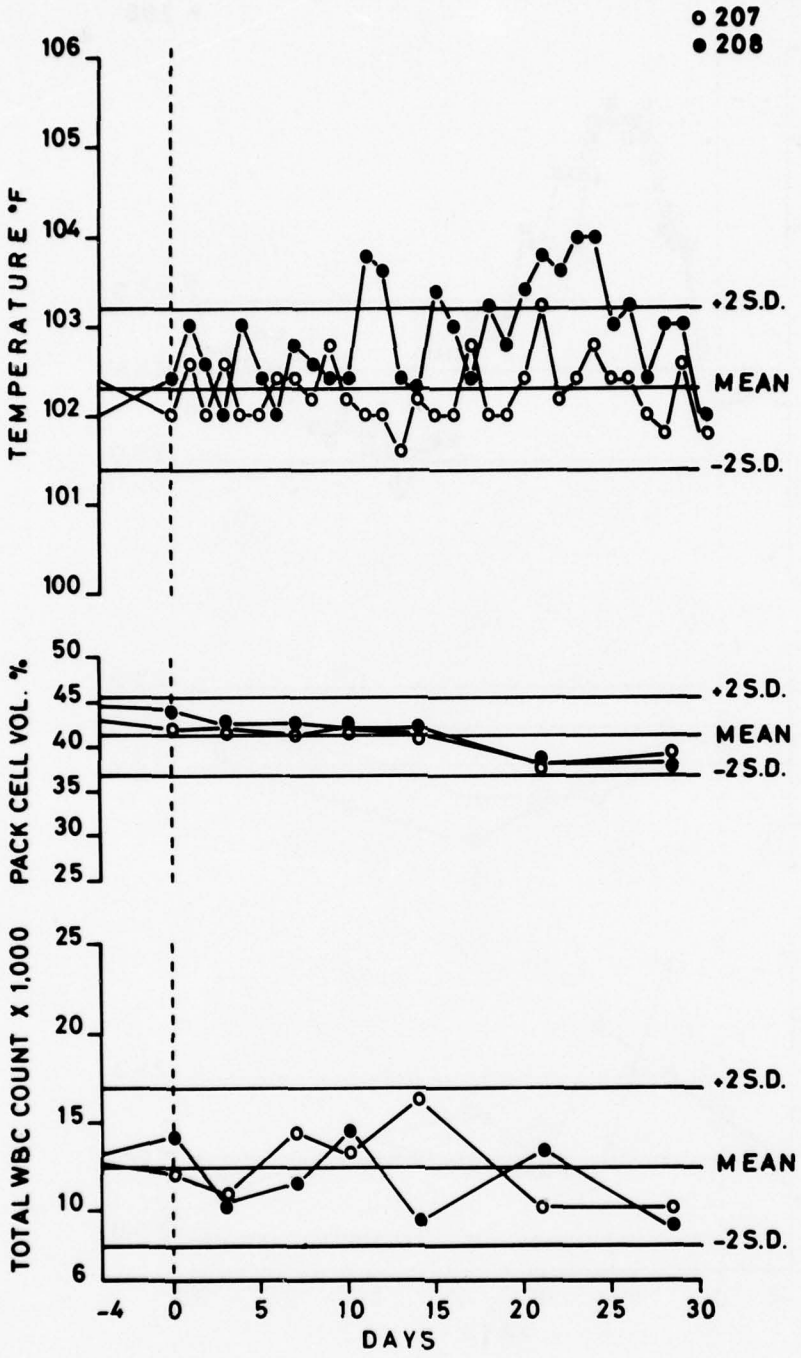


Figure 10. Response of Silvered leaf-monkeys to $10^{1.5}$ $MIPID_{50}$ of the TA 678 strain of *R. tsutsugamushi*.

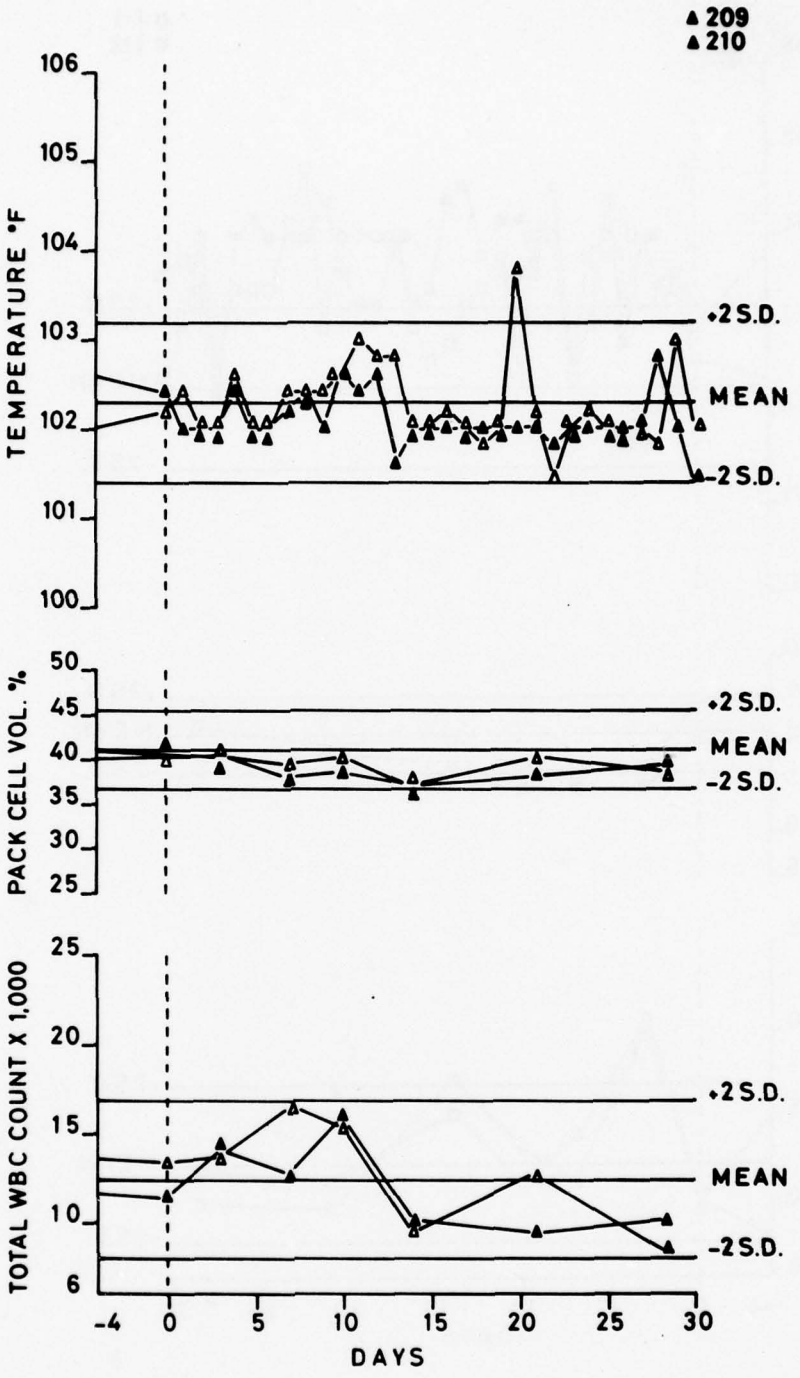


Figure 11. Response of Silvered leaf-monkeys to $10^{3.5}$ $MIPID_{50}$ of the TA 678 strain of *R. tsutsugamushi*.

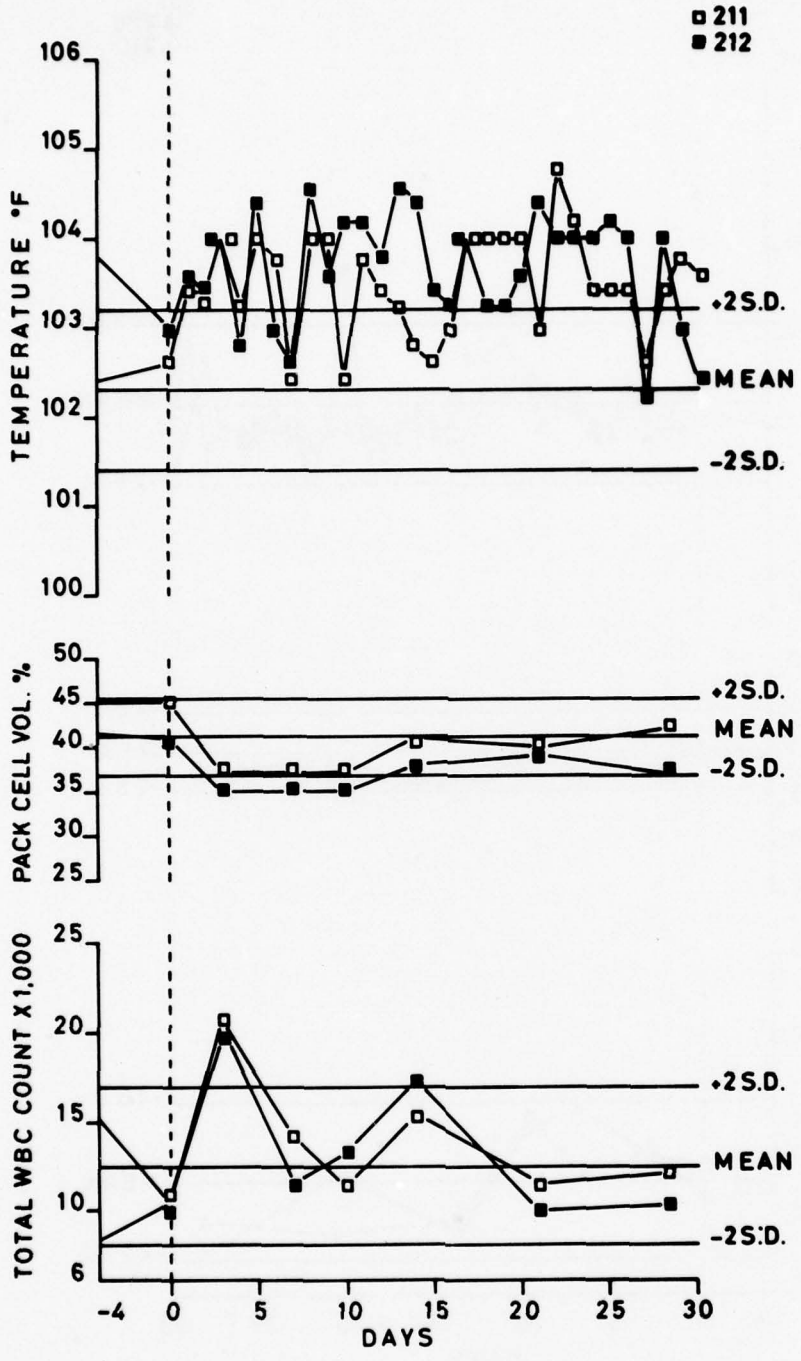


Figure 12. Response of Silvered leaf-monkeys to $10^{5.5}$ $MIPID_{50}$ of the TA 678 strain of *R. tsutsugamushi*.

significant febrile responses and leucopenias, and SLM 212 only had a significant PCV depression. As a group these monkeys were not visibly ill, and other than slightly swollen inguinal lymph nodes on the inoculated side appeared normal on physical examination.

Rickettsemias were detected in all but one of the animals (SLM-195 given $10^{0.9}$ MIPLD50 of Kato strain). Generally there was an agreement between dose of rickettsia inoculated and day of onset of rickettsemia (Table 2), and except in TA 678 in the duration of rickettsemia i.e. animals given larger doses tended to become rickettsemic early and remain rickettsemic longer than those given smaller doses (Table 2).

Serological Responses: All antibody was quantified by means of the indirect fluorescent antibody test with Karp, Kato, Gilliam, TA 686, TA 716, TC 586, TA 678, TA 763, and TH 1817 antigens prepared at WRAIR. Table 3 lists the results obtained when the sera were tested against the homologous strains. At 10 days postinoculation (not shown) 5 of 6 animals given dilutions of the Kato strain were positive at the 1/10 dilution, and 1 of the 5 SLM given the Karp strain was positive at that dilution. All of the remaining animals had no detectable antibody at a 1/10 dilution of sera. The Kato strain elicited antibody more rapidly than the other strains tested, and the titer appeared to wane more rapidly. Few differences were detected in peak titers.

Heterologous reactions were analogous to those observed at WRAIR. SLM inoculated with the Karp strain produced approximately the same level of antibody to the Kato strain. The reverse was not true i.e. SLM inoculated with Kato, with one exception, did not produce high levels of antibody to the Karp strain. SLM inoculated with the Gilliam strain produced broad heterotypic antibodies with especially high levels to the TC 586 strain. Antibodies were not produced by any of the previously described SLM to the TA 678 strain which elicited significant levels of antibody only to itself. These data corraborate those of the Department of Rickettsial Diseases, WRAIR on the heterotypic antibody responses following infection of rabbits with these strains.

SUMMARY TO DATE

A compilation of the pertinent data derived from this experiemnt is presented in Table 4. There are no good parameters to assess severity of infection. Over the 4 $\log_{10}$ dose range encompassed by this experiment the response of the SLM is certainly unpredictable. The lack of any detectable hematologic or febrile response in several animals questions the utility of the SLM as an animal model for the assay of vaccines.

Conversely, the incidence of subclinical scrub typhus in human populations is much greater than has been appreciated. Serological surveys conducted here have found that 73% of certain groups of aborigines have scrub typhus antibody (Annual Report 1971 page 135). It is possible that the response of the SLM mimics the response of

Table 2

Rickettsemias in silvered leaf-monkeys following inoculation of *R. tsutsugamushi* strains

Animal No.	Strain	Inoculums ($MIPID_{50}$)	Rickettsemia on Indicated Day Following Infection									
			-4	3	7	10	14	21	28	35	42	59
189	Karp	$10^{1.4}$	0	0	0	0	+	0	0	0	0	
190	Karp	$10^{1.4}$	0	0	0	+	+	+	+	0	0	
191	Karp	$10^{3.4}$	0	0	+	+	+	0	0	0	0	
192	Karp	$10^{3.4}$	0	0	+	+	+	+	+	+	0	
193	Karp	$10^{5.4}$	0	0	+	+	+	+	0	0	0	
194	Karp	$10^{5.4}$	0	0	+	+	+	+	a			
195	Kato	$10^{0.9}$	0	0	0	0	0	0	0	0	0	
196	Kato	$10^{0.9}$	0	0	0	0	0	+	+	0	0	
197	Kato	$10^{2.9}$	0	0	0	0	+	+	+	0	0	
198	Kato	$10^{2.9}$	0	0	0	+	+	+	0	0	0	
199	Kato	$10^{4.9}$	0	0	+	+	+	+	0	0	0	
200	Kato	$10^{4.9}$	0	0	+	+	+	0	0	0	0	
201	Gilliam	$10^{2.3}$	0	0	0	0	+	0	0	0	0	
202	Gilliam	$10^{2.3}$	0	0	+	+	+	+	0	0	0	
203	Gilliam	$10^{4.3}$	0	0	+	+	+	a				
204	Gilliam	$10^{4.3}$	0	0	+	+	+	+	0	0	0	
205	Gilliam	$10^{6.3}$	0	+	+	+	+	0	0	0	0	
206	Gilliam	$10^{6.3}$	0	+	+	+	+	0	0	0	0	
207	TA 678	$10^{1.5}$	0	0	0	0	0	+	+	0	0	
208	TA 678	$10^{1.5}$	0	0	0	0	0	+	0	0	0	
209	TA 678	$10^{3.5}$	0	0	0	+	+	0	0	0	0	
210	TA 678	$10^{3.5}$	0	0	0	0	+	+	0	0	0	
211	TA 678	$10^{5.5}$	0	0	0	+	+	0	0	0	0	
212	TA 678	$10^{5.5}$	0	0	0	+	+	0	0	0	0	

a. SLM died prior to this sampling time.

Table 3

Homologous antibody responses following initial inoculation with selected strains of *R. tsutsugamushi*

Strain	Animal No.	Dose	Geometric Mean Antibody Response on Indicated Day Postinoculation						
			14	21	28	35	42	150	210
Karp	189, 190	10	10	40	60	80	80	80	40
	191, 192	10	20	40	80	80	40	40	80
	193	10	10	40	40	40	80	80	40
Kato	195-196	10	20	80	80	80	60	40	10
	197-198	10	60	80	80	80	80	80	30
	199-200	10	40	80	80	80	80	80	20
Gilliam	201-202	10	<10	30	40	120	120	40	30
	204	10	<10	20	80	80	80	40	40
	205-206	10	<10	40	80	80	80	20	60
TA 678	207-208	10	<10	20	60	60	60	10	15
	209-210	10	<10	30	40	80	60	30	30
	211-212	10	<10	30	40	40	40	20	15

All titers are reciprocals of highest dilutions showing fluorescence with the homologous strain.

Table 4

Parameters of *Rickettsia tsutsugamushi* infection in silvered leaf-monkeys

Monkey Number	Strain	Dose ($MIPLD_{50}$)	Duration of Fever (Days)	Rickettsemia	Peak Antibody Titer	Eschar	Lymphadenopathy	Significant Changes in		Death
								PCV	WBC	
189	Karp	$10^{1.4}$	0	+	80	0	0	0	0	0
190	"	$10^{1.4}$	5	+	80	0	0	+	+(I,D)[1]	0
191	"	$10^{3.4}$	0	+	80	0	0	+	0	0
192	"	$10^{3.4}$	12	+	80	+	+	+	0	0
193	"	$10^{5.4}$	9	+	80	+	+	0	+(I)[2]	0
194	"	$10^{5.4}$	13	+	NA[4]	+	+	+	0	+
195	Kato	$10^{0.9}$	10	0	80	0	0	+	+(I,D)	0
196	"	$10^{0.9}$	16	+	80	0	0	+	+(I,D)	0
197	"	$10^{2.9}$	13	+	80	+	+	0	0	0
198	"	$10^{2.9}$	3	+	80	0	+	+	0	0
199	"	$10^{4.9}$	2	+	80	+	+	+	0	0
200	"	$10^{4.9}$	1	+	80	0	+	0	0	0
201	Gilliam	$10^{2.3}$	10	+	80	0	0	0	0	0
202	"	$10^{2.3}$	17	+	160	+	+	+	+(I)	0
203	"	$10^{4.3}$	6	+	NA	+	+	+	+(D)[3]	+
204	"	$10^{4.3}$	11	+	80	+	+	+	+(I)	0
205	"	$10^{6.3}$	10	+	80	+	+	+	+(I)	0
206	"	$10^{6.3}$	10	+	80	+	+	+	+(I)	0
207	TA 678	$10^{1.5}$	0	+	80	0	0	0	0	0
208	"	$10^{1.5}$	8	+	40	0	0	0	0	0
209	"	$10^{3.5}$	1	+	80	0	0	0	0	0
210	"	$10^{3.5}$	0	+	80	0	0	0	0	0
211	"	$10^{5.5}$	22	+	80	0	+	0	+(I)	0
212	"	$10^{5.5}$	24	+	20	0	+	+	+(I)	0

1. Increase in WBC count followed by a decrease at a subsequent time period.
2. Increase in WBC count.
3. Decrease in WBC count.
4. Not applicable - animal died.

humans to scrub typhus. i.e. That in both cases clinical scrub fever followed by death is a rare event.

DISCUSSION

Our results with the inoculation of SLM with *R. tsutsugamushi* strains have been at variance with those reported by prior investigators in this unit (see USAMRU-M Annual Reports 1971 and 1972). Profound decreases in PCV have been reported along with deaths in a much higher percentage of animals than we found. In this experiment deaths were 2 of 24 inoculated and could not be related to strain or dose. All but one of the monkeys were rickettsemic at the sampling periods, and all of them converted serologically.

The Silvered leaf-monkey is not an ideal model for acute scrub typhus infection, because of absence of, or when present, a lack of uniform clinical signs. This and the resistance of the monkey to doses 6 $\log_{10}$ larger than those required to kill a mouse indicates that the SLM has little utility over mice in a program designed to study the efficacy of vaccines.

We anticipate using the SLM to study the immunological response of experienced animals to homologous and heterologous challenges. Based on relationships determined by FA (WRAIR Annual Reports 1971, 1972 and 1973) homologous, closely related and unrelated strains will be inoculated into the SLM after their titers have decreased 8 fold or to $< 1/10$. Blood will be drawn at preselected intervals and sera fractionated on G-200 columns. The results will be examined to determine if the response to reinfection produces an anamnestic type antibody response, and to determine the differences, if any, between the responses to the three different types of challenge.

Identification of Mosquito Blood Meals by Polyacrylamide Gel Electrophoresis

by

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A simple precise method of determining that individual mosquitoes collected during field projects have or have not fed on humans would be a useful parameter in explaining the incidence of frank disease and/or antibody conversion in human populations.

The methods presently available to determine the origin of blood meals are of two types 1) serological tests which rely on the reaction of the blood meal components with antisera of specific reactivity and 2) the hemoglobin crystallization test which relies on the ability of the investigator to microscopically identify the crystals formed by extracted hemoglobins.

The serological procedures described include precipitation, Ouchterlony gel diffusion, hemagglutination inhibition, passive hemagglutination inhibition, and fluorescent antibody techniques. They have been employed to detect serum proteins, serum albumins, or red blood cell stroma. All require the production of specific antisera of various species. The precipitin test is the simplest but is unreliable after the mosquitoes have been engorged for 18 hours. The hemagglutination techniques are complicated, but when conducted properly yield data as to the species of host of the mosquito. These techniques require the production and testing of monospecific sera. Our requirements were not this stringent as we wished only to determine human feedings.

The hemoglobin crystallization technique did not prove reproducible in our hands and was cumbersome with extractions and centrifugations. Additionally, it was not possible to distinguish between human and monkey blood meals with this technique.

Several investigators have studied mosquitoes by electrophoresis in polyacrylamide gel as an aid to taxonomic studies. Since these investigators saw reproducible differences between species, we hypothesized that reproducible difference would also occur within the same species of mosquito when blood meals were taken from different hosts.

MATERIALS AND METHODS

The mosquitoes were obtained either from our established colonies or from man-biting collections. Colony mosquitoes were

examined both unfed and after being allowed to feed on a variety of hosts.

Mosquitoes were prepared for electrophoresis by placing individuals into 0.2 to 0.5 ml of distilled water and grinding them with a hand operated glass tissue grinder. A microhematocrit tube was filled from the emulsified mosquito sample and a 2 cm length of fluid (calculated to be 0.02 ml) was applied to the gel column.

For transit and storage, gut tissue was allowed to fill a microhematocrit tube. In the laboratory the contents of the tube were expelled into a tissue grinder containing 0.2 ml of distilled water and further processed as described above.

Blood and tissue spots were prepared by placing a drop of gut tissue or crushing the whole mosquito on Whatman number 3 filter paper. A 3 mm circle was removed with a number 1 cork borer and placed in 0.2 ml of distilled water in a tissue grinder. The pestle was used to extract the material from the filter paper, and samples were further processed as above.

Gel columns were prepared in 6 x 75 mm tubes which has been acid washed, rinsed, and treated with a silicon solution.

The 7.5% separating gel was polymerized by the incorporation of ammonium persulfate and the stacking gel was photopolymerized. The sample was applied directly to the surface of the stacking gel, and buffer was carefully layered on top. No loading gel was used. The gels were then electrophoresed at 5mA per tube until the bromphenol blue tracking dye was within 0.5 to 1.0 cm of the end of the tube.

The gels were removed from the tubes and, the bands were fixed by immersing the gels in 12.5% trichloroacetic acid for 30 min with constant agitation. For staining, the fixative solution was replaced with 12.5% trichloroacetic acid containing 0.05% Coomassie brilliant blue. After a 1 hr stationary staining period the gels were rinsed several times with 7% acetic acid to remove the excess stain and stored. Electrophoretic destaining was not required.

RESULTS

The first figure represents bands found following electrophoresis of unfed and human fed *Aedes aegypti* mosquitoes from the colony.

By comparison of the bands produced by electrophoresis of the unfed mosquitoes and mosquitoes following human feeding with published reports of gel electrophoresis of human sera the following conclusions were drawn. The first band (lower band in photograph) is mosquito in origin, the next band is human serum albumin which is heavy enough to mask a mosquito band which occurs in the same area. The next band(s) (which usually were present as a pair of bands, but the relative proportion of the two components varied) were identified as transferrins and the uppermost series of bands represented a range of heavier serum proteins.

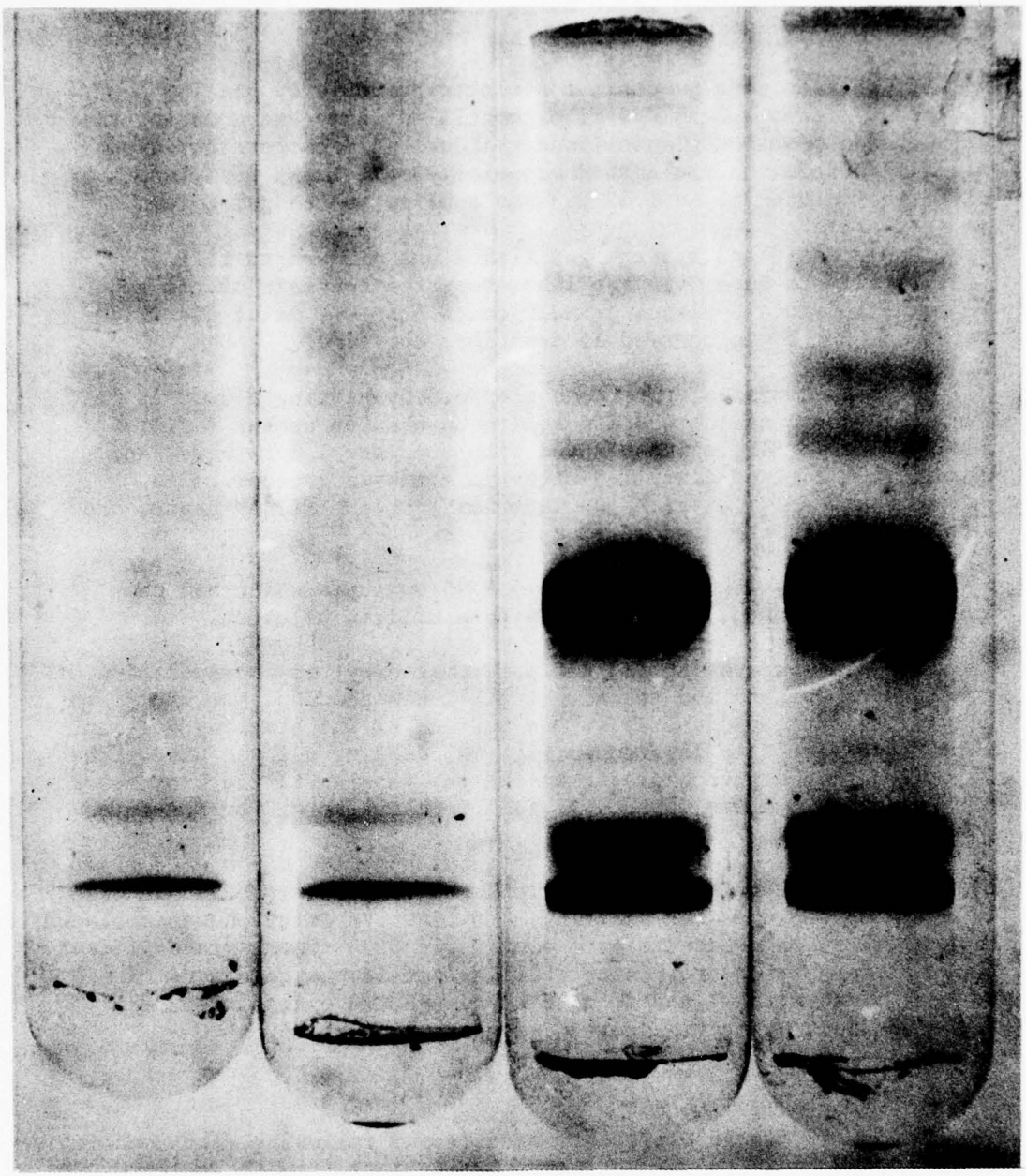


Figure 1. Comparison of Bands Produced by the Electrophoresis of *Aedes aegypti* Unfed and Following a Human Feeding.

This is a reasonable and expected distribution, since generally the migration of any protein into the gel is inversely related to the logarithm of the molecular weight. The molecular weight of human serum albumin, which is homogenous, is 69,000; and the molecular weight of transferrin, which has been reported to be heterogenous, is 88,000. The observed relationship between the migration of these two marker proteins indicate that the top of the gel should contain bands of protein of approximately 120,000 molecular weight. This is well below the molecular weight of macroglobulins and other larger components of sera.

Figure 2 represents replicate samples of fed and unfed *Culex fatigans* mosquitoes. The distance between the two prominent bands in the unfed mosquitoes is much greater than in the previous slide which represented *Aedes aegypti* mosquitoes. Assessing the gels from the samples of fed mosquitoes, the lightest molecular weight (fastest migrating) protein band was mosquito tissue, the next band was human serum albumin, and an additional band now appeared which was a second mosquito tissue band. The next bands which were overloaded, were transferrins. The usual assortment of bands of larger molecular weight material was present above the transferrin bands.

Figure 3 presents the results of the electrophoresis of human fed and unfed *Anopheles maculatus*. The bands are directly comparable with those found in the previous two species. There appeared to be more proteinaceous material immediately above the main transferrin band, but the relationship of the main transferrin band to the main albumin band remained unchanged.

Since human blood meals in these three species resembled each other so closely, the appearance of human blood meals in a range of mosquito species was investigated. Figure 4 illustrates that the species of mosquito did not appreciable affect the location or intensity of the albumin or transferrin bands. In some species additional bands were seen which were later proven to be contributed by the mosquitoes (not shown).

All of the previous work had been conducted on mosquitoes which had been allowed to feed uninterrupted on human volunteers. In wild caught mosquitoes a significant proportion would be expected to have interrupted feedings. Under these circumstances the sensitivity of the assay system becomes important. The electrophoresis of serial 2-fold dilutions of the macerated mosquito is illustrated in Figure 5. The location of the bands did not change. They became less distinct at higher dilutions until at the 1/64 dilution they were no longer discernible. Several repetitions of this experiment were conducted and characteristic patterns were always evident at the 1/16 dilution of the mosquito suspension. In the reverse of this experiment when 10 fed mosquitoes were processed at one time no differences were found in the locations or relationships of the bands although all of them were greatly overloaded.

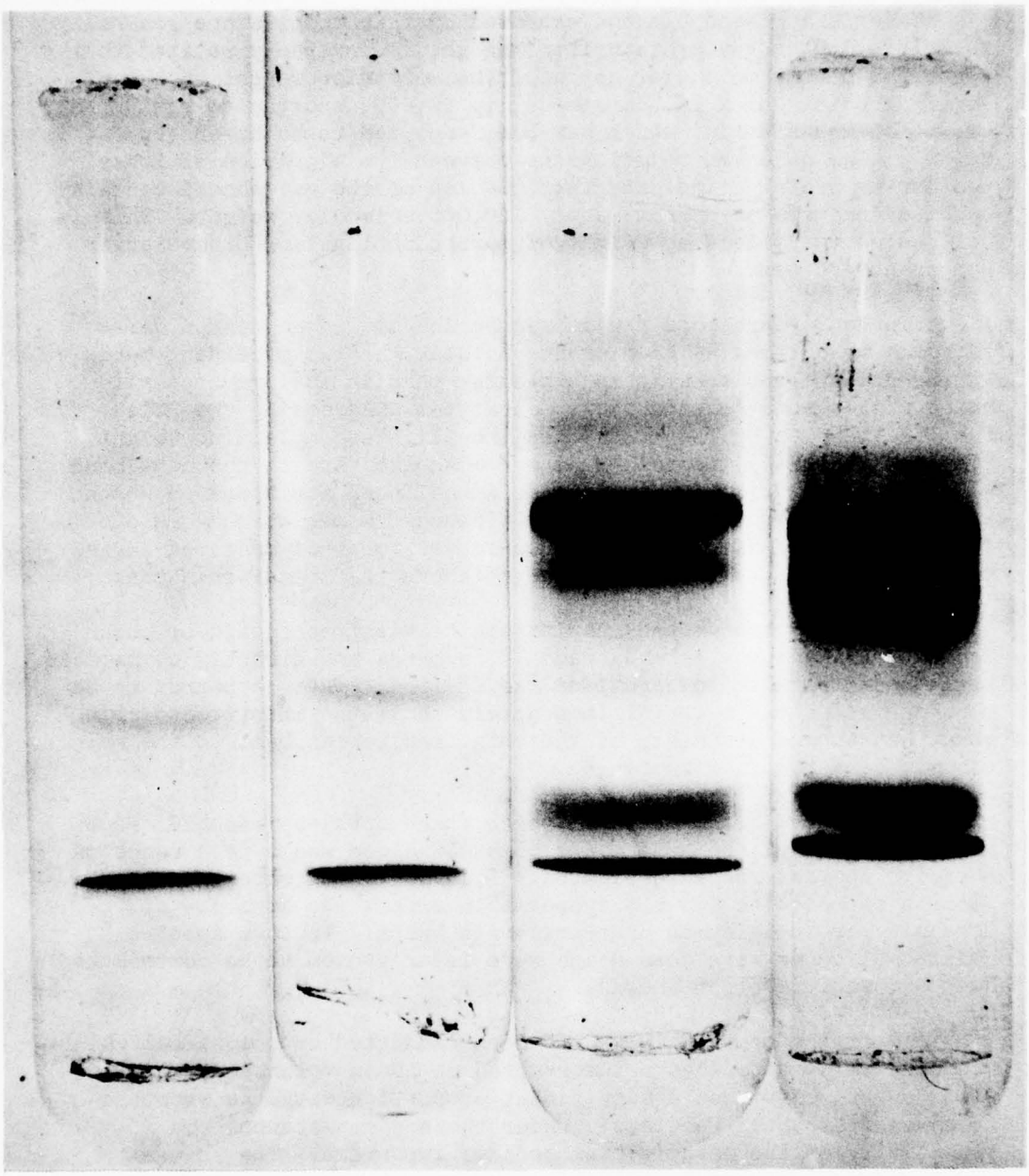


Figure 2. Comparison of Bands Produced by the Electrophoresis of *Culex fatigans* Unfed and Following a Human Feeding.

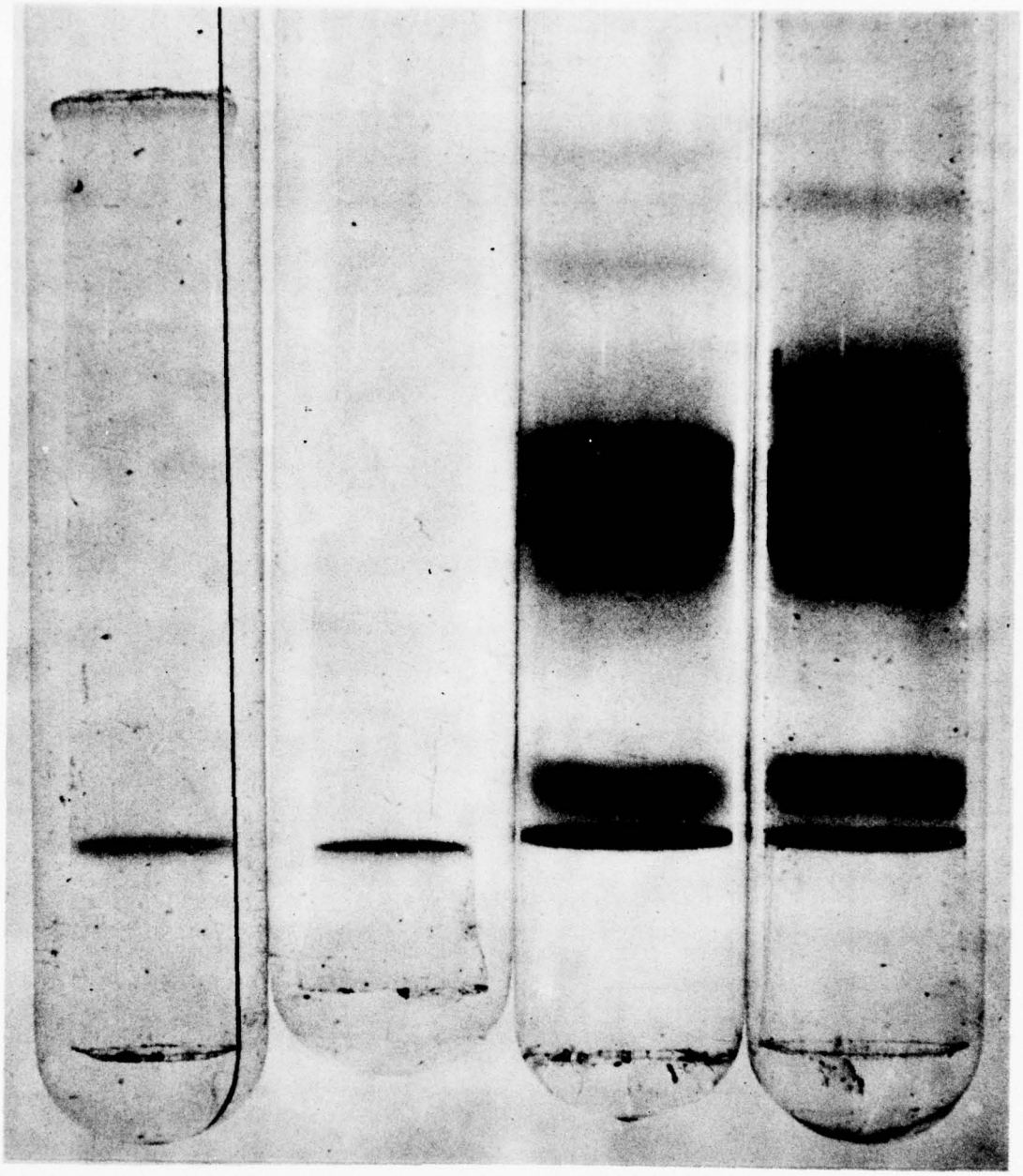


Figure 3. Comparison of Bands Produced by the Electrophoresis of *Anopheles maculatus* Unfed and Following a Human Feeding.

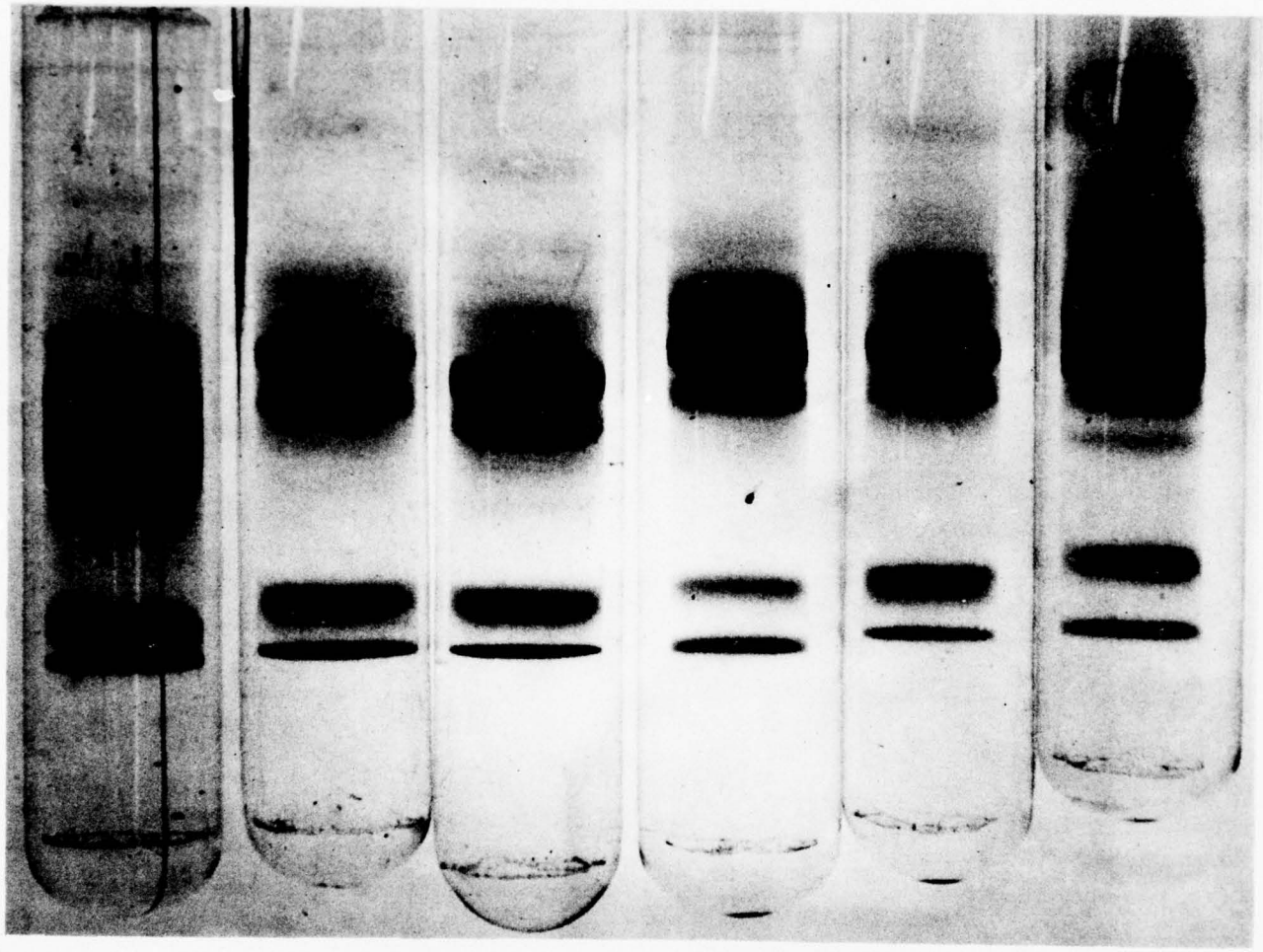


Figure 4. Gel Electrophoresis of Several Common Malayan Mosquitoes Following Human Feeding.

a. *Culex fatigans*, b. *Anopheles maculatus*, c. *Aedes aegypti*, d. *Anopheles barbirostris*, e. *Heizmannia aureschaeta*, f. *Armigeres malayi*.

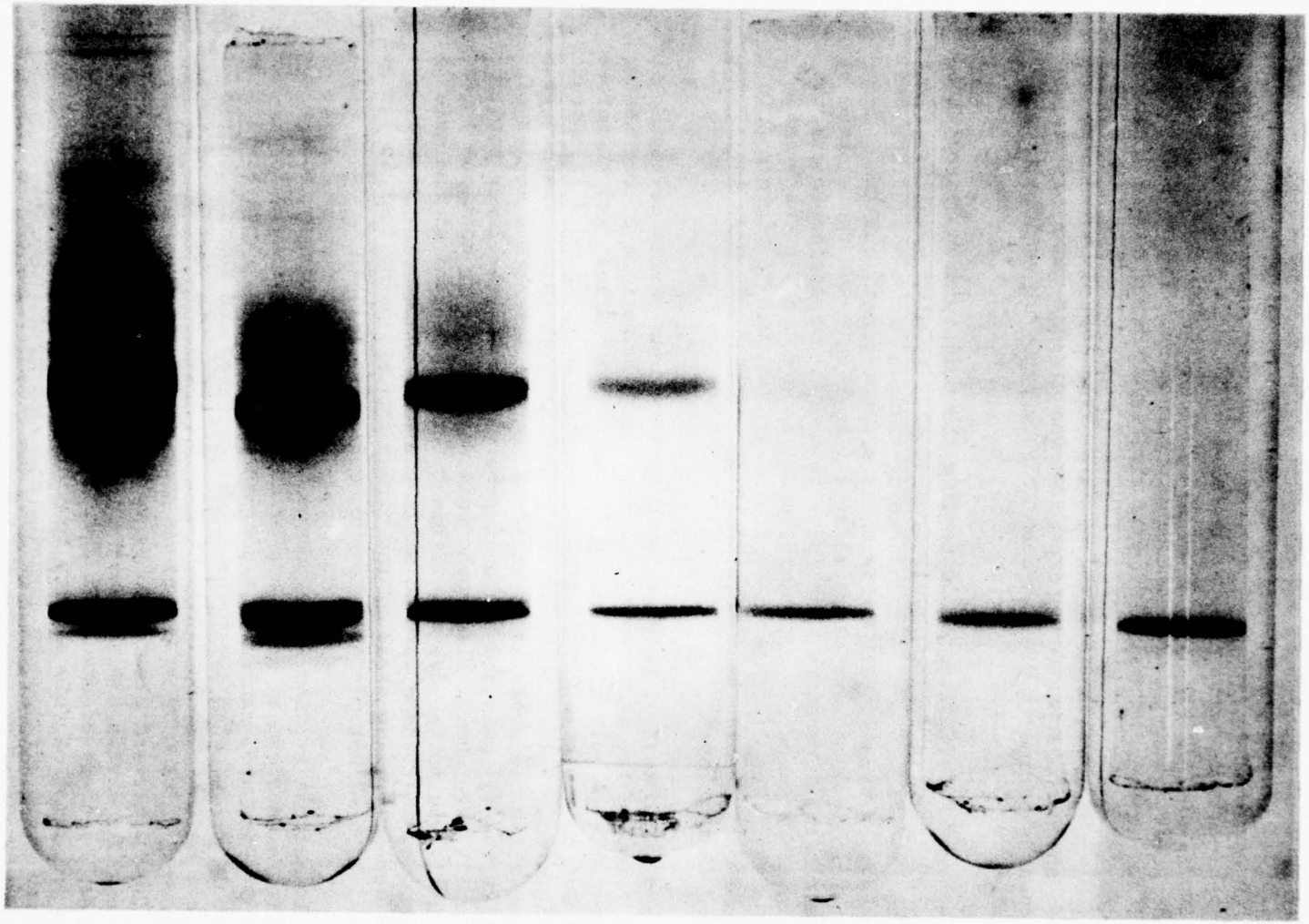


Figure 5. Effect of Dilution of the Macerated Mosquito on the Formation of Bands.

The precipitin test is not reliable after 18 hours post-ingestion of the blood meal. The presence of a cloudy precipitate throughout the mosquito sample interfers with reading. The time sensitivity of gel electrophoresis is presented in Figures 6 and 7. In this experiment mosquitoes were allowed to engorge and then killed by freezing at various predetermined times following engorgement. Figure 6 represents an experiment which shows visible bands up to and including 24 hours following a human feed. Figure 7 represents a guinea pig experiment which yielded results at later time periods. The time related phenomena appeared to depend on the size of the blood meal. Subsequent experiments yielded identifiable bands at various time periods after 24 hours.

We were very interested in testing the ability of polyacryamide gel to differentiate blood meals from closely related species. Figure 8 shows the results of electrophoresing material from mosquitoes fed on monkeys or humans. The differences are not great, but the transferrin from monkeys migrates at a faster rate and the albumin at a slower rate. Therefore, the space between the transferrin and the albumin in the monkey blood meals is not as great as in the human blood meals. The distance the proteins are allowed to migrate into the tube does change these relationships. Additional work is planned in this area.

The usefulness of polyacrylamide gel resides partly in the ability to prepare gels having different concentrations of reactants. This results in gels which have different "pore" sizes; and therefore different characteristics of protein separation. Figure 9 illustrates the migration of identical samples in 10% and $7\frac{1}{2}$% gels. The 10% gels did not allow the proteins to separate into as discrete a series of bands as the $7\frac{1}{2}$% gels and no bands were visible in the lower portion of the 10% gel tubes. Electrophoresis was allowed to continue in each case until the marker dye was within 0.5 cm of the end of the gel. This proved that there were no small pre-albumin proteins present which were migrating ahead of the marker dye and being lost in $7\frac{1}{2}$% gel tubes. We have not yet used less concentrated gels in these studies.

In malaria studies it is common to remove gut tissue from mosquitoes, suspend it in a drop of saline, and examine it for oocysts. The utilization of a portion of this drop in polyacrylamide gel would allow us to determine infection rates and human feedings on the same series of mosquitoes. Figure 10 shows the results of diluting 0.01 to 0.02 ml of the emulsified drop in 0.1 or 0.2 ml of distilled water and electrophoresing the resultant sample. The bands were equally distinct with those obtained with whole mosquitoes. Nor did the locations of the bands and their relative intensities change. As an extension of this ability to field situations, the utilization of blood spots obtained by crushing engorged mosquitoes on, or applying one drop of gut tissue to Whatman number 3 filter paper was studied. Figure 11 presents the results of the electrophoresis of these dried specimens. The albumin and transferrin bands were in their normal relationship and traces of the heavier molecular weight components of sera were occasionally present.

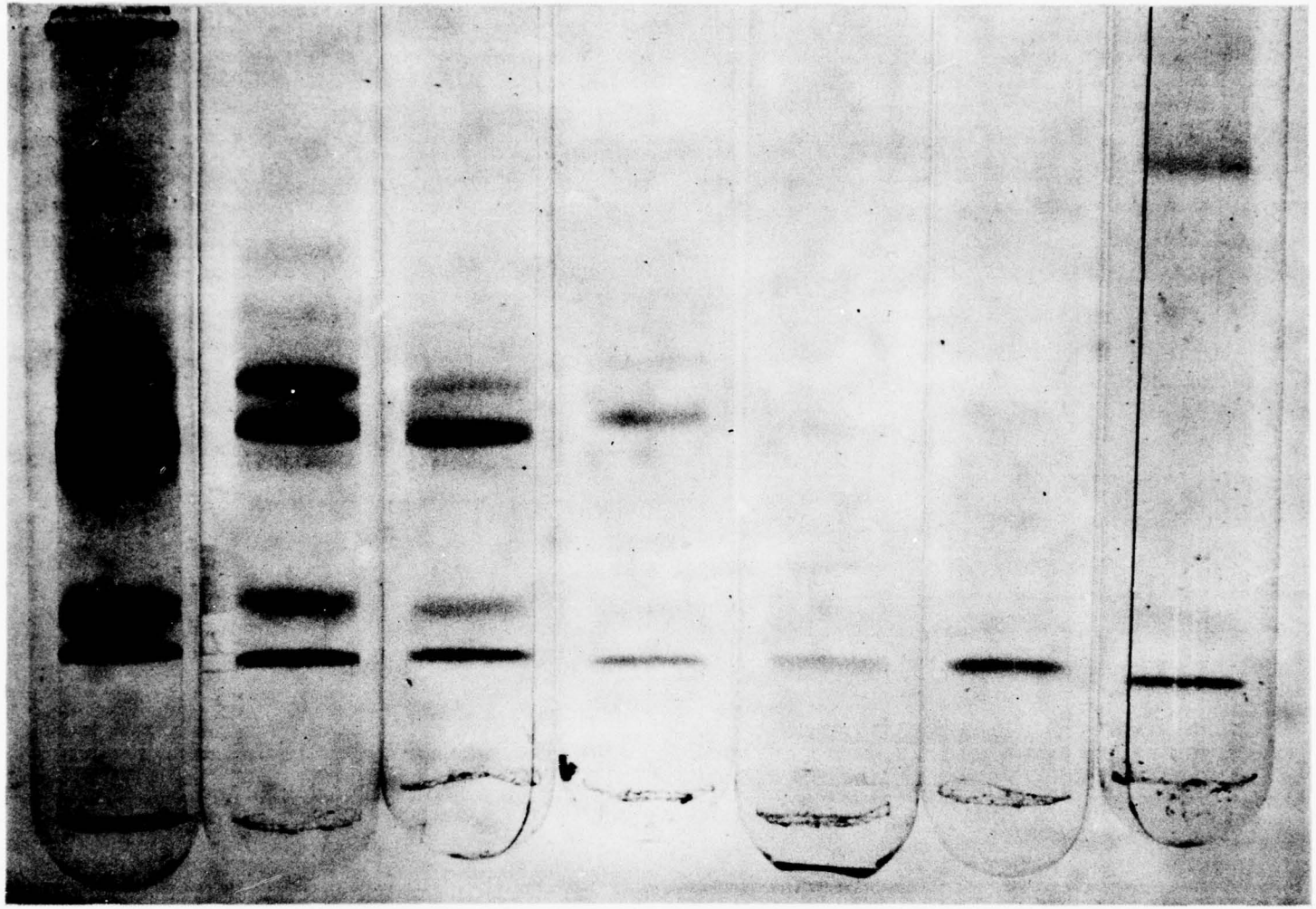


Figure 6. Effect of Time Between Human Feeding and Electrophoresis on the Formation of Bands with *Aedes aegypti*.

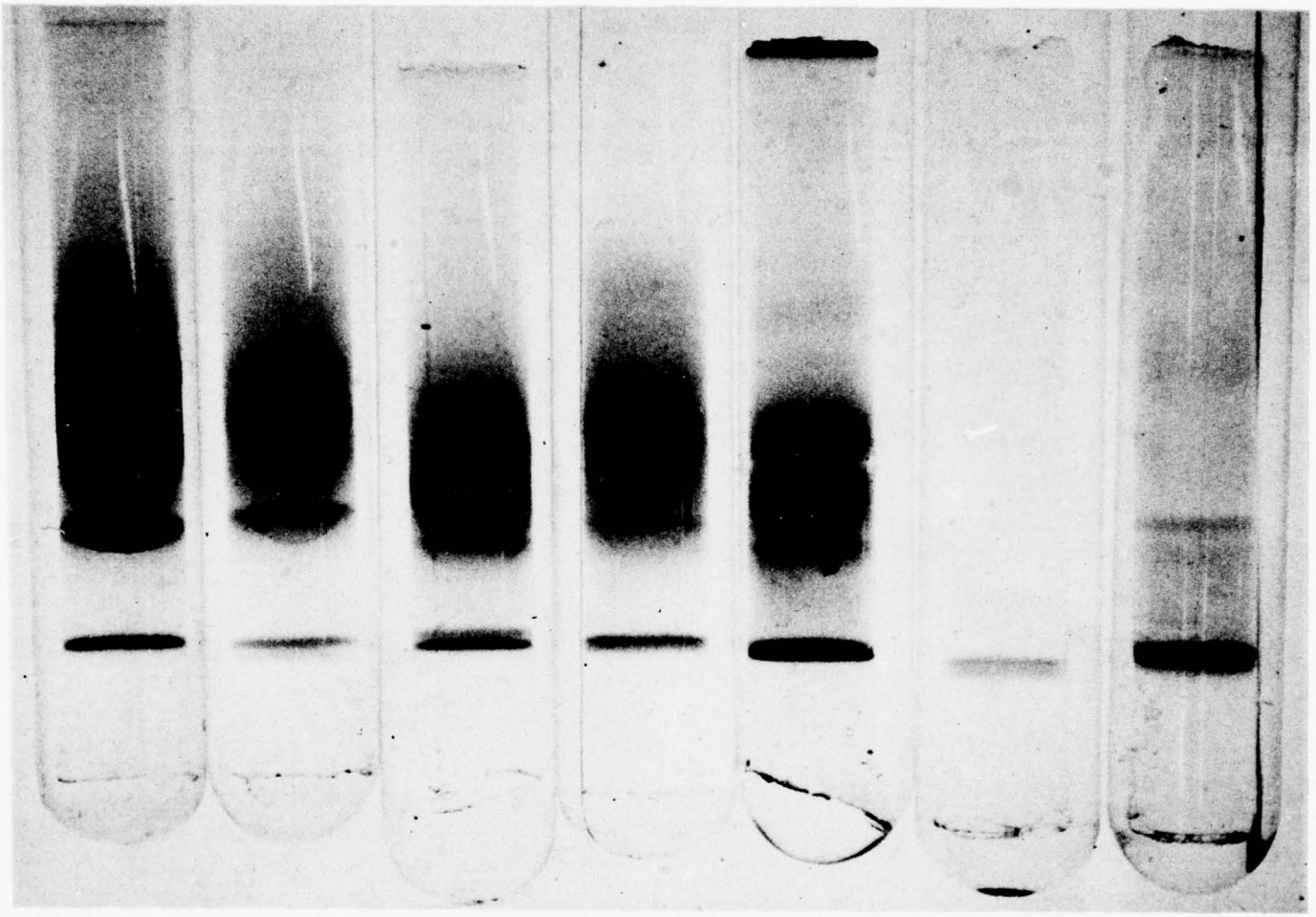


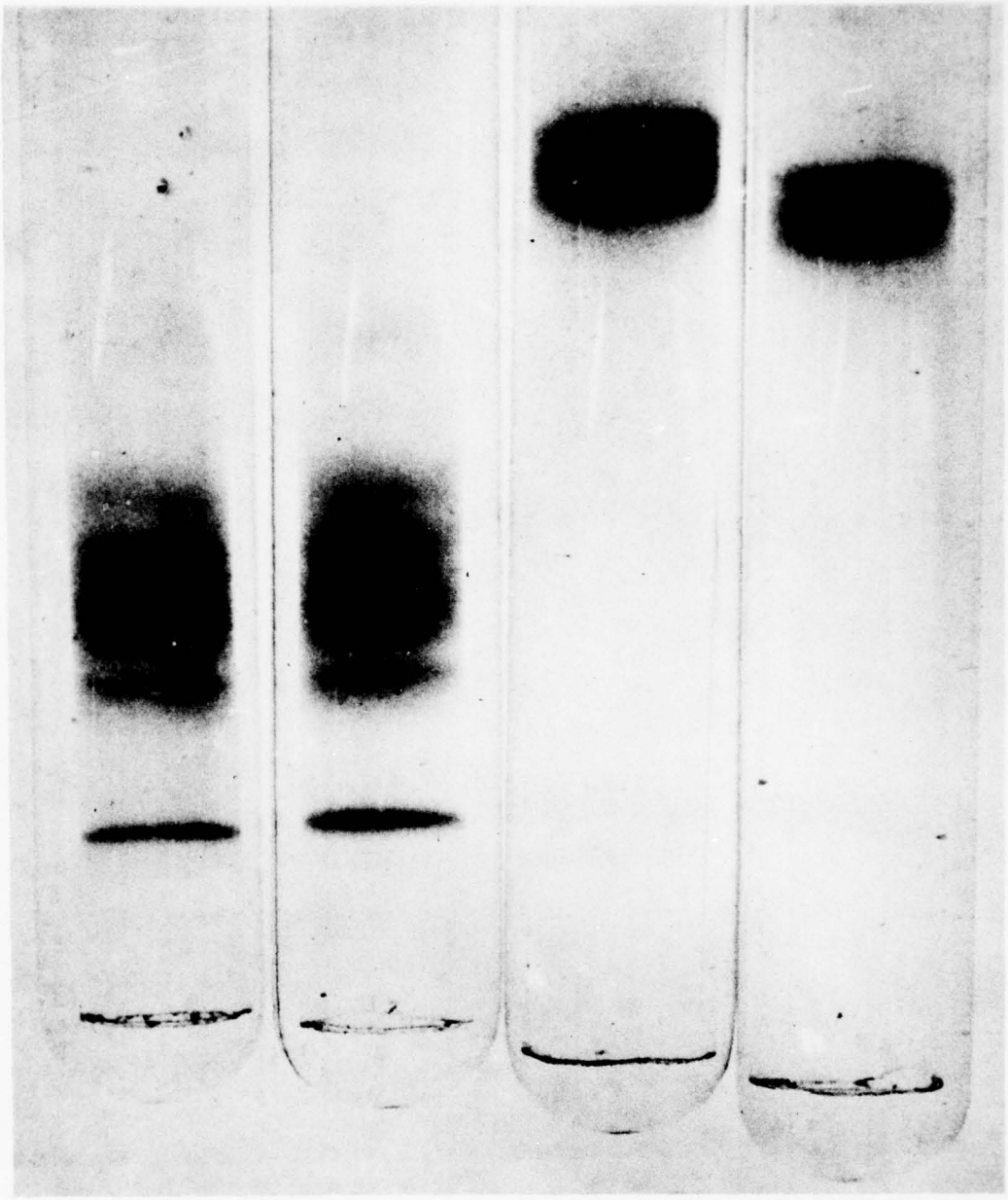
Figure 7. Effect of Time Between Guinea Pig Feeding and Electrophoresis on the Formation of Bands with *Culex fatigans*



An. maculatus *Ae. aegypti* *An. maculatus* *Ae. aegypti*

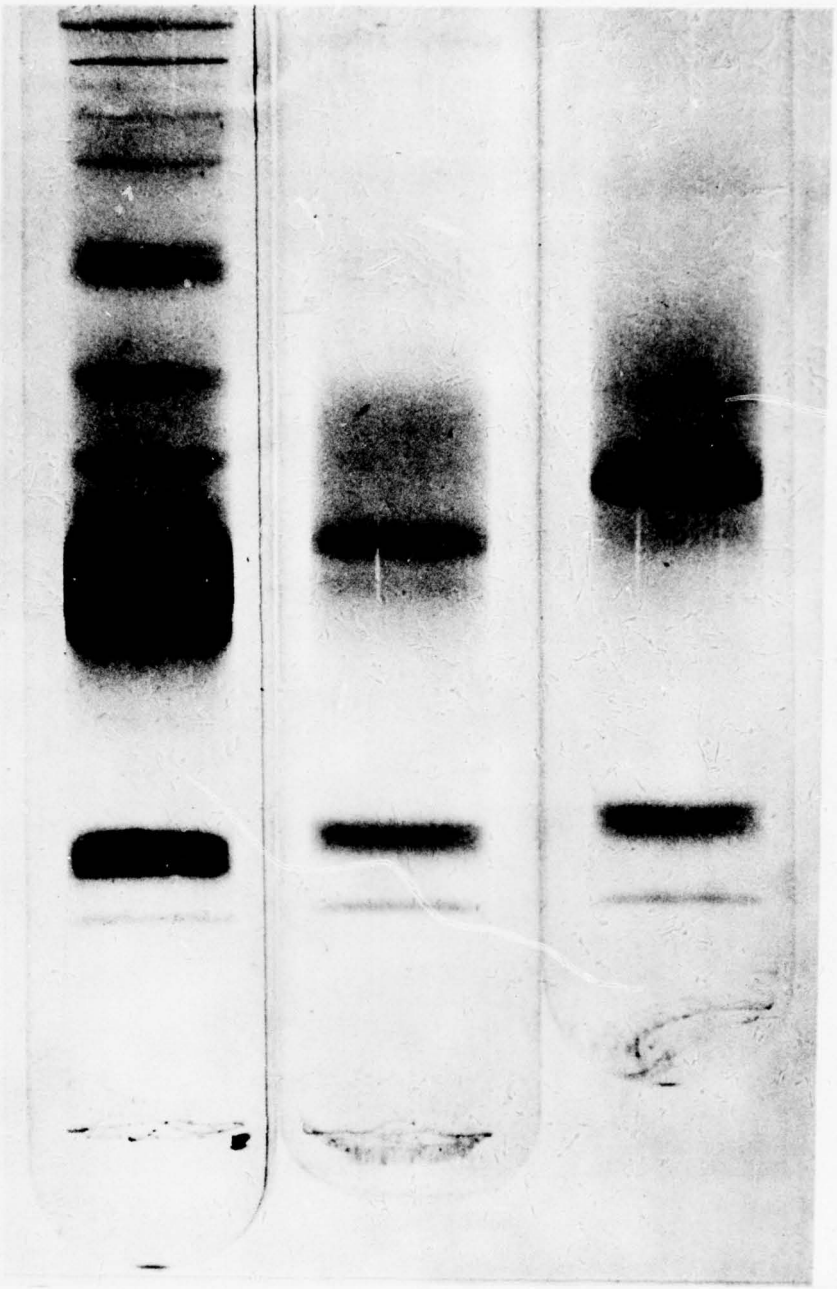
Human Monkey

Figure 8. Comparison of Bands Obtained Following Human or Monkey Feedings



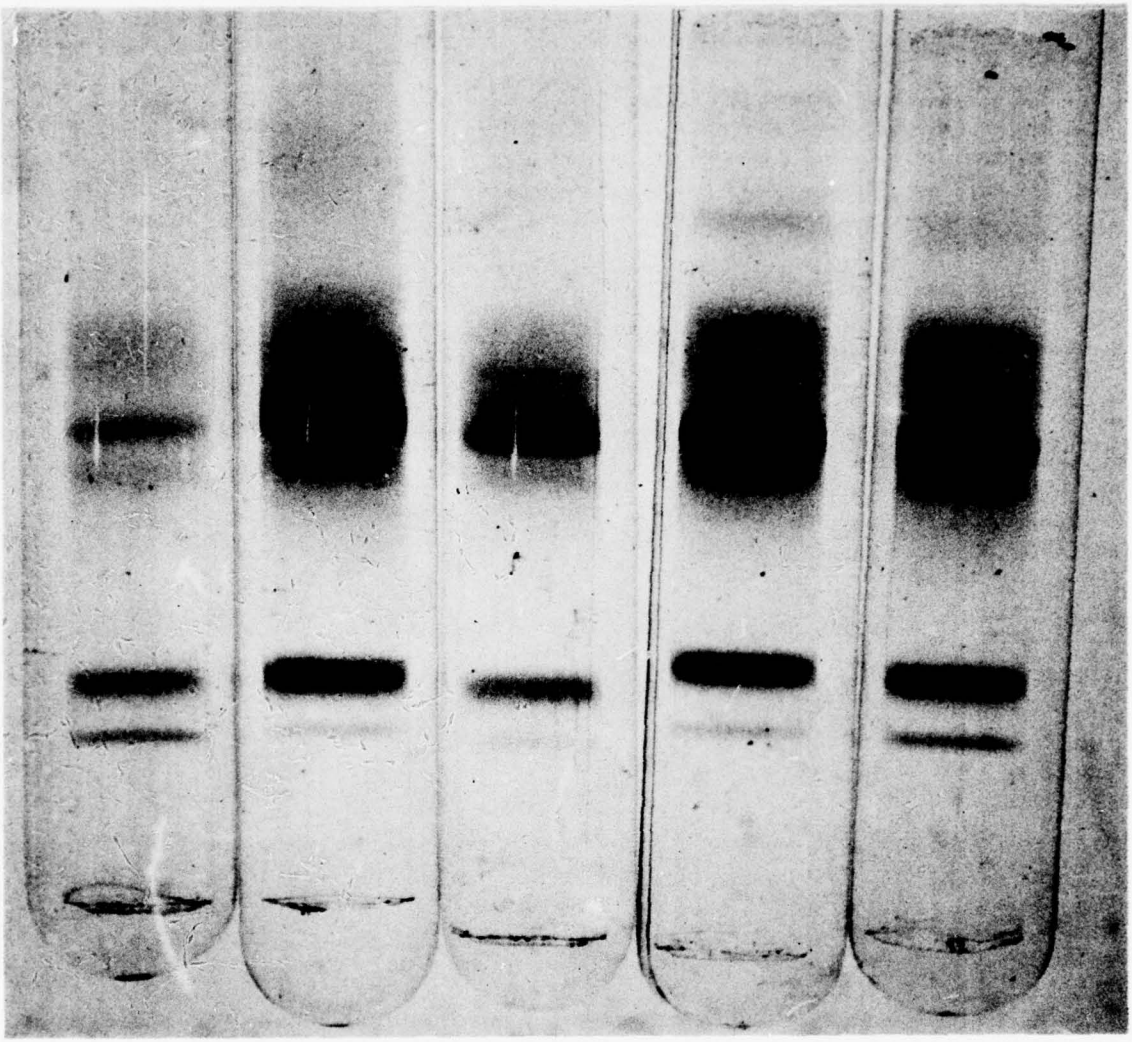
7 1/2 % gel 10% gel

Figure 9. Effect of Increasing Gel Concentration on the Formation of Bands with *An. maculatus* Fed on Guinea Pigs



human feedings

Figure 10. Formation of Bands from *Ae. aegypti* Mosquito Gut Tissue Transported in Capillary Tubes.



human feedings

Figure 11. Formation of Bands from Squashes of Whole Mosquitoes on Filter Paper

DISCUSSION

The initial experiments were designed to determine the feasibility of routinely producing protein bands from the blood meal imbibed by a single mosquito. When this proved practical and reproducible the variation between species became of prime importance. Laboratory workers who have had experience in this technique can very rapidly and with a minimum of uncertainty determine those gels which contain material from human blood meals as opposed to the other vertebrate species studied.

The criticisms of polyacrylamide gel for this purpose are two in number. (i) It requires an appreciable amount of equipment and a laboratory surroundings, (ii) the ultimate decision is subjective. The first criticism can be circumvented by the use of filter paper spots which can be prepared in a field situation and transported to the laboratory, without refrigeration. (Samples for scrub typhus antibody determinations are being shipped to this laboratory from Thailand and Indonesia by international mail.)

The second criticism will require a simple procedure which will affect the migration of human sera but not that of other species. The use of anti-human serum albumin prepared in the rabbit is presently being investigated as a means to this end. If the human serum albumin can be aggregated into a size large enough to not penetrate the gel (> 50°A) the identification could be based on the presence of an albumin band in the control tube and the absence of an albumin band in the sample electrophoresed in the presence of anti-human serum albumin.

Fractionation and Identification of *Pseudomonas pseudomallei* Antigens

by

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BACKGROUND

Early work in this unit resulted in the preparation of a soluble antigen which spontaneously attached to sheep RBC and could be employed in surveys of human and animal sera. Antibody titers were present in from 2 to 16% of a number of individuals grouped on the basis of location or employment. (*Am. J. Trop. Med. Hyg., 18:* 703-707, 1969)

Recent experiments by a member of this group (Donaldson) have produced in disease in silvered leaf-monkeys (SLM) which closely mimics that found in humans. A small number of those inoculated with a dose of bacteria capable of producing disease did not respond with antibody production. Further, as has been found in humans, few of the SLM responding with antibody production exhibited frank signs of the disease. Since all of the monkeys were inoculated with the same strain, the differences in response have been postulated to be due to differences among the individual monkeys.

The present antigen is produced from an autoclaved whole culture. The main constituent of this antigen should be the heat stable endotoxin which is composed of lipopolysaccharide. As an initial approach to the problem, the response of experienced animals to other antigens present in the bacterial cell might give clues to the mechanism of the observed resistance.

This joint project was initiated to prepare and separate additional antigens and to test the response of experimental animals to them.

MATERIALS AND METHODS

Strain: The strain of *Pseudomonas pseudomallei* used throughout these experiments was a locally isolated strain in the smooth colonial morphology.

Antigen: The autoclaved antigen was obtained from the Division of Veterinary Medicine, WRAIR, WRAMC.

Fractionation: Several fractionation techniques have been used and will be fully described in the results section.

Serological Methods: The quantification of antigen by the standard hemagglutination technique is cumbersome. Therefore, we have been using double diffusion in agarose on glass slides to detect antigen. With any eventual procedure developed, the materials resulting will be assayed by both techniques.

RESULTS

Initial work centered on the breakdown of the bacterial cell in such a way as to release components but not destroy many of the fractions which would be degraded by the autoclaving process. Treating a washed suspension of bacterial cells with the nonionic detergent sodium dodecyl sulfate (SDS) at 100 C for various periods of time proved to be effective in breaking down the cells, but the resulting fractions were so broadly distributed that no bands could be identified in polyacrylamide gel electrophoresis. Rather, the proteinaceous material stained by the Coomassie blue R-250 stain was evenly distributed from the origin to the area of the tracking dye. Periods as short as 10 min produced this pattern, but 5 min heating periods differed in that several more densely staining bands were present. To separate these resulting fractions the supernatant from a heated - SDS suspension of bacteria was given a low speed centrifugation and placed on a column of Sephadex G-200. This column was monitored at 280 nm with a continuous flow cell. All the material, as measured by absorbance at 280 nm, eluted in a continuous broad peak beginning at tube 12 (void volume = tube 1) and continuing in decreasing absorbance for 40 tubes. Precipitin bands were formed by material from tubes 15 through 17 only (Figure 1).

A 0.5 ml portion of the same preparation was passed through a G-100 column, (fractionation range 4,000 to 150,000) and the results are presented in Figure 2. The protein, as measured by chemical and spectrophotometric means, was spread broadly from after the void volume through approximately 85 ml of eluent. Only one protein peak was present and no detectable material was present near the void volume. The peak of antigenic activity, as measured by gel diffusion, was present in the area of increasing protein concentration and trailed through 8.5 ml. A protein concentration of 12 μg gave a precipitin line at a $\frac{1}{8}$ dilution.

Figure 3 illustrates a rate zonal sucrose density gradient experiment to determine the characteristics of the SDS antigen by this procedure and the procedure's applicability as a purification technique. It is apparent that none of the antigen penetrated the 5-40% gradient. A subsequent experiment employed 2.5-5%, 2.5-10% and 5-15% gradients (Figure 4). The initial antigen was detected in the fraction with a density of 1.021 in the 2.5-5% and 1.027 in the 2.5-10% gradients. The proteinaceous material or antigen did not penetrate the 5-15% gradient to any appreciable extent.

Additional studies were conducted with precipitation by ammonium sulfate to prepare fractions which were soluble with specific concentrations of salt. Figure 5 presents the results from density

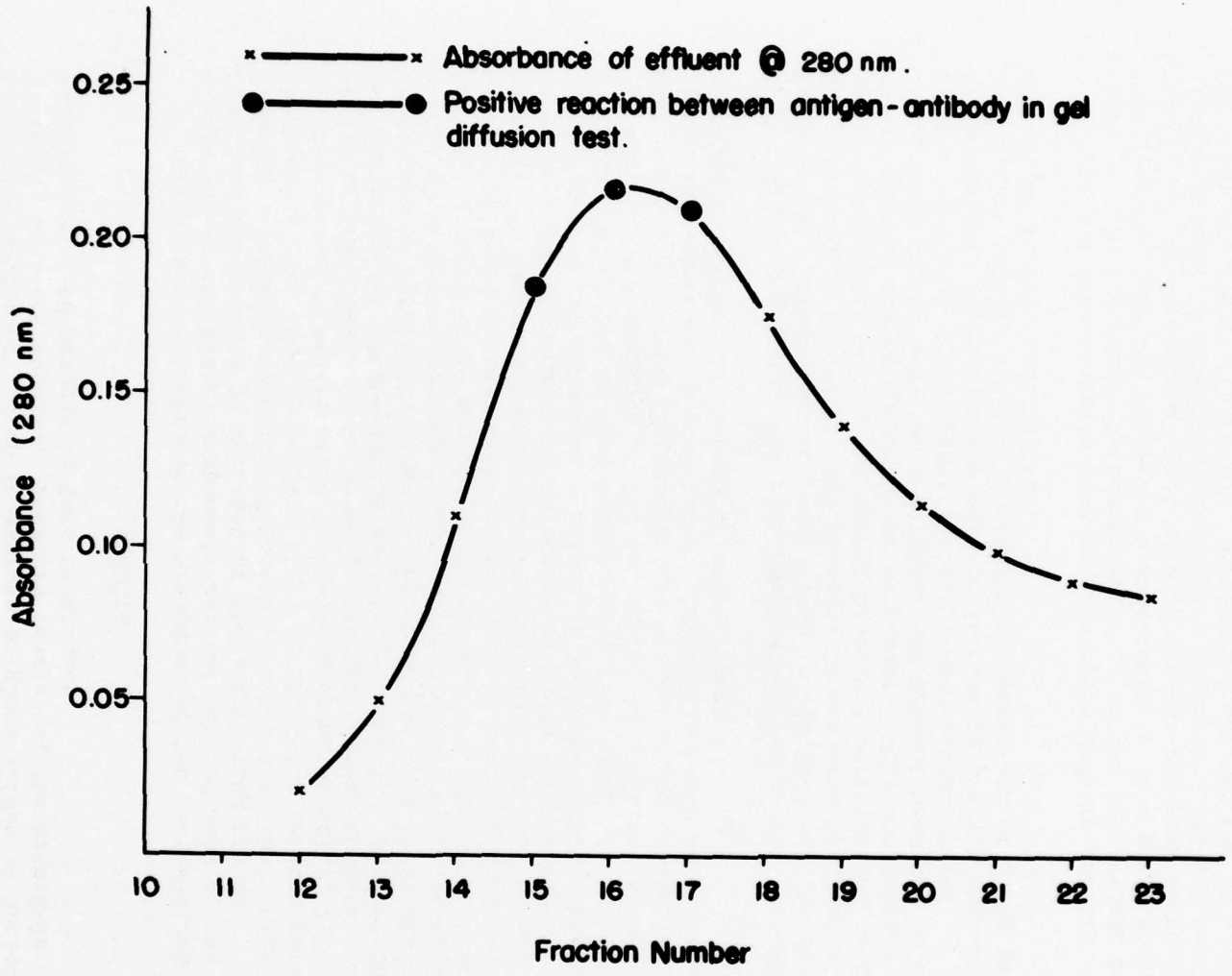


Figure 1. G-200 Column Chromotography of a Heat Treated SDS Lysate of *Pseudomonas pseudomallei.*

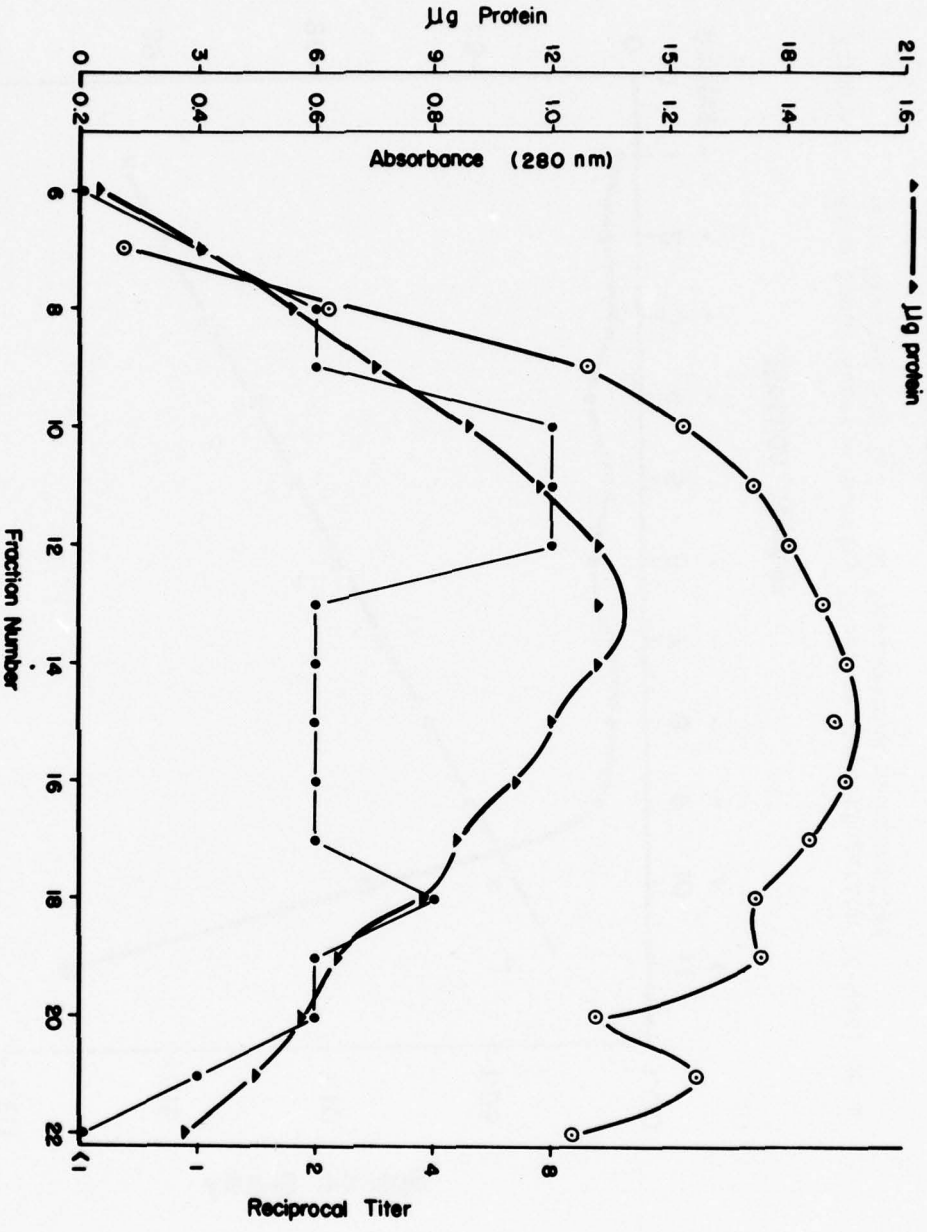


Figure 2. G-100 Column Chromotography of a Heat Treated SDS Lysate of *Pseudomonas pseudomallei*.

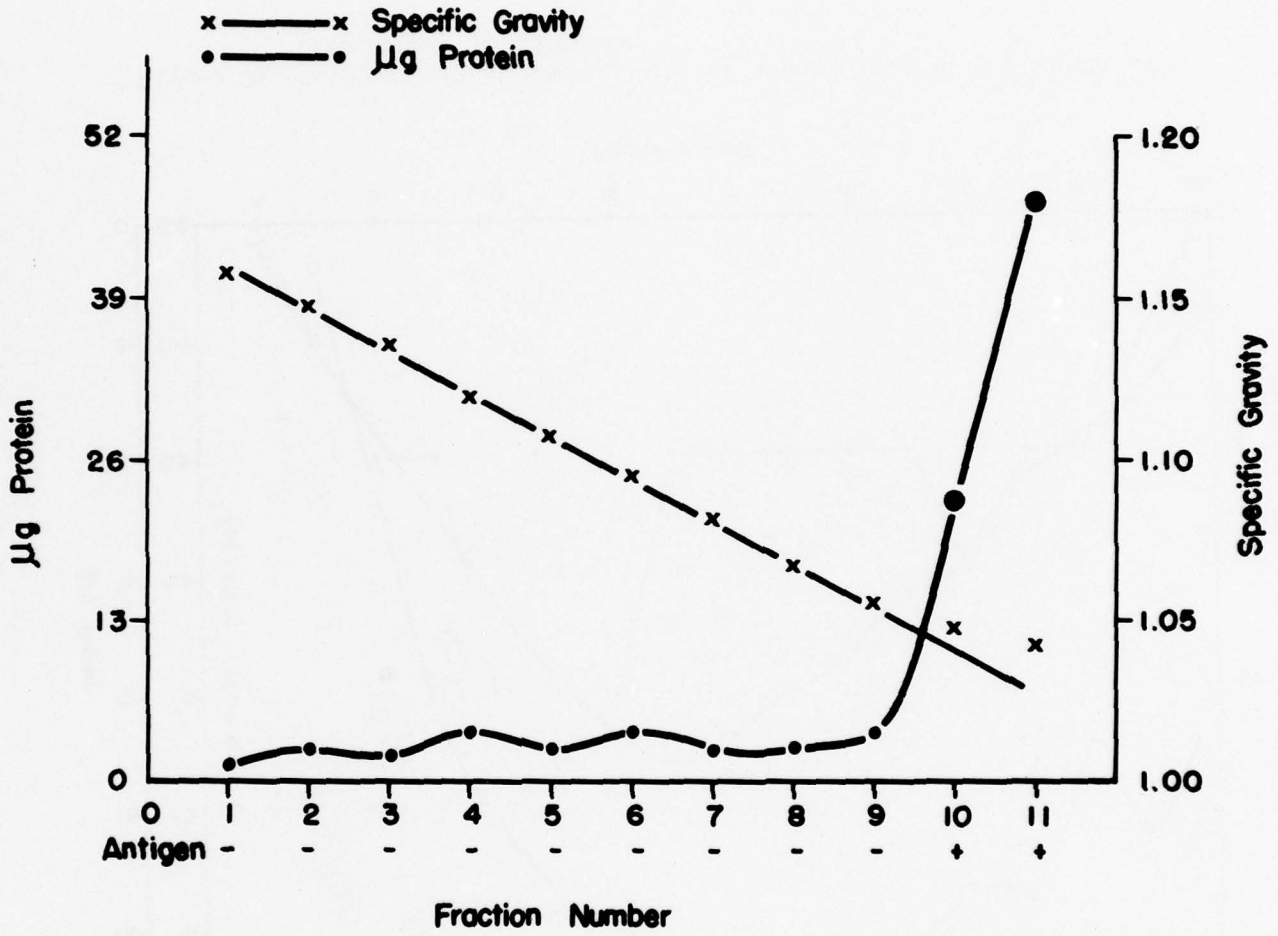


Figure 3. Rate Zonal Sucrose Density Gradient Centrifugation (5-40%) of a Heat Treated SDS Lysate of *Pseudomonas pseudomallei*.

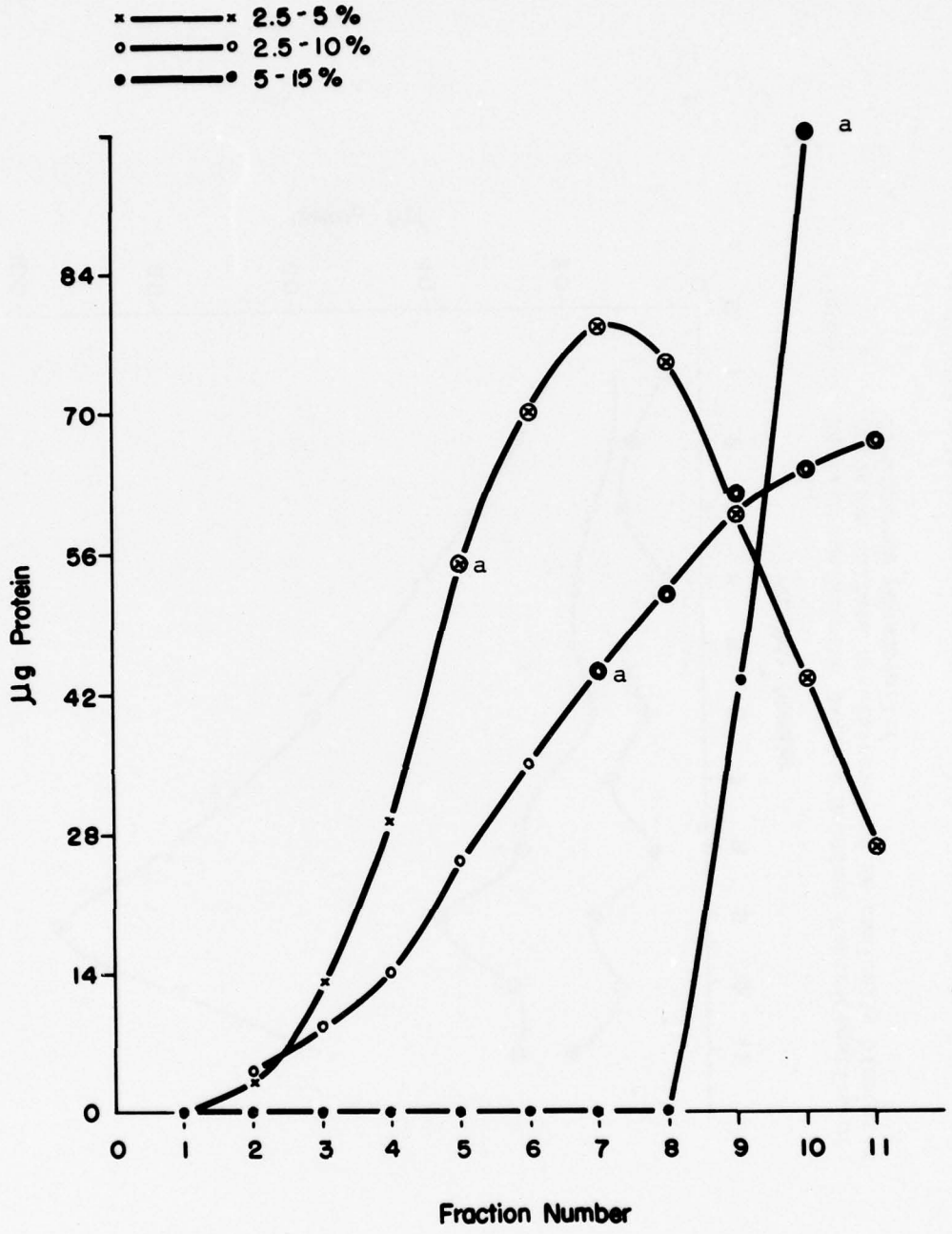


Figure 4. Rate Zonal Sucrose Density Centrifugation Using Gradients With Varying Characteristics.

a; Circled points indicate antigen was detected in fractions.

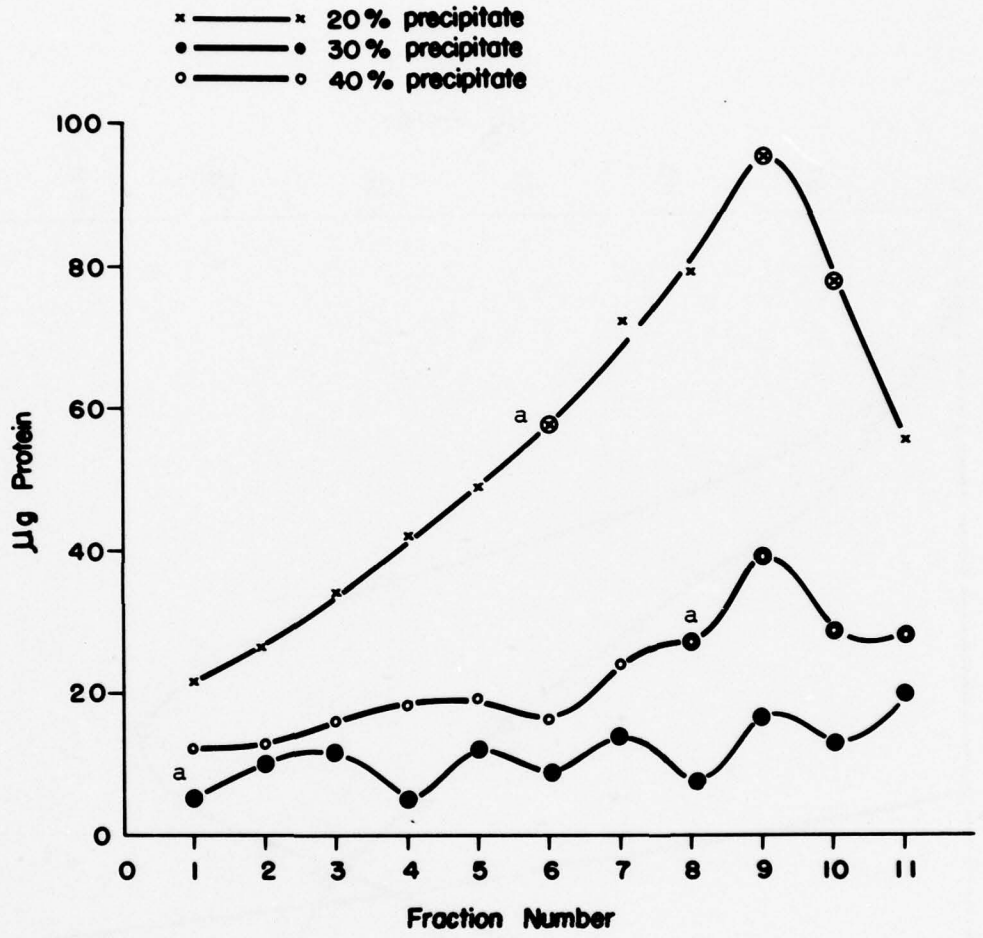


Figure 5. Equilibrium Sucrose Density Gradient Centrifugation of Ammonium Sulfate Precipitates of Mechanically Disrupted *Pseudomonas pseudomallei*.

a; Circled points indicate antigen was detected in fractions.

gradient centrifugation of the precipitates from 20, 30, and 40% concentrations of ammonium sulfate. Antigen was detected in the precipitates in protein that banded at 1.0057 and less in the 20% precipitate, in that banding at 1.0044 and less in the 40% precipitate, and throughout the densities encompassed by the gradient (1.0102-1.0019) in the 30% precipitate. The 30% precipitate contained the smallest amount of protein and the largest amount of antigen. Antigen has never been detected in fractions which did not contain protein as measured by the Lowry reaction.

Recently a ribosomal fraction has been prepared by the method of Jensen *et al.* (*Infect. Immunity, 6:* 156-161). Figure 6 presents the results of studies of the fractions obtained during the procedure.

The complete technique consists of centrifuging a 6-day broth culture at 10,000 rpm for 15 min at 4 C to pellet the cells. An equal volume of 0.01m $MgCl_2$, 0.01m. Tris pH 7.3 (TM buffer) was added to the cell paste along with an equal quantity of sterile sea sand. The cells were disrupted by three 5 min treatments at 16,000 rpm in an Omni-mixer (Sorvall, Newtown, Conn). The menstruum was cooled by immersing the bucket in an ice/salt bath during the grinding and by interspersing the grinding intervals with 5 minute cooling intervals.

The resulting suspension was centrifuged at 10,000 for 15 min to obtain cell free extract (CFE). This CFE was incubated with 10 µg pancreatic DNAse per ml for 1 hr at room temperature and then centrifuged at 27,000 g for 1 hr to yield a crude cell lysate (CCL). The scheme for the fractionation of the CCL is presented in Figure 6. There was purification of higher molecular weight materials as evidenced by staining of the upper parts of the gel columns with Coomassie blue. Although the procedure was designed to prepare ribosomal RNA, the final fraction contains appreciable amounts of protein as evidenced by Coomassie blue staining of bands in the polyacryamide gel tubes. This fraction will also produce precipitin lines with immune sera in gel diffusion. Specific ribosenucleic acid stains will be used to study the distribution of RNA in the gel columns.

DISCUSSION AND FUTURE WORK

Different techniques have been used to prepare three fractions from *Pseudomonas pseudomallei*. These fractions all contain protein and form precipitin lines in gel diffusion with immune sera. Additional fractionation experiments will be conducted with sonicated organisms when the necessary equipment arrives.

We plan to study the composition of these fractions, their immunological relationships to each other, and their ability to protect hamsters from challenge with virulent organisms during the next year.

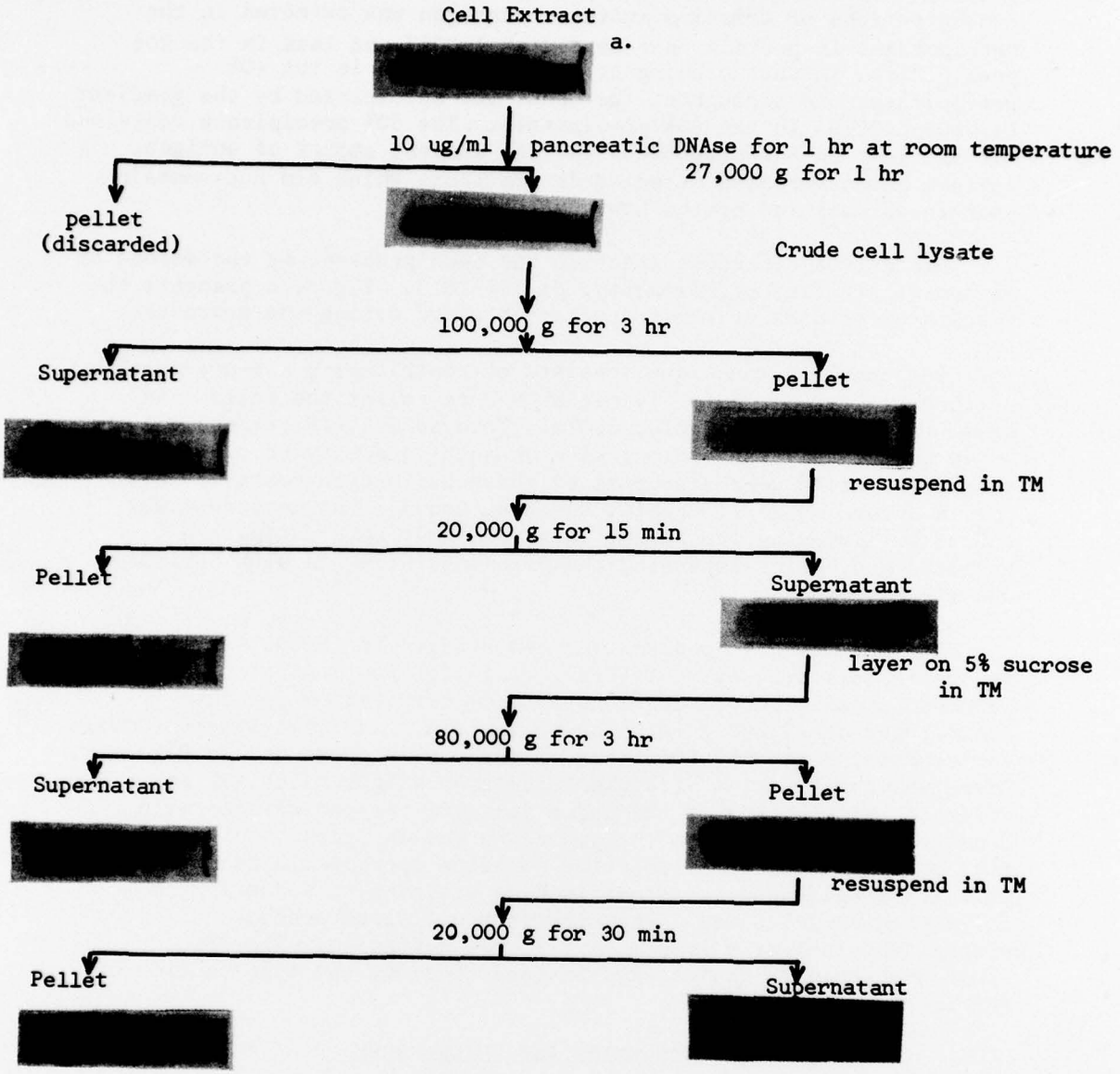


Figure 6. Preparation of "Ribosomal RNA" From a Cell Extract of *Pseudomonas pseudomallei*.

a. All gels electrophoresed in the vertical position with the sample applied to the top of the gel which is to the right in the figure.

Identification of the Antigens Present in Selected Strains of *Rickettsia tsutsugamushi*

by

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Department of Viral and Rickettsial Diseases, USAMRU

Immunity to reinfection with scrub typhus appears to be predicated directly on the relatedness of the strains and the interval between the infections (Shishido, *et al., Jap. J. M. Sc. & Biol., 12:* 391-404, 1959; Smadel *et al., Am. J. Hyg., 56:* 294-302, 1952). For several months following recovery from the disease protection is very broad, and unrelated strains will not produce disease. Later this heterologous immunity wanes and unrelated strains may (or may not, occasionally) produce disease indistinguishable from that associated with the primary infection. The immunity to homologous challenge is retained.

The present grouping of strains is based on the identification of surface antigens by fluorescent antibody methods. Challenge experiments where strains unrelated by this technique are capable of protecting animals indicate that other factors or antigens are important in eliciting protection against challenge. An understanding of the basic antigenic content of the organism is the first step to the understanding of variant challenge phenomenon.

PROGRESS

An area suitable for the preparation of primary cell cultures and the propagation of cell lines and strains has been remodelled. Two cubicles have been built along with a small preparation room/office in this area. Seed stocks of LLC-MK_2, BHK-21 and Vero cells have been obtained from SEATO Laboratory, Bangkok and are being carried.

The Kato, and Gilliam strains of *R. tsutsugamushi* are being passed in duck embryo yolk sacs in an effort to adapt them to this substrate. Generally, the technique involves selecting the yolk sac which has produced the largest number of rickettsia as observed with Machiavello's strain. This yolk sac is processed into a 20% suspension with a hand operated tissue grinder, and the suspension is then inoculated into an additional 5-10 eggs by the yolk sac route. These eggs are harvested at 7 days and the process is repeated.

Efforts to adapt the Gilliam strain to primary silvered leaf-monkey kidney cell culture have been held in abeyance until fresh fluorescent antibody antigens and conjugate sera are prepared for the 9 prototype strains. The reagents presently being employed are

over 2 years old. Dr. Elisberg recommends that antigens over 1 year old not be used.

Initial purification attempts with rickettsia freed from cells in a Sorvall Omni-mixer have been unsuccessful. In a study of the effects of this disruption method on infectivity (Table 1) a two minute intermittent treatment of a chilled suspension in an ice bath destroyed all infectivity. Density gradient centrifugation of disrupted material in sucrose produced no bands of protein. Work has been curtailed on this project also until strains of *R. tsutsugamushi* for use as antigen in the fluorescent antibody test are renewed. (These antigens are produced by the same personnel who prepare rickettsial pools.)

In the near future we will prepare a lot of purified antigen by means of DEAE absorption and ether extraction. This purified lot will be fractionated by appropriate methods and the resultant fractions studied in polyacrylamide gel electrophoresis and gel diffusion.

Table 1

Effect of disruption in an Omni-mixer on the titer of *R. tsutsugamushi*

Time (minutes)	Omni-Mixer Speed	
	50,000 rpm	25,000 rpm
0	$10^{9.2}$[a]	$10^{5.6}$
1	$10^{8.7}$	$10^{6.5}$
2	$10^{6.2}$	$10^{6.8}$
3	$<10^{4.5}$	$10^{4.5}$
4	$<10^{4.5}$	$<10^{3.5}$
5	$<10^{4.5}$	$<10^{3.5}$

a = median lethal doses for mice by the intraperitoneal route.

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13. ABSTRACT

INVESTIGATIONS OF THE DEPARTMENT OF ACAROLOGY

Chiggers and host blood samples on filter paper were collected from 6 areas of North Sumatra, Indonesia. *Leptotrombidium deliense* was the only known vector of scrub typhus found in these areas. Only 13 of 214 mammalian hosts sampled were positive for scrub typhus antibody.

Rattus argentiventer had significantly ($P < .001$) greater numbers of *L. deliense* chiggers per rat than did *Rattus tiomanicus jalorensis*. There was no apparent difference between numbers of chiggers on rats from grassland and those from edge (scrub) habitats. Both host species appeared to be highly efficient as hosts for *L. deliense*. Fluctuations in chigger numbers on these hosts appeared to be correlated with dew-point temperature.

A naturally infected colony of *L. arenicola* was established. The rate of transovarial transmission of *Rickettsia tsutsugamushi* was 100 percent over 3 generations as shown by single feedings of chiggers on mice. The rickettsial strain was found to have Karp and TA 763 as major - and Kato as a minor antigenic component. A partially engorged infected chigger was shown to be capable of transmitting scrub typhus during refeeding on a laboratory mouse. Infection in a *L. arenicola* male was demonstrated.

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14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Chiggers, mites, *Leptotrombidium deliense, L. arenicola, L. fletcheri,* scrub typhus, *Rickettsia tsutsugamushi,* melioidosis, *Ps. pseudomallei, Chromobacterium violaceum, Ps. pseudomallei* indirect hemagglutination, rice padi ecology, silvered leaf-monkey, *Presbytis cristatus,* buffalo leech, *Hirudinaria spp.,* vertical distribution, canopy transect, *Rickettsia tsutsugamushi, Plasmodium, Hepatocystis,* Sabah, Sarawak, Peninsular, malaria vectors, mosquito biology, ULV equipment, *Anopheles balabacensis,* arboviruses, *Anopheles maculatus,* animal model, laboratory animal, cost accounting, guinea pigs, nutrition, new building,human malaria, chloroquine-resistance, epidemiology, *in vitro* testing, Malaysia, *P. falciparum, P. vivax,* urine testing, *Rickettsia tsutsugamushi,* silvered leaf-monkey, *Pseudomonas pseudomallei,* antigens, fluorescent antibody.						

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Rates of spermatophore production by *Leptotrombidium* males were determined. Initial spermatophore deposition occurred an average of 4 days after adult emergence. The average number of spermatophores per day has been 7.9 for *L. arenicola*, 13.0 for *L. deliense* and 11.8 for *L. fletcheri*. Females of one *Leptotrombidium* species did not take up spermatophores from a related species.

Sex ratios of progeny were determined in infected and noninfected colonies of *L. arenicola* and *L. fletcheri*. Infected chiggers produced female offspring almost exclusively, and it appeared that female production and *R. tsutsugamushi* in the chigger are related. Noninfected chiggers had an average ratio of about 2.5:1 (females:male), and even lines producing only females in one generation did not continue to produce females exclusively in the subsequent generation.

Rats of the *R. rajah* group were infested with significantly fewer chiggers than other species in initial field tests. Experimental feedings showed that fewer *L. deliense* chiggers fed successfully on *R. surifer* than on *R. annandalei*. The potential involvement of gamasoid mites in scrub typhus transmission to spiny furred rats was suggested.

INVESTIGATIONS OF THE DEPARTMENT OF BACTERIAL DISEASES

The department continues to provide routine diagnostic support as part of the medical care of U.S. staff and their dependents and locally engaged staff. Support is also afforded to the Aborigine Hospital at Gombak in the form of one technician on detached duty.

The staff of four technicians, only one of whom has received any formal training, was augmented by the appointment, on August 7, of a B.S. degree holder in microbiology. The department remains physically small, occupying one partitioned room (18' x 21').

Much of the year was spent in collecting field material for a reappraisal of the incidence of *Pseudomonas pseudomallei* and *Chromobacterium violaceum* in rice fields. Collaborative work in serology and in the antigenic structure of *Ps pseudomallei* has been established with the Department of Viral and Rickettsial Diseases. The results of the rice field survey indicate a significant reduction in incidence of *Ps pseudomallei* from former years, most likely due to ecologic change; antibody incidence closely parallels recovery rates of the organism, titers of 1:20 or even 1:10 being considered as evidence of prior exposure in the population of an endemic area. The incidence of *Chromobacterium violaceum* is nine-fold greater than that of *Ps pseudomallei*.

Good support from the Department of Laboratory Animal Resources has been received for the above work and in the development of the Silvered Leaf-Monkey (SLM) as a model for melioidosis. The results of

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this latter project show that disease in the SLM reflects the human disease in all its various forms.

The work on leeches of genus *Hirudinaria*, as vectors of *Ps pseudomallei*, has not produced any final conclusion. Tentatively it can be stated that the annelid and the bacterium can co-exist in the same environment but that, in the environment of the leech gut, *Ps pseudomallei* seems incapable of multiplication: this despite, or perhaps because of, the fact that the normal inhabitant of the leech gut is of the same bacterial family. This work is not complete.

Continuing work on a serologic test for *Chromobacterium violaceum* is proposed. Bacterial skin disease in troops has been found to be a major problem in Vietnam and currently poses a problem of morbidity in troops of Australian, New Zealand and United Kingdom (ANZUK) origin in Singapore. This forms the basis for the proposed comparative studies of Malaysian and ANZUK troops during the next fiscal year.

INVESTIGATIONS OF THE DEPARTMENT OF ECOLOGY

Studies of vertical distribution of mammals and their parasites and pathogens within the rainforest with the use of canopy transect walkways are in progress. In this area over 2000 animals have already been captured marked and released. Arboreal species here and elsewhere do not become infected with *Rickettsia tsutsugamushi*, however, blood parasites such as *Plasmodium* and *Hepatocystis* are more common in arboreal than in terrestrial hosts. Large differences in the rates of infections with malarial parasites occur within species of arboreal hosts which seem to be correlated with the habitat types of populations. Nests of arboreal mammals are rich with populations of mesostigmatid mites and other parasites. *Rickettsia tsutsugamushi* in terrestrial mammals appears to be most frequent in forest and lalang grass *(Imperata cylindrica)* habitats. Detailed studies using enclosures in different habitats to test sentinel animals are in progress.

In areas surveyed in Sabah, and especially Sarawak, the rates of transmission of *Rickettsia* seemed to be lower than in Peninsular Malaysia. In arbovirus studies in conjunction with the forest canopy transect walkways, 5 isolates (from 3 arboreal and 2 terrestrial host species) have been made from 823 mammals tested. Serological results are available for 201 samples, with 8 HI positives for Group B arboviruses. Studies of population dynamics have shown that some arboreal species in the primary forest have extremely slow population turnover, infrequent breeding cycles with periods of as long as 17 months with no reproduction at all, and small litters. Data for man-altered habitats is available but not yet analyzed. Several taxonomic and systematic studies remain to be written up.

INVESTIGATIONS OF THE DEPARTMENT OF MEDICAL ENTOMOLOGY

Mosquito surveys in conjunction with chloroquine resistance studies have been conducted at five rubber estates in Pahang and

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Negri Sembilan States, and in the Kuala Brang area of Trengganu. Collections of anophelines have been low at all rubber estates. *Anopheles maculatus*, the presumptive vector of malaria has been collected at all estates. One oocyst-positive *An. maculatus* was found at Paroi estate. In Trengganu, *An. aconitus* was the predominant anopheline collected. In this area the vector situation is very vague and it would be difficult to incriminate any single species at this time.

A total of 30551 mosquitoes were identified, then pooled and screened for arboviruses. From 323 pools, 11 were found positive. The positive isolates will be sent to the U.S. Army Component, SEATO Laboratory, Bangkok, Thailand for further typing.

Other studies in progress include: mosquito surveys in a Federal Land Development Authority (FLDA) scheme to determine the changes in species over a period of years, with special emphasis on the introduction of *Aedes aegypti* in such an area. A study of *An. balabacensis* in Southeast Asia and its relation, if any, to chloroquine resistant malaria.

Future plans include experiments on Ultra Low Volume (ULV) ground aerosol spray evaluations against mosquito populations and possibly malaria incidence. In conjunction with these experiments mosquito life history data will be collected and analyzed and mosquito collection techniques evaluated.

INVESTIGATIONS OF LABORATORY ANIMAL RESOURCES

New cages have been provided for the hamster, rat, suckling mouse, and 25% of the mouse breeding colonies.

Studies have been completed which show the locally produced animal chow to be deficient in ascorbic acid for guinea pigs and in vitamin A for breeding hamsters. The feed processor has been requested to revise his formulations to provide an adequate ration.

Studies have been partially completed to determine growth rate curves for the species bred in this laboratory.

An accounting system has been devised to give the cost of laboratory animals produced in Malaysia. Use of the system should allow more accurate budgeting for laboratory animal expenses.

Redesign of the proposed new animal facility has been accomplished, and construction is now scheduled to begin in January, 1974.

INVESTIGATIONS OF THE DEPARTMENT OF PARASITOLOGY

The major effort of this department during the reporting period has been geographical studies of chloroquine resistant *Plasmodium*

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falciparum and simultaneous searches for evidence of resistant *P. vivax*. Previous field studies in Peninsular Malaysia and Thailand have employed a 7-day follow-up, WHO-recommended in vivo assessment method, and occasionally an in vitro technique. Little resistance had been demonstrated in Malaysia, however past experience here has indicated that the great majority of what resistance there is is the late recrudescing R I type, which would have been missed by follow-up limited to 7 days. We undertook a 28-day follow-up, in vivo, in situ assessment with provisions for estimating the attack rate for new *P. falciparum* infection. The resistance rates found in the studies completed to date have been quite different from past estimates and have been as high as 50%.

The department was invited by Dr. A.N. Lewis and Dr. J.T. Ponnampalam (University of California ICMR and Department of Malaria Research, respectively of the Institute for Medical Research, Kuala Lumpur) to participate in analyzing the results of chloroquine suppression in two groups of control subjects in a prophylaxis study of a different drug. The groups were on either weekly chloroquine suppression or on placebo. In the study area, where at least 85% of the *P. falciparum* infections responded to chloroquine treatment, chloroquine suppression appeared completely ineffective against this parasite. However it still protected against *P. vivax*.

Studies on chloroquine resistance are in progress in North Sumatra, Indonesia, in collaboration with Dr. Kwo Eh Hoa, University of North Sumatra, Medan, Professor (Dr.) J. Sulianti Saroso, National Institute for Medical Research, Jakarta, and Capt. P.F.D. Van Peenan of NAMRU-2, Jakarta, Indonesia.

A preliminary visit was made to Sabah (North Borneo), Malaysia, to assess the feasibility of a chloroquine resistance study there in areas where resistance is strongly suspected. This participation was as a member of an Institute for Medical Research Team, requested by the Director of Medical Services, Sabah.

Long term monthly blood surveillance with treatment of parasitemias has been underway on a rubber estate in Peninsular Malaysia to determine: 1) the seasonality of *P. falciparum* and *P. vivax* incidence in this area; 2) whether a 20% malaria rate (point prevalence) at any given time actually means that closer to 100% of the people are infected at some time during the year; 3) whether there is a sub-group who are repeatedly infected (and to demograph such a group) or whether new infections occur throughout the entire group; 4) malaria histories on a group of people as background for subsequent chloroquine resistance studies on these subjects. This work is in conjunction with the USAMRU-M Department of Medical Entomology, who are conducting parallel entomological surveillance, and in collaboration with Dr. D.R. O'Holohan (Kelinik O'Holohan, Seremban).

Miscellaneous studies in progress, completed, or discontinued during the reporting year include modification of qualitative urine tests for

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chloroquine, comparison of the relative merits of several described urine tests under field conditions, modification of *in vitro* malaria culture techniques for testing of drug resistance, analysis of data collected on filariasis during as assignment at the Institute for Medical Research prior to Active Duty at USAMRU-M, and compiling a review of certain filariasis studies in Southeast Asia.

INVESTIGATIONS OF THE DEPARTMENT OF VIRAL AND RICKETTSIAL DISEASES

The program of the department was divided into three broad areas viz.:

1. support of projects designed and initiated in other departments,

2. combined projects conducted with one or more other departments and

3. projects conducted wholely within the department.

The support of projects of other departments consists of assay of sera or filter paper blood spots for antibody to scrub typhus and isolation of rickettsia from specimens of blood, tissue or vectors. Generally, the specimens for isolation (except for vectors) were harvested by a member of this department. Standard techniques described in prior annual reports (1971; 136-139) were employed for serology, isolation, and identification. Projects of this nature have included the vertical and horizontal distribution of scrub typhus in common Malaysia habitats and support of the chigger colony. Filter paper blood spots submitted from Thailand and Indonesia are also in this category, as is technical support to the HAA project of the IMR.

Combined projects initiated with other departments have included the study of the incidence of arbovirus infection in a circumscribed area (Department of Medical Entomology and Department of Parasitology). Rodents, mosquito pools, and human sera have been processed by suckling mouse inoculation and yielded isolates. These isolates have not been definitely identified, but both group A and B arboviruses are represented. (See Department of Medical Entomology Section)

A study to prepare a purified *Pseudomonas pseudomallei* antigen has been initiated with the Department of Bacteriology. Precipitating antigens have been prepared by three different methods. All contain protein, but differ at least in molecular size. The physical, chemical and immunological relationships of these fractions will be studied during the next year.

The identification of mosquito blood meals was conducted with the Department of Medical Entomology. The project was initiated to determine human feeding rates which could be an aid in explaining the spread of mosquito borne infections. The technique which evolved was based on polyacrylamide gel electrophoresis of the macerated whole

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mosquito. Results indicate that the technique has advantages over those presently in use.

Projects conducted wholely within the department include the fractionation of purified *Rickettsia tsutsugamushi*. Attempts to adapt the organism to growth in locally available subtrates (duck eggs, cell lines, and primary cell cultures) are continuing. The production of cell cultures free of antibiotics, which is required for rickettsial growth, will be enhanced by laminar flow hoods (on order). Several strains have been serially passed in duck egg yolk sacs and are exhibiting sufficient growth for the yolk sacs to be used in purification procedures. Future work will be centered on DEAE absorption of contaminants followed by ether extraction and/or molecular sieve column chromotography.

The response of silvered leaf-monkey (SLM) to various strains and doses of *R. tsutsugamushi* will be continued. Results indicate that clinical signs are mild following inoculation of single strains compared to multiple strain inoculations (Annual Reports 1971, and 1972). Future work will elucidate the specific immunoglobulin response of virgin and experienced monkeys to challenge with a spectrum of strains. Challenges in experienced monkeys will be chosen to include homologous, related, and unrelated strains as assayed by fluorescent antibody techniques. Of particular interest will be the response of experienced SLM to challenge strains unrelated to the originally inoculated strain.