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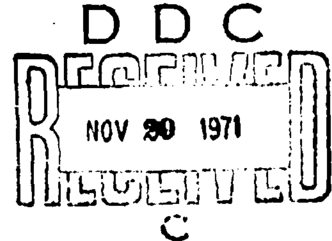

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UNITED STATES ARMY MEDICAL RESEARCH UNIT

INSTITUTE FOR MEDICAL RESEARCH

KUALA LUMPUR, MALAYSIA.

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13. ABSTRACT

INVESTIGATIONS OF BACTERIAL DISEASES

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Temporal Patterns of Pathogen Excretion in Diarrhea

Quantitative Stool Culture Method: The quantitative stool culture method developed here is being used in hospital laboratories. The method was readily accepted and its precision remains good in routine use. Quantitative cultures give more isolations in early diarrhea than routine qualitative methods. Fecal swabs into selenite broth is superior in late diarrhea and carrier states.

Excretion Patterns in Baterial Diarrhea: Distinct pathogen excretion patterns were defined in diarrhea. Different patterns were observed in western (pattern type II) than indigenous patients (patterns types I & III) with diarrhea. In indigenous patients different pathogen excretion patterns occurred in serious diarrhea (elevated, sustained = type I) compared to self limiting diarrhea (transient peak = type III), and could be differentiated by a single quantitative culture by the time of usual admission.

Decreases in pathogen excretion in the absence of antibiotics are associated with an overgrowth in other *Enterobacteriaceae*.

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Bacteriology, diarrhea, aborigines, pathogen excretion patterns, *Shigella*, *Salmonella*, *E. coli*, responsive flora, *Edwardsiella tarda*, *Entamoeba histolytica*, *Trichuris trichiura*, *shigellosis*, *trichuriasis*, necrotizing gastroenteritis, computer descriptors, Gram-stained, fecal smears, fecal exudate, drugs in diarrhea, new pathogens of diarrhea, early diagnosis of diarrhea, public health indices, lymphoma in filariasis, ascariasis in Loeffler's syndrome, Rh negative aborigines, *Orang Asli*, Malaysia, tropical medicine, Septrin, agranulocytosis, blood films in *shigellosis*, prenatal folic acid deficiency in aborigines, tetracycline treatment of melioidosis, deromatomycoses and skin cancer, drug resistant *Pseudomonas*, external otitis, hospital management, public disaster, disease surveillance, *Chromobacterium violaceum*, gibbons, zoo infections, ecology, disease transmission, small mammals, hosts, equatorial ecosystems, anopheline, *Anopheles balabacensis*, canopy mosquitoes, chloroquine resistance, culicine, *in vitro* drug resistance testing, malaria survey, *Orang Asli*, *Plasmodium falciparum*, Tragulid malaria, West Malaysia, endotoxin messengers, endotoxin antagonist, endotoxic shock,cause, endotoxic fever,cause, endotoxic fever & shock, prevention, rickettsia, scrub typhus, *Leptotrombidium deliense*, *R. tsutsugamushi*, tick typhus, hemolymph technique, rickettsia, silvered leaf-monkeys, *Presbytis cristatus*, *Rattus annandalei*, tree shrews, *Tupaia glis*, mouse deer, *Tragulus javanicus*, tropical canine pancytopenia, leptospirosis, melioidosis.						

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Polymorphonuclear leucocytes appear in the stool 48-72 hours after the peak of pathogen excretion.

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Three cases of severe diarrhea (two fatal) were associated with overgrowth of a bacterium in group XIII.

Fecal smear analysis gives an earlier and easier definition of bacterial diarrhea than other methods available.

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

Studies were conducted to ascertain the aspects of the ecology of mammals in equatorial ecosystems that predispose their involvement in disease transmission cycles in general and in specific zoonoses in particular. Because of the cryptic nature of the endemic transmission cycles of many zoonoses it is important to find out more about them to evaluate their potential for epidemics and to implement control measures. The specific objectives of this project are to evaluate ecological factors, such as distribution (geographical, altitudinal, between and within habitats, including vertical and temporal), reproduction (litter size and frequency, seasonality), population dynamics (productivity, survival, and longevity), feeding habits, and other behavior that predispose the involvement of potential host species in zoonotic disease cycles.

Studies were continued on the geographical and altitudinal distribution, temporal and spatial use of the environment, reproduction, population dynamics, feeding habits competition, parasitemia, and taxonomy and systematics. In some areas surveys for scrub typhus antibodies and scrub typhus rickettsia isolation attempts have been begun. Mammals were trapped, netted, shot, or collected from their nests, together with their nesting materials. Pertinent ecological information was recorded. Each animal and nesting materials were examined for ectoparasites. The animals were dissected and endoparasites were collected. Fecal samples were examined for helminth eggs and protozoal cysts (*Eimeria*)(Hooper Foundation). Blood samples were taken for examination for malarial and microfilarial parasites. From some areas, blood samples were tested by fluorescent antibody techniques for scrub typhus antibodies (Department of Serology) and some blood was injected into live mice for rickettsial isolation (Department of Rickettsiology). Reproductive state was determined by dissection and measurement of gonads and embryos when present. Diets were determined from stomach contents. Mark and release studies and other ecological studies are

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continuing in conjunction with an aerial transect (over 200 feet) constructed in the canopy from 30 to 120 feet from the ground in a mature rain forest to determine the vertical distribution of potential hosts, parasites, and pathogens.

Results of these studies indicate that mammalian hosts and potential hosts of zoonotic diseases are not randomly distributed in various equatorial ecosystems. They display varying degrees of altitudinal and habitat specificity. Within habitats they utilize various vertical zones in the forest and are active at specific times. Correlated with this, inter- and intra-specific variations are observed in the parasite patterns. Diets predispose probabilities of endoparasite infections. Species have different patterns of reproductive periodicity, varying from continuous breeders to thos highly seasonal. Population dynamics are also variable. These factors are important in determining whether a given mammalian species would be suitable as a reservoir or an amplifying host for zoonotic diseases. For example, contrary to what has been suggested as a possibility, arboreal species do not seem to be involved in any "jungle tsutsugamushi" (scrub typhus) transmission cycle. Improved rickettsial isolation techniques have made it possible to compare the involvement of mammals in various habitats in the scrub typhus transmission cycle. In Malaysia primary forest species seem to be involved more than species characteristic of scrub habitats, the classical habitat of scrub typhus. Within habitats there appears to be wide interspecific variation in involvement in this disease.

INVESTIGATIONS OF MALARIA

Studies of chloroquine-resistant malaria in West Malaysia were continued during this reporting period. Both the *in vivo* and *in vitro* techniques were used to determine the status of resistant malaria in Perak and Kelantan. Associated anopheline vector surveys were made in Trengganu, Perak, and Kelantan. Emphasis was placed on determining the importance of *Anopheles b. balabacensis* as a vector in these areas and to delineate the gradient between areas having a high percentage of resistant malaria to those having only a low amount. Additional studies using the *in vitro* method were used to try and gain a better understanding of the response of Malaysian-*P. falciparum* strains to chloroquine. Investigations comparing malaria prevalence in *Orang Asli* living on the jungle fringe compared to those dwelling in deep jungle were conducted. Control and prophylatic measures in these areas were evaluated on a periodic basis. It appears that the prevalence of malaria in certain deep jungle areas has been reduced by these measures. One of our technicians contracted a simian malaria, probably *P. knowlesi*, in one of the fringe areas. *Anopheles maculatus* is the main malaria vector in both fringe and deep jungle areas. Studies on the mosquito

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fauna found in the jungle canopy were expanded with the addition of another transect system in less disturbed primary forest. Mosquitoes were collected at different levels within the canopy at heights ranging from 20 to 130 feet above the ground. Certain culicines were found to oviposit in black glasses and bamboo cups at even the highest points along the canopy transect. "Rare" species have been found to be common in the canopy. Colonies of vector mosquitoes are being reared to support work on traguli.d malaria, and additional species of mosquitoes have been added to the reference collection. Electron microscopy has been used to study the erthrocytic and exoerthrocytic stages of *P. traguli*.

INVESTIGATIONS OF MELIOIDOSIS

The Mechanism of Bacterial Endotoxin: Second Messengers and Antagonist

Studies of endotoxic fever and shock have demonstrated a second messenger mechanism of bacterial endotoxin action: endotoxin binds to leucocyte membranes, displaces a preformed second messenger-1, and induces the formation of a delayed second messenger-2. The fever curves of the second messengers together temporally and quantitatively account for the complex fever curve of bacterial endotoxin. The amount of messengers released are proportional to the amount of endotoxin added until the preformed messenger-1 is depleted. At high endotoxin doses, delayed messenger-2 predominates. At high doses the messengers mediate endotoxic shock and death.

A system of messenger-antagonism exists and corrects for inadvertent messenger release. Administered messenger antagonist prevents endotoxic shock and death.

INVESTIGATIONS OF SCRUB TYPHUS

Studies were conducted on the dynamics of scrub typhus in vector mites and rodent hosts, and the bionomics and ecology of vectors.

In preliminary tests, 8 of 75 larvae successfully completed development after being fed on a membrane substrate that was placed over the end of a feeding tube containing rabbit serum. With minor modifications in diet and procedure, this technique can be used in attempts to infect mites with characterized strains of *R. tsutsugamushi*.

One of 5 negative *L. akamushi* nymphs allowed to cannibalize positive prenymphs produced positive offspring (13 of 15 offspring were positive). With one exception, offspring from the other 4

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mites were negative. Microdissection and cryostat methods were developed and are being used to follow rickettsial development in mite organs. Photomicrographs have been made of *R. tsutsugamushi* in mite tissue preparations stained with fluorescin conjugates.

Peak rickettsemia in the skin of white mice was found to occur at approximately 48 hours after IP infection with the Karp strain of *R. tsutsugamushi* at $10^{6.7}$ $MIPLD_{50}$. Previously negative *L. deliense* larvae that fed on infected sensitized mice have been proven positive for *R. tsutsugamushi*. Studies of offspring from the infected mites are being conducted.

Fluorescent antibody analysis using strain specific conjugates revealed that the positive colony, *L. (L.) akamushi*, is infected with Karp and Kato "like" strains of *R. tsutsugamushi*, that one mite can carry more than one strain of *R. tsutsugamushi* and that these strains have remained antigenically stable through 7 generations of vector mites.

Data reveals that the silvered leaf-monkey is susceptible to *R. tsutsugamushi*, as are gibbons, both of which develop eschars which may persist for over 10 days and appear identical to those occurring in humans. Rickettsemias may be demonstrated for as long as 15 days and reach levels of over 10^3 $MIPLD_{50}$'s per 0.2 ml of whole blood. Hyperthermia was demonstrated and may last for over two weeks. In an animal that died a marked hypothermia was present for 10 days preceding death. Chronic infection of silvered leaf-monkeys was demonstrated at 2 and 5 months post inoculation and involved mainly the lymphatic system; however other organs were also found to be infected.

Because of difficulties of obtaining adequate serum specimens from remote jungle areas and from small mammals, filter paper specimens of whole blood, 0.065 ml, were investigated to determine if they could be used in the indirect fluorescent antibody test for scrub typhus. It was determined that not only can they be used to screen human and small mammal specimens in prevalence studies but that accurate titers can be obtained as well.

It was found that *Orang Asli* (Aborigines) living in deep jungle had prevalence ratios of 73% for adults, 20 years old or over, as determined by the indirect fluorescent antibody test using a trivalent antigen to scrub typhus. The prevalence of scrub typhus antibodies decreased in fringe dwelling peoples to 48% while those living in *Kampong* had even lower prevalence ratios (8%). These data indicate that human scrub typhus in Malaysia, at least among the aborigines, is more common in deep jungle than in the "scrub areas" represented by the fringe area data.

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It was found that vertical zonation does affect the prevalence of scrub typhus in small mammals as true arboreal mammals are essentially negative by isolation and serology while semiarboreal and ground dwelling mammals have kidney isolation rates of 23% and a prevalence of 52% as determined by the indirect fluorescent antibody test. It was shown that conjugates can be prepared against wild mammal globulins that allow the indirect fluorescent antibody test for scrub typhus to be used in prevalence studies among small mammals. Kidney isolation ratios were higher (23%) than blood isolations (15%) on a sample size of 1164. The involvement of the various species, over 40, varied with one having an isolation ratio as high as 50% and 6 species had positive serological ratios of over 50%.

It was shown that the laboratory white rat can remain chronically infected for at least up to 16 months post inoculation. Certain animals were positive for *R. tsutsugamushi* at all the time periods tested, 10, 12, 14 and 16 months post-challenge as determined by isolation. Of interest was the fact that chronic infection did not maintain the serum antibody level at a high titer as evidenced by a drop in the indirect fluorescent antibody titer with time. Since the rats were 2-3 months old at the time of challenge and the average life span is approximately 2 years, this data would indicate that once a rat is chronically infected with *R. tsutsugamushi* it remains so for the rest of its life. Challenge dose did affect the rate of chronic infection with $10^{4.3}$ $MIPLD_{50}$'s giving ratios of 4 of 7 while lesser doses gave a ratio of 2 of 15 with an intraperitoneal challenge. This raises some interesting question concerning the dose of rickettsia that a chigger injects into a human or small mammal while feeding.

INVESTIGATIONS OF TICK TYPHUS

Serological and epidemiological studies of tick typhus in Western Malaysia:

No cases of tick typhus were reported during this reporting period. Ticks collected in an area where the last reported cases of tick typhus occurred were negative for rickettsia. Hemolymph slides of 200 *Ornithodorus batuensis* ticks were examined, and at least 1 tick appeared to be positive. The hemolymph technique has also been used to detect *Rickettsia tsutsugamushi* in mite vectors.

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LABORATORY ANIMAL DEVELOPMENT AND ZOONOTIC DISEASES

The laboratory animal management and facilities at the USAMRU were completely reorganized to provide proper management, control and care. In cooperation with the staff at the IMR, the breeding colonies were likewise changed and the facilities and breeding stock updated in such a way that all divisions of the IMR are now assured of an adequate supply of healthy animals. In cooperation with a major animal feed mill in Southeast Asia, laboratory animal chows were formulated and are now available in Southeast Asia for all the common laboratory animals. Caging for the various species was obtained locally following design recommendations of the National Science Council and the American Association for Laboratory Animal Science.

Invaluable experience was gained in the care and management of the silvered leaf-monkey, *Presbytis cristatus*. The problems were investigated starting during capture and followed through conditioning and use in experimental infection with *R. tsutsugamushi*. Early survival rates were very poor, but by changing procedures starting with capture they were increased to 71 percent, and it is felt that they can be increased even more. A preliminary start was made on determining normal physiological and blood cellular values which will be expanded and include blood chemistries. These are being done starting at the time of capture through the conditioning period of approximately 2 months before experimental use. It is extremely encouraging that the normal temperature range in this species is much more narrow than that for the macaque species. In addition our experience has shown that the silvered leaf-monkey is much easier to handle than the macaque species and that serious bites among animal handlers is not a problem.

Colonies of tree shrews and *Rattus annandalei* were started and offspring of *Rattus annandalei* are now being used in scrub typhus investigations.

Procedures, caging and prophylactic therapy has allowed for a survival rate of 68% since December 1970 in newly caught mouse deer. Also several offspring have been conceived, born and reared in the laboratory. The techniques of splenectomy were worked out for this species and splenectomized animals have been used in malaria investigations. Preliminary data revealed that this species, *Tragulus javanicus*, has a red blood cell count of over 70 million per ml which is the highest for any known mammal.

The presence of tropical canine pancytopenia in Malaysia was documented by the presence of the inclusion bodies of *E. canis* in circulating monocytes of naturally infected dogs and

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clinicopathological data from suspected cases.

Serology on over a 1,000 sera of cattle and swine in Malaysia revealed rates of approximately 1% positives for melioidosis which indicates that melioidosis is not a serious problem in domestic livestock in Malaysia. Trouble was experienced with the leptospirosis plate agglutination antigen and the leptospirosis prevalence study in domestic livestock in Malaysia had to be postponed until these difficulties can be overcome.

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US ARMY MEDICAL RESEARCH
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INVESTIGATIONS OF SCRUB TYPHUS

Studies were conducted on the dynamics of scrub typhus in vector mites and rodent hosts, and the bionomics and ecology of vectors.

In preliminary tests, 8 of 75 larvae successfully completed development after being fed on a membrane substrate that was placed over the end of a feeding tube containing rabbit serum. With minor modifications in diet and procedure, this technique can be used in attempts to infect mites with characterized strains of *R. tsutsugamushi*.

One of 5 negative *L. akamushi* nymphs allowed to cannibalize positive prenymphs produced positive offspring (13 of 15 offspring were negative. Microdissection and cryostat methods were developed and are being used to follow rickettsial development in mite organs. Photomicrographs have been made of *R. tsutsugamushi* in mite tissue preparations stained with fluorescin conjugates.

Peak rickettsemia in the skin of white mice was found to occur at approximately 48 hours after IP infection with the Karp strain of *R. tsutsugamushi* at $10^{6.7}$ $MIPLD_{50}$. Previously negative *L. deliense* larvae that fed on infected sensitized mice have been proven positive for *R. tsutsugamushi*. Studies of offspring from the infected mites are being conducted.

Fluorescent antibody analysis using strain specific conjugates revealed that the positive colony, *L. (L.) akamushi*, is infected with Karp and Kato "like" strains of *R. tsutsugamushi*, that one mite can carry more than one strain of *R. tsutsugamushi* and that these strains have remained antigenically stable through 7 generations of vector mites.

Data reveals that the silvered leaf-monkey is susceptible to *R. tsutsugamushi*, as are gibbons, both of which develop eschars which may persist for over 10 days and appear identical to those occurring in humans. Rickettsemias may be demonstrated for as long as 15 days and reach levels of over 10^3 $MIPLD_{50}$'s per 0.2 ml of whole blood. Hyperthermia was demonstrated and may last for over

two weeks. In an animal that died a marked hypothermia was present for 10 days preceding death. Chronic infection of silvered leaf-monkeys was demonstrated at 2 and 5 months post inoculation and involved mainly the lymphatic system; however other organs were also found to be infected.

Because of difficulties of obtaining adequate serum specimens from remote jungle areas and from small mammals, filter paper specimens of whole blood, 0.065 ml, were investigated to determine if they could be used in the indirect fluorescent antibody test for scrub typhus. It was determined that not only can they be used to screen human and small mammal specimens in prevalence studies but that accurate titers can be obtained as well.

It was found that *Orang Asli* (Aborigines) living in deep jungle had prevalence ratios of 73% for adults, 20 years old or over, as determined by the indirect fluorescent antibody test using a trivalent antigen to scrub typhus. The prevalence of scrub typhus antibodies decreased in fringe dwelling peoples to 48% while those living in *Kampong* had even lower prevalence ratios (8%). These data indicate that human scrub typhus in Malaysia, at least among the aborigines, is more common in deep jungle than in the "scrub areas" represented by the fringe area data.

It was found that vertical zonation does affect the prevalence of scrub typhus in small mammals as true arboreal mammals are essentially negative by isolation and serology while semiarboreal and ground dwelling mammals have kidney isolation rates of 23% and a prevalence of 52% as determined by the indirect fluorescent antibody test. It was shown that conjugates can be prepared against wild mammal globulins that allow the indirect fluorescent antibody test for scrub typhus to be used in prevalence studies among small mammals. Kidney isolation ratios were higher (23%) than blood isolations (15%) on a sample size of 1164. The involvement of the various species, over 40, varied with one having an isolation ratio as high as 50% and 6 species had positive serological ratios of over 50%.

It was shown that the laboratory white rat can remain chronically infected for at least up to 16 months post inoculation. Certain animals were positive for *R. tsutsugamushi* at all the time periods tested, 10, 12, 14 and 16 months post-challenge as determined by isolation. Of interest was the fact that chronic infection did not maintain the serum antibody level at a high titer as evidenced by a drop in the indirect fluorescent antibody titer with time. Since the rats were 2-3 months old at the time of challenge and the average life span is approximately 2 years, this data would indicate that once a rat is chronically infected with *R. tsutsugamushi* it remains so for the rest of its life. Challenge dose did affect the rate of chronic infection with $10^{4.3}$ $MIPLD_{50}$'s giving ratios of 4 of 7 while

lesser doses gave a ratio of 2 of 15 with an intraperitoneal challenge. This raises some interesting question concerning the dose of rickettsia that a chigger injects into a human or small mammal while feeding.

INVESTIGATIONS OF TICK TYPHUS

Serological and epidemiological studies of tick typhus in Western Malaysia:

No cases of tick typhus were reported during this reporting period. Ticks collected in an area where the last reported cases of tick typhus occurred were negative for rickettsia. Hemolymph slides of 200 *Ornithodorus batuensis* ticks were examined, and at least 1 tick appeared to be positive. The hemolymph technique has also been used to detect *Rickettsia tsutsugamushi* in mite vectors.

LABORATORY ANIMAL DEVELOPMENT AND ZOONOTIC DISEASES

The laboratory animal management and facilities at the USAMRU were completely reorganized to provide proper management, control and care. In cooperation with the staff at the IMR, the breeding colonies were likewise changed and the facilities and breeding stock updated in such a way that all divisions of the IMR are now assured of an adequate supply of healthy animals. In cooperation with a major animal feed mill in Southeast Asia, laboratory animal chows were formulated and are now available in Southeast Asia for all the common laboratory animals. Caging for the various species was obtained locally following design recommendations of the National Science Council and the American Association for Laboratory Animal Science.

Invaluable experience was gained in the care and management of the silvered leaf-monkey, *Presbytis cristatus*. The problems were investigated starting during capture and followed through conditioning and use in experimental infection with *R. tsutsugamushi*. Early survival rates were very poor, but by changing procedures starting with capture they were increased to 71 percent, and it is felt that they can be increased even more. A preliminary start was made on determining normal physiological and blood cellular values which will be expanded and include blood chemistries. These are being done starting at the time of capture through the conditioning period of approximately 2 months before experimental use. It is extremely encouraging that the normal temperature range in this species is much more narrow than that for the macaque species. In addition our experience has shown that the silvered leaf-monkey is much easier to handle than the macaque species and that serious bites among animal handlers is not a problem.

Colonies of tree shrews and *Rattus annandalei* were started and offspring of *Rattus annandalei* are now being used in scrub typhus investigations.

Procedures, caging and prophylactic therapy has allowed for a survival rate of 68% since December 1970 in newly caught mouse deer. Also several offspring have been conceived, born and reared in the laboratory. The techniques of splenectomy were worked out for this species and splenectomized animals have been used in malaria investigations. Preliminary data revealed that this species, *Tragulus javanicus*, has a red blood cell count of over 70 million per ml which is the highest for any known mammal.

The presence of tropical canine pancytopenia in Malaysia was documented by the presence of the inclusion bodies of *E. canis* in circulating monocytes of naturally infected dogs and clinicopathogical data from suspected cases.

Serology on over a 1,000 sera of cattle and swine in Malaysia revealed rates of approximately 1% positives for melioidosis which indicates that melioidosis is not a serious problem in domestic livestock in Malaysia. Trouble was experienced with the leptospirosis plate agglutination antigen and the leptospirosis prevalence study in domestic livestock in Malaysia had to be postponed until these difficulties can be overcome.

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."

It should be noted that this report covers a nine month period, 1 October 1970 to 30 June 1971.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. Agency Accession	2. Date of Summary	Report Control Symbol
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10. No./Codes	Program Element	Project Number	Task Area Number	Work Unit Number
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19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Microbiology, Public Health

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

23.(U) Technical Objective: (1) To use quantitative methods, developed here, of direct count and culture assays of stool bacteria to study enteric population dynamics and its relation to diarrhea, (2) to apply dimensional descriptor analysis of total bacterial flora of the normal and diseased gut, and (3) to study interrelationships between *Enterobacteriaceae* and intestinal amoebae and helminths and their role in diarrhea.

24.(U) Approach: Fresh stool specimens are examined by fresh smear, stained fecal smear, and qualitative and quantitative stool culture.

25.(U) Progress: (1) The quantitative stool culture method was successfully applied to routine hospital use. The method gave more isolations in early diarrhea than routine qualitative methods. Fecal swabs into selenite broth was superior in late diarrhea and carrier states. Distinct pathogen excretion patterns were defined in diarrhea. Different patterns were observed in western and indigenous patients. In indigenous patients different patterns were observed in serious compared to self limiting diarrhea, and could be differentiated by a single quantitative culture by the time of usual admission. Self limiting diarrhea was associated with a peak of pathogen excretion followed by a peak of other *Enterobacteriaceae*. The appearance of polymorphonuclear leucocytes in the stool was delayed 48-72 hours after the pathogen peak and is not a diagnostic aid in early diarrhea. Effects of antibiotics upon pathogen excretion were markedly influenced by the pre-existent pathogen excretion pattern type. In self limiting diarrhea antibiotics were without observable effect. Quantitative cultures afforded a sensitive assay of clinical drug efficacy, and give results earlier than qualitative methods.

Available to contractors upon originator's approval.

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

(2) Relatively constant descriptor group profiles of bacterial flora were found in normal individuals. Descriptor profiles were markedly altered in diarrhea to predominant forms which were of the same descriptor groups as the identified pathogens from cultures. The shifts were readily apparent on direct microscopic examination. One form of serious diarrhea of unknown etiology was associated with overgrowth of Group XIII. Fecal smear analysis gives an earlier and easier definition of bacterial diarrhea than other methods available.

(3) Although more frequent in chronic trichuriasis, shigellosis showed the same clinical features and excretion pattern as in *Trichuris*-free patients. Shigellosis is the most common cause of hospitalization in chronic trichuriasis patients. Although usually associated with amebic dysentery, *Edwardsiella tarda* was shown to be able to cause transmissible diarrhea on its own with excretion patterns typical of pathogenic *Enterobacteriaceae*. In amebic dysentery *Ed. tarda* excretion was quite different, being elevated, but not episodic nor followed by polymorphonuclear leucocytes nor responsive flora.

Item 22

Bacteriology, diarrhea, aborigines, pathogen excretion patterns, *Shigella, Salmonella, E. coli,* responsive flora, *Edwardsiella tarda, Entamoeba histolytica, trichuris trichiura, Shigellosis, trichuriasis,* necrotizing gastroenteritis, computer descriptors, Gram-stained, fecal smears, fecal exudate, drugs in diarrhea, new pathogens of diarrhea, early diagnosis of diarrhea, public health indices, lymphoma in filariasis, ascariasis in Loeffer's syndrome, Rh negative aborigines, *Orang Asli*, Malaysia, tropical medicine, Septrin, agranulocytosis, blood films in shigellosis, prenatal folic acid deficiency in aborigines, tetracycline treatment of melioidosis, deromatomycoses and skin cancer, drug resistant *Pseudomonas*, external otitis, hospital management, public disaster, disease surveillance, *Chromobacterium violaceum*, gibbons, zoo infections.

INVESTIGATIONS OF BACTERIAL DISEASES

The Department of Bacterial Diseases was formed in 1966 to do medical studies of jungle-dwelling populations of Malaysia. Early investigations demonstrated that several diseases endemic in urban areas caused epidemics in the relatively isolated jungle communities. The first report of an epidemic of whooping cough in an aboriginal people was made here. Measles, chicken pox and pneumococcal pneumonia also cause epidemics with significant mortality. These studies of medical problems in the jungle have continued to the present. The *Orang Asli* (Malaysian aborigine) Medical Service has a very well organized net of radio communications with health personnel stationed at each village. It is possible to remain continually alert to health conditions throughout the country. Ill patients are evacuated by Royal Malaysian Air Force helicopter to the Gombak Medical Center which is in easy driving distance from the U.S. Army Laboratory in Kuala Lumpur. Family members are brought in to the hospital too, and so may be evaluated at the same time as the patient. Hospital staff go regularly into the jungle to carry out preventive medicine. Most departmental research studies are done at the medical center, but helicopter trips to the villages are possible on a priority basis.

During the previous reporting year studies were extended to patients in the Kuala Lumpur General Hospital. Patients at this hospital include each of the racial groups and social classes of Malaysia. At Gombak and GH the department maintains laboratory facilities and joins weekly consultation rounds with the Ministry of Health staff physicians.

A wide range of health problems present at these medical facilities including diseases which are rarely encountered in medical facilities in the U.S.: diphtheria, leprosy, yaws, neonatal tetany, snake bite, typhoid, parasitic infections, tropical mycoses, malaria, and nutritional deficiencies.

Temporal Patterns of Pathogen Excretion in Diarrhea in Malaysia

Introduction: Diarrhea is endemic throughout the Malay archipelago, with over-all case prevalences of pathogens much like that found in other parts of Southeast Asia. The specific area and population distributions of cases, however, are different because urban water supplies here are uniformly pathogen free, and night soil is not used for vegetables sold in most urban markets. Deep jungle mountain tribes take water from streams near their sources and move their villages when the soil becomes poor rather than use fertilizer. In both of these groups acute gastroenteritis is more common than bacterial and parasitic diarrhea. This situation is

reversed, however, in the Malay, Indian, or Chinese in lower income groups and in the aborigines living on the geographical fringe of the jungle. In these groups bacterial or parasitic pathogens may be isolated in over ninety per cent of cases of diarrhea. Multiple infections are quite common. Diarrhea is a major source of morbidity, and stunting of growth and even marasmus are not uncommon in cases of chronic diarrhea. The case mortality is about five per cent as compared to about fifteen per cent here ten years ago and fifty per cent in the general hospital in Jakarta.

Malaysian hospitals are modern and staffed with professionals with full western specialty qualifications. Therapy is based on a standard of western medical practice. The latter is based, however, on experience with cases different enough to be properly considered of a different disease. Such scientific studies as have been done have shown that the prevalence of pathogens as well as the amount of drug resistance here is very high compared to temperate zones. Most of the investigative bases of treatment, including drug efficacy studies, have been done in temperate zones, and the few done here have concentrated on cases among Americans.

The extent and seriousness of diarrhea in the tropics and the paucity of investigative bases of therapy in indigenous tropical populations give such studies high priority in world medicine.

In the very beginnings of studies here on the nature of tropical diarrhea, it became obvious that the essential tools were not available. Essential to any dynamic description is the ability to enumerate units at different times of the process. Existing methods of quantitative stool analyses were both time consuming and imprecise.

In studies here during the previous year the non-homogeneity of stool samples was found to be due to surface bonding between bacteria and stool mucus and debris. A simple method of suspension was devised utilizing hydration shell cleavage of such bonds. This method had a linear standard curve of dilution with a standard deviation of less than 0.1 $\log_{10}$ under research laboratory conditions. *Shigella sonnei* organisms were isolated in a diarrheal stool in million-fold greater numbers than the total of *Enterobacteriaceae* isolated from normal stools. The quantitative suspension was placed on slides, and the Gram-stained fecal smear analyzed by dimensional descriptors. Thirteen descriptor groups of Gram-negative stool bacteria were defined. Histograms of distribution of bacteria by dimensional group showed a consistent profile of "normal" in healthy monkeys under laboratory conditions.

During the present reporting year the methods for obtaining quantitative stool cultures and analyzing fecal smears were applied in routine hospital laboratories to determine the quantitative and

temporal correlates of diarrhea.

Methods: Quantitative stool cultures were performed in clinical laboratories of the Gombak *Orang Asli* (aborigine) Medical Center and the Kuala Lumpur General Hospital. Patients were not selected specifically for the study but rather reflected the usual patterns of hospital admission. Accompanying family members were taken as asymptomatic controls.

All procedures except final identification were performed by hospital staff as well as research staff. The collection and preidentification procedures were done generally by medical students as part of patient workup batteries. Ward physicians were actively engaged in the studies and full clinical data were obtained. Only routine laboratory results were made available to the clinician until the end of the study, and there were no research alterations in the way patients were managed except in those cases in which the research battery indicated a threat to the patient's life which was not available in the routine results.

For quantitative stool cultures, one-half gram of fresh stool was suspended in 50 ml of sterile saline by a Sorvall Omnimixer blender fitted with a sterile closed stainless steel cup and operated at 1500 rpm for 10 minutes. Aliquots were diluted serially and 0.1 ml of dilution, equal to 10^{-5} to 10^{-8} gram stool were spread with bent glass rods onto the surfaces of brilliant green, bismuth sulfate, deoxycholate citrate, and McConkey's agars. Colonies were counted after overnight incubation at 37°C. Identical aliquots were transferred to selenite broth. Swabs from the original stool sample were streaked directly onto each agar plate and into selenite broth. After 24 and 72 hours incubation selenite broth was subcultured onto agar plates and bacterial colonies identified biochemically and serologically.

For bacterial descriptor group analysis, fecal suspensions were placed on glass slides and stained by Gram's method as modified by Preston and Morrell. Slides were examined with a light microscope fitted with an eye-piece micrometer. Slides were screened for major shifts in size distributions of Gram-negative organisms. A few slides were examined by the more detailed photomicrographic method described in the previous annual report.

Quantitative Stool Culture Method

Results: The quantitative stool culture method was accepted readily by hospital staffs. Once premeasured solutions and present measuring devices including bio-pipetters (Schwartz) were made available, the technique required little more work than the

technique already used for quantitative urine cultures. Under clinical working conditions in over 250 case studies, the reproduceability and linearity of the technique were quite good with standard deviations of about 0.2 log_{10} units compared to 2 log_{10} for routine methods.

Of all the methods of initial culture used, quantitative dilutions spread directly upon agars gave the highest number of total isolations of *Enterobacteriaceae*. The optimal method of isolating a particular organism depended, however, on its relative abundance with respect to other organisms growing in the same conditions. Predominant organisms were usually isolated by all the methods used. Organisms constituting less than ten and more than one-half per cent of the total were more often isolated by the quantitative method. Organisms less than one-half per cent of the total were isolated more often by one or another of the non-suspended samples. Of the non-suspended methods, swabs grown in selenite broth were best, but each of the methods was solely responsible for isolations in some cases, and no method could be dropped from the battery without losing diagnoses. Relative abundances also predicted efficacy of agars: McConkey's agar grew the pathogens well when their numbers predominated, but with pathogen frequencies less than 1:200, more inhibitory agars were necessary.

Conclusions: The superiority of the quantitative stool culture method with relative pathogen abundances of greater than 1:200 is probably because four-fold more colonies are inspected on spread agars than streaked agars (200:50). The superiority of streaking methods at lower pathogen ratios (such as seen in the late decline of the pathogen curve and in carrier states) must reflect their two log greater standard deviations.

Excretion Patterns in Bacterial Diarrhea

Results: The temporal patterns of pathogen excretion in bacterial diarrhea fitted a family of curves which can be summarized as three curves labeled types I, II, and III (Figure 1). In type I diarrhea pathogen excretion increased rapidly to high values until the patient succombed or the disease progression was broken by antibiotics. The relationship of excretion to time approximated a rectangular hyperbola. In type II diarrhea pathogen excretion increased to high numbers and then decreased slowly to low values. Type II diarrhea was seen in westerners who got bacterial diarrhea in country. In type III diarrhea pathogen excretion showed a transient peak with a rapid and complete decline. Type III was the most common presentation of diarrhea in our patients. Type IV excretion did not change with time and was found in carrier states.

Clinical signs and symptoms were of limited help in predicting a bacterial etiology or particular pathogen excretion pattern of diarrhea. Type III excretion patterns were the most completely

documented in our series and are illustrated in Figure 2. Numbers of pathogen increased quickly just preceding the onset of diarrhea. At the very start of diarrhea pathogen predominated other flora. At this stage attempts to isolate the pathogen from stool had the greatest chance of success. The appearance of polymorphonuclear leucocytes was delayed, and by the time this diagnostic feature of bacterial diarrhea was present, the optimal time for isolating the etiologic agent had passed. Concurrent to the decrease in pathogen there was an overgrowth of responsive flora including *Escherichia coli*, *Aerobacter spp.*, and Providence Group. We have also developed methods to demonstrate the emergence of bacteriocidal substances in stool fluids during this stage of diarrhea.

Conclusions: The family of curves of pathogen excretion *versus* time are assumed to reflect the balances of pathogen virulence and host defenses. The association of patterns with human populations is interesting. Indigenous individuals are likely to have had previous exposure to nearly all agents and therefore have good specific defenses and type III responses. When specific defenses fail, the patients tend to shift from type III to type I patterns, presumably from deficiencies in general defenses. Westerners tend to get type II disease, not having the specific defenses associated with type III, yet having enough general defenses to avoid type I disease.

In indigenous populations, quantitative stool cultures after day three separate diarrhea types I and III. Because of the delay in appearance of polymorphonuclear leucocytes (polys), they can be used as an important marker when duration of infection is uncertain by history. Polys present and pathogen excretion high indicate type I diarrhea. Polys present and pathogen excretion low indicates type III diarrhea. Cases with polys absent and pathogens absent are probably not due to the pathogens surveyed. Cases with no polys but pathogen present need sequential quantitative follow up.

The transience of pathogen excretion in type III diarrhea dictates that collections of pathogens in tropical surveys be made early in the course of diarrhea.

Efficacy of Drugs in Bacterial Diarrhea

Results: No attempts were made to alter treatment or time of patient discharge, and definitive assessment of individual drugs was not possible. Only in some of those cases of clearly defined appropriate antibiotic and susceptible pathogen, was pathogen excretion clearly altered by treatment. The effect of antibiotic upon excretion of pathogen was markedly influenced by the type of pathogen excretion pattern observed in the case.

Patients with type III diarrhea usually came to the hospital after three or more days of symptoms and pathogen excretion was already into its decline before the start of therapy. The main effect of antibiotics was to interrupt the overgrowth of reactive flora. The data were insufficient to indicate the effect this had on the duration of diarrhea. Experience with type II diarrhea was more limited. Antibiotics speeded the rate of decline of pathogen excretion, but it is unclear whether the final excretion rate or risk of residual carrier states were altered. Even though experience with type I diarrhea was limited, the cases studied were clearly defined. Pre-antibiotic pathogen excretion was high and increasing, and was sharply checked by the addition of antibiotics.

An example of the detailed picture afforded by quantitative measures of pathogen excretion was the case of a 55-year old Malay female who had diarrhea due to combined typhoid and shigellosis (Figure 3). Quantitative cultures showed increasing pathogen excretion after day three. This type I pattern was unaffected by constipating drug treatment, although the patient's diarrhea improved. The patient was still toxic, and after the bacteriological diagnoses of pathogens, chloramphenicol was started. The first post-treatment quantitative culture was taken the morning after the start of therapy and showed a three log drop in pathogen excretion. Routine quantitative methods first showed negative cultures on stool taken on the third day of treatment.

Conclusions: Quantitative stool cultures and patterns of pathogen excretion give information valuable to the decision whether to use antibiotic in a case of diarrhea, and once an antibiotic is given, whether to change it. Using non-quantitative methods, the mere presence of a pathogen does not necessarily mean an antibiotic is needed, an *in vitro* drug sensitivities are at best an indirect way to evaluate the clinical efficacy of a drug. In type III diarrhea antibiotics are probably no help and possibly do harm if indeed normal floral overgrowth serves to inhibit pathogens. A single quantitative cultures at the time of hospital admission should demonstrate the type of diarrhea as early as the next morning. In diarrhea types I and II decreased pathogen excretion is a readily available measure of drug efficacy and, measured by sequential quantitative cultures, has a much shorter delay than routine methods.

Analysis of Fecal Smears by Descriptor Groups

Results: Fecal smears from asymptomatic humans had greater variation in descriptor group profiles than was the case in monkeys under controlled conditions, but there were always multiple sizes and shapes of bacteria present and the general fecal smear profile of asymptomatic man and monkey were much alike.

The appearance of fecal smears in cases of diarrhea was markedly altered from normal. In diarrhea due to the pathogenic *Enterobacteriacea* fecal smears show almost exclusively organisms of groups II and V. Identified pathogens isolated from the same studies fitted groups II and V when suspended and Gram-stained (Group V is the diploid form of Group II; see Annual Report 1970). Fecal smears in late type III diarrhea (during overgrowth of responsive flora) also showed almost entirely group II and V bacteria, but were differentiated from earlier smears by the presence of polymorphonuclear leucocytes. The present method did not differentiate the different *Enterobacteriaceae*.

Some cases of diarrhea were associated with overgrowth of other descriptor groups of enteric bacteria. Three cases of diarrhea were associated with a Group XIII predominant flora. Fecal material examined by our laboratory; Communicable Disease Center, Atlanta; and Dysentery Reference Laboratory, London, failed to demonstrate a classical enteropathogen. Two of the cases died of necrotizing gastroenteritis, and the third case of the series was diagnosed by the fecal smear method within hours of the onset of symptoms and was treated successfully with high doses of ampicillin.

Conclusions: Both fecal smear examinations and quantitative stool cultures show that bacterial diarrhea is associated with a marked overgrowth of pathogen followed by an overgrowth of closely related responsive organisms, not by all of normal flora.

Descriptor grouping of bacteria in fecal smears can define bacterial diarrhea more quickly than bacterial cultures and at an earlier stage of diarrhea than the appearance of inflammatory cell exudate. The method can potentially be automated and linked to computers or used as a quick field or clinical diagnostic aid using only Gram stains and an ordinary microscope. Without additional procedures, however, the method cannot differentiate among *Enterobacteriaceae* nor predict drug sensitivities.

In indigenous tropical patients Gram-stained smears could be used for screening for bacterial diarrhea, then in positives the more time consuming quantitative stool cultures used to differentiate types I and III. Drugs could be started immediately and management reconsidered the next day when the first quantitative agar plates were examined.

In patient groups usually showing type II diarrhea (usually westerners), a complete shift in fecal smear should clearly indicate bacterial diarrhea and the need for antibiotics. In most of our studies ampicillin is the first line drug of choice.

Interrelationship Between *Trichuriasis* and *Shigellosis*

Results: Most of the cases of heavy trichuriasis came from the pediatrics wards of the Kuala Lumpur General Hospital. The association of trichuriasis and shigellosis as described in past studies was maintained. There was also an association between age group of peak prevalence of the two diseases. Patients 5-7 had increased prevalences of both diseases. Patients with salmonellosis were distributed throughout age groups above one year, and peak prevalence of pathogenic *Escherichia coli* was in the age group below eighteen months.

Quantitative stool cultures showed type III diarrhea of *Shigella spp.* excretion and peak of responsive flora in cases of trichuriasis. Type I patterns could not be totally excluded because patients appearing especially sick were treated with antibiotics. Most of the time, numbers of Shigella excreted had started to decline before the start of antibiotics, but occasionally were started early enough possibly to have caused the observed decline.

In chronic cases of trichuriasis most hospital admissions followed episodes of shigellosis. Different species Shigella were usually isolated upon sequential admissions of the same patient. For example, an eight year old Indian boy with heavy trichuriasis was admitted four times during the year but with a different *Shigella* each time.

Conclusions: Although these studies indicate that shigellosis is more likely in the patient with chronic trichuriasis, the nature of the infection was not shown to be different from shigellosis in the absence of chronic trichuriasis.

The changes in species of *Shigella* sequentially infecting an individual patient agrees with prevalence data that increased shigellosis in trichuriasis is not due to an increase in any particular species.

Since superimposed shigellosis is responsible for most hospitalizations of chronic cases of trichuriasis, and since trichuriasis is found in 50-80% of fringe Malay and Indian preschool children, the association of the pathogens is medically important in Malaysia.

Role of *Edwardsiella tarda* in Diarrhea

Results: Isolation of *Edwardsiella tarda* in our series was almost entirely confined to amebic dysentery patients. Stool excretion patterns of *Ed. tarda* in amebic dysentery were type IV: with high numbers being continuously shed. Other *Enterobacteriaceae*

also show flat excretion patterns. Polymorphonuclear leucocytes are shed irregularly and usually are associated with the irregular shedding of *Entamoeba histolytica*.

After amebicide by Flagyl, excretion of *Edwardsiella tarda* slowly declined and ceased. But in one case after treatment with Flagyl and the usual cessation of ameba and *Ed. tarda* excretion, diarrhea recurred. A recurrence of *Entamoeba histolytica* excretion was not found, but *Ed. tarda* recurred in a pattern typical of type III bacterial diarrhea, complete with transient overgrowth of *Ed. tarda* followed by overgrowth of reactive flora and appearance of polymorphonuclear leucocytes. Contacts of the patient were followed closely, and during the time she had type III *Edwardsiella* diarrhea, her younger brother (*E. histolytica* free) began excreting *Ed. tarda* in the stool. *Ed. tarda* excretion rapidly increased and diarrhea started. The older child was better, the family went home, and the younger child was lost to follow up.

Conclusions: Last year an association between *Edwardsiella tarda* and *Entamoeba histolytica* was shown by common isolation, serological titers, and response to therapy. Studies to date suggest an association between *Ed. tarda* and *E. histolytica* which is quite different from the episodic type III shigellosis in chronic trichuriasis. The nature of the association and the individual roles played by each of the two is still unclear, but evidence suggests that *Ed. tarda* is itself a potential pathogen capable of causing transmissible diarrhea.

Miscellaneous Studies

Collaborative work with parasitologists of the Hooper Foundation included this year an intestinal parasite survey. The survey was a repeat of one done five years ago. Presumably associated with the profound economic and medical strides in Malaysia over the period, total cases and case mortality has decreased. Relative prevalences, however, of intestinal parasites have not significantly changed. Also done was a conference on the diagnostic and clinical features of filariasis. In one case of filariasis, lymphoma was demonstrated in the inguinal lymph node of the elephantoid leg. Ascaris larvae were isolated in sputum from cases of Loeffler's syndrome.

Hematological studies included demonstration of the first known cases of Rh negative blood type in an *Orang Asli*, a case of agranulocytosis with Septrin, and a case of probable myeloid leukemia in an aborigine infant. An increase in circulating band forms has been reported in infants with *Shigella*. Our blood films commonly showed increased band forms in shigellosis in pediatric cases of different ages. Our study included children up to the age of ten. Peripheral blood films of maternity cases at Gombak commonly showed normochromic anemia and hyper-segmentation with or without macrocytosis.

A variety of diagnostic and public health referrals came from Commonwealth Forces Hospitals in country, the American International School, the American Embassy, and the Malaysian Ministry of Health. From a small base of departmental laboratory services and a larger base of reference backup in the U.S. and the U.K., we have available a good breadth of low volume support. Additionally, we have access to routine laboratory methods in other sections of the Institute for Medical Research. Laboratory and reference support was linked closely to clinical evaluation so that a composite assessment could be made of individual cases.

Bacteriological and serological diagnostic services in melioidosis and leptospirosis made up a fair portion of referrals, in particular from the Commonwealth military hospitals. At a clinical conference at the RAAF hospital in Butterworth, the history of melioidosis in Malaysia was reviewed as were the diagnostic and clinical features of the disease and ideas on pathogenesis developed here recently (see section on Melioidosis).

Of recent interest from several sources are mycological identifications. A case of chromoblastomycosis was diagnosed which had been listed as squamous cell carcinoma. Fungal diseases of the skin often present in unusual forms here and sometimes cause incorrect impressions of neoplastic disease. External otitis in the *Orang Asli* was commonly associated with isolations of *Pseudomonas spp.* which were resistant to all drugs tested. Possibly a fungus was also associated, and attempts at identification are in progress.

Public health services for the American International School included inspection of the drinking water supply, advice on school health procedures, and public health lectures to the students.

Referrals from the U.S. Embassy included general medical information and medical consultation on individual cases.

Ministry of Health requests this year centered on the massive public health program after the country-wide floods in January. Our staff helped with mass vaccinations in flooded villages and maintained a disease alert. No disease outbreak was documented in spite of massive evacuation of people in striken areas.

Referral work from within the USAMRU included diagnostic bacteriology and vaccine production for Veterinary Medicine and Ecology. Such collaborative work made up between 20 and 50 per cent of total bacteriological work output and has contributed information on laboratory animal care and sources of colony infections. In collaboration with Veterinary Medicine, gibbon deaths due to *Chromobacterium violaceum* at the National Zoo were investigated. The gibbons were kept in groups of several pairs on

small islands surrounded by a diverted stream. It was found that *C. violaceum* was being brought in by the diverted stream, was then impregnating the soil of the islands, and then was infecting the gibbons by dirt contamination of wounds inflicted by competitive pairs of gibbons. Soil contamination was related to height of the islands from the water. Incidence of disease was related more to island density and incidence of trauma than soil contamination. Suitable recommendations to the zoo board were made by the Department of Veterinary Medicine.

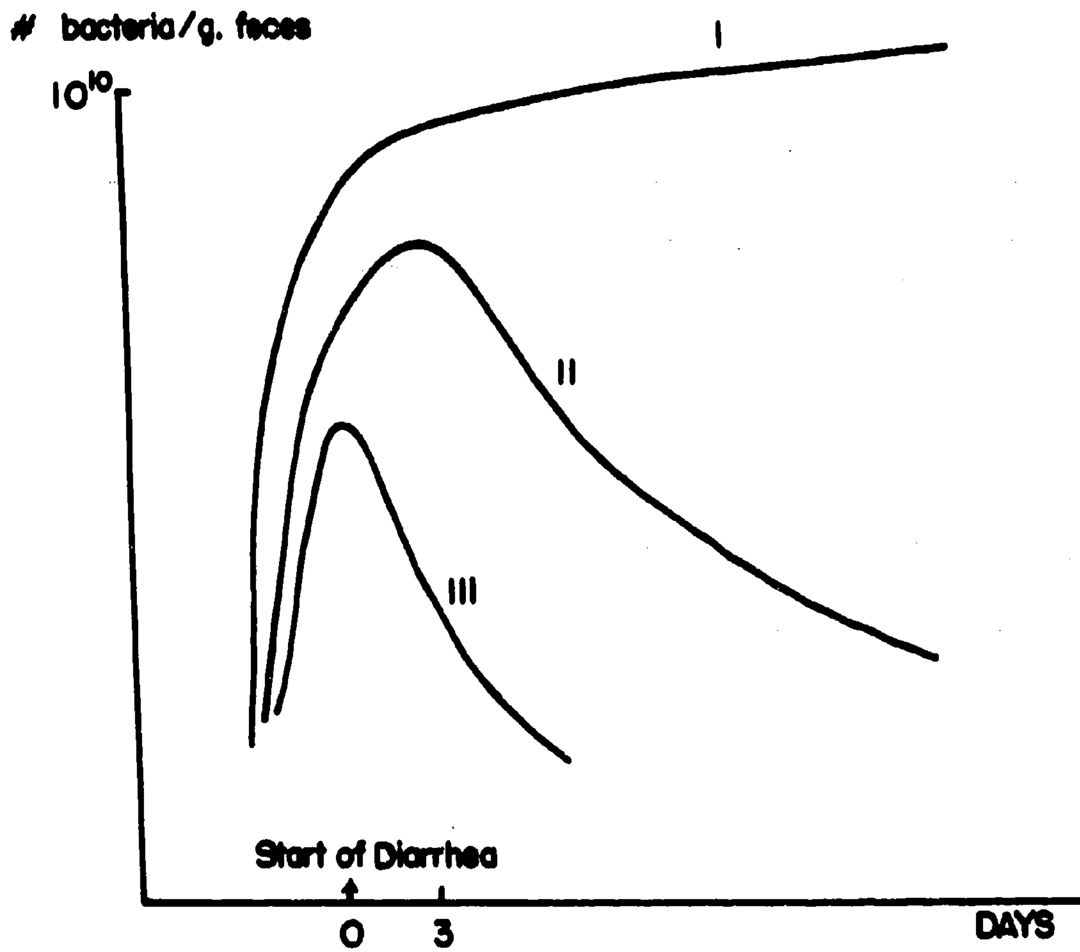

Fig. I Showing quantitative patterns of pathogen excretion in diarrhea

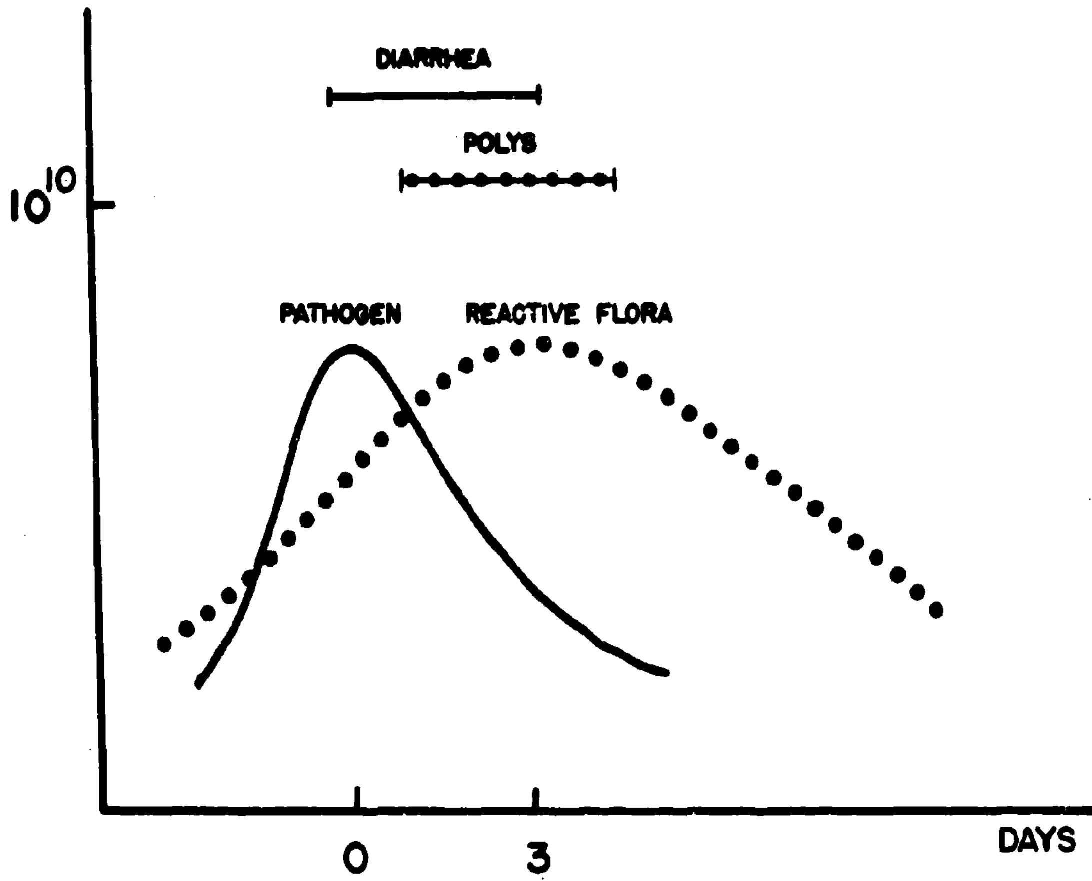

Fig. 2 Showing sequences of changes in type III diarrhea

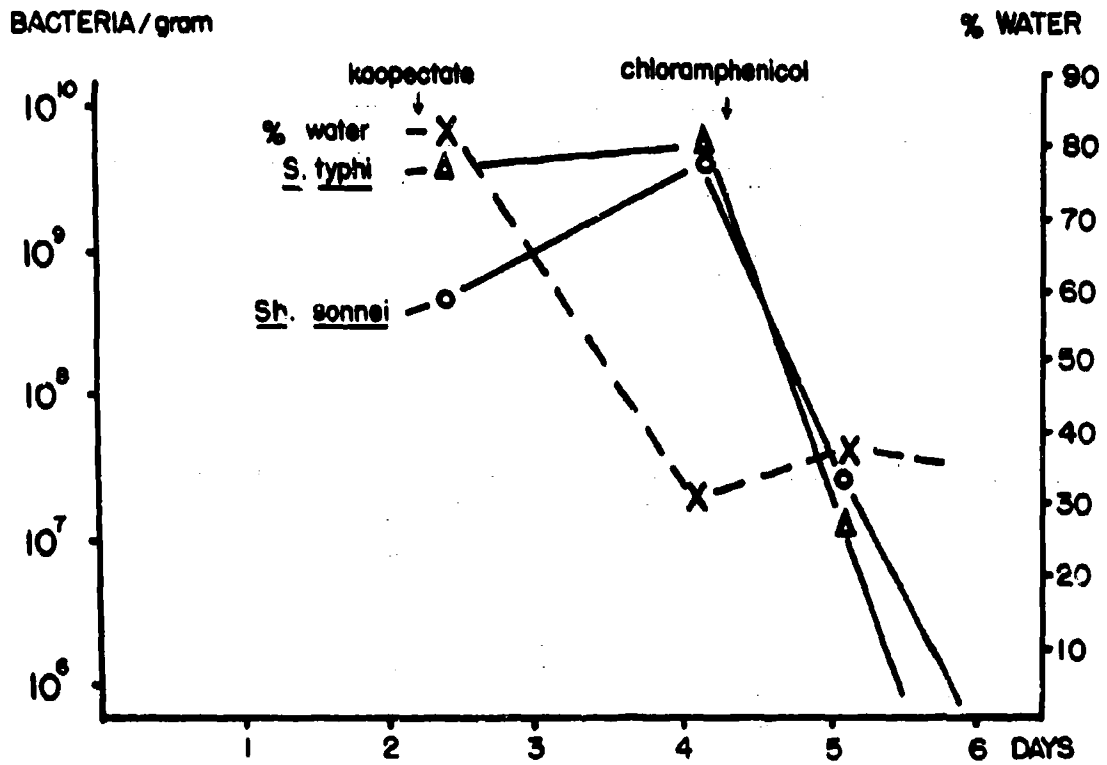

Fig.3 Showing stool pathogens and water content in 55 Y.O. Malay female with severe diarrhea

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY: 30 06 71	REPORT CONTROL SYMBOL DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
30 06 70		U		N/A	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY		[illegible]		
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code): Ecological Studies of Mammals and their Involvement in Transmission of Zoonotic Diseases in Equatorial Ecosystems.

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
10 70	9 71		

17. CONTRACT/GRANT: DADA17-71-G-9332 Mod. P-103
a. DATES/EFFECTIVE: 10 70 EXPIRATION: 10 71
b. NUMBER:
c. TYPE: Y Grant d. AMOUNT: 181
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1.0	21.0
	CURRENT 72	1.0	29.5

19. RESPONSIBLE DOD ORGANIZATION
NAME: US Army Medical Research Unit, Institute for Medical Research
ADDRESS: Kuala Lumpur, Malaysia
RESPONSIBLE INDIVIDUAL
NAME: Dr. Abu Bakar bin Ibrahim, 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: Muul, I., CPT, MSC
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE: Microbiology, Public Health

22. KEYWORDS (Precede EACH with Security Classification Code): Ecology, disease transmission, small mammals, hosts, equatorial ecosystems

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

23.(U) Technical Objective: Studies were conducted to ascertain the aspects of the ecology of mammals in equatorial ecosystems that predispose their involvement in disease transmission cycles in general and in specific zoonoses in particular. Because of the cryptic nature of the endemic transmission cycles of many zoonoses it is important to find out more about them to evaluate their potential for epidemics and to implement control measures. The specific objectives of this project are to evaluate ecological factors, such as distribution (geographical, altitudinal, between and within habitats, including vertical and temporal), reproduction (litter size and frequency, seasonality), population dynamics (productivity, survival, and longevity), feeding habits, and other behavior that predispose the involvement of potential host species in zoonotic disease cycles.

24.(U) Approach: Studies were continued on the geographical and altitudinal distribution, temporal and spatial use of the environment, reproduction, population dynamics, feeding habits competition, parasitemia, and taxonomy and systematics. In some areas surveys for scrub typhus antibodies and scrub typhus rickettsia isolation attempts have been begun. Mammals were trapped, netted, shot, or collected from their nests, together with their nesting materials. Pertinent ecological information was recorded. Each animal and nesting materials were examined for ectoparasites. The animals were dissected and endoparasites were collected. Fecal samples were examined for helminth eggs and protozoal cysts (*Eimeria*)(Hooper Foundation). Blood samples were taken for examination for malarial and microfilarial parasites. From some areas, blood samples were tested by fluorescent antibody techniques for scrub typhus antibodies (Department of Serology) and some blood was injected into live mice for rickettsial

*Available to contractors upon originator's approval.

DD Form 1498, Research and Technology Work Unit Summary,
Item 24 Continued:

isolation (Department of Rickettsiology). Reproductive state was determined by dissection and measurement of gonads and embryos when present. Diets were determined from stomach contents. Mark and release studies and other ecological studies are continuing in conjunction with an aerial transect (over 1200 feet) constructed in the canopy from 30 to 120 feet from the ground in a mature rain forest to determine the vertical distribution of potential hosts, parasites, and pathogens.

25.(U) Progress: Results of these studies indicate that mammalian hosts and potential hosts of zoonotic diseases are not randomly distributed in various equatorial ecosystems. They display varying degrees of altitudinal and habitat specificity. Within habitats they utilize various vertical zones in the forest and are active at specific times. Correlated with this, inter- and intra-specific variations are observed in the parasite patterns. For example, contrary to what has been suggested as a possibility, arboreal species do not seem to be involved in any "jungle tsutsugamushi" (scrub typhus) transmission cycle. Improved rickettsial isolation techniques have made it possible to compare the involvement of mammals in various habitats in the scrub typhus transmission cycle. In Malaysia primary forest species seem to be involved more than species characteristic of scrub habitats, the classical habitat of scrub typhus. Within habitats there appears to be wide interspecific variation in involvement in this disease. Diets predispose probabilities of endoparasite infections. Species have different patterns of reproductive periodicity, varying from continuous breeders to those highly seasonal. Population dynamics are also variable. These factors are important in determining whether a given mammalian species would be suitable as a reservoir or an amplifying host for zoonotic diseases.

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

Background

Ecological studies of mammals in West Malaysia have now been continuing for over three years. Several aspects of the ecology of mammals have been elucidated during this time that are relevant to medical research particularly in regard to zoonoses. Moreover, these studies have demonstrated the feasibility of combining an ecological approach with the more traditional epidemiological approach to better clarify zoonotic disease problems.

The studies of mammalian ecology in West Malaysia have been greatly facilitated by the extensive, earlier work published by H.C. Robinson, C.B. Kloss, F.N. Chasen, J.L. Harrison, J.R. Audy, R. Traub, B.L. Lim, Lord Medway and others. Nowhere else in Asia have mammals been studied as thoroughly as in West Malaysia. This has permitted a general assessment of the involvement of mammals in the transmission cycles of various zoonoses including, for example, scrub typhus, leptospirosis, tick typhus, murine typhus, Q fever, Japanese "B" encephalitis, tick-borne encephalitis, paragonimiasis, strongyloses (particularly *Angiostrongylus cantonensis*).

However, even in West Malaysia very little is known about the ecology of the majority of the 200 species of mammals. Each has its own ecological niche. The differences between the ecological niches of the various species appear to be the bases of the degree to which they become involved in the transmission cycles of various zoonoses (Muul, 1970, Mammalian Ecology and Epidemiology of Zoonoses, SCIENCE, 170: 1275-1279).

Objectives

1. To ascertain the aspects of the ecology of mammals that predispose their involvement in disease transmission cycles in general, and in specific zoonoses in particular.

2. To elucidate ecological phenomena that directly influence the distribution and abundance of mammals and indirectly influence the distribution and frequency of zoonoses.

3. To develop techniques for studying mammalian ecology relevant to zoonotic disease problems.

Approach

Studies were continued on patterns of geographical and altitudinal distribution, temporal and spatial use of the environment, reproductive cycles, population dynamics, food habits, competition, parasitology, taxonomy and systematics. Animals were collected in various localities by traps, nets, or they were shot. Some species were taken directly from their arboreal nests in tree cavities, together with their nesting materials. The nests were immediately sealed in plastic bags in order to retain parasites occurring in them. Nest heights and other characteristics of the nests were recorded. Leaf samples were taken for identification and other ecological information was noted. Animals were placed in cloth bags and killed with chloroform and examined for ecto-parasites (lice, fleas, chiggers, ticks, etc.). Stool samples were examined for helminth eggs and protozoan cysts (*Eimeria*, coccidia). The internal organs were examined for helminths. Blood samples were taken for examination for blood protozoa, microfilariae and for scrub typhus surveys (isolation attempts were also made from kidneys; see section on Rickettsiology). Reproductive condition was noted, embryos counted and reproductive organs were measured and weighed in selected species. Stomach contents of selected species were preserved for later studies.

In studies of vertical distribution of mammals in conjunction with the transect built through the canopy of a primary rain forest (Muul and Lim, SCIENCE, 169: 788-789) the animals were brought back to the laboratory and were etherized for examination, measurements, and collections of blood and parasites. The animals were marked and released the next day at the point of capture. Weekly botanical observations, such as times of flowering, fruiting, and emergent leaves, are continuing to correlate the mammalian data with other ecological information. The trees in the two transect systems are individually designated on the maps of the areas, as are trap locations (Fig. 1 and 2).

Results

Geographical Distribution: The mammalian fauna of Malaysia (including Sabah and Sarawak), Indonesia (Sumatra, Java, Kalimantan), and southern (peninsular) Thailand is closely related and probably represents a former continuous distribution of Sundaland, separated after the last peak in glaciation during the Pleistocene. Studies of the research collections of the Applied Scientific Research Corporation of Thailand and those privately owned by Dr. Boonsong Lekagul indicate that the Sundaland fauna extends at least as far north as Nakhorn Sri Thammarat. Some species, however, extend farther north and some species of the Indo-Chinese region, such as the mongoose new to West Malaysia, *Herpestes urva*, extend into West Malaysia and perhaps farther south. The extant fauna of West Malaysia appears to be composed of at least two waves of invasion from the north and several

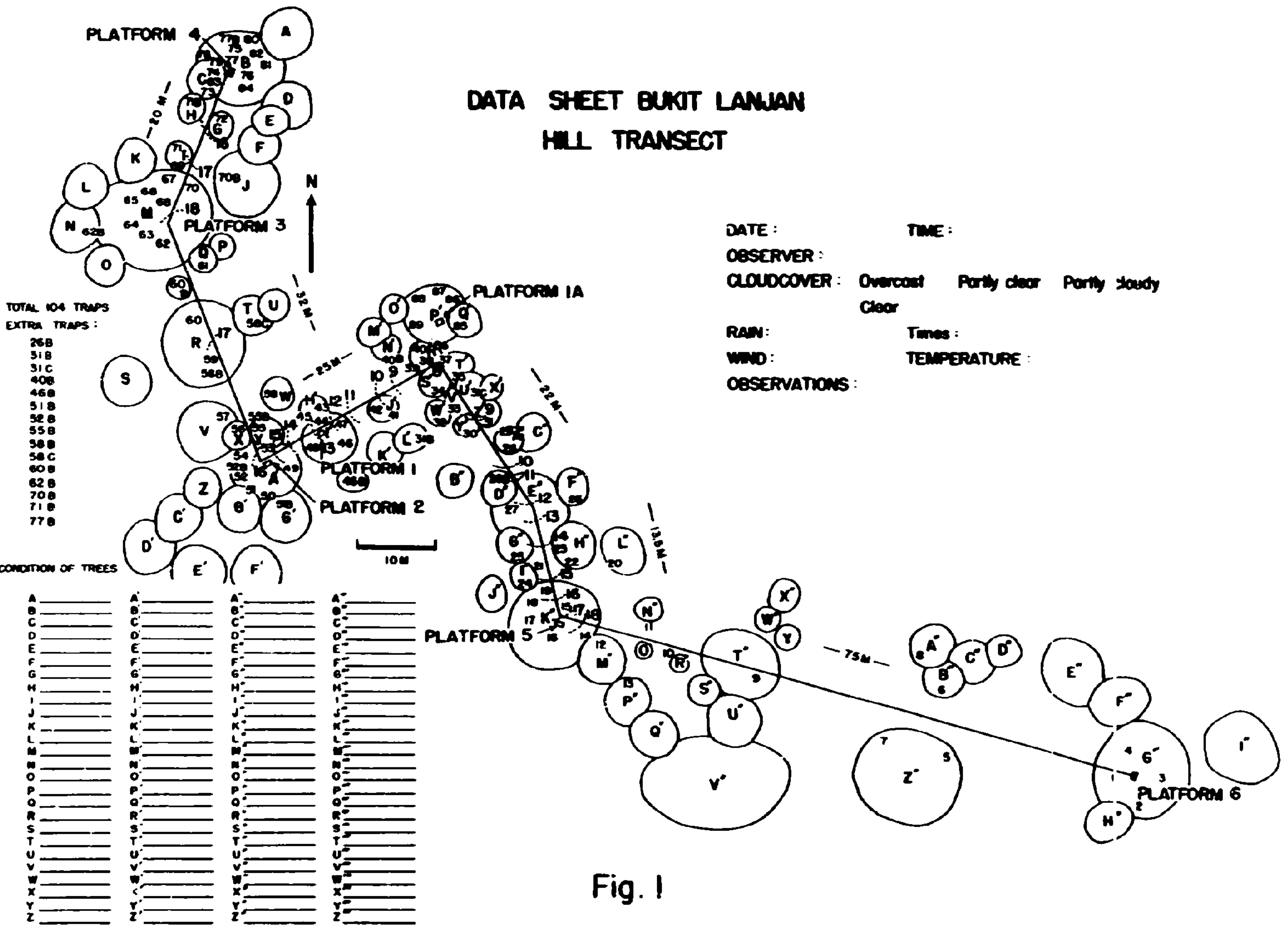

Fig. I

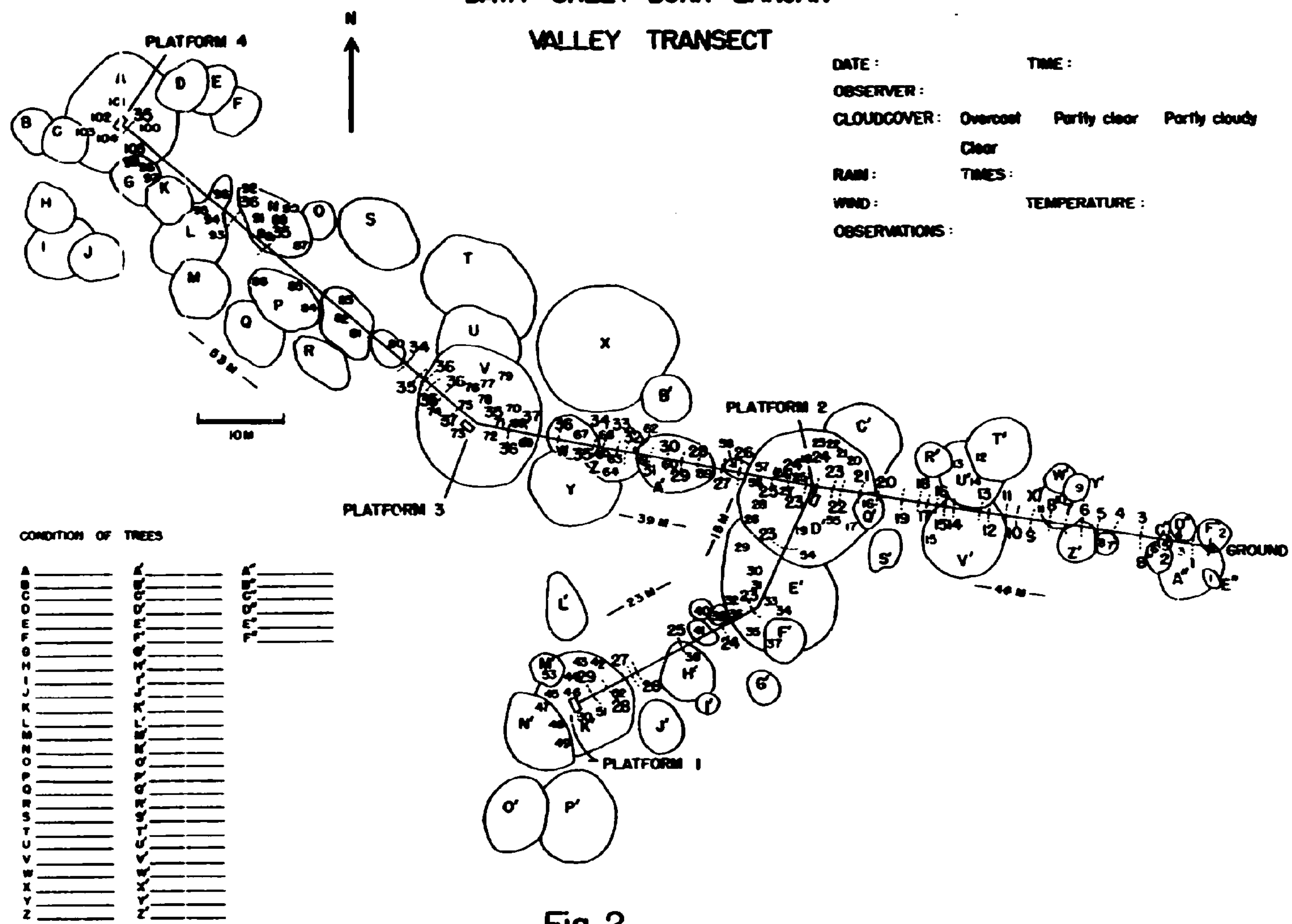

Fig. 2

from the south. The Malay Peninsula, therefore, formed a kind of corridor for faunal exchange between two major zoogeographic areas of the Oriental Region. Having an assortment of habitats in which various forms have been able to survive, West Malaysia supports one of the richest mammalian faunas known anywhere. Several forms from the south and from the north, however, are lacking. In regard to the majority of host species areas in West Malaysia are as favorable for perpetuation of various endemic zoonoses as nearby areas. Why some, such as rabies, have not become a problem in West Malaysia is still not clear.

Altitudinal Distribution: In contrast to the general distribution of lowland species, the communities at elevations above 3000 feet in West Malaysia appear to be remnants of an earlier continuous distribution when climates were cooler, perhaps at times of maximal glaciation in the northern hemisphere during the Pleistocene. (In last year's annual report the highland species were listed.) Apparently as climates became warmer during the post-glacial period, these communities were replaced in the lowlands by communities better adapted for the warmer climate. The hill top communities survived apparently owing to the lower temperatures at high elevations. Not only do the altitudes above 3000 feet have species that do not occur in the lowland areas, but preliminary evidence indicates that there are population variations occurring within species that span the range of altitudes. These variations can easily be misconstrued as taxonomic differences especially in cases in which taxonomic separation is based only on relative size differences. A good example of this is *Rattus argentiventer* (recently found to occur also in highlands above 3000 feet). This species has been frequently implicated in scrub typhus ecology.

Habitat Distribution: There appear to be greater differences in the fauna between adjacent dissimilar habitats, even between mature secondary and primary forests, than between distant geographic areas within West Malaysia (see Tables in the last two USAMRU Annual Reports). Correlated with this are differences in prevalence of disease organisms.

In a preliminary survey of scrub typhus rickettsiae, *Rickettsia tsutsugamushi*, in small mammals taken from various predominant habitats, the samples from relatively undisturbed areas, such as a primary forest had the highest isolation rates (Table 1). The samples from a bamboo forest also included a high proportion of isolations. The latter area was sampled when only kidneys were surveyed and this technique was generally found to yield about twice the number of isolates as surveys of blood. The primary forest, on the other hand, included only blood samples because this is our mark-and-release study area. Seasonal factors may also be involved.

Each of the areas in Table 1 are designated by the predominant habitats. Within the areas represented various specific habitats of

Table 1

Prevalence of Scrub Typhus, *Rickettsia tsutsugamushi*, Isolations from Ground and Semi-arboreal Mammals in Various Predominant Habitats

Predominant Habitat	No. Tested		Percent Positive	
	Blood	Kidney	Blood	Kidney
Bamboo Forest	-	87	-	55
Kampong Rubber	175	-	9	-
Secondary/Scrub/ Relic Trees	154	22	12	5
Mostly Secondary	215	127	19	35
Mostly Primary	45	-	36	-

different sizes, occur side by side. In Table 2 the isolation rates are shown for groups of species according to the specific habitats to which they are characteristic. Between the secondary and primary forests there is a lot of overlap in the ground-dwelling mammalian species, but they occur in the two habitat types in different relative numbers.

Red blood cell protozoa, *Hepatocystis*, appear to be more common in primary forests than in secondary forests (Muul, Lim, Yap, 1970. Arboreal Mammals and their Red Cell Protozoa in Various Habitats in West Malaysia. SE Asian J. Trop. Med. Publ. Hlth., 1(3): 418-419).

Vertical Distribution: About 2/3 of the mammalian species, with the exception of bats which are all tree or cave dwelling, are defined as canopy species (Harrison, J.L. 1957. Stud. Inst. Med. Res. Malaya, 28: 409). Using a transect system built through the canopy of a primary rainforest (Muul & Lim, SCIENCE, 169: 788-789) we have determined that according to trapping results the biomass of mammals in the canopy approximately equals that on the ground. However, many canopy species are not susceptible to trapping.

The differences in disease involvement in various vertical strata within the forest are striking.

Table 3 shows the rates of isolations of *Rickettsia tsutsugamushi* from mammals characteristic of the different vertical strata in forests. Ground dwelling and semi-arboreal species appear to be much more involved in the scrub typhus transmission cycle than the arboreal species. Even though Audy (in May, J.M. ed., Studies in Disease Ecology, Hafner, N.Y.) suggested that there may be a different jungle *tsutsugamushi* transmission cycle, perhaps involving vectors other than the well known *Leptotrombidium deliense*, the prevalence of isolations was much lower in those species that do not commonly harbor this chigger than in those that do.

In general the prevalence of blood protozoa, including *Plasmodium* and *Hepatocystis*, is much greater in the canopy species than in ground species. Preliminary data on this were presented in last year's Annual Report. This year's additional data corroborate these findings.

Seasonal Distribution: Seasonal abundance of mammals depends on the mortality rate, the birth rate, the timing, size and frequency of litters. Some species, such as *Hylopetes spadiceus, Pteromyscus pulverulentus, Petinomys setosus,* were found to breed at infrequent intervals, others, such as *Petinomys vordermanni, Iomys horsfieldi, Rattus tiomanicus jalorensis*, breed nearly continuously. These differences also determine the roles the various species play in disease transmission since pathogens require susceptible individuals in which to propagate. Infected individuals may die or become immune. Either way, they become unavailable to the pathogen. Young susceptibles

Table 2

Prevalence of *Rickettsia tsutsugamushi* Isolations from Ground and Semi-arboreal Mammals Characteristic of Various Habitats (Samples Smaller than Six were Eliminated).

Characteristic Habitat	No. Tested		Percent Positive	
	Blood	Kidney	Blood	Kidney
Village	29	-	3	-
Grasslands	8	-	0	-
Scrub Vegetation	134	81	8	7
Secondary Forest	416	194	15	40
Primary Forest	37	-	40	-

Table 3

Prevalence of *Rickettsia tsutsugamushi* Isolations from Mammals Occurring in the Various Vertical Strata in Forests.

	No. Tested		Percent Positive	
	Blood	Kidney	Blood	Kidney
Arboreal	169	46	0	4
Semi-arboreal	379	136	16	41
Ground	227	100	14	21

are necessary to enable disease organisms to propagate. Several of the Malaysian mammals have been studied in this regard and data are needed for others.

In several species, including the infrequent breeders listed above, that have been studied in detail there appears to be a marked seasonality in reproduction and recruitment into the population. However, at least in the western and southern portions of West Malaysia this seasonality does not necessarily follow an annual pattern. Rather, the periodicity seems to be longer than a year.

A parallel situation may exist in the scrub typhus transmission cycles. The seasonal fluctuations in the abundance of chiggers are thought to correlate with rainfall (Audy, op. cit.). The factors responsible for the reproductive seasonality of mammals are not yet known. Also the correlation of rickettsial isolation rates with any seasonal environmental factors is not yet apparent.

Temporal Distribution: Mammals can be categorized into diurnal e.g. Sciurinae, most Tupaiidae and primates, nocturnal e.g. Muridae, Petauristinae, or crepuscular groups. Within these categories differences have been observed in the prevalence of red blood cell protozoa, particularly *Plasmodium* and *Hepatocystis* (see last year's Annual Report).

In terms of scrub typhus transmission, most species involved are nocturnal. Comparisons cannot be validly made on this basis, however, since diurnal forms are nearly all arboreal.

Population Dynamics: In species that have otherwise similar ecological niches we have observed great differences in rates of turnover of populations. For example, *Petinomys vordermanni* has approximately a six fold greater reproductive rate than does *Hylopetes spadiceus*. Species with lowlevel continuous reproduction, such as most Muridae, would serve well as reservoir hosts since there would be a low, but steady, recruitment of susceptibles into the population. Periodic synchronous breeders, such as the primary forest Petauristinae, would be more likely to serve as amplifying hosts for pathogens in a given season.

Parasite Distribution: In addition to the examples discussed above, various other ecological and behavioral factors predispose the patterns of infection in mammals. Diets have been found to influence prevalence of endoparasites. For example, those species feeding on insects at least part of the time have higher rates of helminth infections than those that feed only on vegetation or fruit. *Rattus sabanus* and *Rattus edwardsii* are both partly insectivorous and harbor more helminths than *Rattus bowersii* which feeds mostly on vegetation.

Species, such as most of the primates, that do not remain in the same nest for long periods, for example, have few fleas. Lice are infrequent in species, such as various Petauristinae, that have elaborate grooming behavior. The presence or absence of such vector may predispose the species involvement in zoonoses.

For example two of the spiny furred rats, *Rattus rajah* and *R. surifer* have seldom been found with vector chiggers *L. deliense*. *R. rajah* had a rickettsial isolation rate much lower than that in other rats. But, *R. surifer* yielded isolates at a rate equivalent to that of other rats. Does this mean that another vector may be involved?

Specimens Examined (Table 4): In conjunction with field activities of the Division of Medical Ecology, IMR, about 5000 specimens of vertebrates were collected and examined during the period covered in this report (9 months). Among the unusual forms collected are *Herpestes urva,* which was hitherto unknown from West Malaysia and *Petaurillus kinlochii,* which was known to science from only the type specimen (lost during WWII when the Federated Malay States Museum was bombed). The new specimens now become the *neotypes*. *Trichys lipura,* which was known from only two specimens is now fairly well represented. Over 800 mammals were collected from various habitats in Sabah (Borneo).

Cooperative Studies

A whole range of new host records and new species of *Eimeria* have been described by F.C. Colley, Hooper Foundation, in the mammals we have collected (Table 5).

Lice have been studied by Dr. K.C. Emerson. Several new species have been found to date (Table 6).

Fleas are being studied by Dr. R. Traub, ticks by Dr. Hoogstral, and nest dwelling mites by Mr. Nadchatram.

Systematic studies of rats, specimens of which we have provided, are being carried out by Dr. G.G. Musser at the American Museum of Natural H' tory in New York. Other systematic studies are in progress in cooperation with various research institutions in the U.S. and Europe. The purpose of these is to define the ecologically independently functioning units in the various habitats within the Sundaland.

Table 4

Mammals collected since 1 October 1970 from West and East Malaysia

Family	Order	West Malaysia	East Malaysia
	Insectivora		
Erinaceidae		77	-
Soricidae		8	-
	Dermoptera		
Cynocephalidae		2	1
	Pholidota		
Manidae		1	1
	Primates		
Tupaiidae		139	149
Larisidae		3	1
Tarsiidae		-	9
Pongidae		8	-
	Chiroptera		
Pteropidae		151	17
Vespertilionidae		31	13
Rhinolophidae		29	-
Nycteridae		2	-
Molossidae		13	-
Emballonuridae		20	-
	Rodentia		
Muridae		1708	384
Sciuridae			
Sciurinae		528	251
Petauristinae		626	15
Rhizomyidae		1	-
Hystricidae		29	-
	Carnivora		
Mustelidae		4	1
Viverridae		9	8
Herpestidae		5	-
Felidae		9	-
	Artiodactyla		
Tragulidae		59	11
Cervidae		1	-

Table 5

Eimeria and *Isospora* species from Malaysian mammals collected and described by Fredrick C. Colley and Steven H. Mullin, Institute for Medical Research, Kuala Lumpur, Malaysia and the G.W. Hooper Foundation, University of California (UC ICMRT), San Francisco, California 94122. Samples provided by the Department of Ecology, USAMRU.

	HOST	NEW SPECIES	NEW RECORD
Family Soricidae	*Hylomys suillus*	*E. bentongi*	
	H. suillus	*I. hylomysis*	
Family Tupaiidae	*Tupaia glis*	*E. ferruginea*	
	T. glis	*E. tupaiae*	
Subfamily Sciurinae	*Callosciurus notatus*	*E. pahangi*	
	C. notatus	*E. notatus*	
	C. prevostii	*E. callosciuri*	
	Sundasciurus hippurus	*E. hippuri*	
Subfamily Petauristinae	*Petaurista petaurista*	*E. malayensis*	
	P. elegans	*E. elegans*	
	Aeromys tephromelas	*E. aeromyis*	
	Hylopetes spadiceus	*E. hylopetis*	
Family Muridae	*Rattus edwardsi*	*E. edwardsi*	
	R. edwardsi	*E. tikusi*	
	R. tiomanicus	*E. pachylepyron*	*E. separata*
	R. sabanus	*E. sabani*	*E. separata*
	R. surifer	*E. surifer*	*E. nieschulzi*
	R. fulvescens		*E. separata*
	R. muelleri		*E. separata*
Family Hystricidae	*Trichys lipura*	*E. lipura*	
	T. lipura	*E. landersi*	
Family Tragulidae	*Tragulus javanicus*	*E. kanchili*	
	T. javanicus	*E. traguli*	
	T. javanicus	*E. pelandoki*	

Table 6

Lice Identified by Dr. K.C. Emerson from Mammals Collected by the Department of Ecology, USAMRU.

Hosts	Lice			
	Neohaematopinus callosciurini	*N. robustus*	*Neohaematopinus* (probably new species)	*Haplopleura* (probably new species)
Family Sciuridae				
Sciurinae				
Ratufa bicolor			1	
R. affinis			1	
Callosciurus notatus	4			
Callosciurus prevostii	1			
Rhinosciurus laticaudatus			1	
Petauristinae				
Hylopetes spadiceus			15	1
Aeromys tephromelas			1	
Petaurista elegans		2		
Pteromyscus pulverulentus			3	
Petinomys vordermanni			2	
P. setosus			2	
Iomys horsfieldii			3	

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. Agency accession:
2. Date of summary: 30 06 71
Report control symbol: DD-DR&E(AR)636

3. Date prev summary: 30 06 70
4. Kind of summary:
5. Summary scty: U
6. Work security:
7. Regrading: N/A
8. Dissem instr'n: NL
9. Specific data-contractor access: ☐ Yes ☐ No
10. Level of sum: A. Work unit

10. No./Codes	Program element	Project number	Task area number	Work unit number
a. Primary		81100 and 831000		
b. Contributing				
c. Contributing				

11. Title (Precede with Security Classification Code): Investigations of Malaria

12. Scientific and technological areas: 010100 Microbiology

13. Start date: 10 70
14. Estimated completion date: 9 71
15. Funding agency:
16. Performance method:

17. Contract/Grant: DADA17-71-G-9332 Mod. P-103
a. Dates/effective: 10 70 Expiration: 10 71
b. Number:
c. Type: Y Grant d. Amount: 181
e. Kind of award: f. Cum. amt.

18. Resources estimate		a. Professional man yrs	b. Funds (in thousands)
Fiscal year	Preceding 71	1.5	15.0
	Current 72	1.0	19.3

19. Responsible DoD organization
Name: US Army Medical Research Unit Institute for Medical Research
Address: Kuala Lumpur, Malaysia
Responsible individual
Name: Dr. Abu Bakar bin Ibrahim, 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: Andre, R.G., CPT, MSC
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Social security account number:
Associate investigators
Name: Cadigan, F.C., Jr., COL, MC
Name: Bolton, M., M.D., M.T.M.&H.
Kyser, K.A., MAJ, MC

21. General use: Microbiology, Public Health

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 Objective: To determine the status of chloroquine-resistant malaria in West Malaysia, to delineate the gradient between a low and a high percentage of resistant malaria, to determine why a gradient in the amount of resistant falciparum exists, to study the vector species of malaria in certain parts of Malaysia, to investigate the mosquito fauna found within the jungle canopy, to study malaria in the *Orang Asli*, and to examine the erthrocytic and exoerthrocytic cycle of tragulid malaria.

24.(U) Approach: Surveys for malaria will be conducted and *P. falciparum* infections will be studied both *in vivo* and *in vitro* for drug resistance. Mosquitoes will be collected by using human bait trap collections, human biting collections, indoor-outdoor collections, light trap (with CO_2) collections, larval collections, and oviposition trap collections. Anophelines and certain culicines will be dissected for parasites. Malaria prevalence surveys in the *Orang Asli* will be made periodically in both fringe and deep jungle areas. Experimental transmission by infected mosquitoes and light and electron microscopy techniques will be used for the tragulid malaria investigation.

25.(U) Progress: Over 1430 schoolchildren in Kelantan and 120 in Perak were surveyed for resistant malaria. The prevalence in the two areas was 19% and 51% respectively. In Kelantan, 6% of the falciparum cases treated with 1500 mg chloroquine base did not respond within seven days. No positive parasitemias were seen on Day 7 in Perak. *In vitro* studies indicated that sensitive and resistant *P. falciparum* strains were present in both areas. Studies on anopheline vectors in the study areas show *Anopheles maculatus* to be the primary vector, however, *A. b. balabacensis* was collected for the first time in Kelantan and may be an additional vector species in this area. Periodic

*Available to contractors upon originator's approval.

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

surveys in *Orang Asli* villages indicate a difference in the predominate species of *Plasmodium* in fringe areas versus deep jungle areas. Control and prophylatic measures have had an effect on the prevalence of malaria in certain deep jungle areas -- 20% reduced to 5%. At the jungle canopy study area over 11,690 adult mosquitoes have been collected. About one-third were caught at canopy level. Seven different species of larvae were found breeding in the bamboo cups and glasses placed within the canopy. Over 20 adults were found to be infected with parasites. Photographs were taken of the erthrocytic stages of *P. traguli* using an electron microscope.

Item 22

Anopheline, *Anopheles balabacensis*, canopy mosquitoes, chloroquine resistance, culicine, *in vitro* drug resistance testing, malaria survey, *Orang Asli*, *Plasmodium falciparum*, Tragulid malaria, West Malaysia.

INVESTIGATIONS OF MALARIA

Malaria research was conducted by the Entomology Section during this past nine months. Collaborative studies were made jointly with the Malaria Division of the Institute for Medical Research, the Gombak Aborigine Hospital, the Hooper Foundation, and with a private clinic in Seremban.

Studies of chloroquine-resistant malaria using both the *in vivo* and *in vitro* techniques were continued, along with associated vector studies. Comparative studies of malaria in Aborigines dwelling in deep jungle versus those residing on the jungle fringe were also continued. Ecological and parasitological studies of the mosquito fauna found within and below the jungle canopy were conducted. Vector mosquitoes are being reared in both indoor and outdoor situations to support certain research projects. Additional species of both larval and adult mosquitoes have been added to the USAMRU reference collection. Investigations of tragulid malaria have been initiated.

Specific Objectives

1. Chloroquine-resistant Malaria:

(a) To determine malaria prevalence within the civilian population in parts of West Malaysia, to determine the frequency with which resistant strains are found, and to characterize the level of resistance by both the *in vivo* and *in vitro* methods.

(b) To determine where the gradient between a high amount of resistance and a low amount of resistance exists, and to determine why this gradient exists.

(c) To determine the principal vectors of malaria found within the study areas and the infection ratio of the anopheline mosquitoes.

2. Malaria in the *Orang Asli* (Aborigines):

(a) To compare the prevalence of malaria in aborigines that are deep jungle dwellers to those *Orang Asli* living on the jungle fringe.

(b) To study falciparum cases for possible chloroquine resistance.

(c) To determine the anopheline vectors in both the deep jungle and the jungle fringe.

(d) To observe the effect of residual spraying and drug prophylaxis in certain aborigine areas.

3. Studies on Jungle Canopy Mosquitoes:

(a) To determine the prevalence of various mosquito species at ground level and at canopy level.

(b) To study the zonation of the mosquito fauna within the jungle canopy.

(c) To determine the infection ratio in certain mosquito species found within the canopy and/or the ground.

(d) To determine oviposition sites and the frequency of oviposition of certain mosquito species found within the canopy.

4. Mosquito Colonies:

(a) To establish and maintain colonies of various vector anophelines and culicines needed for certain projects.

(b) To study the life cycles of some jungle-breeding anophelines under artificial conditions.

5. Reference Collection:

To expand the reference collection of adult and larval mosquito specimens in order to better support taxonomic studies.

6. Studies of Tragulid Malaria:

(a) To determine the exoerythrocytic cycle of *Plasmodium traguli* using both light and electron microscopy.

(b) To experimentally transmit tragulid malaria from infected animals to non-infected animals using various jungle-breeding anophelines.

(c) To study the sporozoites and oocysts of *P. traguli* using both light and electron microscopy.

7. Methods for Investigations of Liver Stages of Malaria:

(a) To develop a method of partial hepatectomy in primates with low associated complication rates.

(b) To set up a histochemical method of demonstrating malaria in liver.

(c) To study liver function in tissue culture.

Chloroquine-resistant Malaria

General Background: Initiation of studies on chloroquine-resistant malaria in West Malaysia was in March 1969 (see Annual Reports dated 1968 and 1969). During this past nine months, investigations have incorporated both the *in vivo* and *in vitro* techniques for determining the presence of drug-resistant plasmodium strains. A chloroquine-resistant malaria survey of *Orang Asli* schoolchildren in lower Perak was conducted. Five schools in Kelantan near the border of Thailand were surveyed to determine the presence of resistant malaria in the Malay schoolchildren. Blood samples from individuals having *Plasmodium falciparum* infections were sent into our lab from the Gombak Aborigine Hospital and from a private clinic run by Dr. D.R. O'Holohan in Negri Sembilan. These samples were tested for the presence of chloroquine-resistant malaria parasites by the *in vitro* method, and some of the individuals involved were followed up for seven days following treatment with chloroquine. Associated vector studies were conducted in the study areas located in the states of Trengganu, Perak, and Kelantan.

Trengganu Studies:

Background: Thus far during this investigation of the status of chloroquine resistance in the state of Trengganu, two surveys to determine the prevalence of malaria in the region have been conducted; one survey using the *in vivo* 7-day study method was done; and finally surveys of the mosquito fauna in the study area have been made periodically over the last one and a half years. During this reporting period, primary emphasis at the study site has been to further delineate the anopheline vectors with respect to their relative importance in malaria transmission. In particular, surveys were made to determine the role of *Anopheles b. balabacensis* in the area, and to try and obtain live specimens for rearing and biological studies.

The survey sites are located about thirty miles southwest of Kuala Trengganu. (See Map). Bordering the study area on the south is the Trengganu River and on the north the Telemong River. Immediately adjacent to the area on the west are the foothills of a main mountain range and also the National Park. Small individual holdings of agricultural land are found throughout the area. Logging is now carried out to a great degree in the areas having secondary and primary forest. Monsoons isolate the region in the months of October through January.

Collections for mosquitoes were made in the latter part of September 1970, and in May 1971. The methods used during the September trip have already been mentioned in last year's annual report; however, the final results of this trip will be commented on in detail in this report. In May a survey was made at the four study villages; Kg. Kuala

Map of West Malaysia

REFERENCE

(1) Kuala Brang
(2) Bukit Lanjan
(3) Fort Kemar
(4) Pos Shean
(5) Satak
(6) Woh (7th Mile Cameron Highlands Road)
(7) Ayer Lanas
(8) Ayer Denak
(9) Lumbok Sempit
(10) Tamok

Dura, Kg. Kuala Jeneris, Kg. Payang Kayu, and Kg. Tapah. Human biting collections (bare leg catches) were made for six nights from 1900 to 2400 hours each night. Five men collected mosquitoes outside at various sites along the perimeter of each village. Mosquitoes were caught by placing a glass vial over them as they started to feed on the collector. A plug of cotton was then placed into the top of the vial to prevent the exit of the mosquito. The vials had been precharged with a small amount of moist tissue paper. Indoor-outdoor collections were made in a similar manner as the human biting collections. At the indoor-outdoor collection sites, one man collected all the mosquitoes coming to feed on him, and one man sat just outside the house collecting those mosquitoes attempting to feed on him. In addition to the adult mosquito collections, extensive larval surveys were made in an attempt to locate the breeding sites of *A. b. balabacensis*.

Results: During the latter part of September over 7300 adult mosquitoes were collected by light trapping, human biting collections, and indoor-outdoor biting collections (see Table 1). At the ground level light trapping sites, 4667 mosquitoes -- consisting of eleven species of anophelines and nineteen species of culicines -- were captured. Another 2497 adults were trapped at canopy level, including ten species of anophelines and eighteen species of culicines. *A. b. balabacensis* was caught in small numbers at both canopy level and ground level. Only 171 anopheline specimens were collected by four to six men collecting for four nights. Dissection results were negative.

Larval collections were made specifically to sample possible breeding sites of *A. b. balabacensis*. Nine species of anophelines were collected, including *A. b. balabacensis* and other members of this *A. leucosphyrus* group. *Aedes lineatopennis, Aedes caecus,* and *Culex (Lophoceraomyia) sp.* were found breeding in the same hootprint collection of water that *A. b. balabacensis* was.

In May extremely dry conditions were encountered at the four study sites. The villages reported that there had only been trace amounts of rain since the beginning of February. Consequently available breeding sites for mosquitoes was greatly reduced. Buffalo wallows which formerly had held water throughout the year were completely dried up. Sites where *A. b. balabacensis* had previously been found breeding were also dry, even those located deep within the jungle. Therefore, only a few anopheline larval breeding areas were found. Some of the larvae were reared to the adult stage and were identified. The two main species were *A. kochi* and *A. barbirostris*. Taxonomic determinations of the larvae preserved in 70% ethanol are not yet finalized.

Only 2179 adult mosquitoes were captured during six nights of collecting using fourteen collectors. Of this total, 1169 adult

Table 1

Summary of Mosquito Collection Results, Kuala Brang, Trengganu
September 1970

Species Collected	Number of Mosquitoes Collected				Total
	Bare Leg Catches		Light Trap Catches		
	Outdoors	Indoors	Ground	Canopy	
Anopheles aconitus	7	6	7	-	20
A. aitkeni	-	-	1	-	1
A. b. balabacensis	-	-	3	4	7
A. barbirostris	1	-	-	2	3
A. crawfordi	15	3	25	7	50
A. indiensis	31	3	6	9	49
A. karwari	52	10	52	50	164
A. kochi	7	11	39	39	96
A. letifer	-	2	-	1	3
A. philippinensis	8	1	2	1	12
A. separatus	10	2	6	39	57
A. tessellatus	2	-	136	39	177
Aedes spp. (3)*	-	-	24	35	59
Armigeres sp. (1)*	-	-	5	6	11
Culex spp. (9)*	-	-	3361	1307	4668
Mansonia spp. (5)*	-	-	964	950	1914
Toxorhynchites sp. (1)*	-	-	1	-	1
Uranotaenia sp. (1)*	-	-	35	8	43
Total	133	38	4667	2497	7335

* Number of different species.

mosquitoes were captured at the human biting collection sites, 368 were captured indoors, and 642 were caught outdoors next to the houses used for indoor collections. *Anopheles spp.* accounted for 156 of the total mosquitoes, and 40 anophelines were caught indoors. Seven *A. maculatus* were captured while attempting to feed indoors, and one *A. b. balabacensis*. Dissection results showed no sporozoites or oocysts to be present within the anopheline mosquitoes. Human biting collection results are listed in Table 2. Indoor-outdoor collection results are found in Table 3.

Kelantan Studies:

Background: In order to delineate the gradient between a high amount of chloroquine resistance in Thailand and a low amount in Malaysia, and also to confirm that this gradient exists, a study was conducted along the Thai/Malaysian Border. The area selected was in the state of Kelantan, only a few hundred feet, in places, from the Thai Border (See Map). The hospital clinic at Ayer Lanas in the Tanah Merah District was utilized throughout the study for sleeping and eating accommodations. A field laboratory was set up in the ambulance garage. No electricity was available and had to be provided for by generators. The state capital, Kota Bharu, is fifty-six miles to the northeast. Four primary schools and one secondary school located in four different villages were included in the chloroquine-resistant malaria survey. Four villages were surveyed for anopheline vectors. Figure 1 shows the relative locations of the study area and of the villages involved in the surveys. The population in this region is made up of primarily Malays; however, some Thai people and a few Chinese are found. Agricultural products, such as rice, rubber, maize, tobacco, and fruit are the main income-producing commodities for these people. Logging companies are also at work within the area. The forested mountains border the study area on the south and on the west. The proposed east-west highway across the northern portion of Malaya will extend through this area and increase the importance of this region.

In March 1971, a survey to determine the status of chloroquine-resistant malaria was conducted. Initially the schoolchildren from the primary schools in Jakar, Ayer Lanas, Gemeng, and Jeli were examined for malaria. Also, included in this prevalence survey were the children attending the Ayer Lanas Secondary School. Over 1400 blood films were examined within two days. The thick blood films were dried in an incubator for eight hours and then stained with diluted Giemsa (3%). Using oil immersion lenses and 6x eyepieces, twenty fields were read; and if no parasites were seen within this number, the individual was considered negative for the purposes of the resistant malaria study. The films were, however, read later at the lab in Kuala Lumpur, with 100 thick film fields being observed. Approximately 7% more positives were picked up during this later screening, but all the additional positives were very light parasitemias.

Table 2

Summary of Human Biting Collection (B.L.C.) Results
Trengganu, from 13 to 18 May 1971

Species Collected	No. of Mosquitoes
Anopheles aconitus	4
Anopheles balabacensis	1
Anopheles barbirostris	3
Anopheles crawfordi	2
Anopheles karwari	18
Anopheles kochi	30
Anopheles maculatus	14
Anopheles tessellatus	7
Anopheles peditaeniatus	2
Aedes spp. (2)*	28
Armigeres sp. (1)*	14
Culex spp. (5)*	453
Mansonia spp. (2)*	593
Total	1169

* Number of different species.

Table 3

Summary of Indoor and Outdoor Biting Collection Results, Trengganu, from 13 to 18 May 1971.

Species Collected	Indoor	Outdoor
Anopheles aconitus	-	8
Anopheles barbirostris	1	1
Anopheles crawfordi	-	1
Anopheles karwari	18	3
Anopheles kochi	-	10
Anopheles letifer	1	-
Anopheles maculatus	7	7
Anopheles peditaeniatus	1	-
Anopheles philippinensis	-	1
Anopheles tessellatus	11	2
Anopheles vagus	1	-
Aedes spp. (3)*	17	16
Aedomyia sp. (1)*	-	1
Armigeres sp. (1)*	4	5
Culex spp. (4)*	62	159
Ficalbia sp. (1)*	1	-
Mansonia spp. (2)*	244	428
Total	368	642

* Number of different species.

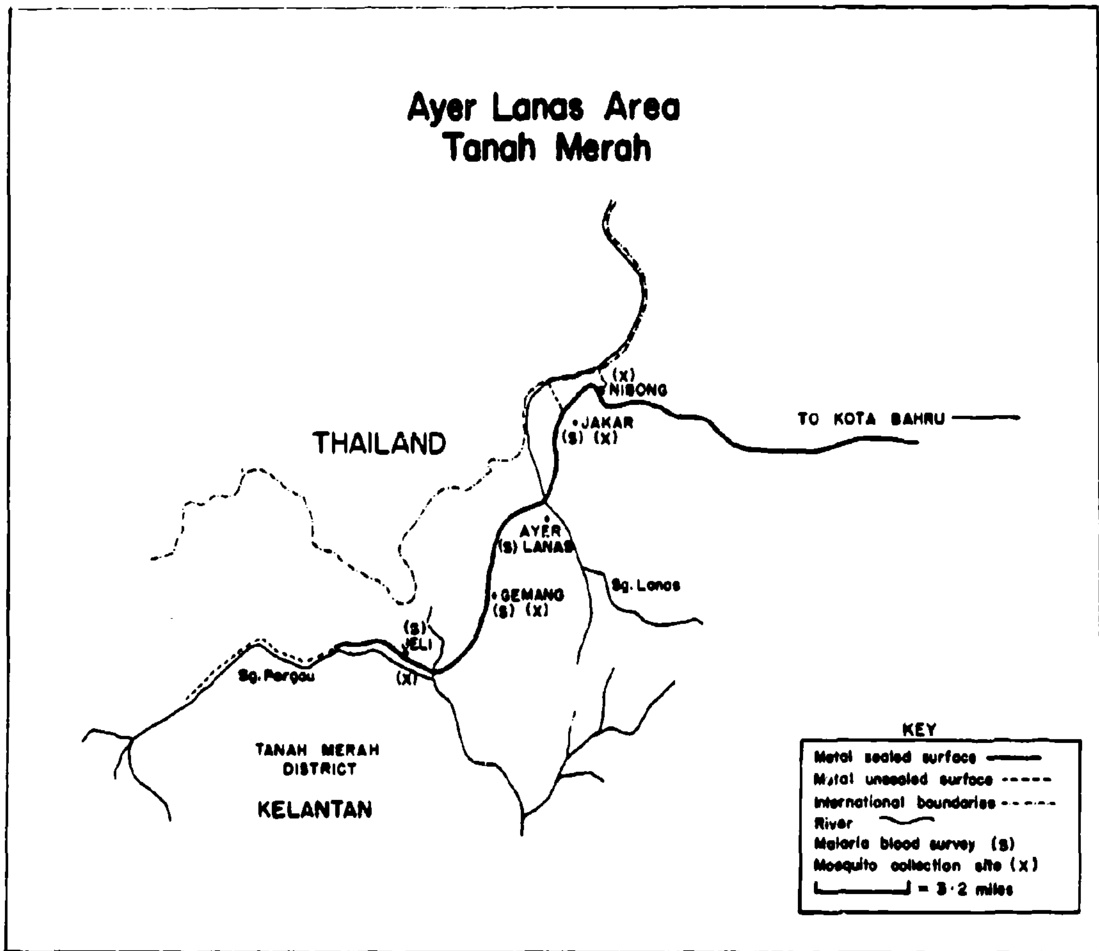


Fig. 1

After the positive individuals were recorded and the treatment lists made, all those having *Plasmodium falciparum* parasites were initially selected for the *in vivo* 7-day study. Those people having *P. vivax* and/or *P. malariae* infections were treated but not included in the drug trial. The selection of the *P. falciparum* infections to be tested for resistance to chloroquine by the 24 hour *in vitro* method was based on the level of parasitemia. Parasitemias consisting of less than five parasites per twenty thick film fields were excluded.

The 7-day *in vivo* study was conducted in the following manner. All the children with *P. falciparum* infections present at school on the first day of treatment were included. The children were given a 3-day dose of chloroquine equivalent to 1500 mg chloroquine base (D_1-600 mg, D_2-600 mg, D_3-300 mg). Age and weight were taken into account so that the child received 25 mg chloroquine/kg. body weight over the three days of treatment. Urines were tested with Meyer's Reagent to determine the presence of chloroquine just before the start of treatment and on the third day of treatment. Any children with a positive urine before receiving treatment or those with negative urines on the third day of treatment were excluded from the final *in vivo* results. Thick and thin blood films were made from each infected person's blood on D_0 (first day of treatment) and on D_7. Any person meeting all the criteria of the test and having asexual *P. falciparum* parasites in his blood on D_7 was considered to have a chloroquine-resistant strain of malaria.

In vitro studies were run by the following method. Students having higher levels of *P. falciparum* parasitemias were selected to be in the *in vitro* trial. Two days following the taking of the first blood film, venous blood was taken from the selected individuals. Approximately 10 cc of blood was withdrawn and placed in a vaccine bottle (50 cc) containing glass beads. The blood was defibrinated for 5 minutes by swirling the bottle. After defibrination, the sample was placed in a cooler containing wet ice. Samples were held at about 4-8°C until processed in the field laboratory. Students included in the *in vitro* trial were treated and observed in the same manner as those in the *in vivo* test, the only difference being that venous blood was taken rather than a drop of blood from the finger. Blood films were made - both thick and thin - using a drop of blood from the syringe sample.

Because of the large numbers of students initially surveyed for malaria, there was an interim of two days between the taking of the first blood film sample and the actual drawing of venous blood for the *in vitro* culture. This delay led to the situation where many bloods were drawn from individuals having extremely low parasitemias; whereas, their levels of parasitemias on the first day had been much higher. This fluctuating level of parasitemias is a normal occurrence in *P. falciparum* malaria, but in this particular type of test was a

big problem. As will be seen later in the results listed in this report, approximately half of the samples tested *in vitro* had to be excluded from the trial because of the lack of sufficient numbers of parasites for accurate analysis. This problem can most likely be solved by one taking venous blood from individuals having high parasitemias on the day of blood withdrawal. Smaller numbers of individuals could be screened for malaria using Rapid Field's Stain and reading the slides on the spot (maximum of 10 fields). Those having good numbers of asexual *P. falciparum* parasites at that moment would give venous blood for the *in vitro* test. A duplicate slide from all individuals could be taken and later stained with diluted Giemsa and read for 100 thick-film fields. This would give a more accurate analysis of the prevalence ratio of malaria in the population tested.

Once the bloods were brought back to the field laboratory, the samples were brought out of the cooler in groups of three and allowed to warm up to room temperature. After swirling briefly, one-ml aliquots of the blood were placed in pre-charged screw-cap, flat bottomed vials (6 cm tall, 1.3 cm internal diameter). The vials contained either glucose (5 mg) -- control vials -- or contained glucose and various amounts of chloroquine -- experimental vials. The following concentrations of chloroquine per ml of blood were used: 0.25, 0.50, 1.00, 1.50, 2.00, 2.50, and 3.00 millimicromoles of base. After addition of blood to the vials the contents were swirled gently. The samples were then placed in an incubator at 38.0 - 40.0°C for 24 hours. Immediately after incubation the vials were swirled gently to mix the contents well and separate thick and thin films slides were prepared. From each series of drug and controls vials for each blood sample, three slides were prepared which contained small thick films from each of the vials in the series. The thin smears were fixed in methyl alcohol and then stained, along with the single thick-films, with diluted Giemsa. The slides containing the multiple thick-films were stained on trays with the blood films facing downwards. This led to fewer films being lost during the staining and washing process, as films made from defibrinated blood tend to wash off the slide easily. One hundred asexual parasites, observed consecutively, in each blood film were then examined and classified according to morphologic characteristics evidence on examination with conventional light microscopy.

Since there was no electricity at the Ayer Lanas Clinic, Honda generators (2.5 kw and 0.3 kw) were used to supply current to the incubator and microscope lights. The incubator was allowed to equilibrate to its set temperature of 39°C for 24 hours before any blood samples were cultured. Two sets of samples were then placed in the incubator over the next two days and allowed to incubate for 24 hours. The generator was refilled periodically with gas and ran continuously for four days. During this time, the maximum-minimum thermometer indicated a change of temperature of only a few degrees

-- the most variance being seen as the samples were placed in the incubator. This is the first time that the *in vitro* test has been run under truly field conditions. The success of this *in vitro* trial proves the feasibility of using this test in the more inaccessible areas of the Malaysia and perhaps other countries. All the equipment and personnel needed to conduct this method can easily be transported by one Land Rover.

Mosquito studies in the Ayer Lanas area were conducted at four villages -- Jeli, Gemang, Jakar, and Nibong -- during February, March, and June of 1971. During the February and June survey, CDC light trap (with dry ice) collections, human biting collections, and indoor-outdoor biting collections were done at each of the four villages for five nights. Ten light traps were placed at 5-6 feet above the ground or 20-30 feet above the ground in the canopy. Each trap was baited with one pound of dry ice which was placed in a styrofoam-insulated wooden box. A rubber tube carried the CO_2 gas from the box to a point 2-3 inches from the intake portion of the trap. A six-volt dry-cell battery operated the trap's fan and light. Traps were turn on at 1830 hours and shut off at 0800 hours. In the human biting collections, five men placed themselves on the perimeter of each village to catch mosquitoes attempting to feed on them. Also, at each village one man collected inside a house while another collected just outside the house. Both the human biting collections and the indoor-outdoor biting collections were made from 1900 to 2400 hours each night. During the March survey, five men from each village collected mosquitoes for three nights both outdoors and indoors.

Larval collections were made during the February and June collection trips. Larval samples were placed in 70% ethanol and the vials labelled for later processing and identification. Some of the larvae were placed in their own breeding water in plastic containers and brought back to the lab for rearing purposes. For the most part only sites thought to be potential anopheline breeding habitats were sampled.

Results:

Human Malaria Studies: A total of 1432 schoolchildren attending schools in the Ayer Lanas study area were surveyed. Of these, 268 (18.7%) were positive for malaria parasites. Over 61% of the infections were *P. falciparum*. There were 72 *P. vivax* cases and 5 *P. malariae* cases. The results of the prevalence survey are shown broken down by school in Table 4. Very few children were seen who had very high parasitemias or fevers. Similar prevalence ratios were seen during a survey conducted by the Institute for Medical Research in 1967 at Jeli, Gemang, and Jakar.

The percentage of resistant cases of falciparum malaria found in the March survey corresponds quite closely to the results obtained

Table 4

Results of Blood Surveys on Schoolchildren for Malaria* in Tanah Merah District, Kelantan, March 1971

School	No. Exam	No. Malaria +ve (%)	Falci-parum	Vivax	Malariae	Mixed	S.U.**
Jeli	135	50(37.0)	25	10	9	2	4
Ayer Lanas (Secondary)	333	39(11.7)	24	12	2	-	1
Ayer Lanas (Primary)	547	74(13.5)	46	21	1	5	1
Gemang	139	62(44.6)	36	12	3	5	6
Jakar	278	43(15.9)	24	17	1	-	1
Total	1,432	268(18.7)	155	72	16	12	13

* Based on 100 thick-film fields examination.
** S.U. species undetermined.

during the IMR survey in 1967. They found that 1% of the people treated with 1500 mg chloroquine base had asexual parasites on the seventh day. We found approximately 6% had not cleared of their parasitemias. This low percentage also agrees with our findings in Trengganu during 1969. In contrast, however, surveys conducted in 1966 by the SEATO Medical Laboratory out of Bangkok indicated that up to 80% of the treated individuals in their surveys in southern Thailand did no respond to chloroquine within seven days. Some of their study areas are only a few miles north of the Kelantan site. This difference is certainly significant and merits much more time and effort to delineate where and why this difference exists.

There were 92 schoolchildren initially included in the 7-day *in vivo* test. Five students were dropped from the final results of the trial because of various reasons; such as, refusal to take the full three-day treatment regimen, non-absorbtion of the drug due to vomiting, and simply not being available for treatment or blood examination. Of the 87 children who were finally included, five (5.7%) were found to be positive for falciparum malaria on the seventh day. As would be expected, several other individuals had gametocytes in their blood on D_7.

The final results of the *in vitro* testing brought out two primary considerations. One was that the *in vitro* system is feasible under field conditions in relatively inaccessible areas. The other is that to obtain a greater number of readible successful cultures, the venous blood should be taken on the same day as the initial blood film is. In order that the microscopist can give an accurate analysis of the morphologic stage of the asexual parasite without too much fatigue, the samples of blood should contain approximately 800-20,000 parasites per cmm. of blood. The best growth to the schizontal stage was noted in samples which contained large ring forms just before culture. Proper defibrination is essential, as otherwise the sample will clot or partially clot rendering it useless. There is no need for taking venous blood from individuals who have taken chloroquine within the last seven days, as very little growth of the parasite will occur *in vitro*. A three-man team can handle 30 to 40 blood samples in one day if only one incubator is used. Constant temperature maintenance and non-disturbance of vials are critical factors in the success of this test.

Fifty-three children were included in the *in vitro* trial. Three samples clotted making them unusable. Half of the 50 samples had too few asexual parasites in 100 thick-film fields to be evaluated. No growth was noted in three of the samples, and six grew to the pre-schizont stage. In five samples, the control vials had less than 5 schizonts out of 100 parasites. Of the completely successful cultures, three were sensitive to chloroquine, six were resistant to chloroquine, and two were intermediate in their response to chloroquine. Table 5 summarizes the *in vitro* and *in vivo* results from the Kelantan survey.

Table 5

Results of Chloroquine-Resistant Malaria Survey in Kelantan, March 1971

In Vitro Results -- Kelantan:

Total Samples	Samples Clotted	Too Few Parasites	No Growth to Schizont Stage	Fewer than 5 Schizonts	Successful cul. Sens	Inter.	R.
53	3	25	9(6 to PS)***	5**	3	2	6*

* One proven resistant *in vivo*
** One had schizonts up thru 1.0 mum chloroquine -- proven resistant *in vivo*
*** PS -- Preschizont stage.

In Vivo Results -- Kelantan:

Total Tested	Total Finally Included	Total Positive on Day 7
92	87	5(5.7%)

Sensitive -- No growth beyond 1.0 mum chloroquine
Intermediate -- Growth beyond 1.0 mum, but not beyond 1.5 mum chloroquine
Resistant -- Growth beyond 1.5 mum chloroquine

Mosquito Studies: Over 13,600 mosquitoes were collected in Kelantan during February and March 1971. Light traps at ground level caught 4,566 adults, and the canopy light traps captured 5,057 mosquitoes. Bare leg catches on the perimeter of the villages accounted for 3,053 mosquitoes. Indoors 380 adult mosquitoes were captured; whereas, immediately outside 557 specimens were obtained. Table 6 shows the results of the light trap (CO_2) collections. The human biting collection results are shown in Table 7. Comparison of the indoor collections to the collections outdoors are seen in Table 8. One *Anopheles maculatus* was found to be positive for malaria parasites.

Armigeres, Culex, and *Mansonia* were the primary culicine genera collected. Fifteen different species of *Anopheles* were caught and identified. *A. b. balabacensis* was collected in this part of West Malaysia for the first time. *A. kochi, A. karwari, A. barbirostris, A. aconitus,* and *A. philippenensis* were the primary anophelines collected. Low numbers of *A. maculatus* were caught. Altogether 34 different species of adult mosquitoes were collected during the February and March trips. The results of the June trip are still pending.

Several breeding areas were found containing vector anophelines like *A. barbirostris*. Breeding sites for six different anopheline species and seven culicine species were located. Of primary interest were those breeding sites containing anopheline larvae within the immediate environs of the villages. Results of the June larval collections are not yet determined.

Perak Studies:

Background: As part of our over-all study of chloroquine-resistant malaria in West Malaysia and as part of our studies of malaria in the Aborigines, a survey of two schools in Lower Perak was made in November 1970. The schoolchildren attending these schools were almost entirely *Orang Asli* (Aborigines). The schools are situated on the mountain road which leads to Cameron Highlands at points seven miles and fourteen miles from Tapah and the main North-South Road (See Map). The Aborigines living in this area are considered to be fringe dwellers and earn their living by rubber-tapping. They tend to stay in one house for a long period of time rather than moving their homes every few years. Also, they do very little fishing and hunting. As their homes are situated on the sides of the forested Cameron Highlands foothills, these aborigines are exposed to the forest-breeding mosquito vectors in addition to *A. maculatus*.

In order to determine the prevalence of chloroquine-resistant malaria in these people, it was felt that surveying the schoolchildren would give an indication of what was occurring in this area. Approximately 120 children were initially surveyed. Finger-puncture blood was taken to make both thick and thin blood films. The films were dried in a incubator overnight, after which they were stained

Table 6

Summary of Light Trap, Ground and Canopy, Collection Results, Kelantan, from 24 Feb. - 5 March 1971

Species Collected	Ground	Canopy
Anopheles aconitus	168	230
Anopheles argyropus	2	-
Anopheles barbirostris	31	26
Anopheles crawfordi	-	1
Anopheles indiensis	111	81
Anopheles karwari	106	234
Anopheles kochi	204	218
Anopheles philippinensis	56	52
Anopheles peditaeniatus	6	3
Anopheles maculatus	-	1
Anopheles tessellatus	12	24
Anopheles sinensis	1	3
Aedes spp. (4)*	50	65
Aedomyia sp. (1)*	-	1
Armigeres sp. (1)*	2	5
Culex spp. (10)*	3546	3761
Mansonia spp. (5)*	277	351
Uranotaenia sp. (1)*	-	1
Total	4566	5057

* Number of different species.

Table 7

Summary of Human Biting Collection (B.L.C.) Results, Kelantan, from February to March 1971

Species Collected	1st Trip 24 Feb-5 Mar	2nd Trip 21-26 March	Total
Anopheles aconitus	69	15	84
Anopheles argyropus	6	26	32
Anopheles balabacensis	2	2	4
Anopheles barbirostris	5	6	11
Anopheles crawfordi	-	4	4
Anopheles karwari	25	12	37
Anopheles kochi	18	44	62
Anopheles maculatus	17	-	17
Anopheles peditaeniatus	10	-	10
Anopheles philippinensis	56	7	63
Anopheles separatus	-	1	1
Anopheles sinensis	-	76	76
Anopheles tessellatus	2	42	44
Anopheles vagus	-	11	11
Anopheles indiensis	54	40	94
Aedes spp. (5)*	73	35	108
Armigeres spp. (3)*	25	1	26
Culex spp. (6)*	1159	291	1450
Mansonia spp. (4)*	678	241	919
Total	2199	854	3053

* Number of different species.

Table 8

Summary of Indoor and Outdoor Biting Collection Results, Kelantan, from February to March 1971

Species Collected	Indoor	Outdoor
Anopheles aconitus	8	9
Anopheles argyropus	1	2
Anopheles barbirostris	4	3
Anopheles crawfordi	3	3
Anopheles indiensis	15	32
Anopheles karwari	8	8
Anopheles maculatus	4	2
Anopheles peditaeniatus	4	12
Anopheles philippinensis	2	4
Anopheles tessellatus	-	3
Anopheles sinensis	3	4
Anopheles vagus	-	1
Anopheles kochi	4	25
Aedes spp. (5)*	6	20
Armigeres sp. (1)*	3	1
Culex spp. (7)*	263	355
Mansonia spp. (3)*	52	73
Total	380	557

* Number of different species.

with diluted Giemsa and read under oil. Thirty thick-film fields were read at the field laboratory that was set up in a nearby Rest House. Final prevalence results, however, are based upon examination of 100 fields for each thick film. All positive children were treated with chloroquine. Those individuals having *P. falciparum* malaria were treated with an equivalent dose to the 3-day 1500 mg base adult dose. Blood films were made on the first day of treatment and on the seventh day following initiation of treatment. Urines were tested with Meyer's Reagent on the first and third days of treatment. Those *P. falciparum* positives not meeting all the criteria of the 7-day *in vivo* test were excluded from the final results.

In addition to the *in vivo* study, most of the children having falciparum malaria were included in the *in vitro* trial. Venous blood was taken from all but four of the children who were tested over the seven days. Before defibrination, approximately 1 cc of blood was placed in a plastic screw-cap tube for scrub typhus antibody analysis. A drop of syringe blood was used to make thick and thin blood films. The blood (5-10 cc) was defibrinated in 50 ml Erlemmeyer flasks containing glass beads. The defibrinated blood was then placed in a cooler with wet ice and taken back to the field laboratory. Once back to the laboratory, the *in vitro* test was conducted in the same manner as described in the section on the Kelantan Studies. The only difference was that the incubator was powered by normal house current rather than generators. A generator was kept in reserve, however, in case of a power failure in this small town. Less of a problem than in Kelantan was noted with the cultures having too few parasites for accurate analysis. This may have been due to there being only one day's separation between the initial blood film and withdrawal of the venous blood sample. The drug concentrations used were 0.25, 0.50, 0.75, 1.00, 1.50, and 2.00 mum of chloroquine per cc of blood.

Limited studies of the mosquito fauna in this area were also conducted; but at a later time, and therefore will be mentioned in the section on malaria in the Aborigines.

Results: There were 126 *Orang Asli* children included in the November survey, with 41 out of 74 (55.4%) at the 7th mile school and 23 out of 52 (44.3%) at the 14th mile school being positive. Most of the positive individuals had *P. falciparum* parasites in their blood. The total number of *P. falciparum* tested in the *in vivo* study was 34, with one individual being excluded because he had taken chloroquine the day before the drug trial started. No positive blood films were seen in samples taken on Day 7. Cases of RI resistance can, of course, not be excluded.

Indications from the *in vitro* results are that resistant strains of malaria are present in this area. *In vitro* cultures totaled 30, but 9 had too few parasites for analysis. Two samples

clotted but were still usable. Only one sample did not have growth in the control; this one being the boy who had taken chloroquine just previous to the drug trial. Of the successful cultures, 9 were considered to be sensitive, 1 to be intermediate in its response to chloroquine, and 3 were classified as resistant. A summary of the *in vivo* and *in vitro* results, along with the prevalence data are found in Table 9. The success of this study shows that it is feasible to use the *in vitro* technique even on people as inaccessible as the Aborigines of Malaya. The equipment and personnel can even be carried into deep jungle areas by helicopter.

Other *In Vivo* and *In Vitro* Studies:

Background: In collaboration with Dr. M. Bolton at Gombak Aborigine Hospital and Dr. D.R. O'Holohan at a private clinic in Seremban, we are analyzing the blood from certain *P. falciparum* infections by the *in vitro* technique. When feasible, these doctors test the patients by the *in vivo* technique. The patients at the Gombak Hospital are almost entirely *Orang Asli* although a few Malays come in as out-patients. Patients coming to the Seremban clinics are Malay, Chinese, or Indians - the majority being rubber and oil palm estate workers. The defibrinated blood samples are picked up by our courier and brought back to our Kuala Lumpur laboratory. The bloods are processed in the same manner as previously mentioned in this report. Results of the test are then sent back to these physicians.

In addition to testing the *in vitro* system using human blood, one trial was made by infecting a gibbon with human *P. falciparum* malaria and testing the innoculated parasites *in vitro*. This is an excellent system for trying to narrow down the varibilities of various *P. falciparum* strains *in vitro*. Unfortunately the scarcity of available gibbons for laboratory use has curtailed this work for the moment. It is hoped that we can innoculate three gibbons with human *P. falciparum*; one with a proven sensitive strain, one with a resistant strain, and one strain intermediate to these. These strains could then be studied both *in vivo* and *in vitro* over a long period of time. With this system, we should be able to correlate our *in vivo* and *in vitro* results in the field with greater confidence, and also gain a better understanding of sensitive and resistant malaria strains.

Results: Approximately 25 samples from the Gombak Hospital and 11 samples from the Seremban clinic have been tested *in vitro*. Some of the samples were shown to be resistant *in vitro* and were later found to be resistant *in vivo*. Sensitive and intermediate responses to chloroquine have also been seen. A representation of the three different responses to chloroquine *in vitro* are shown in Graph 1.

Table 9

Results of Chloroquine-resistant Malaria Survey in Perak, November 1970

In Vitro Results -- Perak:

Total Samples	Samples Clotted	Too Few Parasites	No Growth to Schizont Stage	Fewer than 5 Schizonts	Successful cul. Sen.	Inter.	R.
30	2(OK)	9	3* (2 to PS)***	5**	9	1	3

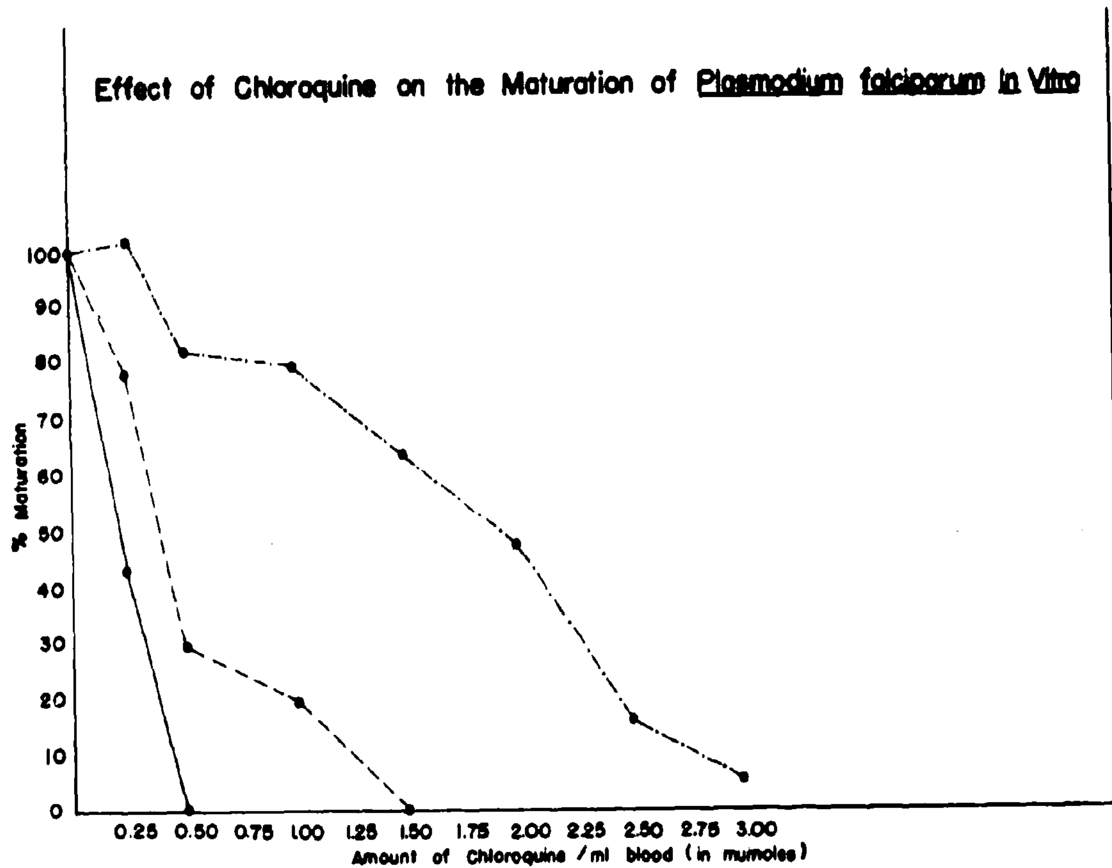
* One had chloroquine in urine.
** One had schizonts up thru 1.5 mum chloroquine.
*** PS -- Pre-schizont stage.

In Vivo Results -- Perak:

Total Tested	Total Finally Included	Total Positive on Day 7
34	33	0

Prevalence Results -- Cameron Highlands Road, Perak:

Location	Total Examined	Total Positive (%)	P. fal.	viv.	mal.	mixed
7th mile school	74	41(55.4)	20	6	-	15
14th mile school	52	23(44.3)	17	2	-	4



Graph I

The one gibbon which was innoculated received its innoculum from a 12 year old Malay girl. The girl's blood had approximately 2000 asexual *P. falciparum* parasites per 500 WBC. Her blood sample was defibrinated, and 0.5 cc was injected IV into the gibbon. Another 0.5 cc was injected IP. The remainder of the blood was tested by the *in vitro* culture. On Day 1 post inoculation, a blood film was made and stained. There were approximately 255 asexual falciparum parasites per 500 WBC. Daily blood films were then taken, almost every blood film taken was positive. Gametocytes were seen for 32 days but at a very low level after the first five days. Graph 2 shows the daily asexual parasitemia level of this gibbon. An *in vitro* culture was made on Day 24 from a small sample of the gibbon's blood. There were approximately 840 parasites/500 WBC. Both the *in vitro* test of the girl's blood and of the gibbon's blood showed this *P. falciparum* isolate to be resistant to chloroquine up to 2.0 mum chloroquine base per ml of blood.

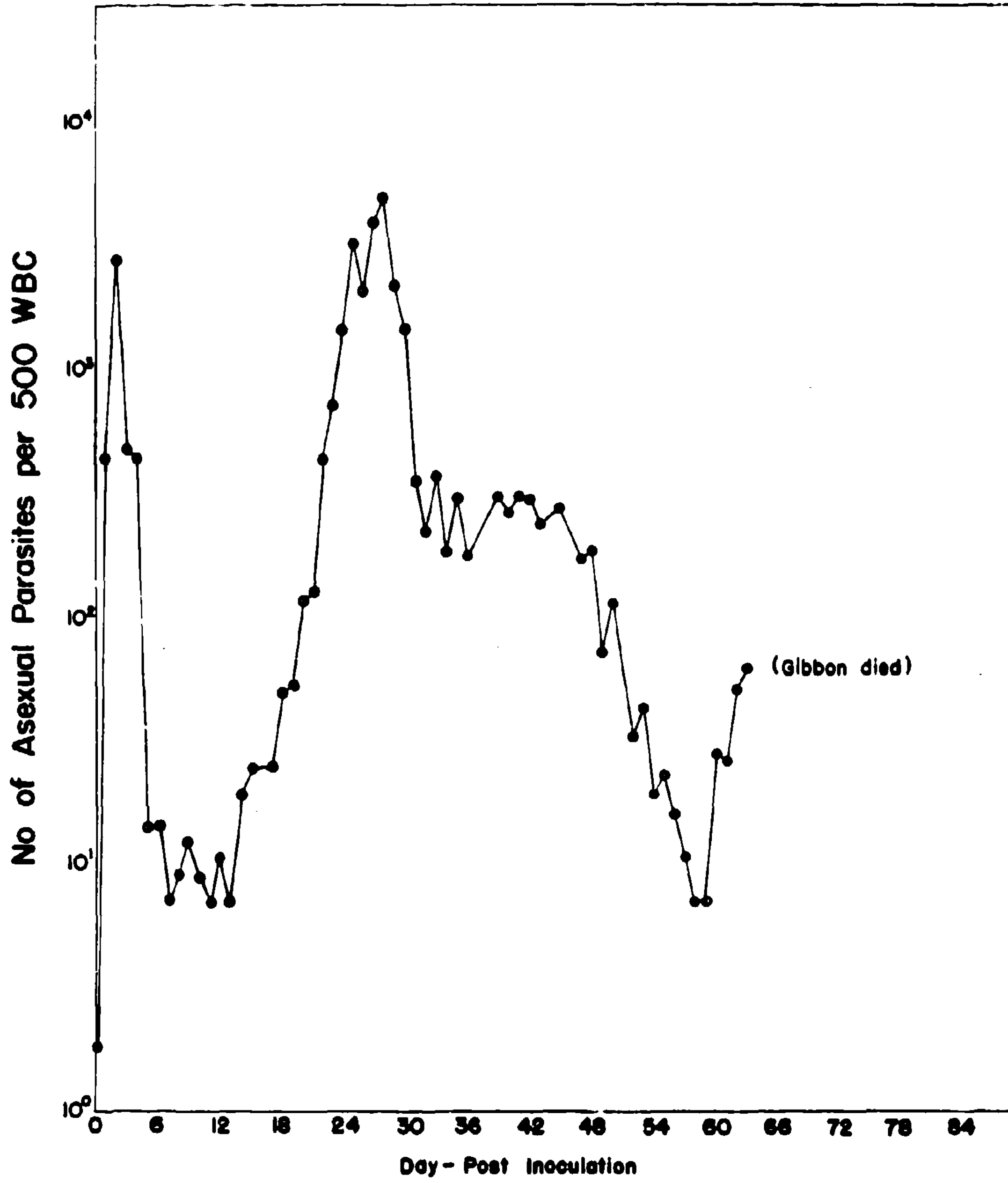
Malaria in the *Orang Asli* (Aborigines)

Background: The Northcentral part of West Malaysia is made-up of primary mountainous terrain covered by dense jungle. For the most part the area is only inhabited by aborigines, with a density of population of only four people per square mile. Medical care for these *Orang Asli* is the responsibility of the Aborigine Hospital at Gombak, Selangor. The planning staff for the Malaria Eradication Program in Malaysia has made the Senior Medical Officer of the Aborigine Hospital responsible for the MEP in the areas inhabited primarily by the *Orang Asli*.

In collaboration with the Gombak Hospital, we are carrying out studies on the prevalence of malaria among the *Orang Asli* people. Blood surveys to obtain baseline and follow-up data on malaria prevalence within the aborigine population just preceding and following residual spraying and initiation of weekly drug prophylaxis have been conducted. Deep jungle villages in the Pos Shean area and the Fort Kemar area are being monitored for changes in malaria prevalence due to the prophylactic and control measures. Fringe villages in the Woh and Satak areas are also being surveyed for malaria periodically. Certain other areas are checked on a one-time basis to evaluate malaria prevalence ratios. See Map for the relative locations of these villages.

Pos Shean and Fort Kemar are surrounded by deep jungle and are completely isolated by mountains ranging up to 7000 feet in elevation. Travel into these areas is feasible only by helicopter. Pos Shean could be reached by a very difficult five day walk and Fort Kemar by a four hour boat ride and a five hour walk. Satak and Woh are accessible by land rover and are located on the jungle fringe. The people living in the deep jungle areas hunt and fish daily for food; whereas, the fringe dwellers buy their food from stores.

Blood Induced Infection of *P. falciparum* in Gibbon G-2



Graph 2

Data is being compiled on malaria, scrub typhus, filariasis, and general health in both the fringe dwellers and the deep jungle dwellers. Also, as mentioned previously in this report, a survey to determine the status of chloroquine-resistant malaria in these people has been conducted. Studies of the mosquito fauna in both the deep jungle areas and the fringe areas have been run. Human biting collections, CDC light trap collections with CO_2 gas, and larval collections have been made. Female anophelines are dissected to detect the presence of sporozoites and oocysts.

Results:

Human Studies: In October 1970, a survey was made of the Pos Shean area. Out of 136 individuals surveyed, 28 (20.6%) were found to be infected with malaria parasites. The majority of the positives had *P. falciparum* infections. Filter paper results of positive scrub typhus antibody carriers are found in the Rickettsia Section of this report. In February 1971, another survey of these people was conducted. There were only 4 (2%) positives out of 171 people examined. This low prevalence may be a seasonal occurrence or it may be due to the anti-malarial measures being carried out in this area. A survey was done in June to try and see if the prevalence is still low. Results are not yet finalized.

During November 1970, studies on malaria prevalence in three groups of aborigines were made. Two groups of schoolchildren were surveyed - the results of which have already been discussed. The *Orang Asli* staff members at Gombak Hospital were surveyed. Malaria smears were made from a drop of syringe blood. The remainder of the venous blood was used for scrub typhus antibody testing. Two individuals were tested for chloroquine-resistant malaria by the *in vitro* method. Altogether 69 aborigines were sampled, with 11 (15.9%) being positive for malaria.

In December 1970, four different *Orang Asli* villages were surveyed. Blood films totaling 152 were made from the aborigines living in Ayer Denak. Approximately 17% were positive, with *P. vivax* predominating. Eleven (15.9%) out of 69 people in Lubok Sempit had malaria infections. No people were found to be positive for malaria in either Chabang Tiga or Fort Kemar - 52 people checked.

In March 1971, a special trip was made to Tamok in the state of Johore. The reason being that one of our technicians contracted a simian malaria while working in this village. Morphologically the malaria parasites looked like either *P. coatneyi* or *P. knowelsi*. Sera results sent by Dr. Coatney from the Center for Disease Control in Atlanta, Georgia, on the technician's blood sample indicate the highest antibody titer against *P. knowelsi*. This is the second recorded case of a simian malaria being contracted by man in Malaysia.

Unfortunately the technician was treated by a local doctor before we could obtain a blood sample to innoculate into a monkey. Out of 77 blood films taken in Tamok, 5 (6.5%) were positive for *P. vivax*. Finding this simian malaria in our technician reinforces the fact that the possibility exists for man to come down with a simian malaria. How often this occurs may be rather infrequent, however, people living in areas of risk are not often surveyed for malaria. Also, only the more experienced microscopist, as in both of the recorded cases of simian malaria in Malaysia, would ever pick out the morphological differences on routine examination.

A summary of all the blood surveys that we have conducted to date of the *Orang Asli* is found in Table 10.

Mosquito Studies: An intensive survey of mosquitoes at Woh Post and at 14th mile, Cameron Highlands Road, was made in April 1971. Collections were made for four nights at each place. Five aborigines at each area were hired to assist in making bare leg catches and indoor-outdoor collections. Light traps with dry ice were placed both within the canopy and near the ground. Larval surveys of each area were also made.

A total of 563 adult mosquitoes were caught attempting to bite the collectors in the jungle. Out of 198 mosquitoes caught at the indoor-outdoor collection sites, a little less than half were caught biting indoors -- including 16 *A. maculatus*. Light traps within the canopy captured 264 adult mosquitoes, and in the ground level traps 175 adults were caught. The light traps were apparently not very effective in attracting *Anopheles spp.* at this particular time, as only three were caught.

Anopheles maculatus was the primary vector of human malaria found during this survey. *Culex* and *Mansonia* were caught in the greatest numbers. Results of the various collections made are listed in Table 11, 12, and 13. Larval collections are still being processed.

Studies On Jungle Canopy Mosquitoes

Background: The area selected for study is located about 12 miles west of Kuala Lumpur near an aborigine settlement. The site itself is a small area of primary forest on the side of a hill which is interspersed with small streams and seepages. Trees 80 to 170 feet tall are found throughout the area. Much low growing scrub is found at ground level. Part of the area could be classified as fringe primary forest and part as relatively undisturbed or deep primary forest -- the taller trees being found in the latter. A system of aluminum ladders has been set up within the jungle canopy to form a walkway which transects both parts of this primary forest. The walkway is constructed horizontally from the hillside using tree

Table 10

Results of Blood Surveys for Malaria among the *Orang Asli*

Location	Date of Survey	Total No. Ex.	Total No. +(%)	P.fal.	viv.	mal.	mixed
Ft. Kemar	Mar. '70	97	9(9.3)	8	-	1	-
Pos Shean	May '70	115	25(21.7)	22	-	3	-
Ft. Kemar	Jul '70	57	6(10.5)	3	1	1	1
Satak	Sept.'70	52	13(25.0)	8	1	4	-
Woh Pos	Sept.'70	55	20(36.4)	12	2	4	2
Pos Shean	Oct. '70	136	28(20.6)	20	5	2	1
7th Mile, CHR*	Nov. '70	74	41(55.4)	20	6	-	15
14th Mile, CHR*	Nov. '70	52	23(44.3)	17	2	-	4
Staff, Gombak Hospital	Nov. '70	69	7(10.1)	6	1	-	-
Ayer Denak	Dec. '70	152	26(17.1)	4	18	-	4
Lubok Sempit	Dec. '70	69	11(15.9)	5	3	1	2
Chabang Tiga	Dec. '70	16	0(0)	-	-	-	-
Ft. Kemar	Dec. '70	36	0(0)	-	-	-	-
Pos Shean	Feb. '71	171	4(2)	4	-	-	-
Tamok	Mar. '71	77	5(6.5)	-	5	-	-
Total		1260	218(17.3)	129	44	16	29

* CHR -- Cameron Highlands Road.

Table 11

Summary of Human Biting Collection Results at 14th Mile, Cameron Highlands Road and Woh Pos, Tapah, from 26 April to 29 April 1971

Species Collected	14th Mile, C. Highlands	Woh Pos, Tapah	Total
Anopheles maculatus	4	33	37
Anopheles umbrosus	-	1	1
Aedes spp. (3)*	30	10	40
Armigeres spp. (12)*	13	30	43
Culex sp. (1)*	60	149	209
Heizmannia sp. (1)*	-	1	1
Mansonia sp. (1)*	154	78	232
Total	261	302	563

* Number of different species.

Table 12

Summary of Outdoor and Indoor Biting Collection Results at 14th Mile, Cameron Highlands Road and Woh Pos, Tapah, from 26 April to 29 April 1971

Species Collected	14th Mile, C. Highlands Outdoor	14th Mile, C. Highlands Indoor	Woh Pos, Tapah Outdoor	Woh Pos, Tapah Indoor
Anopheles maculatus	14	11	8	5
Anopheles aitkeni	1	-	-	-
Aedes spp. (4)*	5	6	21	15
Armigeres spp. (7)*	13	11	10	1
Culex spp. (3)*	1	5	9	18
Mansonia spp. (2)*	11	12	10	11
Total	45	45	58	50

* Number of different species.

Table 13

Summary of Light Trap, Canopy and Ground, Collection Results at 14th Mile, Cameron Highlands Road and Woh Pos, Tapah, from 26 April to 29 April 1971

Species Collected	14th Mile, C. Highlands	14th Mile, C. Highlands	Woh Pos, Tapah	Woh Pos Tapah
	Ground	Canopy	Ground	Canopy
Anopheles maculatus	-	-	-	1
Anopheles asiaticus	2	-	-	-
Aedes spp. (2)*	3	-	-	-
Armigeres spp. (3)*	16	-	-	-
Culex spp. (6)*	85	64	63	278
Orthopodomyia spp. (2)*	5	5	-	-
Uranotaenia sp. (1)*	-	-	1	2
Mansonia spp. (2)*	-	4	-	10
Total	111	73	64	291

* Number of different species.

trunks as vertical supports. It is possible for a person to walk along this system of ladders for more than 1200 feet through the canopy at heights above the ground ranging from 20 to 130 feet. Eleven platforms have been built at various points along the walkway. Meteorological equipment for measuring rainfall, temperature, and relative humidity are located both at ground level and within the canopy. Biting collections, light trap collections, bait trap collections, resting collections, or larval collections can be made at any point along the walkway.

Thus far during this study the collection methods used have been CDC light trap collections with CO_2 gas, human biting collections, and oviposition trap collections. Nine CDC light traps baited with dry ice are placed at various points within the canopy - usually where some shade is available rather than in unprotected open areas. Nine traps are placed at parallel points, to those in the canopy, 5-6 feet above the ground. Traps are turned on at 1830 hours and turned off at 0730 hours. Collection bags are brought back to the laboratory for processing of the captured mosquitoes. Human biting collections have been made by two men collecting at canopy level and two men collecting at parallel points on the ground. Biting collections are made from 1800 to 2230 hours. Bamboo cups and black glasses with filter paper strips are placed along the walkway and at similar points on the ground to determine oviposition sites. Previously periodic checks of cups and glasses were usually done weekly to determine what species were laying eggs. In June, however, the black glasses were checked daily and the cups once a week. Additional cups and glasses were added, so that there are now 24 of each in the canopy and on the ground. Figure 2 shows the placement of the various traps and collection points at the fringe primary forest transect system. Collection points at the deep primary forest transect system are shown in Figure 3.

All mosquitoes are identified and the anophelines are dissected for detection of malaria parasites. *Mansonia crassipes* are dissected to determine the presence of filarial worms. Larval collections are made to determine the breeding habitats of the mosquitoes found within the study area.

Results: Unusual weather caused reductions in the number of collection trips made to Bukit Lanjan and in the relative number of mosquitoes at the site. Extremely heavy rains in January caused extensive flooding in many of the states in West and East Malaysia. These rains washed away many larval breeding sites in the area. Also, our unit was heavily involved in helping the flood victims and in administering vaccine. Therefore, during January very little collecting could be done at Bukit Lanjan. Then during April and May abnormally dry weather hit West Malaysia. The dry period in these two months greatly reduced the number of available breeding areas at Bukit Lanjan and consequently the number of adults.

Overhead View of a Suspended Horizontal Walkway in Fringe Primary Forest Showing Mosquito Collection Sites

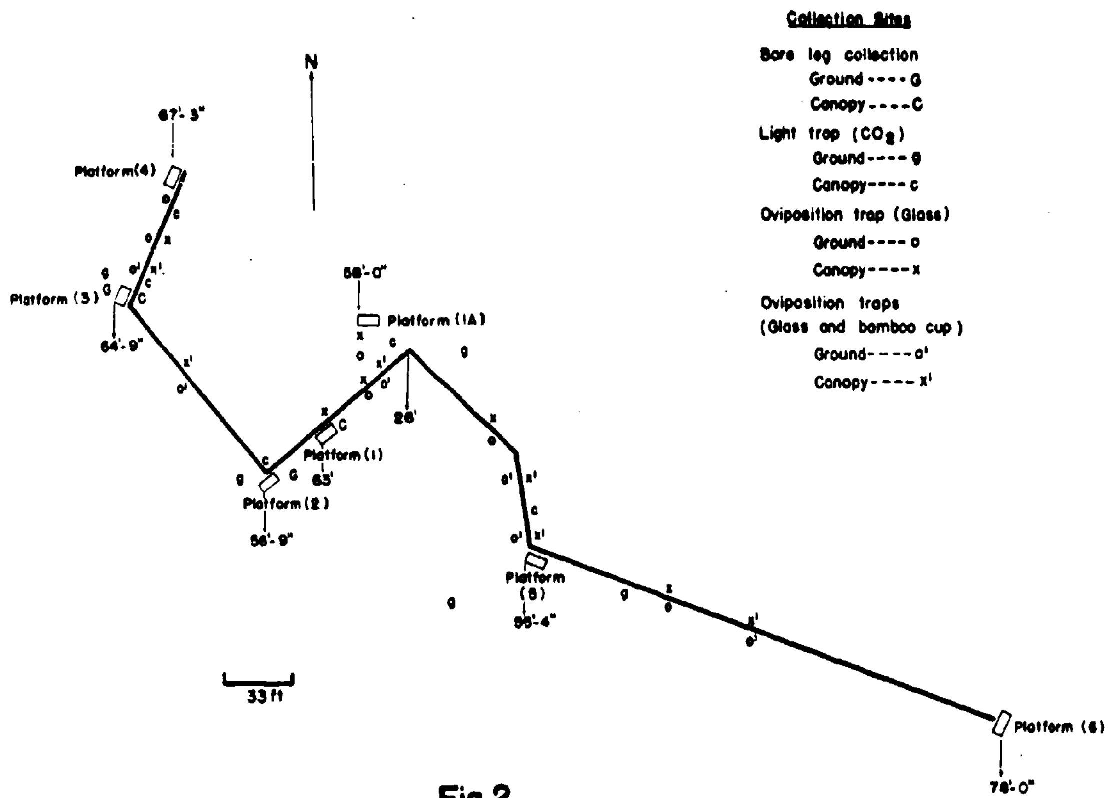


Fig.2

Overhead View of a Suspended Horizontal Walkway in Deep Primary Forest Showing Mosquito Collection Sites

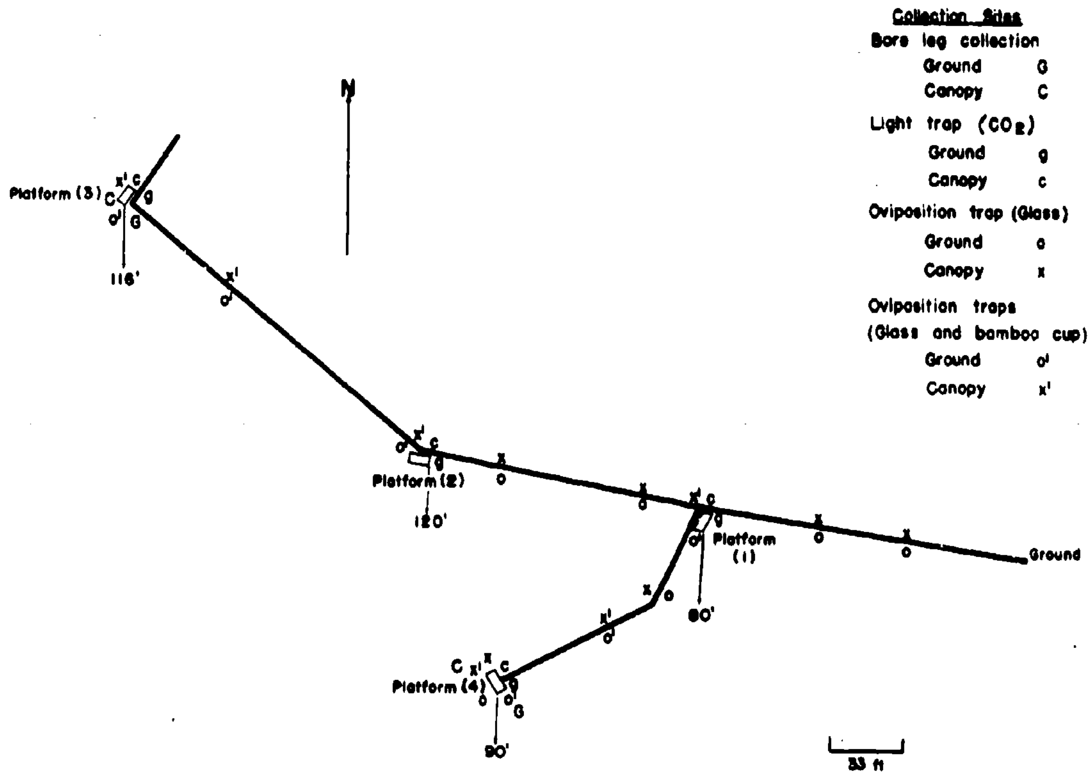


Fig.3

Approximately 11,690 adult mosquitoes have been collected at this study area since October. Collections at the deep primary forest site were started in December. Of the total, 7794 mosquitoes have been captured at ground level and 3900 (33.3%) at canopy level. Light trap collections have totaled 7182 adults and human biting collections accounted for 4509. Table 14 lists the results of the mosquitoes collected by bare leg catches (BLC) both in the canopy and on the ground. Results of the light trap collections are found in Table 15.

Seven different species of larvae were found breeding in the bamboo cups and glasses placed within the canopy. Among others these included *Aedes albopictus*, *Aedes niveus* group, *Heizmannia*, and *Tripteroides*. On the ground *Aedes albopictus*, *Heizmannia*, *Culex minor*, and *Culex brevipalpis* larvae were found in the cups and glasses. Several species of *Toxorhynchites* have been found breeding in the cups and glasses both in the canopy and on the ground.

Two *Anopheles riparis* have been found to have both sporozoites and oocysts. The sporozoites were very short and cigar-shaped and did not seem to resemble human or simian malarial sporozoites. They may have been a tragulid malaria parasite, but further specimens are needed to be sure. One *A. letifer* was found infected. Over 20 specimens of *Mansonia crassipes* have been positive for filarial worms. Most of the positive *Mansonia* were caught in the canopy.

Mosquito Colonies

In order to support various studies being conducted by our Unit, colonies of certain species of mosquitoes were continued or have been started during this reporting period. The indoor insectary was completely torn down and rebuilt. Double panels with insulation in between were used so that temperature and humidity could be held more constant than before. The temperature now stays at 78°F ± 2° and the relative humidity runs about 85% ± 3%. Before rebuilding the temperature would vary as much as 8°F and the relative humidity might rise or fall 20%. The outdoor insectary consists of a screened area which is shaded throughout most of the day by large trees. The floor is dirt and a metal roof protects the rearing area from rain.

Adults are caged in small screened cages, and anophelines are mated artificially. Eggs are laid either in bamboo cups or in paper cups containing wet filter paper. Eggs are placed in porcelain bowls containing rain water. First instar larvae are placed in white enamel pans containing a small amount of sterile soil and rain water. Larvae are fed on a mixture cereal, monkey chow, yeast, ground up dried-beef liver, and vitamin B complex. Pupae are placed daily within cages in porcelain bowls containing rain water. Adults are allowed to feed on cloth wicks soaked in a sugar and vitamin solution. The adult mosquitoes obtain their bloodmeals from guinea pigs and monkeys.

Table 14

Summary of Human Biting Collection (B.L.C.) Results.
Fringe Forest and Deep Forest, Bukit Lanjan,
from October 1970 to June 1971

Species Collected	Ground	Platform	Ground	Platform
	Fringe** Forest	Fringe** Forest	Deep*** Forest	Deep*** Forest
Anopheles aitkeni	1	-	2	-
Anopheles asiaticus	-	-	3	-
Anopheles letifer	16	1	6	-
Anopheles leucosphyrus	4	3	-	30
Anopheles riparis	2	1	-	5
Anopheles watsonii	-	1	-	-
Aedes spp. (8)*	138	7	44	48
Armigeres spp. (4)*	69	2	82	2
Culex spp. (4)*	1722	398	878	148
Heizmannia sp. (1)*	1	-	-	-
Mansonia spp. (3)*	110	92	65	131
Total	2063	505	1580	364

* Number of different species.
** Fringe Forest - Primary trees, a few up to 90 feet tall.
*** Deep Forest - Many primary trees up to 150 feet tall.

Table 15

Summary of Light Trap, Ground and Platform Collection, Fringe Forest and Deep Forest, Bukit Lanjan, from October 1970 to June 1971

Species Collected	Ground	Platform	Ground	Platform
	Fringe** Forest	Fringe** Forest	Deep*** Forest	Deep*** Forest
Anopheles aitkeni	1	-	-	-
Anopheles leucosphyrus	1	2	-	15
Anopheles letifer	6	-	3	-
Anopheles riparis	2	115	1	98
Aedes spp. (2)*	6	34	4	78
Aedomyia sp. (1)*	9	21	2	14
Armigeres spp. (2)*	5	-	3	1
Culex spp. (7)*	2994	1133	439	289
Mansonia spp. (5)*	607	956	63	373
Heizmannia sp. (1)*	-	-	-	1
Orthopodomyia sp. (1)*	3	-	-	-
Uranotaenia sp. (1)*	1	-	-	-
Toxorhynchites sp. (1)*	-	-	1	-
Total	3635	2262	516	769

* Number of different species.
** Fringe Forest - Primary trees, a few up to 90 feet tall.
*** Deep Forest - Many primary trees up to 150 feet tall.

Colonies of *Anopheles le[illegible]* (F_4), *Anopheles maculatus* (F_7), *Aedes aegypti* (F_{21}), and *Aedes albopictus* (F_{14}) are being reared. Several other species of anophelines are now being started in the indoor insectary. Culicines from the egg and larval collections at Bukit Lanjan are also being raised in the insectary.

Reference Collection

During this reporting period nine additional species of adult mosquitoes have been added to the USAMRU reference collection. In last year's Annual Report, the species of adult mosquitoes available at USAMRU for reference and study were listed. In this report, Table 16 lists the species of larval mosquitoes available in the reference collection. Emphasis has been placed on collecting larval anophelines which may or may not be vectors of human malaria.

Studies on Tragulid Malaria

No satisfactory laboratory model for the study of the EE system exists since monkeys are expensive and rodent malarias are difficult to transmit by sporozoites. Little is known of *P. traguli* except that it can be found in the liver of naturally infected animals, that mosquitoes can be infected easily,and that most mouse deer are infected at the time of capture. These latter data suggest that it should be an excellent laboratory model. Previous work was hindered by the inability to maintain the host tragulid in the laboratory. We have been successful in maintaining and breeding these animals in captivity as is described elsewhere in this annual report. Work to date includes only familiarization with the stages of this minute erythrocytic parasite, electron microscopy of this parasite and *H. fuldi* and colonization of *Anopheles letifer* and *A. maculatus* to do transmission studies. Microfilaria and what may be a new blood parasite have also been found and are being studied.

Methods for Investigations of Liver Stages of Malaria

Method of Partial Hepatectomy: A method of partial hepatectomy was developed for monkeys using Sparine and Sernylan anesthesia, midline incision, partial resection using Doyens crushing clamps, and then suture of the crushed serosal surfaces with both discontinuous and running 4-0 sutures. Just greater than 50% of the liver mass was removed. Monkeys were placed back into their normal single cages and allowed to take water and food *ad lib.*

In none of twelve cases were there surgical complications of hemorrhage, infection, or dehiscence. One monkey early in the study died postoperatively. Autopsy showed no discernible surgical error. The monkey had been bled the evening before the surgery to obtain a pre-hepatectomy serum. Subsequent monkeys were bled one week prior to surgery and weekly after surgery.

Table 15

Species of Mosquito Larvae in USAMRU Reference Collection

1.	*Anopheles aconitus*	1.	*Culex annulus*
2.	*Anopheles baezai*	2.	*Culex bitaeniorhynchus*
3.	*Anopheles barbirostris*	3.	*Culex brevipalpis*
4.	*Anopheles bengalensis*	4.	*Culex fragilis*
5.	*Anopheles crawfordi*	5.	*Culex fraudatrix*
6.	*Anopheles fragilis*	6.	*Culex fatigans*
7.	*Anopheles hackeri*	7.	*Culex fuscanus*
8.	*Anopheles indiensis*	8.	*Culex gelidus*
9.	*Anopheles insulaeflorum*	9.	*Culex hutchinsoni*
10.	*Anopheles karwari*	10.	*Culex halifaxi*
11.	*Anopheles kochi*	11.	*Culex mammilifer*
12.	*Anopheles lesteri*	12.	*Culex mimulus*
13.	*Anopheles letifer*	13.	*Culex minor*
14.	*Anopheles leucosphyrus*	14.	*Culex nigropunctatus*
15.	*Anopheles maculatus*	15.	*Culex obscurus*
16.	*Anopheles montanus*	16.	*Culex papuaensis*
17.	*Anopheles peditaeniatus*	17.	*Culex perplexus*
18.	*Anopheles philippinensis*	18.	*Culex pseudovishnui*
19.	*Anopheles pujutensis*	19.	*Culex fuscocephalus*
20.	*Anopheles pollicaris*	20.	*Culex scanloni*
21.	*Anopheles separatus*	21.	*Culex spathifurca*
22.	*Anopheles sinensis*	22.	*Culex tritaeniorhynchus*
23.	*Anopheles subpictus*	23.	*Culex variatus*
24.	*Anopheles sundaicus*	24.	*Culex whartoni*
25.	*Anopheles stricklandi*	25.	*Culex (Lophoceraomyia) sp.*
26.	*Anopheles tessellatus*	26.	*Culex (Mochtogenes) sp.*
27.	*Anopheles umbrosus*	27.	*Aedes albolineatus*
28.	*Anopheles riparis*	28.	*Aedes albolateralis*
29.	*Anopheles roperi*	29.	*Aedes albopictus*
30.	*Anopheles vagus*	30.	*Aedes albotaeniatus*
31.	*Anopheles asiaticus*	31.	*Aedes amesii*

Table 16 (Continuation)

Species of Mosquito Larvae in USAMRU Reference Collection

32. *Aedes butleri*
33. *Aedes chrysolineatus*
34. *Aedes caecus*
35. *Aedes fumidus*
36. *Aedes jugraensis*
37. *Aedes lineatopennis*
38. *Aedes litoreus*
39. *Aedes longirostris*
40. *Aedes mediopunctatus*
41. *Aedes masculinus*
42. *Aedes novoni-eus*
43. *Aedes pseudoniveus*
44. *Aedes w/albus*
45. *Aedes (Finlaya) sp.*
46. *Uranotaenia campestris*
47. *Uranotaenia lateralis*
48. *Uranotaenia longirostris*
49. *Uranotaenia lutescens*
50. *Uranotaenia micans*
51. *Uranotaenia sp.*
52. *Armigeres durhami*
53. *Armigeres malayi*
54. *Armigeres moultoni*
55. *Armigeres flavus*
56. *Armigeres subalbatus*
57. *Armigeres sp.*
58. *Aedomyia catasticta*
59. *Ficalbia chamberlaini*
60. *Ficalbia elegans*
61. *Ficalbia fusca*
62. *Malaya genurostris*
63. *Malaya jacobsoni*
64. *Heizmannia complex*
65. *Hodgesia malayi*
66. *Mansonia crassipes*
67. *Topomyia sp.*
68. *Toxorhynchites sp.*
69. *Tripteroides aranoides*
70. *Orthopodomyia albipes*

Modified Giemsa Stain of Liver Tissue Sections: A method of liver histochemistry has been developed combining the modified Giemsa methods of Price and of Garner. The method allows clear definition of hepatocyte and malarial structure.

Liver Cultures Using Homologous Post-hepatectomy Serum: Heterologous and homologous serum, one-week post-hepatectomy, was added to tissue culture medium 199 and used to feed Macaque liver biopsy pieces. The tissue culture looked good at four days growth with polygonal cells growing away from the tissue mass. Glucose was added and forty-eight hours later the tissue was assayed for incorporation of labeled glucose into glycogen. Glycogen was extracted and purified by the Pfluger method.

About a microgram of nswly synthesized glycogen was present after six days of tissue culture in each group of ten tissue fragments fed homologous post-hepatectomy serum, compared to one-hundredth that value for heterologous serum.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

Field	Value
2. Date of summary	30 06 71
Report control symbol	DD-DR&E(AR)636
3. Date prev summary	30 06 70
5. Summary scty	U
7. Regrading	N/A
8a. Dissem instr'n	NL
10. No./Codes	Program element / Project number / Task area number / Work unit number

11. Title: Investigations of Melioidosis

12. Scientific and technological areas: 010100 Microbiology

13. Start date	14. Estimated completion date	15. Funding agency	16. Performance method
10 70	9 71		

17. Contract/Grant: DADA17-71-G-9332 Mod. P-103

a. Dates/Effective: 10 70 Expiration: 10 71

b. Number:

c. Type: Y Grant d. Amount: 181

e. Kind of award: f. Cum. amt.:

18. Resources estimate		a. Professional man yrs	b. Funds (in thousands)
Fiscal year	Preceding 71	1.0	7.8
	Current 72	1.0	8.7

19. Responsible DOD organization

Name: US Army Medical Research Unit
Institute for Medical Research

Address: Kuala Lumpur, Malaysia

Responsible individual

Name: Dr. Abu Bakar bin Ibrahim, Director

Telephone: Institute for Medical Research

20. Performing organization

Name: Institute for Medical Research

Address: Kuala Lumpur, Malaysia

Principal investigator

Name: Kyser, K.A., MAJ, MC

Telephone:

Social security account number:

Associate investigators

Name:

Name:

21. General use

Microbiology, Public Health

22. Keywords: Endotoxin messengers, endotoxin antagonist, endotoxic shock, cause, endotoxic fever, cause, endotoxic fever & shock, prevention

23.(U) Technical Objective: (1) To determine the mechanism(s) of death due to fulminating sepsis and endotoxic fever and shock. (2) To find a drug or natural factor which blocks endotoxic fever and shock.

24.(U) Approach: (1) The binding of bacterial endotoxin to leucocytes is studied *in vitro*. Fever studies are carried out in rabbits after administration of bacterial endotoxin or leucocyte products released *in vitro* by bacterial endotoxin. (2) Various drugs and tissue extracts are tested *in vitro* and *in vivo* to block endotoxic fever and shock.

25.(U) Progress: The Mechanism of Bacterial Endotoxin: Second Messengers and Antagonist. Studies of endotoxic fever and shock have demonstrated a second messenger mechanism of bacterial endotoxin action: endotoxin binds to leucocyte membranes, displaces a preformed second messenger-1, and induces the formation of a delayed second messenger-2. The fever curves of the second messengers together temporally and quantitatively account for the complex fever curve of bacterial endotoxin. The amount of messengers released are proportional to the amount of endotoxin added until the preformed messenger-1 is depleted. At high endotoxin doses, delayed messenger-2 predominates. At high doses the messengers mediate endotoxin shock and death.

A system of messenger-antagonism exists and corrects for inadvertent messenger release. Administered messenger antagonist prevents endotoxic shock and death.

*Available to contractors upon originator's approval.

INVESTIGATIONS OF MELIOIDOSIS

The Mechanism of Bacterial Endotoxin: Second Messengers and Antagonist

Introduction: Death due to melioidosis is due to classical bacterial endotoxin. Not only in melioidosis, but in most forms of sepsis, endotoxemia is the ultimate cause of death. Studies on the mechanism of endotoxic fever and shock were started to gain information as to possible therapeutic tools, now almost entirely lacking.

A second messenger mechanism of bacterial endotoxin was proposed in the 1970 Annual Report from this Unit. A pyrogenic lipopolysaccharide was extracted from mammalian tissues and shown to have a different molecular structure from bacterial endotoxins.

This year's report presents the discoveries of two separate second messenger systems and an antagonistic system which mediate the fever and shock of endotoxemia.

Methods: Bacterial endotoxin was added to suspended leucocytes to study second messenger release *in vitro*. Endotoxin was prepared from *Salmonella typhimurium* by a modified Westphal extraction. Labeled endotoxin was prepared by the addition of marker sodium chromate.[1] Free chromate was removed by dialysis and the labeled endotoxin checked for pyrogenicity in rabbits.

A leucocyte suspension was made from citrated blood of the Malaysian water buffalo (*Bos bubalis*). We found this blood to have a naturally rapid erythrocyte sedimentation rate, obviating the need for dextran which would have interfered with polysaccharide analysis later. All animals were young adults and were inspected by a state veterinarian and found free of disease. Leucocyte suspensions were examined and found free of parasites and bacteria. Normal white blood cell counts were $9,000/mm^3$ to $11,000/mm^3$.

Leucocyte suspensions were equilibrated with bacterial endotoxin and centrifuged to yield a supernatant (SUP A). The sediment was resuspended and incubated at 37°C. An incubate supernatant (SUP B) was separated after centrifugation. Solutions used and the durations of equilibration and incubation were varied as were the subsequent centrifugation speeds. Antagonist was separated from the SUP A of lysed leucocytes by high speed centrifugation.

1. Collaborative study with Dr. Dharmalingam, Chief of Nuclear Medicine, Kuala Lumpur, Malaysia.

Supernatant fractions and sediment extracts were analysed for pyrogenicity in young adult rabbits and for chemical composition.

Results: Bacterial endotoxin, identified by label or characteristic fever curve, added *in vitro* to leucocyte suspensions disappeared from the plasma and bound to leucocytes. Once bound, the endotoxin was not extractable by the typical methods but could be demonstrated by the presence of label. Bound endotoxin decreased the amount of protein leakage from leucocytes during incubation. The protection was graded and proportional to the amount of endotoxin added (Figure 1).

Simultaneous to the binding of endotoxin to leucocytes there appeared in the plasma a non-labeled pyrogen (second messenger or SM-1). The equilibration was complete within an hour of standing.

By two hours of incubation at 37°C there appeared a separate pyrogen (SM-2) in the leucocytes and in the plasma which had not been present at one hour. The production of this delayed pyrogen (unlike SM-1) required leucocyte integrity and only occurred under careful conditions of incubation.

In the absence of bacterial endotoxin, neither SM-1 or SM-2 were spontaneously released. SM-1 was extracted from the leucocyte pellet only prior to treatment with bacterial endotoxin. SM-2 was extracted from the leucocyte pellet only at a specific time period between 1½ and 2 hours after the addition of bacterial endotoxin.

Pyrogenicity studies in rabbits (Figure 2) demonstrated peaks of bacterial endotoxin at 1 hour and 3-3½ hours, a single peak of SM-1 at 1 hour, and a single peak of SM-2 at 1½ hours after injection into rabbits. The summed fever curves of SM-1 and SM-2 (if a delay for SM-2 synthesis was allowed) accounted for the complex fever curve of bacterial endotoxin.

The relative sizes of the 1 hour (SM-1) and 3-3½ hour (SM-2) components of the fever response to different doses of bacterial endotoxin indicated that *in vivo* release of SM-1 but not SM-2 soon reached a fixed limit. At low doses SM-1 predominated; at higher doses, although SM-1 released was not reduced, it was overshadowed by the SM-2 response (Figure 3). The *in vivo* limit of the one hour peak approximated that amount of preformed SM-1 predicted from *in vitro* studies.

A substance antagonistic to messenger was discovered in experiments which allowed leucocyte lysis. In these experiments low speed supernatants containing SM-1 did not cause fever in rabbits unless centrifuged for 100,000 g for one hour. Messenger antagonist was demonstrated only after cellular lysis; no antagonist was released with SM-1 when endotoxin was added to intact leucocytes.

Studies in which amounts of messenger and antagonist were separately varied showed that antagonist excess depressed rabbit body temperature, that a messenger-antagonist balance evoked no temperature change, and that a messenger excess caused fever (Figure 4).

High doses of messenger caused not only fever but also shock and death. The same amount of messenger in the presence of antagonist caused only fever.

Conclusions: A second messenger model for the mechanism of bacterial endotoxin may be outlined now in some detail (Figure 5). Endotoxin binds to leucocytes. As a lipopolysaccharide endotoxin is likely to bind to or within the cellular membranes, and direct evidence for such a site is membrane stabilization. A membrane site is ideal for displacement reactions between cellular and non-cellular substances. Evidence for SM-1 release by displacement includes: 1) SM-1 is preformed, 2) it is released immediately upon the addition of endotoxin, and 3) cellular structure and conditions are not critical for release. A simple binding site displacement mechanism is also consistent with extraction data that both SM-1 and endotoxin are lipopolysaccharides, and that endotoxin binds more firmly than SM-1. SM-2 formation is specifically induced by bacterial endotoxin. The one to two hour delay and the critical requirements of cellular structure and function may indicate a genetic induction of SM-2 biosynthesis by endotoxin.

The evidence that the endogenous pyrogens are indeed second messengers or mediators of endotoxin is simply that they are specifically released by endotoxin, and they do what endotoxin is supposed to do. They cause both fever and death, depending upon dose, and account for the fine details of the temporal and quantitative response of the body to endotoxin.

The discovery of a messenger antagonist is not only a discovery of another of the body's many control systems, but also is another confirmation of the messenger hypothesis. The presence of a preformed messenger implies a built-in control against its inadvertent release such as occurs with leucocyte lysis.

The stoichiometry of antagonism and the ability of antagonist to prevent endotoxic death indicate its possible therapeutic importance.

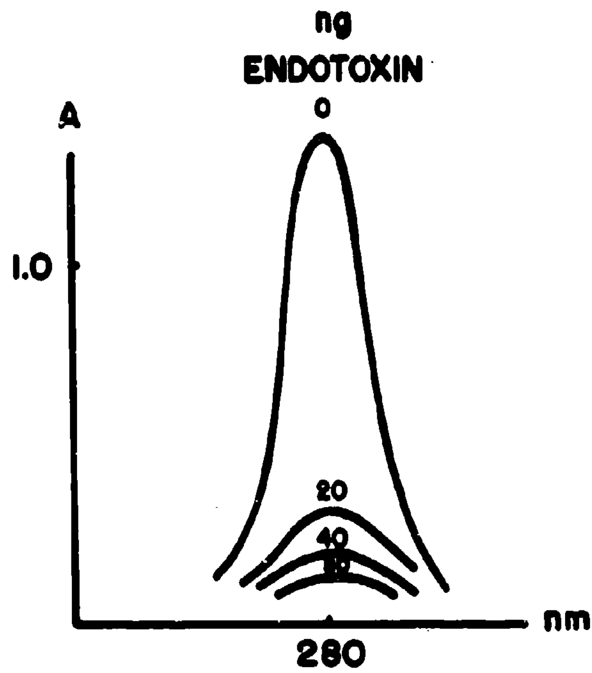


Fig. I Showing effect of endotoxin on cell protein leakage

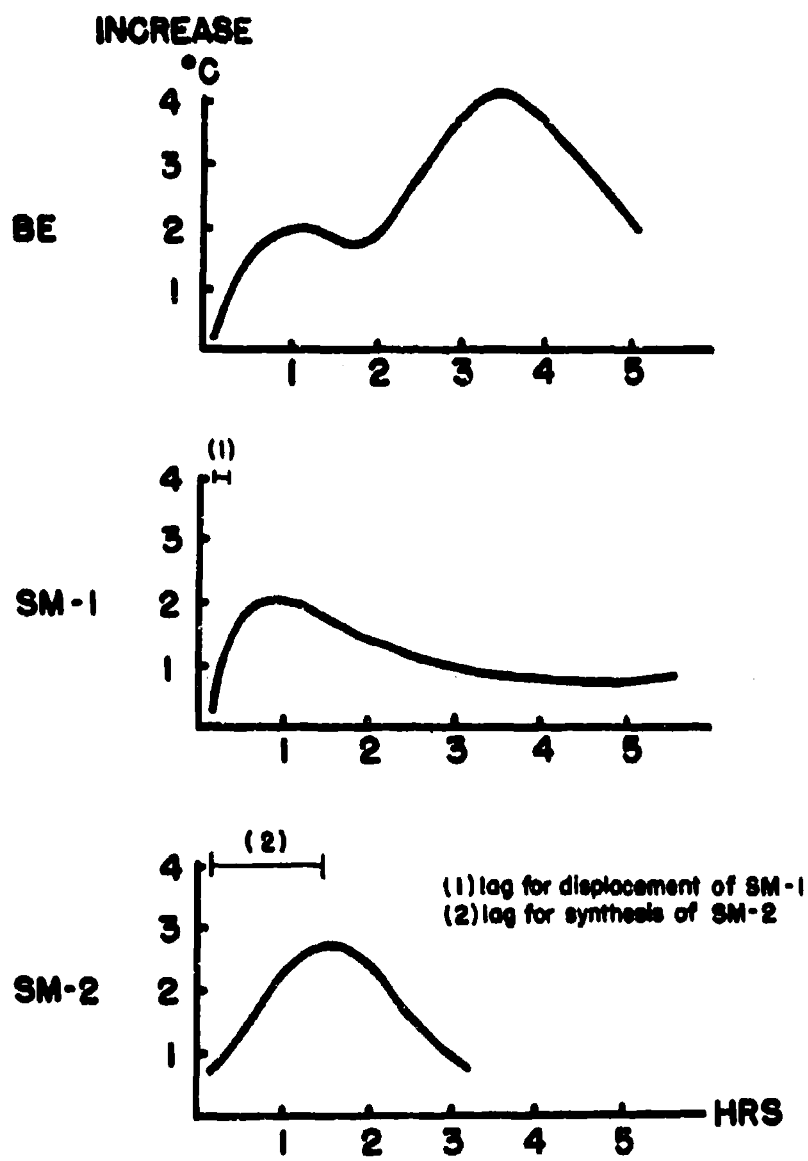


Fig. 2 Showing fever curves in rabbits of bacterial endotoxin and messengers

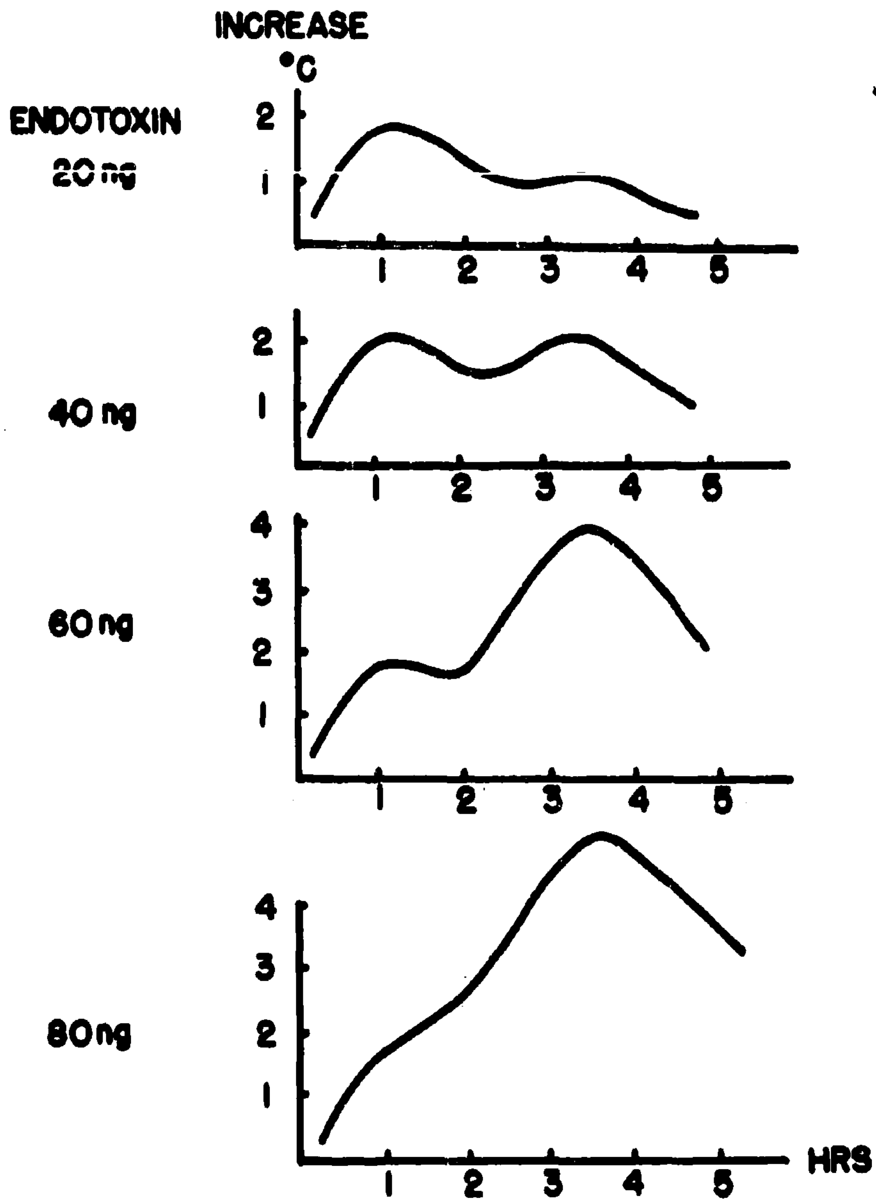


Fig. 3 Showing fever curves from different amounts of bacterial endotoxin

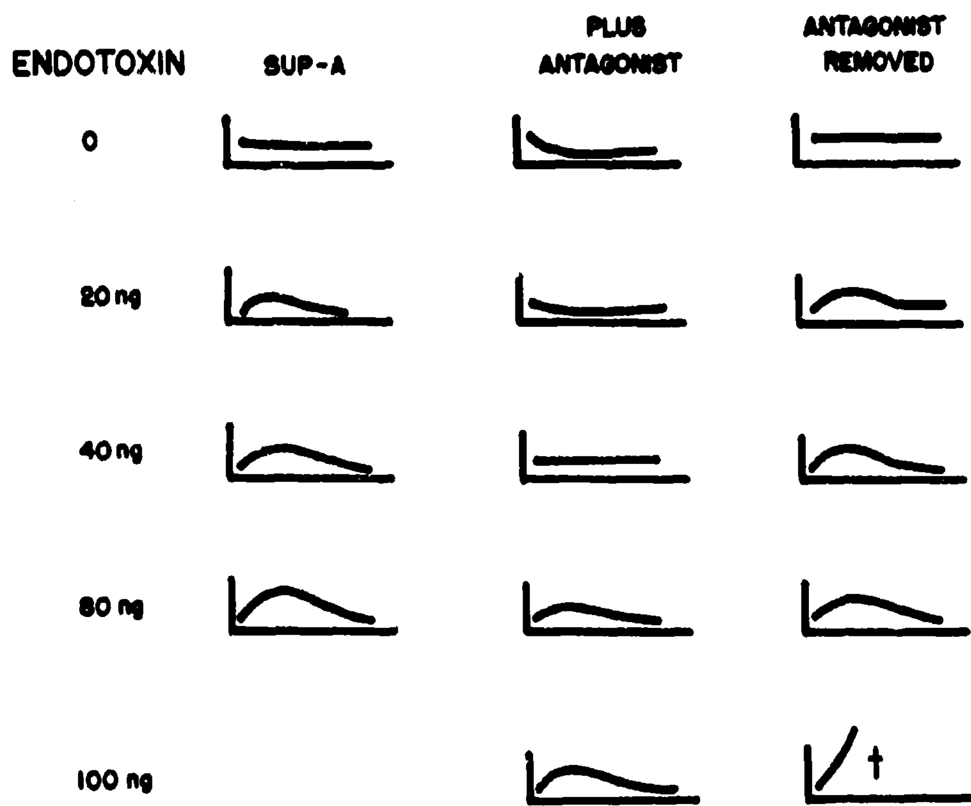


Fig. 4 Showing the stoichiometry of messenger ANTAGONIST

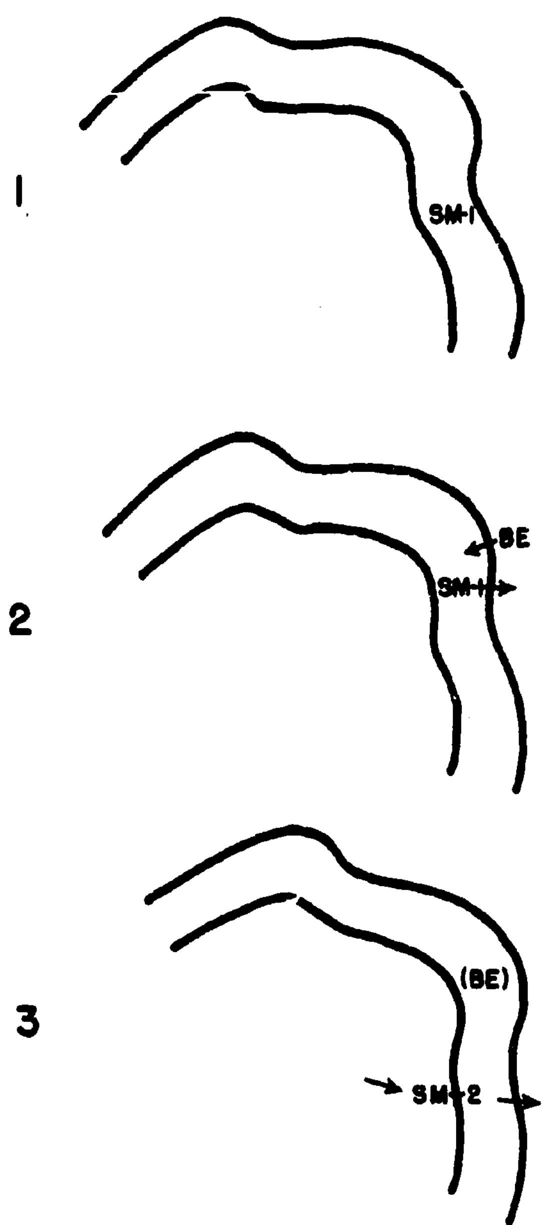


Fig. 5 Showing the second messenger (SM) mechanism of bacterial endotoxin (BE) action

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	30 06 71	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
30 06 70		U		N/A	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY		81100 and 831000		
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)
Investigations of Scrub Typhus

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
10 70	9 71		

17. CONTRACT/GRANT
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b. NUMBER: DADA17-71-G-9332 Mod. P-103
c. TYPE: Y Grant — d. AMOUNT: 181
e. KIND OF AWARD: — f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1.0	43.4
	CURRENT 72	1.0	45.8

19. RESPONSIBLE DOD ORGANIZATION
NAME: US Army Medical Research Unit
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RESPONSIBLE INDIVIDUAL
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ADDRESS: Kuala Lumpur, Malaysia

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

ASSOCIATE INVESTIGATORS
NAME: Roberts, L.W., CPT, MSC
NAME: Gan, E., B.A.

21. GENERAL USE

22. KEYWORDS (Precede EACH with Security Classification Code)
Rickettsia, scrub typhus, *Leptotrombidium deliense*, *R. 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 Objective: To determine the antigenic stability of *R. tsutsugamushi* in vector mites, small mammals and primates and the dynamics of scrub typhus in vector mites, small mammals and primates, and to continue studies of vector and small mammal bionomics and ecology.

24.(U) Approach: In order to develop means for infecting vector mites with laboratory characterized strains of rickettsia, the following approaches will be made: an artificial feeding technique for trombiculid larvae will be developed; cannibalism as a means of mite to mite transmission will be studied under laboratory conditions; microdissection and cryostat methods along with FA techniques will be used to study rickettsia in various organs of mites at each stage of development; characteristics of *R. tsutsugamushi* infection in white mice will be determined; attempts will be made to infect vectors by allowing them to feed at the optimum time on sensitized, infected mice; characteristics of the infection in small mammals and primates as well as the antigenic stability of the organisms will be determined; the effect of habitat and ecosystem on the prevalence of scrub typhus in humans and small mammals will be determined.

25.(U) Progress: In preliminary tests, 8 of 75 larvae successfully completed development after being fed on a membrane substrate that was tied over a feeding tube containing rabbit serum. With minor modifications in diet and procedure, this technique can be used in attempts to infect mites with characterized strains of *R. tsutsugamushi*.

*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

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

One of 5 negative *L. akamushi* nymphs allowed to cannibalize positive prenymphs produced positive offspring (13 of 15 offspring were positive). With one exception, offspring from the other 4 mites were negative. Microdissection and cryostat methods were developed and are being used to follow rickettsial development mite organs. Photomicrographs have been made of *R. tsutsugamushi* in mite tissue preparations stained with fluorescent conjugates.

Peak rickettsemia in the skin of white mice was found to occur at approximately 48 hours after IP infection with the Karp strain of *R. tsutsugamushi* at $10^{5.7}$ $MIPLD_{50}$. Previously negative *L. deliense* larvae that fed on infected sensitized mice have been proven positive for *R. tsutsugamushi*. Studies of offspring from the infected mites are being conducted.

Fluorescent antibody analysis using strain specific conjugates revealed that the positive colony, *L. (L.) akamushi*, is infected with Karp and Kato "like" strains of *R. tsutsugamushi*, that one mite can carry more than one strain of *R. tsutsugamushi* and that these strains have remained antigenically stable through 7 generations of vector mites.

It has been shown that the silvered leaf-monkey is susceptible to *R. tsutsugamushi* and that the response is similar to that of humans to scrub typhus. These studies are being expanded.

Because of difficulties in obtaining adequate serum specimens from remote jungle areas and from small mammals, filter paper specimens were investigated to determine if they could be used in the indirect fluorescent antibody test for scrub typhus. It was determined that not only can they be used to screen human and small mammal specimens in prevalence studies but that accurate titers can be obtained as well.

It was determined that among *Orang Asli* (Aborigines) living in deep jungle, that adults had high (73%) ratios of positives to scrub typhus, while fringe area peoples had lower ratios (48%) and village dwelling peoples had the lowest ratios (8%).

It was determined that vertical zonation does affect the prevalence of scrub typhus in small mammals as true arboreal mammals are essentially negative by isolation and serology while semiarboreal and ground dwelling mammals have kidney isolations of 23% and a prevalence ratio of 52% as determined by the indirect fluorescent antibody test. Kidney isolations were higher (23%) than blood isolations (15%) on a sample size of 1164.

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

It was determined that infecting dose did affect the ratios of chronic infection in laboratory white rats and that positive isolations were obtained from certain animals at 10, 12, 14 and 16 months post inoculation, and that chronic infection did not seem to maintain the IFAT titer at a high level.

INVESTIGATIONS OF SCRUB TYPHUS

The investigations of scrub typhus represent a combined effort by the Divisions of Entomology and Rickettsiology. Objectives for FY 71 are listed below.

Studies of Scrub Typhus Vectors

1. Artificial Feeding: To develop an artificial feeding technique for trombiculid larvae.

2. Cannibalism Study: To investigate cannibalism as a means of rickettsial transmission to previously uninfected mites.

3. Microdissection of Vectors: To develop techniques for dissecting out organs from all stages of vector mites.

4. Vector Colonization: To ctoninue investigations of vector bionomics with respect to rickettsial transmission in the colonies of *Leptotrombidium (L.) akamushi, L. (L.) deliense* and *L. (L.) arenicola.*

5. Miscellaneous Vector Surveys To continue vector distribution studies in West Malaysia.

Studies of Scrub Typhus Vectors

Artificial Feeding:

General: To study the genetic stability of *R. tsutsugamushi* in mites it is necessary to infect mites with laboratory characterized strains. Previous attempts to infect larval mites by allowing them to feed on an infected host have been largely unsuccessful (Annual Reports 1968, 1969). Thus, a technique of artificial feeding was devised, using a membrane substrate.

Methods: Unengorged chiggers are placed on shaved areas on the backs of laboratory mice as in the capsule feeding method (Baker & Hubert, 1968). Each mouse is anesthetized with Nembutal so that the chiggers can more readily become attached. The mouse is then killed and 1 square inch of skin with the chiggers attached is removed. The skin is stretched and tied over the top of a cylinder made from a 2½ cc plastic disposable syringe with the tip cut off. Rabbit serum is pipeted into the other end of the syringe. Pressure is applied with the syringe plunger until the fluid causes the membrane to bulge outward slightly. The feeding tube is then placed in the original plastic case for the syringe, and cotton is placed

around the tube to keep it from moving. The case is then capped and placed in a water bath set at the approximate body temperature of laboratory mice (39°C).

Results: Initially, 100 *L. (L.) akamushi* larvae were placed on mice, 94 attached and 75 partially engorged larvae were recovered. Of the larvae recovered, 8 developed to adults. The technique is being repeated with modifications designed to improve the survival rate. However, it was shown that *Leptotrombidium spp.* can complete their life cycle with artificial feeding, and this technique is feasible for infecting mites with characterized rickettsial strains.

Cannibalism Study:

General: It was observed that nymphal mites fed upon prenymphs of the same species when both stages were present in a laboratory rearing container. The possibility of cannibalism as a means of mite to mite transmission of *R. tsutsugamushi* was considered.

Methods: A preliminary study was initiated in which 7 uninfected nymphal *L. (L.) akamushi* were allowed to feed individually on prenymphs that were taken from a pool of infectious mites. The seven mites were allowed to complete development. Four of the seven were females, and these were mated and allowed to oviposit. Their offspring were fed individually on mice and checked for rickettsia.

Results: One of the 4 females produced 15 larvae, 13 of which were positive for rickettsia. With one exception, the offspring from the other females were all negative. The experiment is being repeated with larger numbers of nymphs, and if positive offspring are obtained, cannibalism as a means of transmission under field conditions will be investigated.

Dissection: Dissection and cryostat sectioning techniques have been devised for study of the internal organs of vector mites. Individual organs and sections through organs can be examined for rickettsia using fluorescent antibody (FA) technique. In addition, the internal anatomy of these vectors can be studied in different stages of development.

Dissection Method: The dorsum of the mite is fixed to a parafin coated dissecting dish. A minuten insect pin imbedded in a wooden applicator is used to tease the integument from the opisthosomal region of the mite. Ovaries or testes, accessory glands, gut and other organs are removed en masse with the teasing needle and placed on a slide with a drop of phosphated buffer solution. The individual organs can then be separated, and smears can be made for fluorescent antibody examination. The entire dissection can be completed in 10 - 15 minutes.

Cryostat Method: The mite tissue is placed in 1 ml. of 10% gelatin solution and frozen in an alcohol - dry ice mixture. The preparation is transfered to the freezing cabinet (-15°C) of the cryostat apparatus, and sections 1-2 microns thick are cut. The sections are then fixed on a microscope slide with acetone and treated with fluorescein isothiocyanate - labeled immunoglobulins so that they can be examined by fluorescent microscopy for rickettsia.

Results: Using the above methods, *R. tsutsugamushi* organisms have been located and identified from the gut and salivary glands of larvae and from the ovaries, gut, salivary glands and epidermal tissues of adults.

Vector Colonization:

General: Colonies of *L. (L.) akamushi*, *L. (L.) deliense* and *L. (L.) arenicola* are maintained for genetic stability studies of rickettsia, transovarial passage and other studies. They are also used for teaching and demonstration purposes; USAMRU Entomologists will again be assisting in the teaching of 2 medical entomology courses at the Institute for Medical Research, Kuala Lumpur.

L. (L.) akamushi: The *L. (L.) akamushi* colonies were initiated in 1964. The *R. tsutsugamushi*-infected (positive) colony has remained infectious into the 10th generation. Following an extensive report by Rapmund, et al. (1969) on transovarial transmission of rickettsia, the rearing procedure was changed from individual maintenance to pooled rearing of approximately 20 mites per container. For additional transovarial and other studies, seven of the pools were again separated in the F_8 generation, and subsequent offspring are being maintained individually. Of 70 F_9 offspring checked, all but one female were positive. No male offspring have ever been produced by positive females from this colony (Annual Report 1968). Isolated unmated females infectious for *R. tsutsugamushi* have not reproduced parthenogenetically. In the few instances where negative females were produced in the positive colony, negative male offspring have been obtained from these females. Normally, males are obtained from the noninfectious colony. The negative colony is also used for vector infection studies, bionomic and other investigations.

L. (L.) arenicola: In 1968, infectious *L. (L.) arenicola* were collected for the first time in West Malaysia, and transovarial transmission in this vector was demonstrated. However, there were no positive offspring produced after the F_2 generation, which included a positive male, no offspring were infectious. For additional study, 200 larvae were collected from the same locations where positive mites were previously obtained. These larvae were fed in pools of 10 mites. One of the 20 pools proved

to be infectious. Four adult females from the positive pool produced offspring. The offspring were fed individually on mice, and mouse passage results are incomplete. Of 18 F_1 offspring checked, however, no positive individuals have been found. If necessary, additional attempts to establish an infectious *L. (L.) arenicola* colony will be made.

Other Colonies: Colonies of *L. (L.) bodense* and *Blankaartia acoscutellaris* are also being maintained for study and demonstration. As potential vectors are collected they will be colonized for transmission studies.

Miscellaneous Vector Surveys:

Distribution Studies of L. (L.) deliense and L. (L.) akamushi: Hubert and Baker (1963) stated that *L. (L.) deliense* and *L. (L.) akamushi* in West Malaysia have distinct, separate habitats, with *L. (L.) deliense* being restricted to forests and *L. (L.) akamushi* being confined to lalang grass. Observations by Traub and Wiseman (1968) showed that *L. (L.) deliense* could be found in new lalang fields arising in recently cleared areas, but they stated that *L. (L.) akamushi* replaces *L. (L.) deliense* after a few years. In recent studies by this Unit in Sabah, East Malaysia, neither of the above findings occurred (Annual Report 1970). Large, 10-15 year old, open lalang fields had mixtures of the 2 species, and in fields near forests, *L. (L.) deliense* was the predominant species.

As a result of these findings, a large lalang field in Pahang, West Malaysia was chosen for study (Figure 1). The field is located in the jungle on a former military post, Pos Shean, and it is at least 25 years old. Rodent and black plate collections of chiggers were made in this field in February, April and June, 1971. As shown in Table 1, *L. (L.) deliense* was the predominant species with 1236 being collected, and *L. (L.) akamushi* was not found in any of the collections. It appears that *L. (L.) deliense* has a broader ecological niche than *L. (L.) akamushi* in Malaysia and is not restricted to the forest areas only. The absence of *L. (L.) akamushi* was probably because of the relative isolation of the post.

Swampy Forest Collections: With the assistance of the Department of Medical Ecology, chiggers were collected from rodents near Mt. Kemandul, Klang, Selangor. The collection site was a swampy forest habitat that surrounds a forest of rubber trees (Figure 2). There is at least some standing water in this area during most of the year, and preliminary blackplating showed that *L. (L.) deliense* chiggers were present on the small areas of ground that remained dry. Rodents were trapped daily from 10 March to 8 April. As indicated in Table 2, *L. (L.) deliense* was found on 11 different hosts but was much less abundant than several other species. The largest total number of *L. (L.) deliense* were

Table 1

Number of Chiggers Collected on Three Ocassions from Rodents and Black Plates at Pos Shean, Pahang.

Chigger Species	Location/No. examined	Black Plates/12	*Hylomys suillus*/4	*Rattus tiomanicus*/58	Total
Ascoschoengastia (Lau) indica		-	-	2	2
Eutrombicula wichmanni		-	-	33	33
Gahrliepia (W.) disp. disparunguis		-	-	1	1
Leptotrombidium (L.) bodense		-	-	8	8
Leptotrombidium (L.) deliense		86	2	1148	1236
Walchiella oudemansi		-	-	8	8

Table 2

Chiggers from Rodents Collected from 10 March to 8 April in Swampy Forest Habitats near Bukit Mandol, Klang, Selangor

Chigger Species / Host and No. Collected	*Callosciurus notatus* (129)	*C. nigrovittatus* (9)	*C. prevostii* (11)	*Hylopetes spadiceus* (4)	*Iomys horsfieldi* (14)	*Petaurillus kinlochii* (1)	*Petinomys setosus* (4)	*Rattus bowersi* (1)	*R. canus* (1)	*R. muelleri* (2)	*R. rajah* (9)	*R. sabanus* (174)	*R. whiteheadi* (77)	*Rhinosciurus laticaudatus* (7)	*Total*
Ascoschoengastia (Lau) audyi	2413	453	225	120	293	-	25	-	-	-	-	69	12	4	3614
A. (Lau) indica	-	-	-	-	-	-	6	-	-	-	-	-	-	-	6
A. (Lau) lorius	185	3	41	3	16	-	4	-	-	-	-	6	-	-	258
Gahrliepia (G.) ciliata	-	1	-	-	-	-	-	-	-	-	-	2	-	-	3
G. (G.) decora	-	-	-	-	-	-	-	-	-	-	-	3	1	1	5
G. (G.) fletcheri	20	-	-	1	-	-	-	-	-	3	-	1402	-	-	1426
G. (G.) insigne	-	-	-	-	-	-	-	-	-	-	9	11	-	-	20
G. (G.) rutila	-	-	-	-	-	-	-	-	-	-	-	7	-	-	7
G. (W.) disp. disparunguis	4	-	-	-	-	-	-	-	-	-	-	1	351	-	356
G. (W.) lewthwaitei	2	-	-	-	-	-	-	-	1	-	-	6	976	-	985
G. (W.) rustica	-	-	-	-	-	-	-	-	-	-	-	16	-	-	16
G. (W.) turmalis	1	-	-	-	-	-	-	-	-	-	67	876	27	2	973
Leptotrombidium (L.) bodense	3	-	-	-	1	-	-	-	-	-	-	2	-	-	6
L. (L.) deliense	41	2	1	-	5	2	-	45	1	3	-	273	7	86	466
Microtrombicula spicea	15	-	-	2	2	-	-	-	-	-	-	-	-	-	19
Trombiculindus hastata	-	-	-	-	-	-	-	-	-	-	-	14	1	-	15
Walchiella impar	-	-	2	2	-	-	-	-	-	5	-	3	-	-	12
W. oudemansi	46	-	2	-	1	-	-	-	-	20	3	27	2	19	120

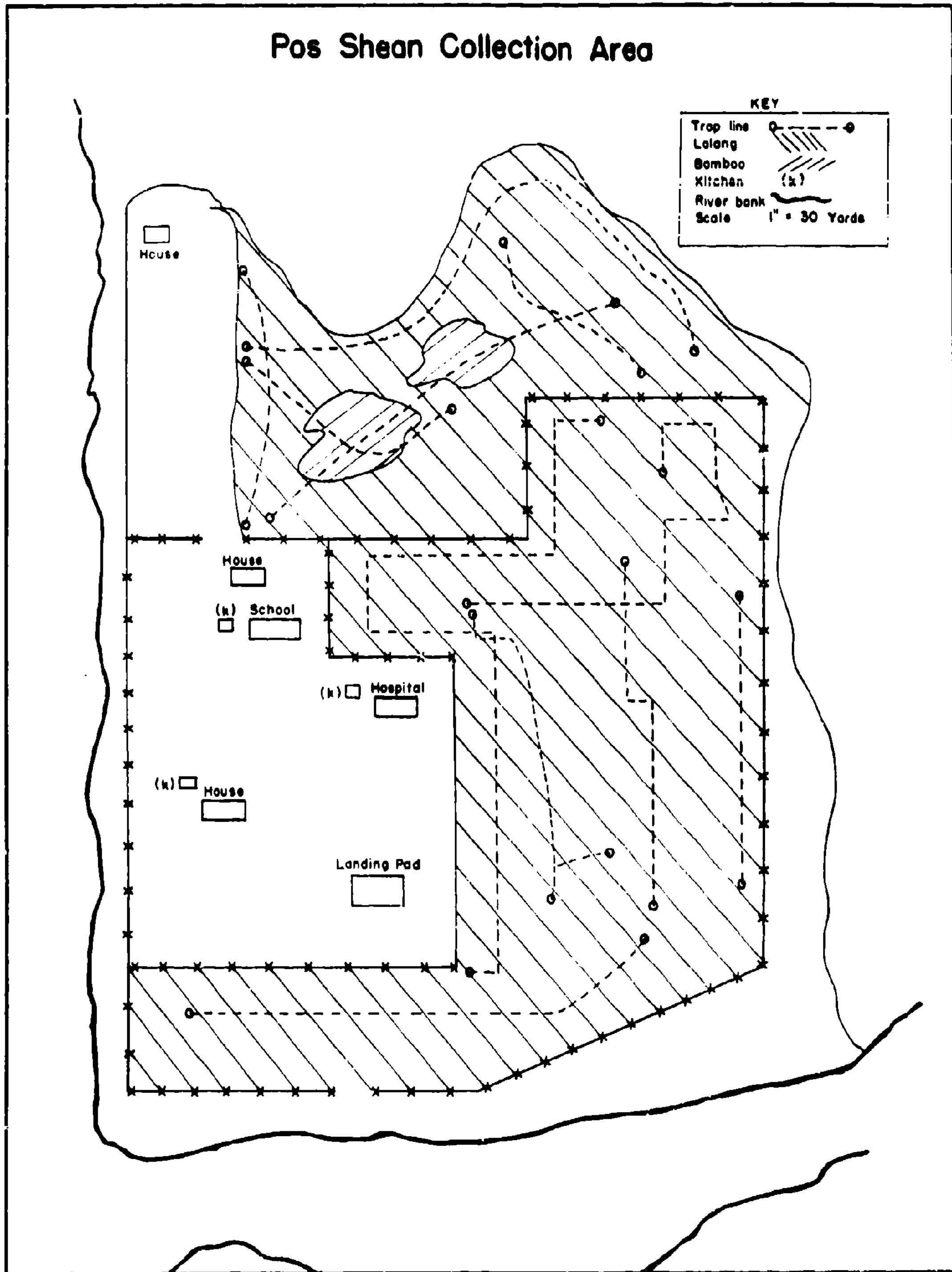

Fig. 1

Bukit Kemandul Collection Area

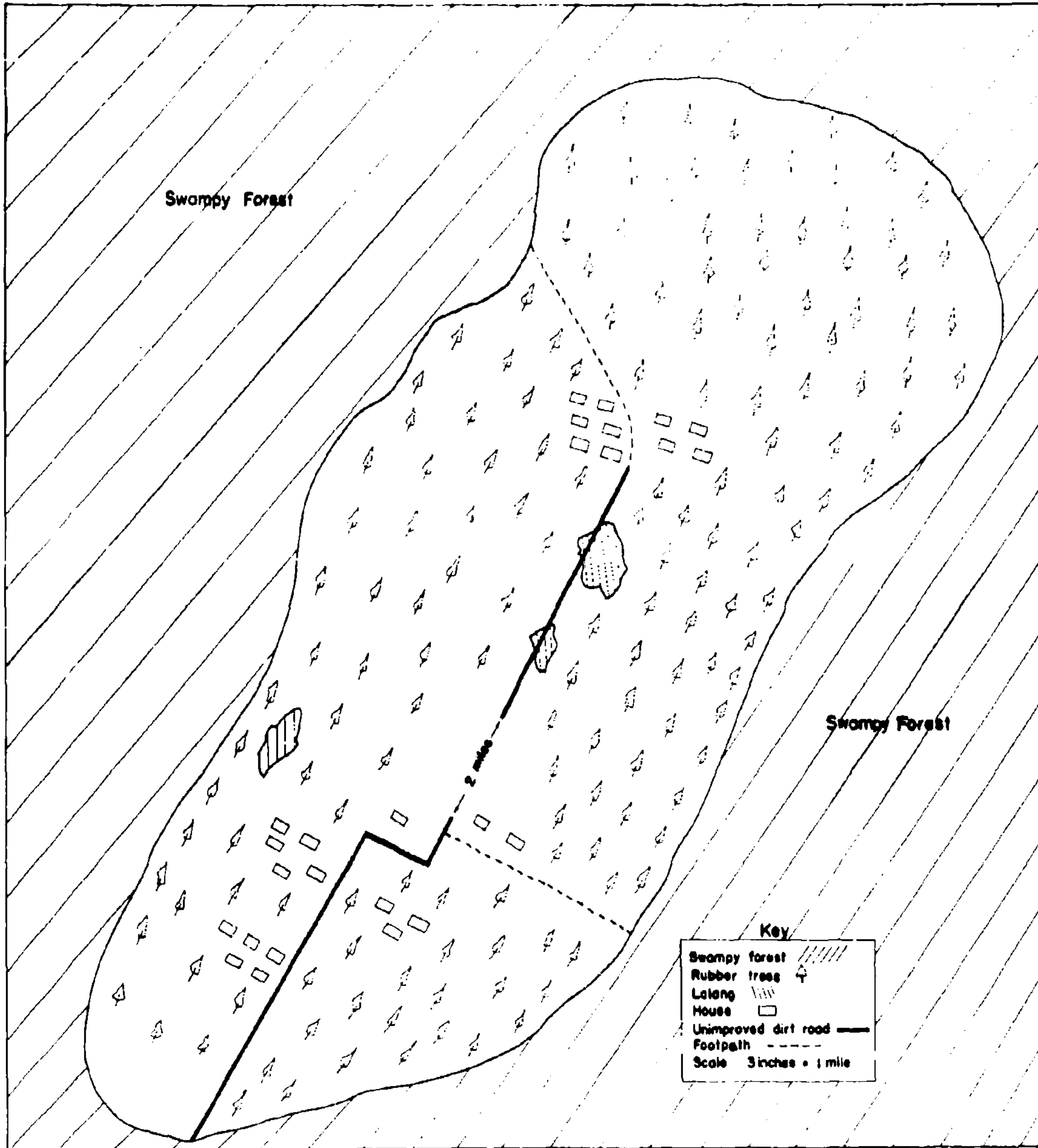

Fig.2

collected from *Rattus sabanus*, which was the most common host collected. The single *R. bowersi* hosted 45 *L. (L.) deliense*, but this rat is not common in the lowland forest areas. Compared to other forest habitats surveyed, the *L. (L.) deliense* population was low at this time, but during drier periods the population in the swampy areas may be higher.

Mt. Brinchang Collections: Leptotrombidium (L.) scutellare is a vector of scrub typhus in Japan and elsewhere, but has not been found to be infectious in West Malaysia. Mt. Brinchang in the Cameron Highlands, Pahang was surveyed for *L. (L.) scutellare* in June 1970, and although large numbers (3,500) were collected, none were infectious for rickettsia (Annual Report 1970). Additional collections were made in October 1970 in an attempt to obtain infectious mites. As shown in Table 3, only 4 *L. (L.) scutellare* were collected. Heavy rains just prior to the October collections probably caused the low number collected. Since a positive case of scrub typhus was reported from this area in 1966, there is still reason to suspect that this mite is a vector. Attempts will be made to reestablish a laboratory colony of *L. (L.) scutellare* for transmission studies.

Studies of *Rickettsia tsutsugamushi*

1. Infection of Leptotrombidium larvae with known characterized strains of *R. tsutsugamushi*.

2. Antigenic stability analysis: To make an antigenic analysis of the strain(s) of *R. tsutsugamushi* present in the positive *L. (L.) akamushi* colony through 7 generations.

3. Silvered Leaf-Monkeys: To determine the susceptibility and response of the Silvered Leaf-monkey to *R. tsutsugamushi*.

4. Filter Paper Technique: To develop and test a technique of collecting blood samples on filter paper for use in the Indirect Fluorescent Antibody test for scrub typhus antibodies.

5. *Orang Asli*: To determine if the *Orang Asli* (Aborigines) that live in different areas such as deep jungle, fringe and rural *kampongs* have the same or different prevalence ratios as determined by serology for scrub typhus.

6. Rodents: To determine if prevalence ratios, as shown by isolation and serology, varied according to vertical zonation and what the relationships were between blood and organ isolations and serology.

7. Chronic Infection in Rodents: To determine if route of inoculation and challenge dose affect the development of chronic infection in laboratory white rats and how long chronic infection persists and in what organs.

Table 3

Chiggers on Rodents and Black Plates Collected from 21 - 23 October at Mt. Brinchang (5,500 ft.), Cameron Highlands, Pahang

Chigger Species / Location and No. Surveyed	*Gahrliepia (G.) cetrata*	*G. (G.) disp. disparunguis*	*G. (G.) fletcheri*	*Leptotrombidium (L.) brinchangense*	*L. (L.) deliense*	*L. (L.) keukenschrijveri*	*L. (L.) scutellare*	Total
Black plates (27)	-	-	-	13	-	68	4	85
Rattus bowersi (1)	-	-	17	-	9	-	-	26
R. cremoriventer (1)	-	-	-	-	11	-	-	11
R. fulvescens (4)	-	12	-	1	-	-	-	13
R. muelleri (1)	-	-	-	-	5	17	-	22
R. sabanus (1)	1	-	2	-	3	15	-	21
Vivera tangalunga (1)	-	-	-	-	-	28	-	28
Total	1	12	19	14	28	128	4	

[illegible]

Infection of *Leptotrombidium* Larvae with Known Characterized Strains of *R. tsutsugamushi:*

General: It is generally accepted that the *Leptotrombidium* mites serve as reservoirs in nature for scrub typhus. If so, this is one of the three possible places in the disease cycle where genetic changes in the pathogen can take place (the other two being in small mammals and in man himself). Previous attempts to infect *Leptotrombidium* larvae in the laboratory with *R. tsutsugamushi* have been unsuccessful except for recent work at the University of Maryland, and some of the techniques used in that study left questions yet unanswered.

Study of Rickettsia in Mice: Burgdorfer, *et al* showed that transmission of rickettsial infections to ticks in the laboratory is directly related to the time of rickettsemia in the test rodent and to the level of rickettsemia.

Preliminary studies were necessary to determine the relationships in mice between the route of infection, infecting dose and the time of occurrence, level and duration of rickettsemia, and the rickettsia level in the skin since chiggers feed on tissue fluid in the skin rather than on blood. NCI mice were infected by two routes (IP and IV), and two mice were sampled every 12 to 24 hours by serial sacrifice. Skin was removed from the abdominal region and the subcutaneous tissue; blood vessels removed under a dissecting microscope. The remaining outer layers of the skin were then weighed and Snider's diluent at 4°C was added at a rate of 1:10 (wt./vol.). The preparation was then minced and ground in a tissue grinder. From this, further 1:10 dilutions were made, and 0.2 cc were inoculated IP into 5 mice at each dilution. The same procedure was followed for whole blood, except that a straight 1:10 dilution (vol/vol) was made. As shown in Tables 4-6, both challenge dose and route of challenge affected the level, time of occurrence, time of peak rickettsemia, occurrence and level of rickettsia in the skin as well as time to death. Because of these data, it was decided to challenge mice with 10^{6-7} $MIPLD_{50}$ using the IP route and to place the chiggers on the mice at 48 hours post-challenge.

Infection Methods: Host hypersensitivity to the saliva of *Leptotrombidium spp.* may be important in the normal infection of chiggers. If an animal is sensitive to the salivary secretions of the chigger, cells of the host may move into the feeding site, carrying rickettsia with them. To test this theory, 4 mice were subjected to ear feedings every 2 weeks by 20 *L. akamushi* larvae from the negative colony. Five other mice were subjected to capsule method feedings on the back by 50 *Blankaartia sp.* chiggers. The feedings were made at 2 week intervals over 4½ months prior to inoculating the mice with rickettsia. In the infection attempts, both the *L. akamushi*- and

Table 4

The Effect of Route of Challenge and Challenge Dose of the Karp Strain of *R. tsutsugamushi* on the Rickettsemia in Laboratory Mice.

Route IP

Sampling Time Post-challenge	Challenge Dose, $MIPLD_{50}$ $10^{8.7}$ Blood	Skin	Blood	Skin	$10^{6.7}$ Blood	Skin	Blood	Skin	$10^{4.7}$ Blood	Skin	Blood	Skin
12 hours	2.0*	±**	2.3	+	Neg	-	Neg	±	Neg	±	Neg	-
1 day	1.5	+	2.5	+	Neg	±	Neg		Neg	-	Neg	±
2 days	2.0	+	1.6	+	Neg	±	1.5	+	Neg	+	Neg	+
3 days	3.0	+	3.7	+	2.2	+	1.7	+	Neg	+	Neg	+
4 days	3.8	+	3.4	+	2.2	+	2.5	+	1.4	±	1.8	±
5 days	MD***		MD		2.4	+	1.8	+	2.0	+	1.2	+
6 days	-		-		2.5	+	MD		2.0	+	1.7	±
7 days	-		-		4.3	+	MD		2.5	+	2.3	+
8 days	-		-		-		-		2.0	±	2.4	±
9 days	-		-		-		-		2.6	±	MD	

Route IV

Sampling Time Post-challenge	$10^{8.7}$ Blood	Skin	Blood	Skin	$10^{6.7}$ Blood	Skin	Blood	Skin	$10^{4.7}$ Blood	Skin	Blood	Skin
12 hours	3.6	±	3.0	+	2.5	+	3.0	-	Neg	-	Neg	-
1 day	3.2	+	3.0	±	2.0	±	2.0	-	Neg	-	1.2	+
2 days	3.0	+	2.7	+	3.0	+	2.0	±	Neg	±	Neg	±
3 days	5.4	+	3.9	+	3.0	+	3.8	+	Neg	-	3.0	+
4 days	3.8	±	3.2	+	2.2	±	1.7	+	Neg	-	1.5	-
5 days	MD		MD		2.5	±	4.2	+	1.6	±	1.4	±
6 days	3.3	+	3.0	+	1.7	±	4.5	+	2.5	+	1.5	+
7 days	3.8	+	MD		MD		MD		1.2	±	3.5	+
8 days	3.0	±	MD		3.0	+	MD		2.0	±	2.0	±
9 days	MD		MD		3.3	+	MD		2.4	+	1.7	±

2.0*: $10^{2.0}$ $MIPLD_{50}$/0.2 ml of whole blood.

±**: Results of a 1:10 dilution of minced and ground skin injected into 2 mice; IP; + both mice died, ± one of two died, - both survived.

MD***: Mice dead at sampling time.

Table 5

Effect of Challenge Dose of the Karp Strain of *R. tsutsugamushi* and Route of Challenge on the Time to Death of 10 Mice at each Challenge Dose.

Route of Challenge	Challenge Dose,* $MIPLD_{50}$	Day of Death													
		0	1	2	3	4	5	6	7	8	9	10	11	12	Number Surviving
IP	$10^{8.7}$	0	0	0	0	0	10**								0
	$10^{6.7}$	0	0	0	0	0	0	4	6						0
	$10^{4.7}$	0	0	0	0	0	0	0	0	3	5	1	0	1	0
IV	$10^{8.7}$	0	0	0	0	1	5	1	1	1	1				0
	$10^{6.7}$	0	0	0	0	0	0	1	0	1	0	2	0	0	7
	$10^{4.7}$	0	0	0	0	0	0	0	0	1	0	0	0	0	9

Dose*: Same dilutions of the same inoculum used on the same day as for the mouse challenge in Table 4.

10**: Number of mice that died on that day.

Table 6

The Effect of Route of Challenge on the Level of Rickettsemia and Level of Rickettsia per 100 mg of Skin at a Challenge Dose of $10^{6.9}$ $MIPLD_{50}$'s of the Karp Strain of *R. tsutsugamushi*.

Sampling Time Post-challenge	Route of Challenge			
	IP		IV	
	Blood	Skin	Blood	Skin
12 hours	1.7*	3.6**	3.4	3.4
1 day	2.5	3.2	3.0	2.4
1½ days	2.4	3.3	4.6	4.1
2 days	3.2	3.5	3.5	2.5
2½ days	3.0	4.2	2.7	1.8
3 days	3.0	4.0	5.0	4.2
3½ days	3.3	4.0	3.7	3.0
4 days	3.8	3.9	3.2	3.3
4½ days	3.1	3.6	3.3	2.8

1.7*: $10^{1.7}$ $MIPLD_{50}$/0.2 ml of whole blood, mean titer of 2 animals.

3.6**: $10^{3.6}$ $MIPLD_{50}$/100 mg of skin from which the subcutaneous tissue and blood had been removed under a dissecting microscope, mean titer of 2 animals.

[illegible]-sensitized mice had 20 larvae placed in each ear and 20 placed on the back, using the capsule method. The larvae were obtained from the negative laboratory colonies of *L. akamushi* and *L. deliense*. Each vector species was fed on 2 mice from each of the sensitized groups (i.e. 2 *L. akamushi*-sensitized mice and 2 *Blankaartia*-sensitized mice). The mice were held in wire cages over pans of water, and a divider was placed in each pan so that ear-fed and capsule-fed larvae were kept apart after they had engorged and dropped from the mouse. The larvae were then collected from the water and kept in separate pools by mouse and by site of feeding. The challenge dose was $10^{6.7}$ $MIPLD_{50}$ of the Karp strain of *R. tsutsugamushi*. Periodically, 2 *L. akamushi* and 2 *L. deliense* from each group were ground separately in Snider's diluent and injected IP into 2 separate mice to determine if the mites had become infectious. In this way, larvae (24 hours after engorgement), nymphs, teliophanes and adults from each group were injected into mice. The remaining mites are being maintained in laboratory rearing containers and their offspring will be checked for transovarial transmission. All first passage mice that survived were back-challenged with the Karp strain at a dose of 10^4 $MIPLD_{50}$. Liver and spleen preparations of those mice which were dying were passed into second passage mice on chloramphenicol. The second passage mice were then back-challenged at 28 days to determine if *R. tsutsugamushi* was the cause of death in the first passage mice.

Table 7 shows the results to date. At present, prenymphs from 3 different groups have been proven positive for *R. tsutsugamushi*. Two different mice were involved and all of the infected prenymphs were *L. (L.) deliense*.

If any of the adults being maintained produce positive offspring, genetic stability studies of the rickettsial strain will be made through at least 3 generations. This experiment has been repeated using 2 challenge strains, Karp and Gilliam. The specimens for the post larval (24 hours), prenymphs (7 days) and nymphs (13 days) have been injected into mice; however no results are available as of this date.

Antigenic Stability Analysis:

General: Although it is highly desirable to infect the various vectors of scrub typhus with antigenically stable laboratory strains of *R. tsutsugamushi*, and every effort is being made to do so, it was decided to take advantage of available material from the positive *L. (L.) akamushi* colony while the techniques for the above were being worked out. A brief history and current status of the positive *L. (L.) akamushi* colony is given in the scrub typhus vectors portion of this report. Frozen material (-70°C, 2nd mouse passage) was available for all the generations including the original parent.

Table 7

An Attempt at Laboratory Infection of Two Species of *Leptotrombidium* with the Karp Strain of *R. tsutsugamushi*.

L. (L.) deliense

Mouse No. & Sensitization.	Site of Feeding	Development State and Time After Feeding										No. of Adult ♀	No. Laying Eggs
		Post Larval (24 hrs)		Prenymph (7 days)		Nymph (13 days)		Teliophane (31 days)		Adults (45 days)			
1B*	Ear	-**	-	Pos***	-	-	-	-	-	OT+	OT	4	1
"	Back	-	-	-	-	ND++	ND	ND	ND	ND	ND	0	0
2B	Ear	-	-	-	-	-	-	-	-	OT	OT	8	4
"	Back	-	-	-	-	-	-	ND	ND	ND	ND	2	1
3A	Ear	-	-	-	-	-	-	-	-	-	-	9	2
"	Back	-	-	-	-	-	-	ND	ND	ND	ND	2	1
4A	Ear	-	-	-	Pos	-	-	-	-	OT	OT	7	1
"	Back	-	-	Pos	-	-	-	ND	ND	ND	ND	4	0
L. (L.) akamushi													
5B	Ear	-	-	-	-	-	-	-	-	-	-	7	1
"	Back	-	-	-	ND	-	ND	ND	ND	ND	ND	0	0
6B	Ear	-	-	-	-	-	-	-	-	-	-	5	0
"	Back	-	-	-	-	-	ND	ND	ND	-	ND	0	0
7A	Ear	-	-	-	-	-	-	-	-	-	-	9	0
"	Back	-	-	-	-	-	-	ND	ND	ND	ND	3	0
8A	Ear	-	-	-	-	-	-	-	-	-	-	6	2
"	Back	-	-	-	-	-	-	ND	ND	ND	ND	2	0

1B*: B = *Blankaartia sp.* sensitized mouse: A = *L. (L.) akamushi* sensitized mouse.

-**: Negative

Pos***: Positive including back-challenge with Karp strain.

OT+: Currently on test, results not completed.

ND++: Not done.

Methods: Samples were removed for selected specimens from each generation, thawed and 0.2 ml of the frozen spleen-liver suspension from 2nd mouse passage was injected IP into four NCI mice. On the second day of illness the four mice were euthanized and a 20% suspension in Sniders diluent was made from their pooled spleens and livers. This suspension was immediately injected (IP, 0.2 ml) into five mice and (IC, 0.1 ml) three guinea pigs. The remaining material was labelled and frozen at -70°C.

The five mice per specimen were euthanized on day 9 or 10 post inoculation and five peritoneal smears were made from each mouse for a total of 25 smears for each specimen. These were labelled, fixed in acetone, dried and stored at -20°C. The three guinea pigs per specimen were held for 28 days and then bled. The serum was collected, labelled and frozen at -20°C.

The antigenic analysis involves two separate and different techniques, direct immunofluorescent antibody and strain specific complement fixation. To date only three strain specific conjugates, Karp, Gilliam and Kato, have been available for use. These conjugates were supplied to us by the Department of Rickettsial Diseases, WRAIR. Details on their preparation and specificity are given in the Rickettsial Disease section of WRAIR Annual Reports of 1970 and 1971. The strain specific fluorescein conjugates were titrated against their homologous as well as the two heterologous antigens and a determination made of the dilution where specific staining occurred with the homologous antigen and no staining occurred with the two heterologous antigens. For the Karp conjugates this was at a dilution of 1:256, Gilliam 1:128, and Kato 1:128. A control conjugate made from normal rabbit serum was used for the nonspecific staining control at a dilution of 1:128.

One slide of each of the specific antigenic strains (Karp, Gilliam and Kato) prepared from yolk sac early in the studies or later peritoneal smears, were included as controls each time a group of unknown slides were stained. Each slide, unknown as well as control, had four [illegible] it and each of the four areas stained with a different conjugate, Karp, Gilliam, Kato and normal rabbit serum. Thus, each slide served as its own control plus the three specific antigen control slides.

The guinea pig hyperimmune sera were shipped to the Department of Rickettsial Diseases, WRAIR for CF analysis.

Results: Preliminary results to date on the first continuous line selected through seven generations is given in Table 8. From the data several tentative conclusions can be drawn. First, it appears that this particular continuous line is infected with both Karp and Kato-like organisms and that the Gilliam antigenic component is not present. Second, it appears that Karp was the dominant antigenic component and Kato the minor one in the parent, which changed in F-1,

Table 8

The IF Antigenic Strain Analysis of *R. tsutsugamushi* Present in the Positive *L. (L.) akamushi* in One Line Continuous Through Seven Generations.

Generation	Number of Specimens Analyzed Per Generation	The "Antigenic-like" Present Strain	Relative Expression of the Antigenic Type* RE
Parent	1	Karp	56
		Kato	8
F-1	1	Karp	10
Progenitor of F-2		Karp	10
F-2	1	Karp	20
		Kato	20
Progenitor of F-3		Karp	20
		Kato	20
F-3	5	Karp	7
		Kato	11
Progenitor of F-4		Karp	8
		Kato	4
F-4	5	Karp	4
		Kato	12
Progenitor of F-5		Karp	4
		Kato	4
F-5	5	Karp	3
		Kato	14
Progenitor of F-6		Karp	8
		Kato	28
F-6	5	Karp	6
		Kato	16
Progenitor of F-7 Line 1		Kato	5
Progenitor of F-7 Line 2		Karp	24
		Kato	40
F-7 Line 1	2	Kato	18
F-7 Line 2	2	Karp	1
		Kato	7

* Relative expression (RE) was calculated using the following formula:

$$RE = \frac{P}{T} \times K$$

P = the sum of the intensity of fluorescence for each slide plus the relative number of organisms on each slide staining, both of which are recorded on a scale of 0 to 4.

T = total number of slides examined for each generation or progenitor (1 slide per mouse, 4-5 mice per specimen)

K = a constant of 10.

F-2 to equal presence and expression. This reversed itself in the remaining generations through F-7 to Kato being the major component and Karp the minor one. In the F-7 generation it was interesting to note that the progenitor of Line 1 contained only the Kato like antigen and this was also true of the two descent pools (F-7 Line 1). The other F-7 progenitor and its two F-7 pools, F-7 Line 2, all contained both Kato- and Karp-like antigens. Unfortunately, neither F-7 Line 1 nor F-7 Line 2 were continued into the F-8 generation so there is no material available to determine if they remained pure Kato-like through three generations once the two strains became separated.

The complement fixation analysis, in summary, revealed that the parent, 1854, contained both Karp and Kato-like antigens while sera prepared against specimens from the remaining generations reacted only with Kato. This is explained at the present time as a lack of sensitivity on the part of the CF test or that it is more strain specific, or both. For details on the complement fixation results, see the 1971 WRAIR Annual Report.

Extensive work is currently in progress on a larger sample of two additional continuous lines and larvae are now being individually reared at the 9th generation so that individuals will be available for testing at the 9th and 10th generation.

Silvered Leaf-Monkeys (*Presbytis cristatus*)

General: In this Unit's Annual Report of 1970, the rationale for beginning a preliminary investigation into the susceptibility of the silvered leaf-monkey was given as well as caging, diet and some very preliminary challenge data. During this reporting period, two monkeys were examined for chronic infection at five months and three at two months post-challenge. An additional 16 animals were challenged with *R. tsutsugamushi*. Extensive work and man hours had to be expended in investigations of the diet, husbandry practices and diseases acquired in the laboratory before any extensive or expanded investigations could be conducted with any degree of reliability. A full report on this is included under the Laboratory Animal Development and Zoonotic Diseases section.

Results: Serological data on the immune status of this monkey in the jungle (within 24 hours of capture) is being accumulated against several human pathogens including *R. tsutsugamushi*. To date, sera from 42 silvered leaf-monkeys at the time of capture has not revealed any detectable antibodies at a 1:40 dilution using the indirect fluorescence antibody test employing yolk sac antigens of the Karp, Gilliam and Kato strains of *R. tsutsugamushi*. This is in direct contrast with the high rate of positives found in the different *Macaca sp.*, see Annual Reports 1968 and 1969.

Of the 16 monkeys challenged, four were challenged by intradermal inoculation with a dose of $10^{4.4}$ $MIPLD_{50}$'s of the Karp strain while the

remaining 12 received an intradermal challenge of approximately 10^4 $MIPLD_{50}$'s of various combinations of the three strains, Karp, Gilliam and Kato. The data on the 12 monkeys challenged with the various combinations of three strains are incomplete as of this date and will not be reported except as relates to mortality rate. Of the 16 monkeys challenged, only two died from an infection of scrub typhus for a death rate of 12% at challenge dose of approximately 10^4 $MIPLD_{50}$'s which is considerably lower than the rate reported in the Annual Report of 1970 with the same or lower dose. It is felt that part of this discrepancy may be due to prior conditioning of the monkeys before challenge and control of miscellaneous diseases, particularly diarrheas, in the laboratory. The detailed data on the four monkeys challenged with $10^{4.4}$ $MIPLD_{50}$'s of the Karp strain, intradermal, in separate sites on both thighs is given in Table 9.

With four animals one can draw only tentative conclusions realizing that they may be subject to change as more data become available. The following observations or conclusion could be drawn: (1) that all four animals run a rickettsemia with the average duration time of 12 days and the animal that died ran a continuous rickettsemia for 21 days before succumbing to the infection, (2) all four animals developed eschars in both sites of inoculation and these varied in severity from very minor ones that lasted only a few days to severe ones that lasted for up to 13 days, (3) that all four developed some significant degree of hyperthermia which varied in onset and degree between monkeys, (4) that the one animal that succumbed to the infection developed a severe hypotheremia beginning 10 days before death. This had been immediately preceded by a significant hypertheremia of five days duration and a rickettsemia level of over 10^3 $MIPLD_{50}$'s per 0.2 ml of blood for five days. It appears from incomplete data now available on the other 12 monkeys that were challenged, that in general the same conclusions will hold for them.

Since man develops a chronic infection or carrier status following scrub typhus infection if not treated or inadequately treated, it was desirable to determine if the phenomenon occurred in silvered leaf-monkeys following untreated infections with *R. tsutsugamushi*. It was also desirable to determine what organs were involved at death and during chronic infection and what the rickettsial level per unit weight of tissues was. Table 10 gives the data on organ isolation from two monkeys at the time of death, for two monkeys at five months and three monkeys at two months post-challenge.

The data in Table 10 would appear to indicate that: (1) when silvered leaf-monkeys die of scrub typhus infection that most if not all the organs of the body are involved and some of them may have very high organism levels per unit weight of tissue, (2) the silvered leaf-monkey does develop a chronic infection or carrier status (four out of five) and that the lymphatic system is the one most

Table 9

The Response of Four Silvered Leaf-Monkeys to a Challenge with the Karp Strain of *R. tsutsugamushi**

Monkey No. & Status	Response	DAYS POST CHALLENGE 1-4	5-9	10-14	15-19	20-24	24-28
1 Survived	Temp.	103.2	102.8	102.8	102.5	102.3	101.8
	Eschar						
	R	-	R+(4)	-	-	-	-
	L	-	L+(4)	-	-	-	-
	Rick.	-	1.5(2)	-	-	-	-
	Titer	-(0)	-(7)	80(14)			160(28)
2 Dead, day 29	Temp.	103.8	103.0	103.1	104.0	98.8	96.2
	Eschar						
	R	-	R+(4)	R+(5)	R+(5)	-	-
	L	-	L+(4)	L+(5)	L+(4)	-	-
	Rick.	-	1.5(1)	2.5(5)	3.1(5)	2.3(5)	1.6(5)
	Titer	-(0)	-(7)	-(14)			80(28)
3 Survived	Temp.	105.0	104.4	105.4	104.4	103.7	103.2
	Eschar						
	R	-	R+(5)	R+(5)	R+(3)	-	-
	L	-	L+(5)	L+(4)	L-	-	-
	Rick.	-	1.5(2)	2.2(5)	1.6(4)	-	-
	Titer	-(0)	-(7)	40(14)			80(28)
4 Survived	Temp.	102.8	103.4	103.0	102.0	102.2	102.3
	Eschar						
	R	-	R+(4)	R+(4)	-	-	-
	L		L+(4)	L+(4)	-	-	-
	Rick.	-	1.6(3)	2.0(5)	1.4(5)	1.5(2)	-
	Titer	-(0)	-(7)	40(14)			160(28)

*Note: Challenge dose was $10^{4.4}$ $MIPLD_{50's}$, 0.1 ml, ID, in both thighs. Temp. = rectal temperature, °F average over days indicated. Eschar, R = right thigh (days duration) and L = left thigh, + = positive, - = negative; Rick. = Rickettsemia $\log_{10}$ $MIPLD_{50's}$ per 0.2 ml of whole blood (days duration), if of more than one days duration, titer shown is the mean titer. Rickettsia isolated on two differen. days were injected into mice on chloramphenicol and back-challenged with 10^4 $MIPLD_{50's}$ of the Karp strain at 28 days, in all cases the organisms isolated were confirmed as being *R. tsutsugamushi*. Titer = IFAT titer using a trivalent yolk sac antigen of Karp, Gilliam and Kato, (day postinoculation titer was determined).

Table 10

Organs Isolation of *R. tsutsugamushi* from Silvered Leaf-Monkeys at Deaths, 2 and 5 Months Post-challenge

MONKEY*

ORGANS		A Death	2 Death	1 2 Months	3 2 Months	4 2 Months	B 5 Months	C 5 Months
Blood	Titer, isol.**		N.D.	<1.0	<1.0	<1.0	N.D.	N.D.
	Confirmation***		+	-	-	+	-	-
Brain	Titer, isol.	4.3		<1.0	<1.0	<1.0	N.D.	N.D.
	Confirmation	N.D.		-	-	-	-	-
Lung	Titer, isol.	5.2	N.D.	<1.0	<1.0	<1.0	N.D.	N.D.
	Confirmation	N.D.	+	-	+	-	-	-
Liver	Titer, isol.	1.4	1.6	<1.0	<1.0	<1.0	N.D.	N.D.
	Confirmation	N.D.	+	-	+	-	-	-
Spleen	Titer, isol.	<1.0	2.0	<1.0	<1.0	<1.0	N.D.	N.D.
	Confirmation	N.D.	+	-	-	+	-	-
Left Kidney	Titer, isol.	1.5	2.2	<1.0	<1.0	<1.0	N.D.	N.D.
	Confirmation	N.D.	+	-	-	-	-	-
Right Kidney	Titer, isol.	1.5	2.0	<1.0	<1.0	<1.0	N.D.	N.D.
	Confirmation	N.D.	+	-	-	-	-	-
Axillary Lymph Nodes								
Left	Titer, isol.		N.D.	<1.0	2.7	2.5	N.D.	N.D.
	Confirmation		+	+	+	+	-	+
Right	Titer, isol.		N.D.	<1.0	3.3	2.6	N.D.	N.D.
	Confirmation		+	-	+	+	-	+
Inguinal Lymph Nodes								
Left	Titer, isol.		N.D.	<1.0	3.4	2.5	N.D.	N.D.
	Confirmation		+	-	+	+	-	+
Right	Titer, isol.		N.D.	<1.0	2.7	3.1	N.D.	N.D.
	Confirmation		+	-	+	+	-	+
Immune Status								
	IFAT Titer		00	80	160	80	160	160

* Monkey: Challenge data are as follows; A, Karp strain, ID, $10^{4.3}$ $MIPLD_{50's}$, B, Sankaran isolate, ID, $10^{3.5}$ $MIPLD_{50's}$; C, Sankaran isolate, ID, $10^{2.7}$ $MIPLD_{50's}$; 1, 2, 3, & 4, Karp strain, ID, $10^{[illegible].4}$ $MIPLD_{50's}$: For complete data on course of disease in monkeys 1, 2, 3 & 4 see Table 6.

** Titer, isol.: Log_{10} $MIPLD_{50's}$ per 100 mg of tissue or 0.2 ml of blood.

***Confirmation: If mice survived at the 10^{-1} dilution they were back-challenged at 28 days inoculation with 10^4 $MIPLD_{50's}$ of the lethal Karp strain to see if any protection existed. If a titer was present then a spleen and liver suspension from mice at 10^{-1} was passed into mice on chloramphenicol and back-challenged at 28 days with 10^4 $MIPLD_{50's}$ of the Karp strain.

Symbols: N.D. = not done, + = positive, - = negative.

often involved (four out of four positive); however, in two out of four positives, other organs were also involved. Also of interest was the fact that not only were the lymph nodes draining the site of inoculation were positive, but also the axillary lymph nodes. This indicates that the entire lymphatic system was involved not just the local lymph nodes draining the inoculation site.

The data on the one gibbon for which complete data are available will be given in this section, Table 11. The gibbon was susceptible to *R. tsutsugamushi*, ran a hyperthermia, developed eschars and had a demonstrable rickettsemia which was continuous for 14 days after which it was intermittent until day 28, the last day on which it was checked. That animal was rechallenged in the latter part of the reporting period with Kato and Gilliam strains and survived the infection. The data are incomplete at this time but it appears that the infection was modified. A second gibbon was challenged also at that time with the Karp strain and we felt would have died if not treated with chloramphenicol, again the data are still incomplete.

Discussion: Although the data are still limited, it begins to appear that the silvered leaf-monkey can serve as an animal model for human scrub typhus. Extensive work is planned in the coming year to provide a broad foundation on which to build this primate model. Many more parameters will be measured on large groups and the dose response for as many strains as possible will be determined as well as the gross and histopathology. The data on the gibbon are interesting but probably will not be expanded to any degree because the limited number of gibbons that could be made available more than likely would not be enough to adequately test a vaccine before going to human trials.

Filter Paper:

General: Last year a preliminary report was made on the development of this technique. It greatly simplifies the collection and transport of specimens from remote areas and in those cases where only a limited amount of blood can be obtained it allows for a more accurate specimen.

Method: Sufficient blood is obtained by a finger puncture in the human or other primates and by cutting off the end of the tail in rodents, to cover an area of 13 millimeters in diameter on No. 4 Whatman filter paper. No special treatment is necessary for the preservation of the samples as they are allowed to dry and are stored at ambient temperature until testing. However, in the tropics they must be kept in insect proof containers or otherwise insects will eat them. As many as 12 individual specimens are placed on one 10 x 4 inch piece of filter paper.

At the laboratory, 3 millimeter discs are cut from the specimens on the filter paper using a No. 1 cork borer. The discs are then

Table 11

The Response of One Gibbon to a Challenge with the Karp Strain of *R. tsutsugamushi**

Gibbon Number	Response	DAYS POST CHALLENGE 1-4	5-9	10-14	15-19	20-24	24-28
	Temp.	102.9	103.6	103.2	99.8	101.1	102.2
1	Eschar						
Survived	R	R+(3)	R+(5)	R+(5)	R+(5)	R+(2)	-
	L	L-	L+(5)	L+(5)	L+(5)	L+(2)	-
	Rick.	2.2(4)	1.6(5)	2.1(5)	1.0(2)	1.0(1)	1.0(1)

* Note: Challenge dose was $10^{7.5}$ $MIPLD_{50's}$, 0.1 ml, ID, in both thighs. Temp. = rectal temperature, °F averaged over the days indicated. Eschar, R = Right leg (days duration) L = left leg (days duration), + = positive, - = negative. Rick. = Rickettsemia $\log_{10}$ $MIPLD_{50's}$ per 0.2 ml of blood (days duration), if of more than one day's duration titer shown is the mean titer. Rickettsia isolated on two different days were passed into mice on chloramphenicol and back-challenged with 10^4 $MIPLD_{50's}$ of the Karp strain at 28 days, both confirmations were positive.

placed directly on the individual antigen spots which have 0.025 ml of IF diluent dispensed by means of a microtiter constant delivery pippette. This gives an effective dilution of 1:50. The antigen used in this study was a trivalent one composed of yolk sac suspensions of the Karp, Gilliam and Kato strains of *R. tsutsugamushi*. A suspension composed of the three antigens is placed in spots of 0.01 ml quantity with eight spots being made on each slide. The antigen spots are then rapidly dried in a dehumidified room and fixed in dry acetone at room temperature. After fixing, the slides are dried in a dehumidified chamber at 37°C and stored at -20°C until use.

The antigen and specimen are incubated together at 37°C for ½ hour in a high humidified chamber. They are then washed and antispecies globulin is added that has been conjugated with fluorescein isothiocynate. The anti-species globulin used was varied depending on the species being tested for scrub typhus antibodies. After incubation the slides are again washed and mounted in buffered glycerine at pH 7.3 for examination with a fluorescence microscope. A known negative and positive control serum of which the titer was known were included each time the test was set up.

Quantitative studies comparing filter paper and the corresponding serum titers were conducted on paired specimens. The only difference between the procedures was that instead of placing the filter paper disc on the antigen spot, it was eluted and the eluate, in varying 2 fold dilutions starting at 1:40, was added to the antigen spot. This was accomplished by taking five discs of three millimeters diameter each and placing all five of them in 0.1 ml of IF diluent for 10 minutes, at ambient temperature, giving an effective dilution of 1:[illegible]. From this, two fold serial dilutions were made. The corresponding serum sample was handled in the usual manner.

Results: Figures 3 and 4 give the serum and corresponding filter paper results. The data in Fig. 3 are from human specimens while Fig. 4 are primate and rodent data. The screening titers on the human filter paper specimens were recorded at a 1:50± or 1:50. The criteria for a 1:50 reading were numerous organisms with bright fluorescence distributed uniformly throughout the antigen spot. A 1:50± reading was defined as fewer organisms with dim fluorescence which were located primarily around the edge and not uniformly distributed throughout the antigen spot. Figure 3 shows that the majority of the specimens with a screening titer of 1:50± had a serum titer of 1:40 while those with a screening titer 1:50, in general, had a serum titer of 1:80 or greater. All specimens with a serum titer of 1:160 or greater had a screening titer of 1:50.

Table 12 gives the comparison data for 173 humans, 28 rodents, and 6 non-human primates. Both the false positives and false negatives at a screening titer of 1:50 for filter paper specimens are very low, 1%. This is better than most currently used serological tests and does not represent a problem.

COMPARISON OF THE I F A TITER OF HUMAN SERUM AND FILTER PAPER PAIRED SPECIMENS

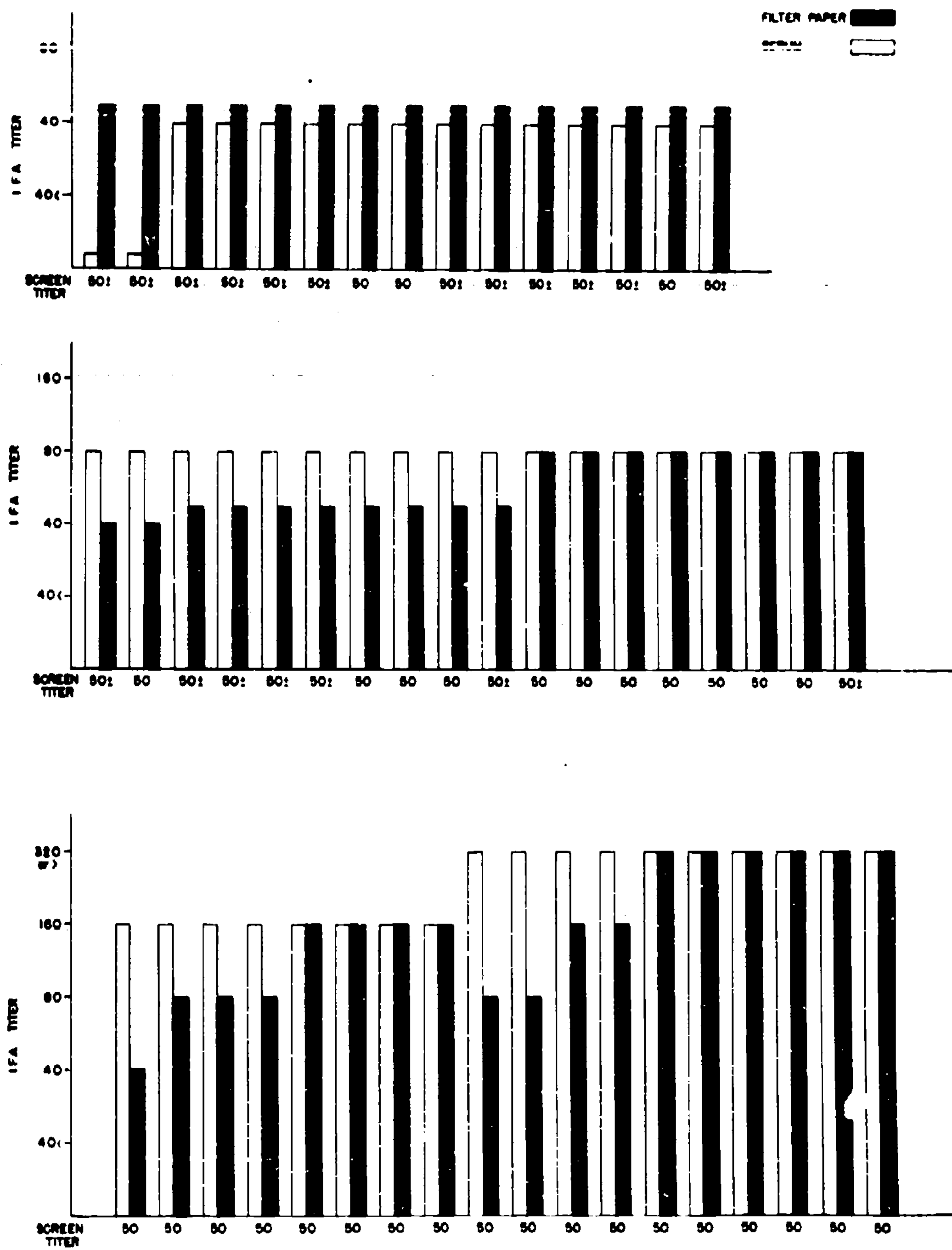

Fig. 3

COMPARISON OF THE I F A TITER OF ANIMAL SERUM AND FILTER PAPER PAIRED SPECIMENS

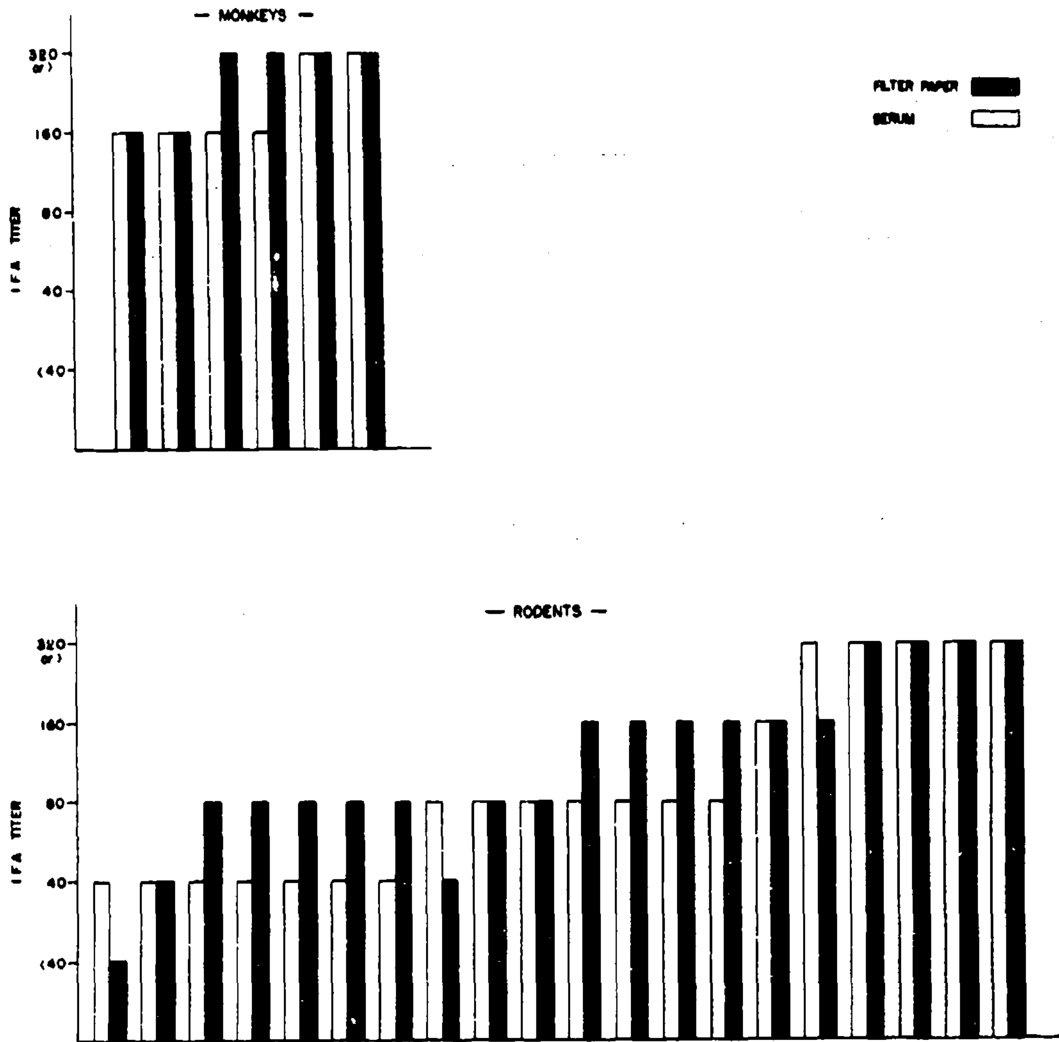

Fig.4

Table 12

Comparison of Serum (1:40) and Filter Paper (1:50)
Paired Specimens in the IFA. Test

RESULTS	NUMBER OF SERA Human	Rodent	Primate
Both samples negative	91	8	0
Both samples positive	78	19	6
Serum positive and filter paper negative	2	1	0
Filter paper positive and serum negative	2	0	0
Total	173	28	6

To test the reproducibility of the technique, we tested 35 individuals on which we had two filter paper specimens and one on which we had five individual specimens. Table 13. Each specimen was run on a separate day. Using serum, 74 percent gave the same titer while only 52 percent of the filter paper specimens had the same titer. No filter paper or serum specimen varied over four fold. The greater variation in filter paper specimens could be accounted for by the fact that it is extremely difficult to get the same amount of blood on the smears each time and also that the elution process is not that accurate, indeed it is surprising that there is not greater variation.

Conclusions: The filter paper technique of collecting blood specimens for scrub typhus indirect fluorescent antibody titers works as well as serum, is reliable and reproducible. It works equally well on human, rodent and primate specimens. Not only can it be used to screen sera at a 1:40 or 1:50 dilution in a prevalence survey but it works equally well where one wants to determine the specific titer that the human or animal has against *R. tsutsugamushi*. Therefore, the technique permits both a qualitative and quantitative analysis for scrub typhus antibodies with a specimen of approximately 0.065 ml of blood which offers an advantage over conventional tests based on larger quantities of serum. It provides a simple method of collection and transportation of specimens that bridge the gap between the availability of modern diagnostic techniques in large control or research laboratories and the application of these techniques to problems in remote endemic areas. To date over four hundred human specimens and one thousand rodent specimens have been collected using this technique that otherwise might not have been because of the quantity of blood needed and the remote collection areas made the collection of serum difficult because of the equipment, needles, syringes, tubes, etc., needed.

Orang Asli (Aborigines):

General: In previous years this Unit has reported that large concentrations of known vector mites of scrub typhus were found in deep jungle sites and further that many of these were carrying *R. tsutsugamushi*. Other work of this Unit has shown that large concentrations of infected vector mites occur on the edge of sandy beaches of West Malaysia. Yet unfortunately, the name 'scrub typhus' still ties the disease to scrub habitats. This section presents data on the prevalence of human sero-positive cases arranged by the different habitats the individuals lived and worked in. The following section and the medical ecology part of this report presents data on rodents from various habitats.

Methods and Descriptions: Due to the difficulties in obtaining, separating, preserving and transporting whole blood specimens obtained in remote areas this data was obtained using the filter paper technique. For a complete description of this technique, its comparison to serum results, accuracy and reproductability see page 126, Filter Paper Technique of the scrub typhus section of this report.

Table 13

Comparison of IFA. Titers from Two or More Filter Paper Samples from the Same Individual

	NUMBER OF SPECIMENS	
	TWO (N = 35)	FIVE (N = 1)
TITER	PERCENTAGE	
Same		
Serum	74	100
Filter Paper	52	100
2 Fold		
Serum	23	0
Filter Paper	29	0
4 Fold		
Serum	3	0
Filter Paper	19	0

Bloods were collected from *Orang Asli* adults and children as part of a malariometric survey. *Orang Asli* were studied that lived and worked in deep jungle areas, fringe areas and rural *kampongs* (villages). The deep jungle areas are primary forest with only a few small disturbed or semicleared areas around their villages and along some of the trails running through the jungle. The people live a very primitive life where a great deal of the food is obtained by hunting and gathering forest fruits; thus, the entire family spends much or all of its time in the forest. Generally, these areas can not be reached by boat or Landrover. Fringe areas, as the name implies, are on the edge of the jungle with secondary growth and scrub habitats. The fringe areas correspond with the so-called "scrub areas" from which scrub typhus received its unfortunate name. These areas are easily reached by Landrover and the people lead a fairly settled life, do considerable farming and only occasional hunting. Hunting, when it is done, is generally done by the men and the family spends little time in the jungle per se. The *'kampong' Orang Asli* live in settled villages away from the jungle in a fashion almost identical to most rural Malays. Farming and occasional outside work is their livelihood with only a rare hunting trip to the jungle. Most of the vegetation to which they are exposed is cultivated with a few secondary trees. For the most part, secondary brush is kept down or cleared.

The age ranges which were used in tabulating the data are only approximate since few *Orang Asli* know (or care) what their ages are. Because distinct seasons do not occur in Malaysia nor other phenomena by which they can easily compute long periods of time and since they have no reason whatever to wish to do so, they are quite content with recognizing somewhat less even than the seven ages of man which Shakespeare describes. Our age determinations, therefore, are rough estimates based on height and general appearance coupled with information as to the relative age of other members of the family or community. For these reasons the data are presented in ten year increments to take up some of the inherent errors.

Results: A total of 690 persons were studied. Of these, 245 were classified as adult (over 20 years of age) and 445 were below 20 years of age. The division between deep jungle, fringe and village was almost equal, being 223, 256 and 211 respectively, see Table 14. In the fringe or scrub area which is the 'textbook' habitat for scrub typhus, 18% of those under 20 years of age and 48% of adults had antibodies to *R. tsutsugamushi*. In the *kampong* areas, 0% of those under 20 and only 8% of those over 20 had antibody. In the deep jungle, on the other hand, 56% of those under 20 and 73% of those over 20 had antibody. Arranged in order of *kampong*, scrub and deep jungle the percentages in children were 0, 18 and 56 respectively. For adults the percentages were 8, 48 and 73% respectively.

The age of acquisition of scrub typhus appears to vary with the habitat of the people. By ten year increments, the percentages in

Table 14

Prevalence Rates of Scrub Typhus Antibodies in *Orang Asli* Arranged According to Habitat

			FRINGE AREAS[1]			
Age	1-9	10-19	20-29	30-39	40+	Total
Total	109	86	25	23	13	256
Positive[2]	18	17	9	13	7	64
%	17	20	36	57	54	25
	18%			48%		
			KAMPONG AREAS[3]			
Total	69	58	28	21	35	211
Positive	0	0	2	1	4	7
%	0	0	7	5	11	3
	0%			8%		
			DEEP JUNGLE AREAS[4]			
Total	59	64	54	27	19	223
Positive	27	42	38	21	14	142
%	46	66	70	78	74	64
	56%			73%		

1. Fringe Areas: Totals from four separate areas of West Malaysia; Woh, Tapah, Tamok and Kudang.

2. Positive: Positive using the filter paper collections, 1:50 dilutions at a reading of ± or greater in the indirect fluorescent antibody serological test employing the trivalent yolk sac antigens of the Karp, Gilliam and Kato strains of *R. tsutsugamushi*.

3. *Kampong* Areas: Totals from two separate *Kampongs* in West Malaysia, Ayer Denok and L. Sempit.

4. Deep Jungle Areas: Totals from three separate areas of West Malaysia - Shean, Kamar and Satak.

fringe people are 17, 20, 36, 57, 54 whereas those in deep jungle are 46, 66, 70, 78, 74.

Discussion and Summary: It is not known for certain how long the antibodies against *R. tsutsugamushi* demonstrated by the IFAT persists in human, so that the percentages positive are minimum figures. Since in humans, rickettsial infection is virtually synonymous with disease, we assumed that each positive represents at least one episode of disease.

It can be seen from the data that not only do deep jungle people acquire scrub typhus, but they are more likely to do so than are fringe or *kampong* peoples. We do not know exactly, yet, where these deep jungle people acquire their infections. Possibly they are acquired in the small clearings where they build their houses rather than in the jungle itself. On the other hand, one would still have to explain why the risk should be that much higher in a small clearing in the jungle than in a similar clearing in the fringe or scrub forest. The data on rodents presented in the next section and in the medical ecology part of this report help substantiate our opinion that the risk of a scrub typhus infection is greater in the deep jungle than in other eco-systems in this country except possibly the beaches of West Malaysia which were not included in this survey.

Rodents:

General: Numerous studies have been done and reported by this Unit and others on the prevalence ratios of scrub typhus, generally isolations; however, the effect of vertical zonation in a habitat had not been adequately investigated nor had the indirect fluorescent antibody technique been successfully applied to rodent sera. In addition, for the first time, this Unit had a highly trained and experienced ecologist-zoologist on the staff to supervise the collections and document them in large numbers. Therefore, it was decided to take advantage of the situation and in cooperation with the Department of Medical Zoology to investigate prevalence ratios in a large number of rodents taking into account not only habitat but also vertical zonation within a given habitat. All of the basic data are given in this section; however, part of it is also presented in a different way in the Medical Ecology section stressing the effect of various eco-systems on the prevalence ratios of isolations of *R. tsutsugamushi*.

Methods: Rodents were trapped, tagged, catalogued by species and habitat, bled and euthanized by the Department of Medical Ecology before being brought to the Department of Rickettsial Diseases where in the case of organ isolations, the organ in question was immediately removed aseptically. The carcasses were then returned to the Department of Medical Ecology for identification and other studies later that same day. Immediately upon obtaining blood, it was injected undiluted (IP, 0.2 ml) into two mice. In the case of the organs (kidneys), they

were immediately removed aseptically following euthanasia. They were weighed and then Snider's diluent added in the ratio of 1:5, wt/vol, and ground in a hand operated tissue grinder. The resulting tissue homogenate was injected (IP, 0.2 ml) into each of two mice. The whole procedure was carried out on an assembly line basis in less than 2 hours from euthanasia to injection of the mice with the tissue homogenate and less than 15 minutes in the case of blood. The remaining blood was kept at 4°C and sent to the serology department where the serum was separated and stored at -20°C until tested.

The two mice were observed daily and if no deaths occurred they were sacrificed on day 14, their spleens and livers were pooled, weighed and Snider's diluent added at 1:5, wt./vol., then ground in a sterile hand operated tissue grinder. The entire procedure was carried out at 4°C to the extent possible. The resulting homogenate was then injected into six mice for second passage (IP, 0.2 ml) which were observed daily for 28 days. If no deaths occurred, these six mice were back-challenged between 28 and 38 days with the mouse lethal Karp strain of *R. tsutsugamushi* (10^4 $MIPLD_{50}$'s, yolk sac, dose 0.2 ml, IP) and observed daily for another 28 days to determine if protection existed and whether the strain was a non-lethal mouse strain. In the case of death either at the 1st or 2nd passage, the remaining mouse (or mice) was sacrificed, a homogenate made of the spleen and liver and injected into six mice (IP, 0.2 ml) which were given chloramphenicol (2.5 mg./ml of drinking water from day 3 post-infection to day 24) to prevent mouse lethal strains from killing the mice and held for 28 days at which time they were back-challenged using the identical procedure outlined above. This procedure confirmed that the organism isolated in the mice was *R. tsutsugamushi* and not a viral or bacterial organism because in either case there would be no immunity and the mice would die while if *R. tsutsugamushi* was isolated the animals would be immune and survive the challenge. In addition, all homogenates from the original animal and both mouse passage were cultured in Thio-glycerate to determine if any bacteria were present. Only those animals where the back-challenged mice survived the lethal challenge with the Karp strain were considered positive.

The first problem with the indirect fluorescent antibody test was the anti-species conjugates. Since it would have been impossible or very time consuming to produce a conjugate against the globulins of each species (over 30) they were grouped into 12 groups, phylogenetically, by the Department of Medical Ecology and conjugates prepared against each of the groups. To date, successful conjugates have been prepared against six of the groupings (Table 15). When more than one species was present in a grouping, a pool was made of representative sera using equal amounts before fractionation. The pooled sera were fractionated using the standard ammonium sulfate method making a total of 3 precipitations. The final precipitate of the serum globulins was diluted in PBS (pH 7.3) such that five ml

Table 15

Species Groupings for the Production of Anti-species Antibodies

Group	Species
1	*Callosciurus caniceps* *Callosciurus nigrovittatus* *Callosciurus notatus*
2	*Rattus bowersi* *Rattus muelleri*
3	*Rattus argentiventer* *Rattus rattus diardii* *Rattus tiomanicus jalorensis*
4	*Rattus annandalei*
5	*Rattus rajah pellax* *Rattus surifer* *Rattus whiteheadi*
6	*Rattus edwardsi* *Rattus sabanus*

of the suspension equaled 2.5 ml of the original serum pool. The globulin suspensions were then divided into 3.5 ml aliquots and stored at -20°C until use. Rabbits were used to produce the anti-species antibodies with one rabbit used per group. Each rabbit received a total of 2.5 ml of globulin suspension. Two milliliters of the suspension were homogenized with two ml of complete Freund's adjuvant. This was injected in one ml quantities, IM, in each rear leg and one ml, subcut, in each side of the neck for a total of four ml of the homogenate. The remaining 0.5 ml of the globulin suspension was injected using the IP route. On day 14, another 2.5 ml of the globulin suspension was injected using the same procedure. On day 28 the rabbit was bled from the ear, the serum fractionated and the globulins labelled with fluorescein isothiocynate (see below for procedure). The resulting conjugate was block titrated against at least four known positives of that group, and the fluorescent titer and optimum dilution of the conjugate determined. If the resulting conjugate did not titer at least 1:2 then the rabbit received a third dose of the globulin suspension and the testing procedure was repeated 14 days following the third injection. Most groups required three injections and one group four, before an acceptable conjugate was obtained.

The serum to be labelled with fluorescein isothiocynate was fractionated with ammonium sulfate. To a measured amount of undiluted serum, an equal amount of cold saturated ammonium sulfate solution was added. The resulting mixture was allowed to stand overnight at 4°C. It was then centrifuged at 4°C and the resulting sediment was disolved with a quantity of distilled water equal to the original volume of serum. This globulin solution was reprecipitated three times and centrifuged each time. The resulting globulin was then labelled with fluorescein isothiocynate using essentially the procedure of Riggs. It was then dialized against phosphate buffer, pH 7.3 overnight to remove the majority of the uncombined dye. It was then further purified and refractioned using a Sephedex column; (grade course 50 with phosphate buffer pH 7.5). Following column purification and fractionation,it was absorbed with acetone dried guinea pig liver powder using the wet procedure to remove any non-specific staining properties that remained. The conjugate was then block titrated against known positives of that group, dispensed in 0.5 ml quantities and stored at -20°C until use.

The procedures for indirect fluorescent antibody test were the same as outlined in the filter paper technique section. All sera were screened at a dilution of 1:40 of the unknown test serum. Any sera showing a reaction of ± or + at a 1:40 dilution was considered positive. A trivalent antigen was used that was composed of the Karp, Gilliam and Kato strains of *R. tsutsugamushi*.

Results: Differences in the rates of isolations and percentages of sero-positives are immediately obvious when one compares arboreal species to semiarboreal and ground species (see Table 16).

Table 16

Prevalence of *R. tsutsugamushi* Isolations and Sero-positives from Mammals in Various Vertical Strata

ARBOREAL

Species	Isolation				Serology, IFAT	
	Blood		Kidney			
	Sample Size	% Positive	Sample Size	% Positive	Sample Size	% Positive
Callosciurus caniceps	8	0	-	-	17	6
Callosciurus nigrovittatus	7	0	13	0	11	0
Callosciurus notatus	124	0	67	3	220	3
Hylopetes spadiceus	3	0	13	0	-	-
Sundasciurus tenuis	10	0	5	0	-	-
Other*	20	0	4	0	-	-
Total	172	0	102	2	248	4
SEMIARBOREAL						
Lenothrix canus	19	0	2	0	10	0
Rattus annandalei	90	21	54	33	234	68
Rattus cremoriventer	5	0	-	-	15	7
Rattus rattus diardii	13	15	-	-	7	0
Rattus edwardsi	-	-	7	29	7	57
Rattus exulans	22	0	3	0	23	0
Rattus sabanus	173	20	207	21	370	52
Rattus tiomanicus jalorensis	91	8	12	8	90	36
Tupaia glis	16	6	12	50	-	-
Others**	9	22	1	0	-	-
Total	438	15	298	23	756	52
GROUND						
Rattus argentiventer	9	33	2	0	10	50
Rattus bowersi	36	14	15	47	61	62
Rattus muelleri	88	15	88	30	210	49
Rattus rajah pellax	53	11	21	5	89	52
Rattus surifer	19	37	7	29	24	46
Rattus whiteheadi	49	10	27	7	59	32
Others***	4	0	10	10	-	-
Total	258	15	170	23	453	49

Others*: Includes the following species: *Arctogalidia trivirgata, Nycticebus coucang, Hylomys suillus, Iomys horsfieldii, Petinomys vordermanni, Ratufa bicolor,* and *Tupaia minor.*

Others**: Includes the following species: *Chiropodomys gliroides, Hapalomys longicaudatus,* and *Pithechir parvus.*

Others***: Includes the following species: *Lariscus insignis* and *Rhinosciurus laticaudatus.*

For all essential purposes the arboreal species are negative while the semiarboreal and ground species both had isolations of 23% from the kidneys and a sero-positive rate of 52 and 49% respectively. The rate of isolations from the kidneys was higher, 23%, than that from whole blood, 15%, in the case of both the semiarboreal and ground species. This is a difference of 8% on a combined sample size of 1164, so it is real difference. When one looks at individual species the differences in isolations are very striking, *Tupaia glis*, for example, had a rate of 6% for blood isolation vs. 50% for kidney isolations. The same order of magnitude of differences were exhibited by *Rattus bowersii*, 14% vs. 47%. Other species, primarily *Rattus rajah pellex* and *Rattus surifer* had the reverse situation 11 vs. 5 and 37 vs. 29 for kidney isolations on an adequate sample size.

In any epidemiologic or medical ecological study, it is always necessary to compare the results of the techniques used to obtain the prevalence rates. Table 17 compares the rates of isolations of *R. tsutsugamushi* from the blood and kidney to the rates of sero-positives and negative animals for seven species where an adequate sample size of at least 20 paired specimens (isolation and serology samples on the same animal) in either the blood or kidney isolations were available. The percentages where both isolation and serology were positive on the same animal are essentially the same for both methods of isolation, blood, 11%, and kidney, 12%. Also the percentage of animals that were sero-positive but isolation negative was the same 35 and 36 percent. This means that regardless of the source of isolation (blood or kidney) that at least 35% more animals are going to be found positive on serologic testing than will be by isolation. The only place where there was a major difference was in animals which were isolation positive and sero-negative. For blood isolations this comparison was 2% while for kidney isolations it was 9%. Both figures are low and present no problem in a prevalence study. The possible reasons for this are discussed below.

Discussion and Summary: The differences in prevalence ratios of isolation and serology between arboreal and the semiarboreal or ground species are striking and are new findings as adequate samples of arboreal species had never before been tested for *R. tsutsugamushi* infection. One is tempted to explain this difference by lack of exposure to the vector *Leptotrombidium* mites which live on the ground. The arboreal animals don't come to the ground, therefore they do not get mites and therefore do not develop scrub typhus infections. In fact, we originally theorized that the arboreal species might even be more susceptible to infection with *R. tsutsugamushi* since they had never been exposed to the organism and therefore had no chance to develop an innate resistance over the past several thousands of generations. To date, we have tested this hypothesis to a limited extent. In one species, *Callosciurus notatus*, preliminary data show the reverse from what we hypothesized, in that they are extremely resistant to infection (challenge dose $10^{7.5}$ $MIPLD_{50}$'s Karp strain, route IP, 0.2 ml). Only one out of twenty died and the nineteen

Table 17

Comparison of the Rates of Isolations of *R. tsutsugamushi* from Blood and Kidney to the Rates of Sero-positive and Negative Animals.

BLOOD ISOLATIONS

Species*	Total Paired Specimens	Isolation & Serology Both Positive %	Isolation & Serology Both Negative %	Isolation Positive & Serology Negative %	Isolation Negative & Serology Positive %
Callosciurus notatus	110	0	99	0	1
Rattus annandalei	70	19	17	1	63
R. bowersii	30	13	13	0	74
R. tiomanicus jalorensis	70	6	73	0	21
R. muelleri	66	16	23	3	58
R. sabanus	126	16	37	4	43
R. whiteheadii	23	9	78	4	9
Total	495	11	52	2	35

KIDNEY ISOLATIONS

Species*	Total Paired Specimens	Isolation & Serology Both Positive %	Isolation & Serology Both Negative %	Isolation Positive & Serology Negative %	Isolation Negative & Serology Positive %
Callosciurus notatus	60	0	90	2	8
Rattus annandalei	47	23	17	4	56
R. bowersii	14	29	29	21	21
R. tiomanicus jalorensis	11	9	45	0	41
R. muelleri	83	11	49	17	23
R. sabanus	185	14	29	7	48
R. whiteheadii	20	5	70	5	20
Total	420	12	43	9	36

* Species, only those species with at least 20 paired blood or kidney samples were included.

survivors apparently did not develop chronic infection, since *R. tsutsugamushi* was not isolated from blood, liver, spleen or kidneys at four weeks post inoculation. Data on two other arboreal species *Callosciurus nigrovittatos* and *Hyaopetes spadiceus* are incomplete but indicate that they might be more susceptible than *C. notatus*. Whether or not they are as susceptible as *Tupaia glis* or the commonly involved *Rattus* species remains to be determined, however, the indications are that they are not. This leaves one with a triple explanation why the arboreal species are not involved in the scrub typhus cycle. They do not get exposed to the vector mites. They are extremely resistant to infection with *R. tsutsugamushi*. They do not develop chronic infections, thus, do not become carriers of the disease. In answering the question as to their susceptibility, several equally intriguing questions became apparent. Why are *C. notatus* so resistant to establishment of an infection with *R. tsutsugamushi*? Why don't they become chronically infected as the *Rattus* genera do? What type of selection pressure in nature has allowed this innate resistance to develop since they are not exposed to the vector mites and therefore *R. tsutsugamushi*?

The higher overall ratios of isolations from kidneys rather than blood are not surprising and indeed was expected. It is known that during the chronic infection stage, various organs become infected and may remain so for several months while the blood becomes negative. What is not known is how long the blood remains positive in various wild species nor how long certain organs remain positive for *R. tsutsugamushi*. Data on the latter part are very limited, however, the next section of this report gives some interesting data on chronic infection in the laboratory rat and future work is planned with *Tupaia glis* and *Rattus annandalei*. In view of the high frequency (15%) of isolations from whole blood on over 600 animals, it would seem that they have rickettsemia for prolonged periods of time, probably for weeks, otherwise, such ratios would have been unlikely. Data recently obtained on silvered leaf-monkeys indicate that some animals do have rickettsemia for prolonged periods and the same probably holds for the small mammals involved in the scrub typhus cycle in nature. We are currently addressing ourselves to that question.

The explanations for why 2% of the animals positive by blood isolations were negative serologically may be different from why 9% of the animals that were positive by kidney isolations were negative serologically. The probable reason is that the first case involves acute infection, while the latter involves chiefly chronic infections - the mechanisms of which are different. The case of 2% blood isolation positive, serology negative can best be explained by the fact that animals when first infected with *R. tsutsugamushi* may run a rickettsemia of several days before they became sero-positive by IFAT. In the case of silvered leaf-monkeys this may be as long as seven to 16 days and one can assume that the same would hold true for most other mammals. The 9% isolation positive and serology negative for kidney isolations, involving chronic infection, is more difficult to explain but again

seems to be characteristic of scrub typhus infections. In other words, it seems that it may be possible for an animal to have a chronic infection of *R. tsutsugamushi* in face of a falling or negative titer after several months. Again, the data are limited but for example three monkeys from which organisms were isolated at two and five months post inoculation all had declining or falling titers of four-fold or greater from their peak titers. In fact, two of the animals at two months had IFAT titers of only 1:80 in face of a chronic infection. Work is currently underway that should at least answer the question as to whether it is possible 12+ months post inoculation for an animal to be chronically infected yet serologically negative.

In summary, the isolation procedures used gave a high recovery frequency, in certain species, as high as 47 to 50 percent. For the first time, it has been demonstrated that adequate conjugates can be produced against wild rodent species and that the indirect fluorescent antibody test, IFAT, can be used to determine the prevalence of scrub typhus infection in small mammals involved in the infection cycle in nature, and that vertical zonation of species does affect the prevalence of scrub typhus in species found in the three zones - arboreal, semiarboreal and ground. For rearrangement of the data according to several different eco-systems involving the semiarboreal and ground species and the epidemiological and ecological significance of the data, see the Medical Ecology portion of this report.

Chronic Infection in Rodents:

General: Only a very brief comment will be made for the rationale behind these studies as the reasons were dealt with in detail in the discussion and summary portion of the previous section. Unfortunately, the challenge doses and number of animals per route of inoculation are not parallel in all cases as the animals were those left over from the preliminary studies on the pathogenesis of rodent infections. When the current investigator took over in September, 1970, it was decided that these animals could yield a significant amount of preliminary data which could be useful in planning future experiments.

Methods: The challenge strain was the mouse lethal Karp strain of *R. tsutsugamushi* and the routes of challenge were IP, 0.2 ml and IV, 0.1 ml of varying doses which ranged from $10^{1.4}$ to less than 10^{1} $MIPLD_{50}$'s for the IV route and from $10^{4.3}$ to $10^{1.3}$ $MIPLD_{50}$'s for the IP route. Isolation, organ titration and serological procedures were identical to those described in the preceding sections.

Results: The data arranged by route of challenge, dose and time post challenge and given in Tables 18 and 19. The white laboratory rat can become chronically infected as evidenced by positive isolations at 10, 12, 14 and 16 months post inoculation. All the positive isolations we: from animals inoculated via the IP route

Table 18

Chronic Infection of the Laboratory White Rat with the Karp Strain of *R. tsutsugamushi* at 10 and 12 Months Post-challenge

Challenge Dose $MIPLD_{50}$ Log_{10}	Blood	Brain	Lung	Liver	Spleen	Kidney	Axillary L. Nodes	Inguinal L. Nodes	IFAT Titer
10 Months									
Route of Challenge-IP									
4.3	-	-	-	-	-	+*	-	-	320
4.3	-	-	-	-	-	-	-	-	320
3.3	-	-	-	-	-	-	-	-	320
2.3	-	-	-	-	-	-	-	-	160
1.3	-	-	-	-	+	-	-	-	320
1.3	-	-	-	-	-	-	-	-	320
Route of Challenge-IV									
1.4	-	-	-	-	-	-	-	-	40
1.4	-	-	-	-	-	-	-	-	-
0.4	-	-	-	-	-	-	-	-	-
0.44	-	-	-	-	-	-	-	-	40
12 Months									
Route of Challenge-IP									
4.3	-	-	-	-	+	-	-	-	40
4.3	-	-	-	-	-	-	-	-	80
3.3	-	-	-	-	-	-	-	-	80
2.3	-	-	-	-	-	-	-	-	80
1.3	-	-	-	-	-	-	-	-	-
1.3	-	-	-	-	-	-	-	-	40
Route of Challenge-IV									
1.4	-	-	-	-	-	-	-	-	-
0.4	-	-	-	-	-	-	-	-	80
0.04	-	-	-	-	-	-	-	-	-

* All isolations confirmed by back-challenge, see preceding section for procedure.

Table 19

Chronic Infection of the Laboratory White Rat with the Karp Strain of *R. tsutsugamushi* at 14 and 16 Months Post Challenge

14 Months

Route of Challenge-IP

Organs

Dose $MIPLD_{50}$ Log_{10}	Blood	Brain	Lung	Liver	Spleen	Kidney	Axillary L. Nodes	Inguinal L. Nodes	IFAT Titer
4.3	-	-	-	-	+	-	-	-	40
4.3	-	-	-	-	-	-	-	-	40
3.3	-	-	-	-	-	-	-	-	40
2.3	-	-	-	-	-	-	-	-	-
1.3	-	-	-	-	-	-	-	-	80
1.3	-	-	-	-	-	-	-	-	80
Route of Challenge-IV									
1.4	-	-	-	-	-	-	-	-	-
0.4	-	-	-	-	-	-	-	-	80
0.04	-	-	-	-	-	-	-	-	-

16 Months

Route of Challenge-IP

Dose $MIPLD_{50}$ Log_{10}	Blood	Brain	Lung	Liver	Spleen	Kidney	Axillary L. Nodes	Inguinal L. Nodes	IFAT Titer
4.3	-	-	+	-	+	-	-	+	80
3.3	-	-	+	-	-	+	-	-	80
2.3	-	-	-	-	-	-	-	-	80
1.3	-	-	-	-	-	-	-	-	40

and in four of the six positive animals at a dose of $10^{4.3}$ $MIPLD_{50}$'s. Of the remaining two animals, one received 3.3 $MIPLD_{50}$'s and the other one $10^{1.3}$ $MIPLD_{50}$'s. None of the 10 animals challenged via the IV route were positive at any time, however, it must be pointed out that they received doses that were one to two log_{10} less in the majority of the cases than the IP challenged animals in which the lowest IP dose yielded only one positive out of seven rats.

There are obvious differences in organs from which organisms could be isolated. In no case were organisms isolated from blood, brain, liver or axillary lymph nodes. In those organs from which organisms were isolated, (lung, spleen, kidney and inguinal lymph nodes) the frequency of isolation varied from four out of six for the spleen to one out of six for the inguinal lymph nodes. The total isolation frequency for the IP route of challenge (the challenge dose ranged from $10^{1.3}$ to $10^{4.3}$ $MIPLD_{50}$'s) was six out of 22 and at a dose of $10^{4.3}$ $MIPLD_{50}$'s, four out of seven.

Time did have an effect on the serological IFAT titers. There was a decrease in titer between 10 and 12 months via the IP route. Of interest, is the observation that chronic infection does not appear to maintain the IFAT titer at a high level as evidenced by titers of only 40 and 80 in chronically infected animals at 14 and 16 months.

Discussion and Summary: As stated earlier these studies were unfortunately not comparable in all cases because the animals used were left over from a prior study. However, several interesting observations are still possible. One of the more interesting is that chronic infection, at least, in the white laboratory rat, does not maintain the IFAT titer at a high level and is similar to our limited data, on the silvered leaf-monkey. These observations also support our findings that in kidney isolation attempts from a large number of rodents (see preceding section) in 9% of them isolations can be made although serologically the animal is negative by IFAT.

The effect of challenge dose on the establishment of chronic infection was not unexpected and probably explains why the animals challenged by the IV route were negative. It is expected that this effect will be found in other species. The levels necessary for establishment of chronic infections is expected to vary from species to species and raises some interesting questions as regards the dose that chiggers inject.

These data also help explain why the kidney isolation rates were higher than the blood isolations for the Rodent Prevalence study. We selected the kidney for isolation studies based on prior work of others with scrub typhus and the knowledge that in chronic systematic bacterial infections the pathogen can often be isolated from kidneys when other organs are negative.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. Agency Accession	2. Date of Summary	Report Control Symbol
	30 05 71	DD-DR&E(AR)636

3. Date Prev Sum'ry	4. Kind of Summary	5. Summary Sct'y	6. Work Security	7. Regrading	8a. Disb'n Instr'n	8b. Specific Data-Contractor Access	9. Level of Sum
30 06 70		U		N/A	NL	☐ Yes ☐ No	A. Work Unit

10. No./Codes	Program Element	Project Number	Task Area Number	Work Unit Number
a. Primary		01100 and 031000		
b. Contributing				
c. Contributing				

11. Title: Investigations of Tick Typhus

12. Scientific and Technological Areas: 010100 Microbiology

13. Start Date	14. Estimated Completion Date	15. Funding Agency	16. Performance Method
10 70	9 71		

17. Contract/Grant: DADA17-71-G-9332 Mod. P-103

a. Dates/Effective: 10 70 Expiration: 10 71

b. Number:

c. Type: Y Grant d. Amount: 181

e. Kind of Award: f. Cum. Amt.

18. Resources Estimate		a. Professional Man Yrs	b. Funds (In thousands)
Fiscal Year	Preceding 71	.25	15.4
	Current 72	1.0	21.7

19. Responsible DoD Organization

Name: U.S. Army Medical Research Unit Institute for Medical Research

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Responsible Individual

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Principal Investigator

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Social Security Account Number:

Associate Investigators

Name:

Name:

21. General Use: Microbiology, Public Health

22. Keywords: Tick typhus, Hemolymph technique, Rickettsia.

23. Technical Objective, 24. Approach, 25. Progress

23.(U) Technical Objective: To make serological and epidemiological studies of tick typhus in Western Malaysia.

24.(U) Approach: Ticks will be collected from areas where tick typhus cases have occurred; a hemolymph test will be used to detect rickettsia in ticks.

25.(U) Progress: No cases of tick typhus were reported during this reporting period. Ticks collected in an area where the last reported cases of tick typhus occurred were negative for rickettsia. Hemolymph slides of 200 *Ornithodorus batuensis* ticks were examined, and at least 1 tick appeared to be positive. The hemolymph technique has also been used to detect *Rickettsia tsutsugamushi* in mite vectors.

*Available to contractors upon originator's approval.

INVESTIGATIONS OF TICK TYPHUS

General

This Unit conducts serological and epidemiological studies of all reported cases of tick typhus in Western Malaysia. No cases were reported during this reporting period.

Tick Survey

In June, 1970, a total of 99 ticks were collected from rodents and leaf litter in an area near Mersing, Johore, where the last reported tick typhus cases occurred. The ticks were triturated in phosphated buffer solution and injected into white mice and guinea pigs. Sera were collected at 28 days and examined using fluorescent antibody technique. None of the ticks were positive for rickettsia.

Hemolymph Technique

Background: Burgdofer (1970) described a hemolymph test for detection of rickettsiae in ticks. The Departments of Entomology and Rickettsiology are using a modification of this technique to screen potential vector species in West Malaysia.

Methods: The distal portion of one of the tick's legs is severed, and a small spot of hemolymph is placed on each of 3 slides. One slide is stained with Giemsa and examined for rickettsia. If rickettsia appear to be present, the remaining slides are examined using fluorescent antibody technique. The ticks are kept alive in rearing containers, and those which are shown to be positive can be retained for transmission studies.

Preliminary Results: Hemolymph slides have been made from approximately 200 *Ornithodorus batuensis* ticks (Acarina, Argasidae). Screening of the Giemsa slides is incomplete at this time, but at least 1 tick appears to be positive for rickettsia as indicated by FA examination. The tick was a male and was injected into a guinea pig. Serum will be collected at 28 days.

Projected Use: As ticks are collected they can be rapidly screened for rickettsiae. Attempts will also be made to use this technique for screening mites for scrub typhus. If successful, this technique could be used instead of slower, more costly methods for rickettsia diagnosis.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. Agency Accession	2. Date of Summary	Report Control Symbol
	30 06 71	DD-DR&E(AR)636

3. Date Prev Sum'ry	4. Kind of Summary	5. Summary Scty	6. Work Security	7. Regrading	8. Dissem Instr'n	9. Specific Data-Contractor Access	10. Level of Sum
30 06 70		U		N/A	NL	☐ Yes ☐ No	A. Work Unit

10. No./Codes	Program Element	Project Number	Task Area Number	Work Unit Number
a. Primary		81100 and 831000		
b. Contributing				
c. Contributing				

11. Title: Laboratory Animal Development and Zoonotic Diseases

12. Scientific and Technological Areas: 010100 Microbiology

13. Start Date: 10 70 | 14. Estimated Completion Date: 9 71 | 15. Funding Agency: | 16. Performance Method:

17. Contract/Grant: DADA17-71-G-9332 Mod. P-103
a. Dates/Effective: 10 70 Expiration: 10 71
b. Number:
c. Type: Y Grant d. Amount: 181
e. Kind of Award: f. Cum. Amt.

18. Resources Estimate		a. Professional Man Yrs	b. Funds (In thousands)
Fiscal Year	Preceding 71	1.0	6.0
	Current 72	1.0	7.2

19. Responsible DoD Organization
Name: US Army Medical Research Unit
Address: Institute for Medical Research
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Responsible Individual
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20. Performing Organization
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Principal Investigator
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Telephone:
Social Security Account Number:
Associate Investigators
Name:
Name:

21. General Use

22. Keywords: See continuation sheet

23. Technical Objective, 24. Approach, 25. Progress

23.(U) Technical Objective: To update the management, facilities and diets of the research and breeding colonies of laboratory animals, to study and develop procedures that could be used for maintaining the silvered leaf-monkey, to start a laboratory colony of tree shrews and develop procedures that would allow for colonization of the wild rat, *Rattus annandalei*, and to further develop the procedures for colonization of mouse deer, to demonstrate the presence of tropical canine pancytopenia in Malaysia and determine the prevalence of leptospirosis and melioidosis in cattle and swine in Malaysia.

24.(U) Approach: In order to update the laboratory animal colonies, they will be reorganized, new diets obtained commercially and new caging built; groups of silvered leaf-monkeys will be procured and extensively studied during the conditioning period in order to determine the problems of maintaining this species and the solutions or procedures that work best; a colony of tree shrews will be started following procedures determined by others and to attempt to use similar caging, diet and procedures for colonizing the wild rat, *Rattus annandalei*; to study mouse deer that die shortly after arrival at the laboratory, attempt to determine the cause of death and initiate preventive measures and to start several breeding pairs to determine if they will breed and conceive in the laboratory; to examine blood from several suspected cases of tropical canine pancytopenia, to determine if the inclusion bodies of *E. canis* could be found and if the clinicopathological results of these animals were in agreement with published data; to obtain serum samples on large numbers (over 2,000) of cattle and swine in Malaysia and determine what the prevalence ratios were to leptospirosis and melioidosis antigens.

*Available to contractors upon originator's approval.

DD Form 1498, 1 Mar 68. 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

25.(U) Progress: The management of both the research and breeding colonies was reorganized in such a way that responsibility was defined and standardization could be implemented throughout. For most species complete new caging was built following the recommendations of the National Science Council and the American Association for Laboratory Animal Science. For the standard laboratory animal species, diets were formulated and a local feed manufacturer in Singapore is now producing them commercially. For the new species, silvered leaf-monkeys, tree shrews, *Rattus annandalei* and mouse deer, various diets were tested and have reached a stage of development where these species can be maintained and, in some cases, bred in the laboratory. It was demonstrated that mouse deer and *Rattus annandalei* can be bred successfully in the laboratory. The presence of tropical canine pancytopenia was documented in Malaysia. Serological data indicate that melioidosis is not a major problem in domestic livestock in Malaysia.

Item 22

Silvered leaf-monkeys, *Presbytis cristatus*, *Rattus annandalei*, tree shrews, *Tupaia glis*, mouse deer, *Tragulus javanicus*, tropical canine pancytopenia, leptospirosis, melioidosis.

LABORATORY ANIMAL DEVELOPMENT AND ZOONOTIC DISEASES

During this reporting period the Department of Laboratory Animal Development and Zoonotic Diseases devoted most of its effort to laboratory animal problems, care and development as the need was extremely urgent in support of programs in the Departments of Rickettsial Diseases, Bacterial Diseases and the Malaria program. A small effort was made to begin a program on Tropical Canine Pancytopenia and to continue the Leptospirosis and Melioidosis programs.

The objectives of the program were:

1. Reorganization and Animal Diets: To reorganize the laboratory animal management and facilities at the USAMRU in such a way that proper management, control and care could be provided and to obtain and, if needed, develop optimum diets for the various species.

2. Breeding Colonies: To work with the staff at the IMR in reorganizing the breeding colonies and institute procedures and methods which would assure all divisions of the IMR of an adequate supply of healthy animals.

3. Silvered Leaf-monkeys: To study and develop procedures that would allow the silvered leaf-monkey to be used as a laboratory animal.

4. Mouse Deer: To further refine the colonization procedures used for the lesser mouse deer, *Tragulus javanicus*, and determine if they could be bred successfully in the laboratory.

5. Colonization of *Tupaia glis* and *Rattus annandalei*: To study and determine if a small mammal, *Rattus annandalei*, could be colonized, bred and used as a laboratory animal and to start a colony of *Tupaia glis* following procedures developed elsewhere because both of these species appear to be involved in the disease cycle of scrub typhus in nature and are needed for laboratory studies involving the antigenic stability of *R. tsutsugamushi*.

6. Tropical Canine Pancytopenia: To determine if the inclusion bodies of TCP could be found in dogs in Malaysia and if chronic cases could be located.

7. Leptospirosis and Melioidosis: To continue a program started during the last reporting period on determining the prevalence of leptospirosis and melioidosis in cattle and swine in Malaysia in cooperation with the Veterinary Research Institute of Malaysia.

Reorganization and Animal Diets

Problem: Upon arrival of the present investigator, the need for laboratory animals had increased in both numbers and species. In addition, preliminary work in other departments had developed to the degree that their demand had also increased. Neither the research colony of the Unit nor the breeding colonies could handle this demand under the existing setup. An updating of facilities and caging was urgently needed before any expansion could be considered. In past years the demand for laboratory animals had not been as great and each department had been responsible for the procurement and care of their own animals. This divided responsibility obviously could not be continued in the face of increased demands.

Reorganization and Improvements: The first step was to place all of the animal men directly under and responsible to the Chief of the Department of Zoonotic Diseases who is now responsible for maintenance and care of all laboratory animals in the experimental colony regardless of which department is utilizing them. This has allowed better utilization of existing facilities and standardization of diets, cleaning, disinfecting and caging. Any of the above are modified to the investigator's requirements for particular needs.

Diet: In cooperation with the feed manufacturer, diets were formulated and standardized for all of the common species of laboratory animals. The only diet available previously was a mouse and rat chow, which was found to be inadequate both in formulation and pellet durability. There are now three diets being produced, a mouse and rat chow, a primate chow and a rabbit and guinea pig chow. The problem of durability of the mouse and rat pellet was solved by adding approximately 8-9% tapioca to the formulation. It is an excellent binder, provides a readily available carbohydrate source and its palatability is good. The diets have been in use now for 8 months and all animals appear to be doing well.

The availability of three commercial diets greatly reduced the number of supplements needed and in the case of rabbits and guinea pigs provided an obviously healthier animal. Table 1 gives the current species maintained and their diets. The diet for the silvered leaf-monkeys still needs improvement; however, PCV (packed cell volume) results show that progress has been made on the diet. In one group of eight animals on the previous greens diet the mean PCV was 32 on arrival at the laboratory and 36 after 5 weeks of conditioning while four monkeys which started eating primate chow increased their mean PCV by over 10 points (from 31 to 43) during the same period. When the Unit first started working with this monkey, the diet consisted mostly of greens, including green beans, *kangkong* (similar to spinach) and sweet potato leaves. It became evident that this was not adequate and it was changed (see Table 1). The only supplement given now is a multivitamin which contains lysine. This is added to the drinking water daily.

Table 1

Current Species of Animals in the Experimental Colony and their Diets

Species	Diet
Dogs	1) Dog chow[1]
Primates	
Pigtail macaques	1) Primate chow[2] 2) 1 orange and 1 apple twice a week
Longtailed macaques	1) Primate chow[2] 2) 1 orange and 1 apple twice a week
Silvered leaf monkeys	1) Primate chow[2] 2) Sweet potatoes, green beans and a few sweet potato leaves daily
Tree shrews	1) Dog chow[3] 2) Mouse and rat chow[2]
Mice	1) Mouse and rat chow[2]
Rats (white laboratory)	1) Mouse and rat chow[2]
Rabbits	1) Rabbit and guinea pig chow[2] 2) A few leaves of *kangkong* daily
Guinea pigs	1) Rabbit and guinea pig chow[2] 2) A few leaves of *kangkong* daily except in the breeding colony where mustard greens are substituted for *kangkong*
Mouse deer	1) Rabbit and guinea pig chow[2] 2) *Kangkong* and green beans 3) Sweet potatoes 4) Apples and oranges
Wild rats *(R. annandalei)*	1) Dog chow[3] 2) Mouse and rat chow[2]

Dog Chow[1]: Lucky Dog Dinner[R], I.G.Y. Veterinary Products, a Division of Marrickville Holdings, Ldt., Sydney, Australia.

Primate, mouse and rat, rabbit and guinea pig chow[2]: Produced in pellet form by Gold Coin Mills, Singapore - For formulations see TB MED 255, October 1967 and the Purina Laboratory Manual. Any differences in concentrations between the two recommendations were resolved in favor of the higher concentration.

Dog Chow[3]: Chuck Wagon, Soft Moist dog food; Diamond Foods Ltd., Osborne Park, West Australia.

Kangkong which is added to the diet of rabbits, guinea pigs (other than breeders), and mouse deer is a green closely related to spinach. It provides mainly fiber (needed for rabbits and mouse deer) as well as vitamins, particularly Vitamins C and K. For the breeding guinea pigs, mustard greens are used in place of *kangkong* as they are more closely equivalent to kale which is used in guinea pig colonies elsewhere.

The diet for the mouse deer was not changed during the last year except to add rabbit and guinea pig chow and the multivitamin-lysine preparation to the drinking water. This was because one objective was to determine if they would breed under laboratory conditions and, therefore, manipulation of the diet was contra-indicated. Experiments for next year are planned to determine the optimal diet for the mouse deer. Preliminary results indicated that rabbit and guinea pig chow, *kangkong* and a vitamin supplement may be all that is needed.

The diet used for tree shrews and wild rats is one that was worked out previously at SEATO Medical Laboratories. These species also receive the multivitamin and lysine supplement in their drinking water. For the tree shrews, the diet is mixed at the ratio of three parts dog chow to one part mouse and rat chow. For the wild rats it is a 1:1 ratio. Neither species will eat hard dog chow which confirms the findings of the SEATO Medical Laboratory.

Breeding Colonies

The breeding colonies at the IMR furnish animals for all the divisions of the IMR. The mouse breeding colony had undergone a complete change of breeding stock (NCI mice) during the last reporting period. The breeding system followed at SEATO Medical Laboratory had been instituted also during that time. No changes were required in breeding stock or procedures during this period. Previously, the diet consisted of five different items (mouse and rat chow, green peas, bran, rolled oats and grass). Following the availability of an adequate diet from a commercial source, they were put on a diet of mouse and rat chow only. No disease or nutritional problems were encountered in the mouse colony during this reporting period. There are currently 1700 breeding females averaging a litter size of 9.3 with a weekly production of 2,000 weaned mice and 200 mouse litters, two days old, for viral isolation work.

New programs and the demands of old ones made it evident that the rabbit and guinea pig colonies had to undergo complete changes in caging, breeding stock, diet and procedures in order to meet the increased demand. The cages that were being used were of a 20 year old design, constructed of wood and were not amenable to proper sanitation procedures nor feeding practices. The breeding stock was old and no new stock had been introduced for several years.

During this reporting period complete new caging was made locally for both colonies following designs recommended by the National Science Council of the U.S. and the American Association for Laboratory Animal Science. Completely new breeding stock was obtained for both colonies and they were increased from 10 to 30 breeders for the rabbit colony and from 20 to 140 for the guinea pig colony.

Following consultations with the Chief of the Department of Veterinary Medicine at NAMRU-2 in February 1971, the breeding procedures for the rabbit colony were completely changed to those followed at NAMRU-2 except for their artificial insemination procedure which hopefully can be instituted within the next year. The NAMRU-2 procedure is essentially one of separating the mother from her young when they are born and then placing her in with her young three times a day, holding her while the young nurse and then removing her. This is done for 10 days at which time the mother is allowed full freedom of the nesting box. In our Unit, during the last reporting period, the average litter size weaned was approximately 2.5. Following a complete change of breeding stock, cages, diet and procedures, the average litter size is now 5.5 and it is anticipated that following the institution of artificial insemination it will increase to over seven.

The diet for the rabbit colony was changed from mouse chow, green peas, bran and grass to one of rabbit and guinea pig chow with a daily supplement of *kangkong*.

Disease problems in the rabbit colony were limited to otoacariasis (ear mites) which was severe and involved practically all of the old breeding stock. Not only were the ears involved, but the feet and toes as well. A fungus infection had developed under the mite incrustations making the condition worse. The animals were dipped in Malathion (25 ml of 57% solution in 4 gallons of water) once a week for three weeks and the cages sprayed with D.D.T. 25%. The fungal infection was treated with Catallenis solution. These measures served only as stop-gap procedures until the old breeding stock could be replaced and new caging procured. During the last five months only an occasional case of otodectic mange has occurred and these have responded dramatically to treatment with a mineral oil and rotenone mixture applied locally to the affected site once a day for seven days.

The guinea pig breeding colony underwent a complete change of breeding stock, diet and caging. In addition, it was expanded from 20 breeders to 140. The breeding stock procured was all local multicolored stock as they were immediately available. However, it is planned that within the next few months laboratory albino stock will be introduced from the colony at the SEATO Medical Laboratory. Pullout tray type cages are used for the breeding stock with four females and one male per tray. The trays are of stainless steel

construction and fit into aluminum frames. The diet was changed from mouse pellets, grass and green peas to one of rabbit and guinea pig chow plus mustard greens. Before the above changes the average litter size was two and is now approximately three weaned offspring. After consultations with laboratory animal personnel at the SEATO Medical Laboratory and at NAMRU-2, we doubt that litter size can be increased much beyond 3 to 3.5 in the tropics without elaborate air conditioning and air handling equipment.

Since there has been little demand for either hamsters or white laboratory rats, the colonies have not been brought up to standard yet. The diet was changed to rat and mouse chow. It is planned to improve these two colonies next year.

Silvered Leaf-Monkeys (*Presbytis cristatus*)

Management and Care: Last year's Annual Report described the caging designed for the silvered leaf-monkey. The three main points on caging are: 1) It must be at least five feet high and contain a perch that is approximately 3½ feet from the floor of the cage. 2) All feeders and water devices must be at perch level or higher and greens must be placed on top of the cage. 3) All feeding and watering devices must be designed to be placed inside the cage so that the animal does not have to reach through to obtain his food. This is only one of the major differences between the *Macaca sp.* and the silvered leaf. The silvered leaf will go hungry to the point of starvation before he will reach his hand through an opening to obtain food; therefore, all feeders have wide open tops. It was also found that the common type watering devices used for the *Macaca species* are unsatisfactory. The current watering method involves an open pail (4 quarts) placed at one end of the perch. If it is placed on the floor of the cage, they will only occasionally come to the floor to drink. All of those requirements are derived from this monkey's arboreal habits. Another type cage which was made from specifications for the "large primate cage" designed and reported several years ago at the SEATO Medical Laboratory was tried. This is an excellent cage and has great versatility as it can be used for silvered leaf-monkeys, gibbons and long tailed macaques. The only change made in the specifications was that the door was moved from the end of the cage to the side. This allows greater access to the interior of the cage and facilitates catching of the animals.

They are housed three to five per cage depending on the size of the monkeys. Fights are rare between females and only occasionally occur between males. In fact, they sit very close together on the perch and it is often difficult to count them. The same type of social behavior has been noted in nature. They are even easier to catch than gibbons and all that is required is a pair of "primate" gloves. During this reporting period we have had a total of 89 silvered leaf-monkeys with varying numbers at any one time. These have

been handled on almost an every other day basis and in the case of 16 every day for a period of two months without a single serious bite. In fact there have been only a few minor skin scratches among the animal handlers.

Diet: The diet is composed of primate chow, green beans (long beans), raw sweet potatoes and a few sweet potato leaves (see Table 1). All greens are washed in a Chorox[R] solution. During the last year several changes were made and it is not felt that even the current diet is optimum.

In one group of 30 animals, it was observed that several animals suddenly collapsed and died within an hour or two following feeding. No evidence of illness had been noted. Upon obtaining blood samples from one animal that had collapsed it was observed that the blood was dark chocolate brown, almost black, in color. It was suspected to be a case of metahemaglobinemia and the animal was treated with methylene blue (4% solution, 10 cc, IV) with immediate clinical response. That sample plus samples from the remaining leaf monkeys were sent to the Division of Biochemistry at the IMR for analysis of hemoglobin and metahemoglobin levels (see Table 2), 75% showed increased levels of metahemoglobin (over 1%). All were treated with methylene blue successfully. A nitrate poisoning was suspected as the silvered leaf-monkey has a rumen-like type digestion and should be able therefore to convert nitrate to nitrite as the bovine species do. Upon consultation with local chemists and nutritionists, *kangkong* was suspected as being involved. A local primate exporter had also observed that feeding of *kangkong* was lethal to silvered leaf-monkeys. The feeding of *kangkong* was immediately discontinued and no further deaths have occurred due to what appears to be metahemoglobinemia. It is planned to investigate this further.

Since 53 animals from whom blood samples were obtained at the time of capture were all negative serologically for Salmonella and Shigella antibodies it is assumed that they have never been exposed to these human diarrheal pathogens; therefore, they would be expected to be extremely sensitive to infection with these organisms. Because of this, we wash and soak all greens and sweet potatoes in a chlorine solution before feeding and, in addition, during the first four weeks, soak all greens and sweet potatoes in a solution of tetracycline (60 mg per liter of water). The tetracycline is also added to the drinking water (125 mg/liter) for five weeks. The results of our investigation into the disease problems of this species and isolations are discussed below.

Survival and Disease Problems in the Laboratory: Since there is little known about conditioning of silvered leaf-monkeys it was decided to bring a large group into the laboratory and determine the best procedure as the animals were urgently needed for the scrub typhus program. Thirty animals were procured from a local exporter.

Table 2

Metahemoglobin Levels on a Group of Silvered Leaf-Monkeys Following a Suspected Case of Metahemoglobinemia

Number	Physical Condition	Color of Blood	Metahemoglobin (% of total hemoglobin)
1	Collapsed state	Chocolate	10.5
	Normal-12 hours later following treatment	Lt. brown	1.81
2	Normal	Brown	2.04
3	Normal	Brown	2.00
4	Normal	Brown	1.40
5	Normal	Lt. brown	1.31
6	Normal	Brown	1.27
7	Normal	Lt. brown	1.24
8	Normal	Slightly darker than normal	0.82
9	Normal	Red	0.79
10	Normal	Red	0.64
11	Normal	Red	0.51
12	Normal	Red	0.20

In most cases, the animals were delivered to our laboratory within 72 hours of capture. Twenty five other animals were kept at the dealer's primate holding facility for conditioning. Thus a total of 55 monkeys began the conditioning period during February and March 1971. For details on the survival of the 30 in our laboratory see Table 3.

Table 3

Survival Rates and Causes of Death in the First Group of 30 Silvered Leaf-Monkeys brought into the Laboratory

Number	Status	Percent
12	Survived	40
18	died	60
30		

Cause of Death	Number	Percent
Diarrhea	12	67
Exhaustion*	2	11
Metahemoglobinemia**	2	11
Suspected Viral***	2	11
	18	100

* Exhaustion: Arrival at the laboratory in a state of exhaustion and dying within five days of arrival.

** Metahemoglobinemia: Animals that died suddenly on the same day that the diagnosis of metahemoglobinemia was made on one case that was in a state of collapse, see Table 2.

*** Suspected Viral: Diagnosis made on the basis of clinical impression and a leukopenia.

In summary, it can be seen that only 14 (12 in our laboratory and 2 at the exporters) of the 55 (26%) survived. Twelve (12) of 30 (40%) survived in our laboratories where they received intensive veterinary care while only 2 of 25 (8%) survived at the exporter's facility. Although even our experience was poor, valuable data were collected on the 30 in our laboratory. The causes of death, as seen in Table 3, could be broken into several etiologic categories.

One cause (metahemoglobinemia) has been discussed above under the heading of "diet". The most common cause of death was diarrhea and accounted for 67% of those dying in our laboratory. For a listing of isolates, see Table 4.

Table 4

Isolates Obtained from Five Monkeys which had Enteric Pathogens

Monkey	Isolate
1	*Salmonella* D
2	Pathogenic *E. coli* O111:B4
3	*Salmonella* B
	Salmonella D
	Salmonella E1
4	*Salmonella* E1
5	Pathogenic *E. coli* O119:B14

Isolates of enteric pathogens were made from 5 of the 19 animals cultured. Many of those dying from salmonellosis had an acute peritonitis due to perforations of the gastointestinal tract. Also because the animal handlers were inexperienced in passing a stomach tube correctly, several died from aspiration pneumonia caused by kaolin and pectin being inadvertently injected into the lungs. After several isolates were made, no further bacteriology was done because of pressing requirements of the programs in the Department of Bacterial Diseases. It is hoped that a complete study of this can be done within the coming year. However, a bacterin (10^8 organisms/ml) composed of four of the isolates obtained from the monkeys, was made by the Department of Bacterial Diseases, see Table 4, and is currently in use. Of interest was the fact that several antispasmodics were tried for the prevention of excessive fluid and electrolyte loss by diarrhea, but without success. The animals appearred to respond to this class of drugs and tranquilizers differently than *Macaca sp.* and man in that they require doses of 10X or higher for even a minimal effect.

The animals also develop what appears to be very similar to the human "common cold". No investigations into the etiology of this have been made to date. They generally respond to symptomatic treatment of antihistamines and nose drops, a few develop pneumonia

which in most cases responds to treatment with penicillin. Penicillin is now used prophylactically to prevent pneumonias for the first three weeks following capture.

A smaller number (2) of animals died very suddenly of an unknown cause. Since few if any lesions are found on gross pathology and there is a leukopenia before death, it may be viral in etiology. Histopathology (Department of Experimental Pathology, SEATO Medical Laboratory) has not been able to give us a clue yet. It is hoped that during the next year an investigation into this can be done in collaboration with the Division of Virology at the IMR.

A second group of 41 animals were brought into the laboratory in late May and early June 1971. All of the procedures, diet, etc., mentioned above were instituted at this time and in addition, the procurement procedure was changed. Rather than have the local exporter deliver the animals to the laboratory, arrangements were made to have one of our technicians present when the animals were caught. The technician was to immediately take charge of them, and bring them directly to the laboratory so that the time from capture to our laboratory was not longer than six hours. At the time our technician took possession of them in the jungle he did the following: 1) took fecal and blood samples, 2) gave them injections of penicillin, promazine and a bacterin composed of previous isolates, see Table 4, 3) marked each animal for future identification, 4) placed them in cages which were high enough (2½ ft.) to allow them to stand up and move about and which had a water pail in it so that they could immediately drink. Upon arrival at the laboratory they were immediately fed and watered. If they appeared to be in a state of exhaustion and dehydration from capture they were encouraged to drink by manually placing their heads into water pail every hour. Nothing further was done for two days other than treat wounds resulting from capture. On day three, they were given another injection of long acting penicillin and 1 cc of B complex if they were exhibiting anorexia. On day seven they were tattoed and weighed, and the temperature, blood and fecal specimens taken. They were given more injections of penicillin and the bacterin. Then once every two weeks for the rest of the conditioning period, they were weighed, samples taken, temperature taken, etc. The bacterin is repeated again at 28 days (the third dose). Table 5 summarizes the data on this group.

Table 5

Survival Rate and Cause of Death in a Group of 41 Silvered Leaf-Monkeys

Status	Number	%
Survived	29	71
Died of Exhaustion	10)	29
Suspected "Viral"	2)	
	41	100

The differences between the first and second group are interesting. First, there were no clinical diarrheas which had accounted for 67% of the deaths in the first group of 30, nor were there any cases of metahemoglobinemia in this group. However, the number and percentage of deaths due to exhaustion did increase. This was the result of our receiving them immediately upon capture and in seven cases the investigator's instructions not being carried out. In the prior group of 30, we assume that the exporter had already lost most of the cases of exhaustion before we received them at the laboratory. Upon detailed questioning it was revealed that in three cases of exhaustion death out of 24 animals handled according to the above protocol, that the animals had been pursued in the jungle for over 30 minutes before they were caught. In the remaining 17 animals, there was a break in procedure in that the catchers had run the monkeys down the day before and held them under very poor conditions with limited food and water overnight before we picked them up. The results were that seven out of 17 (41%) died of exhaustion. It is felt that most of these deaths due to exhaustion can be eliminated by strict adherence to our protocol and setting a time limit whereby if a monkey is not caught within 30 minutes no further pursuit is made.

Normal CBC Data: Since little or nothing is known about this animal in the laboratory it has been necessary to obtain data on "normal values". The data presented in Table 6 are preliminary in nature. It appears that the animals are anemic in the wild as evidenced by the increase in packed cell volume, PCV, after a few weeks in the laboratory. At least a portion of the anemia appears to be microcytic as the RBC counts do not increase parallel to the PCV. In the future hemoglobin determinations can be performed on micro samples. Other "normal" blood chemistry values will be studied also. The increase in WBC count is not significant as the upper limit of the monkeys on arrival and the lower limit after conditioning overlap.

Table 6

Blood Cell Values and Temperatures on a Group of Silvered Leaf-Monkeys during the Five Week Conditioning Period

	On Arrival (N=17)	After 5 Weeks (N=12)
White Blood Cell Count cells/ml		
Mean	7,756	10,558
Range	(6,436 - 9,076)	(8,622 - 12,594)
Red Blood Cell Count (X 10^6) cells/ml		
Mean	4.48	4.27
Range	(4.10 - 4.86)	(4.03 - 4.51)
Packed Cell Volume (%) %/ml		
Mean	32	37.8
Range	(30.8 - 33.2)	(35.6 - 40.0)

Temperature (4 readings on 12 monkeys over a five week period)

Mean (N=48)	101.2°F
Range	(101.2 - 102.1)

Differential on arrival (N=17)

Cell Type	Percent	Absolute Counts (Mean)
Basophiles	0.3	21
Myelocytes	1.0	110
Eosinophiles	1.0	110
Monocytes	3.0	200
Lymphocytes	35.2	2,680
Neutrophiles		
Segmented	59.0	4,619
Bands	0.3	21
Juveniles	0.2	13

It may be that with more animals this will become significant. It is extremely encouraging that the standard error for the rectal temperature is so small, which is in contrast to the *Macaca sp.* This means that hyper or hypothermia in this species can be accurately measured an even fairly small changes could be significant.

Mouse Deer

General: Last year this Unit reported on the successful maintenance of the lesser mouse deer, *Tragulus javanicus*, in the laboratory. During this reporting period the studies were expanded to include some improvements in diet (see Table 1, and discussion under diets), cage improvement, investigations into the cause of the high mortality rate upon arrival at the laboratory and its prevention, the first serious attempts at breeding in the laboratory and very preliminary work on normal blood values.

Cage Improvement: The original cages were built following a design worked out for the larger species of mouse deer. It was observed that if the cages were decreased in height which was excessive that they could be stacked 3 high thus allowing more animals to be kept per unit of floor space. Also, it was observed that the cages were too large for newly arrived animals in that they injured themselves in racing about the cage. Two sizes of cages were designed, built and are now being successfully used. Both are the same height, 18 inches, and are stacked three high to each unit for an overall height of 70 inches. The regular maintenance and breeding cages are 2 feet wide, 4 feet long and 18 inches high. They are designed so that a sliding panel can be inserted from the outside, converting them to two smaller cages. The cages used to hold the mouse deer for the first four weeks in the laboratory are 2 feet wide and 2 feet long with no projections inside the cage on which the animals can harm themselves. This rapidly proves to the newly caught animal that he cannot get away and they settle down much faster than before.

Bedding consists of wood shavings which are placed in the cage to a depth of at least two inches. It was found that anything less than this would allow the animals to slip and injure themselves and was not enough to keep the cage dry. Feeders and waterers consist of two small bowls placed in wooden holders with an aluminum band two inches wide being bent in the shape of an arch over the water bowl. The aluminum arch is necessary to keep the animals from defecating in their water bowls.

Survival Rates: Survival rates when the study was started two years ago were very poor (see Table 7). Several necropsies were performed on animals dying without any gross pathology being evident during the first week of captivity. Bacterial cultures done prior to the arrival of the present investigator had also failed to demonstrate

any pathogen. In addition, the history of the animals appearing to be in good condition that morning and being dead before the next morning was not consistent with most infectious organisms. Following several weeks of direct observations of the animals by the investigator, it was concluded that they appear to be in a state of shock which had a rapid onset and short duration. The condition seemed to be similar to a condition in the toy breeds of dogs in which they develop severe hypoadrenocorticism, concomitant hypoglycemia and very rapidly go into a state of shock and total collapse following an abnormal period of stress. Other similar conditions in domestic cattle are "milk fever" and acetonemia. All of the above conditions are treated similarly during the acute state, parenteral administration of calcium gluconate with or without dextrose plus adreno-corticosteroids. In toy breeds of dogs the oral administration of adreno-corticosteroids from 24 hours before until 48 hours after periods of abnormal stress has been shown to prevent the condition. Therefore, it was decided to try similar prophylaxis with the mouse deer. See Table 7 for overall results.

Table 7

Survival Rate of Mouse Deer in the Laboratory

Time Period	Total No. of Animals	Number Surviving*	%
Oct 69 to Dec 70	67	15	22
Dec 70 to June 71**	25	17	68

* An animal that lives beyond 2 months in the laboratory.

** Prophylactic treatment given upon arrival. Sick animals received treatment for hypoadrenocorticism and hypoglycemia.

Mouse deer receive calcium gluconate (10%, 10 cc, IP, upon arrival and at the first signs of shock) and prednisolone acetate (25 mg, subcutaneous).

Beginning in December, animals were divided into four groups based on accession; one group to receive prednisolone, one to receive gluconate, one to receive both and the last to be a control. The technicians responsible for administering the drugs didn't follow the outline in detail and the group sizes are not equal (see Table 8 for treatments and results). This was because the study wasn't a double

blind study and the technicians recognized early in the experiment that calcium gluconate prevented death and therefore biased the study. This makes the results even more valid since the animals in the worst shape were treated while the healthy ones were used as controls. It is too early yet to state positively whether one of the two drugs or the combination is best. However, there is no question that treatment is effective.

Table 8

The Effect of Different Prophylactic Treatments on the Survival Rates of Mouse Deer in the Laboratory

Prophylactic Treatment	Total No. of Animals	No. Died	Day of Death	No. Surviving/ Total
Calcium gluconate	11	2	1,1	9/11
Prednisolone	9	3	1,14,30	6/9
Calcium gluconate) plus prednisolone)	2	0	-	2/2
Untreated controls	3	2	24,47	1/3

Histopathological results on a few animals (Department of Experimental Pathology, SEATO Medical Laboratory) confirmed that the animals were dying of shock and histochemical staining of the tissues is now being done.

Breeding: During this reporting period 9 offspring have been conceived and born in the laboratory (see Table 9 for the results of 5 breeding pairs kept under close observation). The other 3 offspring were from mothers kept under semi-natural conditions in a small pen. One pair have produced two offspring during this reporting period. No evidence of twinning, common in the true deer, has been observed yet. One neonatal death occurred when the mother was under severe stress and one abnormal fetus from a mother who was pregnant upon capture caused a case of dystocia. A caesarean section was performed after 24 hours of labor and the dead fetus removed. The mother only lived another eight hours because of the prolonged labor before surgery. The two deaths that occurred in animals several weeks old occurred at the time of the floods and heavy rains in January 1971. During this time it was impossible to provide proper food, care and observation and it is felt that they died of "stress shock" which went undetected and untreated.

Table 9

Production Results from 5 Breeding Pairs of Mouse Deer

Pair Number	Date Put Together	Number of Offspring	Current Status of Offspring
1	Oct 69	2*	Both died**
2	June 70	1	Living
3	June 70	1	Living
4	Feb 71	1	Living
5	Feb 71	1	Living
		6***	

2*: First birth was 24 June 70 and second one was 15 June 71.

Both Died**: The one born 24 June 70 died during the floods of Jan 71 due to what was considered "stress shock", the other one fell out of the cage and fatally injured itself.

6***: In addition 3 additional offspring (1 still living) were conceived and born in another facility. One of the two died during the floods of "stress shock" and the other one died within five minutes of birth.

Surgery: The techniques of splenectomy were worked out and two splenectomized animals furnished to the Entomology Department for malaria studies.

Normal Blood Values: Preliminary observations were made on 15 animals during this reporting period, WBC 7,560, range 6,800 to 7,900; PCV 50, range 36.5 to 55.5; RBC count over 70 million per milliliter. Techniques of counting RBC's at this level still need perfecting before accurate counts can be made. The RBC count in this species is the highest known for any mammal. Studies have been initiated on the size and shape of the RBC's and in cooperation with the Department of Experimental Pathology, SEATO Medical Laboratory on the RBC development series in the bone marrow. It is also hoped that the hemoglobin type and content of the cells can be determined.

Summary: It has been demonstrated that this species can be maintained in the laboratory (21 months to date), that survival rates of wild caught animals can be made very acceptable (68), and that they will breed and produce live offspring in the laboratory, as all breeding pairs to date have produced offspring.

Plans: Work with this species in the malaria project will be expanded during the coming year and it is hoped that a number of animals can be challenged with the common bovine diseases to determine if they can be used to study these in the laboratory.

Colonization of *Tupaia glis* and *Rattus annandalei*

General: The studies of the antigenic stability of *R. tsutsugamushi* and the study of chronic infection in animals frequently infected in the wild both required laboratory reared animals. Wild caught animals could have been used except that there is no way to be sure that they have not had an infection by *R. tsutsugamushi*. The first species selected was the tree shrew, *Tupaia glis*, because of its predicated involvement in the disease cycle in nature and the fact that it had been successfully colonized previously. The other species, *Rattus annandalei*, was selected after data became available from a recent study of over 40 species of small mammals for prevalence of infection (see the scrub typhus section of this report).

Caging: Cages were constructed locally of the design developed at the SEATO Medical Laboratory for tree shrews.[1] This cage has been used for both *Tupaia glis* and *Rattus annandalei*. Two females and one male are placed in each cage.

Survival and Production: No losses occurred in the colony of 21 tree shrews nor in the 13 *R. annandalei*. Both colonies were started in early 1971. Presumably the diet outlined in Table 1 is adequate. The rats started eating the diet immediately. On the other hand, it took 10-14 days to get the tree shrews to change over. Diets are supplemented during this time with fresh fruits but these are gradually decreased during the 2 week period.

To date no offspring have been produced by the tree shrew colony. However, recent consultation with one of the developers of the first colony at SEATO Medical Laboratory revealed that it often took 6-9 months with wild caught animals before any production was realized and that wild caught animals never produced as well as the F-1 generation.

The *R. annandalei* colony has produced 29 offspring to date from 8 females. The average litter size weaned was 3.6 and ranged from a low of 1 to a high of 7. Of 23 weanlings sexed, there were 9 males and 14 females. Until recent work done by the Department of Medical

Ecology of this Unit, this rat was considered a rare species. To our knowledge this is the first time that this species has been colonized.

Tropical Canine Pancytopenia

General: This was a new project and due to other urgent requirements little progress was made. It is hoped that another veterinary corps officer will be assigned to the Unit so that more extensive work can be done.

Results: Our first objective was to document the presence of TCP in West Malaysia. This has been done by demonstrating the inclusion bodies of *E. canis* in suspected cases. The clinicopathologic data are in agreement with published results. Figure 1 shows inclusion bodies in circulating lymphocytes from a recently documented case.

Melioidosis and Leptospirosis

General: Work on the project this year was confined to serologic surveys of cattle and swine in West Malaysia to determine prevalence ratios. Since the leptospirosis hemolysis (HL) test is not reliable for cattle and swine sera, plate antigen was obtained from Difco Laboratories for all six pools. When we tried to standardize the leptospirosis plate test, we discovered that 4 of the 6 pools were defective in that they agglutinated with negative control sera or saline or both. Antigens have been reordered and will be checked by the WHO Reference Laboratory, Division of Veterinary Medicine, WRAIR before shipment to us. Recent consultation with the WHO Reference Laboratory indicated that difficulties with these antigens were becoming common and that it might be some time before a satisfactory lot would be available.

Results: Table 10 gives the results using the melioidosis hemagglutination (HA) antigen. The ratio of positives is much lower than was expected.

Plans and Summary: During next year, another 1,000 sera which are already on hand will be tested. The leptospirosis plate agglutination antigen will also be used if it is available. These results suggest that melioidosis in domestic livestock, cattle and swine, is not a problem in Malaysia.

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Table 10

Prevalence Rates of Melioidosis in Cattle and Swine in Malaysia as Determined by the Hemagglutination Serological Test

Species	Total Number Tested	% Negative	% Positive (1:40 or >)
Swine	202	99%	1%
Cattle	907	99%	1%

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APPENDIX

List of Investigators

U.S. Army Medical Research Unit

Commanding Officer	-	COL Francis C. Cadigan, Jr., MC
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Medical Officer	-	MAJ Kay A. Kyser, MC
Veterinary Officer	-	MAJ Jerry S. Walker, VC
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Ecologist	-	CPT Illar Muul, MSC
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Administrative Officer	-	CPT George A. Kirschbaum, Jr., MSC[3]

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Dr. Lim Teong Wah, Senior Virus Research Officer, IMR.

Mr. Cheong Weng Hooi, Senior Entomologist, IMR.

Mr. M. Nadchatram, Acarologist, IMR.

Mr. Yap Loy Fong, Senior Laboratory Assistant, IMR.

Dr. Malcolm Bolton, Senior Medical Officer, Department of *Orang Asli* Affairs, Kuala Lumpur.

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1 - Departed for USA, December 1970
2 - Arrived from USA, March 1971
3 - Arrived from USA, February 1971