VECTOR BIOLOGY & CONTROL

Vector Biology & Control Project
1611 North Kent Street, Suite 503
Arlington, Virginia 22209
(703) 527-6500

Telex: 248812 (MSCI UR)
Cable: MSCI Washington, D.C.

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MALARIA IN NEPAL: INTERIM SUMMARY OF LONGITUDINAL RESEARCH IN SINDHULI AND KANCHANPUR DISTRICTS

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by

William K. Reisen, Ph.D.

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Author

William K. Reisen, Ph.D., is a Research Entomologist and the Director of the Arbovirus Field Station, School of Public Health, University of California, Berkeley, P. O. Box 1564, Bakersfield, CA 93302, USA.

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EXECUTIVE SUMMARY

At the request of USAID/Kathmandu and the Nepal Malaria Eradication Organization (NMEO), I conducted my second consultant visit as technical advisor to longitudinal research projects on malaria ecology in Sindhuli and Kanchanpur Districts during April/May 1988.

With the cooperation of the Department of Entomology, Walter Reed Army Institute of Research, the ELISA test to detect sporozoites was brought to the NMEO NHQ in Kathmandu and a trial assay performed. The test worked well and the falciparum capture antibodies recognized local strains of the parasite. A protocol for the use of the ELISA in Nepal was written.

Field study areas were visited and the ongoing research evaluated. Problems in data collection and administrative support were recognized and reported to NMEO and USAID. Data from the first eight months of research are summarized and interpreted briefly. Of the 5,650 females in nine species that were dissected, only four An. fluviatilis from Bardia Goth, Sindhuli District, collected during the current visit were found to be sporozoite positive. Low sporozoite rates were supported by low man biting rates, and none of the 493 freshly fed females of four species were found to have fed on man when assayed by a precipitin test. Further research will be needed to explain continued malaria transmission by seemingly inefficient vectors.

Protocols were written for three additional research activities to expand data collection and interpretation: (1) ELISA testing of dissected and undissected females, (2) nocturnal human bait and resting catches, and (3) malaria vector competence studies. It was recommended that the research project be extended for an additional four to six months to replicate data collected during the critical May-July period.

I. INTRODUCTION

Continued malaria transmission in the Central and Far Western Regions of Nepal despite ongoing control efforts has stimulated the need for basic entomological investigations designed to elucidate the ecology and vectorial role of each anopheline species and thereby improve control effectiveness. An intense short term entomological and epidemiological study in Kanchanpur District during the monsoon transmission season of 1986 failed to directly incriminate any of the common anophelines[1] and stimulated the development of in-depth longitudinal research protocols designed to directly or indirectly incriminate the common anopheline species.[2] Comparable studies initiated in Sindhuli District were designed to study the influence of insecticide spray on the behavior of the reputed anopheline vectors and to determine the importance of forest transmission in malaria maintenance.[2]

Field studies were organized collaboratively to include participation by the Nepal Malaria Eradication Organization (NMEO), USAID/Kathmandu and the Vector Biology & Control (VBC) Project. NMEO was to provide field staff, transportation and supervision by NMEO Headquarters staff, USAID/Kathmandu was to provide supplies, equipment and technical assistance during three short-term consultancies. In February 1987, the VBC Project requested me to serve as technical consultant for this project (AR-026) which consisted of 3 short term (two - three week) visits to Nepal. During the first visit (March 23 - April 8, 1988), I reviewed NMEO research proposals, wrote a detailed work plan and outline a short term training course at Hetauda for the NMEO field research teams.[2] My scope of work for the present trip (visit 2) was to: (1) identify changes in the accepted work plan[2], (2) visit the study areas to provide on-site advice on study area selection, quality control and additional training, (3) summarize data collected in both Sindhuli and Kanchanpur Districts from August 1987 to April 1988, and (4) recommend course corrections to improve data quality. My itinerary and list of persons contacted during visit 2 are presented in Annexes 1 and 2, respectively.

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[1]Reisen, W.K. 1986. Malaria in Nepal: Observations during a short-term entomological consultancy. VBC Activity AR-023:33.

[2]Reisen, W.K. 1987. Malaria in Nepal: Development of a detailed working plan for entomological research in Sindhuli (Central Region) and Kanchanpur (Far Western Region) Districts with comments on Japanese Encephalitis. VBC Activity AR-026:36.

II. METHODOLOGY ALTERATIONS AND ADMINISTRATIVE PROBLEMS

A. Methodology

In general, the research is progressing according to the working plan described in detail previously.[2] Following are unprotocolled technical modifications in sampling and data collection:

1. The study areas at Sindhuli have been modified. Samples are being taken at the Tallo Khutepani cattle shed, Dovan Goth; however, few villagers at Tallo Ranibas were found to keep cattle in the forest. As a replacement, the Bardia Goth was sampled. This Goth also serves as an overnight stopping place for villagers traveling to and from Janakpur and has been the site of repeated malaria transmission in the past (J.D. Shrestha, personal communication). This change in protocol seems well-justified.

2. Pit shelters were in place at Sindhuli, but not in Kanchanpur District which hampered the sampling of outdoor resting mosquitoes. The construction of pit shelters in Kanchanpur study villages is needed to augment searches of natural outdoor shelters because of the relatively poor resting habitats available for sampling. These marginal outdoor resting sites have produced relatively few specimens and were devoid of mosquitoes during the present visit.

3. Supplementary human bait collections were not being made at the houses of recent fever cases, although fixed and random collection sites were selected on the basis of past case clustering.

4. Biting collections at both Sindhuli and Kanchanpur were not always conducted according to the protocol. At Sindhuli, night collections frequently were terminated before dawn due to "heavy wind and/or rain". While gale force winds or heavy rains undoubtedly affect outdoor catches, indoor observations should be continued throughout the night regardless of weather conditions. In addition, if the wind or rain subsides, the outdoor collection activities should be resumed. During four months, observations at Kanchanpur purposely were terminated at midnight. Termination of collections early in the night is not acceptable because it (a) prevents the estimation of the man-biting rate necessary to calculate vectorial capacity for indirect vector incrimination, and (b) precludes the definition

of the biting curve useful in determining the actual time of transmission.

5. Larval collections were not done as specified. First and second instars were not counted precluding the estimation of relative abundance (numbers per dip). Dip sampling was not expedited by using the sample concentrator described previously[2], but rather fourth instar larvae were transferred laboriously by eye-dropper to collection bottles thereby elongating sampling time.

6. Parity dissections have been expanded to include freshly fed females from resting collections thereby increasing sample sizes. Ovaries of unfed females were not being examined for tracheation, but rather the ovarial pedicels were observed for dilatations. The number of dilatations were not being counted. Increasing ovarity sample sizes will increase the precision of survivorship estimates needed for vectorial capacity calculations.

7. The ovaries of females collected biting were not being scored to Christophers egg stages as described in the working plan.[2] This precluded the detection of gonotrophic discordance (more than one blood meal within a single gonotrophic cycle) which seems to occur commonly among host-seeking anophelines in Kanchanpur District.

8. Pools of females were not being evaluated routinely for sporozoites using the concentration method specified in the working plan[2]. The method was tried unsuccessfully during August 1987 and discontinued. However, most females were being dissected by conventional methods (see below) and with the introduction of the ELISA test (see below) discontinuance seems justified.

9. It was not possible to determine how many females of what species had been examined cytogenetically for subspecific status. However, neither field team had ovarian fixative or vials in which to preserve the ovaries for later cytogenetic study. The Kanchanpur field team had been instructed to only save Anopheles culicifacies and ignore An. annularis and An. fluviatilis, contrary to what was specified in the working plan[2].

10. No insecticide susceptibility tests were conducted at the study villages during 1987. Monitoring the susceptibility of the common Anopheles species to insecticides used by NMEO will be critical if these villages will be used for future spray evaluation

trials. In addition, the protocol for Sindhuli District is targeted at evaluating the impact of spray on anopheline behavior. Response of the common species to the insecticide used should be done both pre- and post spray.

B. Administration

The quality of any research project depends on the level of supervision, training and morale of the field teams which are responsible for data collection. Careful and timely administrative support is needed to ensure their maximum performance under field conditions. The following administrative deficiencies were observed during the present study:

1. Staffing

The integration of the NMEO staff into the Public Health Program has resulted in the transfer of the Principal Investigator, Mr. Shambu L. Shrestha, and the coinvestigators, Messrs. Janak D. Shrestha and Shambhu R. Shrestha. It is recommended that Mr. Jitendra P.B. Shrestha, Interim Chief Officer NMEO, become the Principal Investigator, and Mr. Rhiddi G. Vaidya, Senior Entomologist NMEO, become the Co-investigator. Mr. S.P. Pradhan, USAID Malaria Advisor, and myself will remain as collaborating coinvestigators.

In both Sindhuli and Kanchanpur Districts, the daily activities of the four insect collectors are supervised by Malaria Inspectors who in general were doing an adequate job. It is recommended that Messrs. Sambhu B. Shrestha, Central Region, and Gautam R. Sharma, Far Western Region, be retained as team leaders to provide consistent supervision for the remainder of the research project. In the Far West, two additional Malaria Inspectors are required to assist the Team Leader and should be transferred as soon as possible.

The composition of the field teams in both Sindhuli and Kanchanpur Districts has been less than the proposed eight men per team. The shortage of collectors has resulted in the reduction in the number of human bait collections and forced the same collection team to sample both indoor and outdoor resting sites. I recommend that 1-2 villagers be hired on daily wages to assist each of the field teams in all night collections.

Finally, continued frequent field visits by the USAID Malaria Advisor and Senior NMEO staff is urged to ensure the quality of the research. In addition, feed-back of laboratory results to the field staff is recommended to stimulate greater interest in research activities.

2. Transportation

The two drivers and vehicles budgeted in the original NMEO protocol were not in use, which required that the field teams transfer supplies and equipment between study villages by ox cart. In addition, the two Honda trail bikes purchased by USAID have not been distributed to the teams, requiring that the collectors hike for more than two hours from the village to the forest collection sites, remain awake all night collecting mosquitoes and then hike two hours carrying the collections to the base camp for identification and dissection. Improvement of transportation would allow additional time in the field to be spent on entomological activities.

3. Supplies/Equipment

Microscopes ordered over a year ago finally were delivered to the field teams at the time of the present visit to Sindhuli and Kanchanpur. While these new microscopes vastly improved the dissection capability of the field teams, they have arrived in an untimely fashion eight months after the study was initiated.

4. Rent and Allowances

Rent budgeted for field housing by the entomological teams by NMEO was not being utilized and the team members were forced to pay for housing with their own per diem. In addition, NMEO does not have funds to pay overtime. It is recommended that USAID contribute to overtime pay for the field teams to increase morale and thus data quality.

5. Reporting

NMEO staff have not been preparing quarterly reports for the internal staff and the USAID and VBC collaborators as specified in the working plan. Data collation has been left in the hands of the field staff and the USAID Malaria Advisor with final summarization hand-tallied by the VBC consultant for the present report. I recommend that copies of the field sheets be sent by the field staff directly to NMEO NHQ to be entered into a computerized database system for rapid collation and statistical analyses. Data entry should be done from the actual field sheets and not collated in the field to preclude computational and transcription errors.

III. INTERIM DATA SUMMARY

A. Study Areas

Maps of the four main study villages are presented in Figures 1-4. Included in the figures from Sindhuli are the locations of the fixed human, mixed dwelling and cattle shed resting collection sites, the fixed sites for human and animal bait catches, and the pit shelters. In Sindhuli larval breeding sites were restricted to the seeps, canals and rice fields bordering the Kamala Nadi River, while natural outdoor shelter collections were made in erosion cuts along the hillsides of the Churia Range. Between August 1987 and March 1988, 60% of 141 malaria cases at Tallo Ranibas were clustered at houses bordering the hillside resting sites. Malaria transmission at Tallo Khutepani was scanty with only four cases observed during our study period.

At Musepani, breeding was restricted to permanent seepages along river beds to the east of the study village and to irrigation related to rice farming. At Baidapur, breeding was restricted similarly to pools along the Mahakali River and to rice fields and irrigation ditches. Natural resting sites at both villages in Kanchanpur District were restricted to the margins of river beds which become dry prior to the monsoon rains.

B. Malaria Prevalence

Malaria prevalence at Tallo Ranibas increased markedly after withdrawal of the Ficam spray in 1987, with the annual parasite incidence (API) increasing from 32/1000 during 1986 to 280/1000 during 1987. In contrast, the API at Tallo Khutepani which was sprayed twice with Ficam during 1987 remained essentially the same at 22/1000 during 1986 and 26/1000 during 1987. With minimal duration residual activity, it was difficult to understand how a round of Ficam in Khutepani during August 1987 could control transmission for the succeeding eight-month period.

Despite the withdrawal of DDT spray from the study villages in Kanchanpur, the API at Musepani has continued to decrease from 127/1000 in 1986 to 47/1000 in 1987. Transmission at Baidapur has continued at a consistent low level (25/1000 in 1986 and 33/1000 in 1987) and has changed little since the withdrawal of spraying.

C. Mosquito Abundance Patterns

1. Sindhuli District

A total of 4,971 anophelines comprising 20 species were collected at the four study areas in Sindhuli District from August 1987 to April 1988 (Table 1). An. maculatus Theobold was the most common species comprising 47% of the total, followed by An. splendidus Koizumi (16%) and An. fluviatilis James (8%). Based on comparative indoor and outdoor resting and human and animal bait catches, it was possible to group the common species (>100 specimens collected) according to their resting and feeding habits. Of the three indoor resting habitats samples, most anopheline females were collected resting in cattle sheds and fewest were collected resting in houses. Resting site selection agreed well with host selection patterns which were decidedly zoophilic for all species. Although difficult to compare directly, it was apparent that many An. fluviatilis, An. jamesii Theobald, An. maculatus, An. pallidus Theobald and An. tessellatus Theobald rested diurnally in natural outdoor shelters.

With the exception of An. maculatus at Khutepani, the human biting rate remained less than 0.5 bites/man/night for all species throughout the observation period. In agreement, none of 19 An. annularis, 8 An. fluviatilis and 176 An. maculatus freshly fed females were positive for human blood when tested by a gel diffusion precipitin test at the NMEO NHQ. During my visit, anopheline mosquitoes were not observed biting during four evenings camping at Ranibas. However, the biting rate increased markedly at both Bardia and Dovan Goths, even though the ratio of man to cattle appeared to be considerably less than in the village environment.

Temporal changes in the numbers of malaria cases and the relative abundance of the common anopheline species have been compared graphically in Figure 5. Although most malaria cases were contracted during July before mosquito sampling commenced (Figure 5a, f), the reputed vector, An. fluviatilis, was rare at villages during this period (Figure 5b,h). An. maculatus was abundant biting cattle and resting at natural outdoor shelters at Tallo Ranibas (Figure 5c), but not at Khutepani (Figure 5i), during August. The abundance of An. annularis (Figure 5g) and An. pallidus (Figure 5h) biting bovids also was highest during the August transmission period in Khutepani.

A total of 2,151 anopheline females in seven species (43% of total collected) were dissected for sporozoites in Sindhuli District (Table 2). Emphasis was placed on the reputed vector species An. annularis, An. fluviatilis and An. maculatus and 70% of these females were dissected. Overall, only four specimens of An. fluviatilis collected at Bardia Goth during our visit in

April 1988 were found to be positive for sporozoites. The sporozoite rate at this time was 4.9% (n=82), the biting rate was 18 An. fluviatilis females/man/night, and the parity rate was slightly elevated at 0.71 (n=70 females dissected). Slides were stained with Giemsa for confirmation and the corpses of the females were retained for later parasite identification using ELISA (see below). None of 48 An. maculatus or 13 An. culicifacies collected concurrently were infected. Our results supported the earlier findings of J.D. Shrestha who found single specimens of An. maculatus and An. fluviatilis naturally infected at this locality in 1986. The repetitive finding of infected females at an isolated cattle shed, but not in the village of Tallo Ranibas, is perplexing and will require further study to understand the importance of cases contracted in the goths to the maintenance of low level transmission at villages.

Overall, 2,061 females of four species from Sindhuli District were dissected to determine oviparity (Table 3). Parity rates ranged from 0.49 to 0.78 and were variable among species over both time and space. Further data will be needed to elucidate seasonal trends and estimate survivorship.

2. Kanchanpur District

Overall, 4,848 Anopheles females comprising 11 species were collected from three localities in Kanchanpur District from August 1987 to April 1988 (Table 4). An. culicifacies was the most abundant species comprising 39% of the total, followed by An. subpictus Grassi (21%) and An. maculatus (19%). Although most species were most abundant resting in cattle sheds, females of An. culicifacies and An. subpictus also were collected frequently in both houses and mixed dwellings. An. maculatus was collected most frequently in cattle sheds and was the only species collected in good numbers resting in natural outdoor shelters.

All anopheline species collected in Kanchanpur were decidedly zoophilic and few were collected biting man. Man biting rates for all species remained less than 0.05 bites/man/night at both villages and less than 0.5 bites/man/night at the Amthala Goth in the forrested Churia Hills. Females rarely were taken at human bait in the villages, although both An. culicifacies and An. subpictus frequently rested in houses. These species apparently egressed from these diurnal shelters at dusk, fed on cattle out of doors and then ingressed at dawn. In agreement with the low man biting rates observed during the present and previous[1] all night collections, no human positive blood meals were detected by precipitin assay for 32 An. maculatus, 226 An. culicifacies and 32 An. fluviatilis.

The temporal patterns of malaria case prevalence and anopheline relative abundance in Kanchanpur were compared

graphically in Figure 6. Similar to Sindhuli District, malaria cases were most prevalent during the July-August monsoon period (Figure 6a,g), while the relative abundance of the reputed vectors, An. fluviatilis and An. maculatus, were comparatively low (Figure 6d,e,j,k). The abundance patterns of An. annularis (Figure b,h) and An. subpictus (Figure 6f,l) most closely paralleled the occurrence of malaria cases.

Overall, 3,499 females collected from Kanchanpur were dissected for sporozoite infection with negative results (Table 2). Again, emphasis was placed on the four reputed and potential vector species, and 90% of these females were dissected.

A total of 1,856 females were dissected for oviparity (Table 3). Parity rates ranged from 0.43 to 0.85 and, in general, were lower than observed at Sindhuli District in inner terai. In both Districts, the parity rate of An. maculatus was higher in forested cattle shed habitats than at cleared villages, indicating survivorship may be related to the quality and availability of outdoor resting sites.

D. Relationship to Malaria Transmission and Control

Malaria transmission in both Sindhuli and Kanchanpur Districts was difficult to explain epidemiologically. Case prevalence was highest during midsummer, while the abundance of the reputed vectors (An. fluviatilis and An. maculatus) was extremely low, except at isolated cattle sheds where seldom more than 10 people resided. Although travelers could possibly keep reintroducing malaria into these forested areas, it would seem to be a dead end for continued transmission. The few, sporadic cases occurring at Khutepani, but not Ranibas, could be explained by repeated introduction. The extensive malaria epidemics experienced in the Far West during 1985 and 1986 most certainly occurred as a result of widespread indigenous transmission.

Transmission in villages would seem unlikely under the present conditions of low man biting rates by the Anopheles species which were numerically abundant during the summer monsoon season. In agreement, none of 3,961 females collected in villages were positive for sporozoites. Continued dissections throughout the summer will hopefully shed further insight into this apparent enigma.

Mosquito control by residual house spraying presumes that the target species will bite man and then rest indoors at night or ingress and rest on sprayed surfaces during the day. Most females in both Sindhuli and Kanchanpur Districts were collected biting bovid hosts and to some degree resting inside cattle sheds. Of the few females collected biting man, only 13% (n=146 total collected) in Sindhuli and 25% (n=12) in Kanchanpur

villages were taken at human bait positioned indoors. Thus, the reputed vectors were markedly exophilic and exophagic, and presumably would be further selected by residual spray for these behavioral tendencies. Only species such as An. culicifacies, An. subpictus and perhaps An. vagus seemed to obligatorily rest indoors and would be dramatically effected by residual spray; however, these species have evolved resistance to organcloride insecticides[1] in Nepal, and organophosphorus compounds throughout India making control progressively more expensive and finally, difficult at best.

IV. PROTOCOL CHANGES

To enhance and/or augment the current field research, four additional activities are recommended:

1. ELISA Test for Sporozoites

Background - Low *Anopheles* infection rates during epidemic malaria transmission in Nepal has stimulated the need to provide important back-stopping and quality control to the field sporozoite dissection program. The enzyme-linked immunosorbent assay (ELISA) for sporozoites (see Annex 3) seems well-suited to augment, but NOT replace, the current dissection program. Tests can be performed on pools of up to ten females, and thus large numbers of specimens can be processed in a relatively short time period thereby increasing the chances of detecting low sporozoite rates. In addition, performing both the *P. falciparum* and *P. vivax* assays will allow the identification of parasites detected by positive sporozoite dissections. This is especially critical in remote forested areas such as Bardia Goth where primate malaria may be transmitted by the same anophelines involved in human transmission. Finally, the ELISA tests can be used as a quality control tool to recheck negative dissections to ensure that light infections are not being missed during field dissection.

An ELISA kit capable of performing 2,000 tests was provided by Dr. R.A. Wirtz, Department of Entomology, Walter Reed Army Institute of Research, and hand-carried to Nepal during the current visit. Additional equipment and supplies were provided by the Vector Biology & Control Program. A preliminary assay was conducted successfully at NMEO NHQ on April 14-15, 1988. Plates for both *falciparum* and *vivax* were set up with two serial dilutions of positive antigen controls as well as 15 *An. maculatus* and 20 *An. fluviatilis* from the Hetauda colonies which had been killed 14 days after feeding on a *falciparum* gametocyte carrier and stored dry at room temperature for five days. The test worked well with substrate color intensity paralleling the antigen dilution series (Annex 4). In addition, three (20%) *An. maculatus* and seven (35%) *An. fluviatilis* females gave a strong positive reaction in the *falciparum*, but not in the *vivax*, plate. These results indicated the *falciparum* monoclonal antibodies reacted well with *falciparum* parasites from Nepal. The reagents and equipment have been deposited at the Parasitology Laboratory of NMEO NHQ for future assays.

Methodology for Field Use in Nepal - Since field infection rates are low (see above), it is recommended that whole mosquitoes be tested to detect both salivary gland and late gut infections and thereby increase the chances of detecting at least

some positives. Thus, it should be noted that the circumsporozoite antigen positivity rate from the ELISA test should be higher than the sporozoite rate determined by dissection. Three groups of females should be saved for ELISA testing:

a. Thoraxes and abdomens (including the gut) of females used for salivary gland and ovarian dissections, but not used for blood meal tests, should be pooled by locality, date and species into lots of $\leq$10 females each.

b. Thoraxes and abdomens of all females positive for salivary gland dissection should be saved individually for ELISA testing with the ELISA tube number recorded on the dissection sheet.

c. When collections are large, extra females not dissected should be killed, dried, and then pooled by locality, date, collection method and species into lots of $\leq$10 females each.

It is important to make sure that the females are well dried prior to storage and that they do not become moldy prior to testing. Vials should be numbered appropriately and with the completed data sheet (Annex 5) sent in a timely manner to Kathmandu for testing at the NMEO NHQ after sufficient specimens have been collected. All ELISA tests should be set up and performed with appropriate controls as outlined in the protocol in Annex 3. A second confirmatory test should be performed on all positive samples.

2. Nocturnal Bait and Indoor Resting Catches

Background - Since many of the anopheline species in both Sindhuli and Kanchanpur Districts were found to rest outdoors during the day and infrequently enter houses at night to bite man (see above), a modification of the human bait collection protocol is necessary to provide more specimens for dissection and to verify quantitatively how many host-seeking females of what species enter houses. Quantifying house entering behavior is critical to predicting the impact of residual spray for malaria control. Certainly, if the reputed vectors both rest and bite outdoors, a house spraying program would be expected to have minimal impact on malaria transmission.

Methods - All night biting catches should be performed at two fixed locations in each village by two three-man teams. One collector will sample mosquitoes coming to cattle bait as described in the working plan[2]. A second collector will collect mosquitoes attracted to his exposed legs while seated either indoors or outdoors depending upon the sleeping habits of the people during the sampling period. A third collector will sample

females attracted to sleeping villagers as well as collect females resting indoors according to the following protocol:

- Afternoon resting collection. Two collectors will collect all females resting in the house for ca 20 minutes starting 30 minutes before sunset. This collection should be labeled appropriately and kept in a separate carton.

- Nocturnal biting and resting. From sunset to sunrise, one collector will check the residents of the home (either sitting, eating or sleeping) for mosquito biting activity four times each hour. Once each hour, the same collector will catch all mosquitoes resting indoors during a 15 minute period. Biting and resting catches should be kept separate, but catches may be combined over time throughout the night. Care should be taken to record ovarian condition to separate host-seeking from resting females.

- Morning resting collection. Starting 15 minutes after sunrise, two collectors will sample indoor resting mosquitoes for 10 minutes. This collection also should be kept separate.

Anticipated Results - Removing the females resting in the house during late afternoon should provide an indication of what species remained resting in the house throughout the day. Collections throughout the night should provide an indication of what species entered the house for biting and rested there before or after blood feeding. Resting collections after sunrise should provide information on what species entered the house in the morning for resting only. This information will be critical to more clearly assess what proportion of a normally exophilic and exophagic vector population would contact an indoor residual house spray.

3. Vector Competence Studies

Background - Vector incrimination can be accomplished directly by repeatedly finding sporozoites in the salivary glands of females frequently collected during the transmission season or indirectly by a combination of ecological and/or experimental methods. The comparative susceptibility of candidate vector species to strains of both falciparum and vivax are important in understanding their physiological capacity to acquire and transmit infection. Previously, colonized strains of three anophelines (annularis, fluviatilis and maculatus) were found to develop sporozoites when fed on gametocyte carriers infected with chloroquine resistent strains of P. falciparum (J.D. Shrestha, unpubl. data). However, comparative vector competence studies were not conducted on other potential vector species such as An.

culicifacies and An. subpictus, and chloroquine susceptible strains of P. falciparum or P. vivax have not been evaluated.

4. Methodology

Species to be evaluated - If possible, strains of the potential vector species from both Sindhuli and Kanchanpur Districts should be tested. Species to consider include field strains of An. annularis, culicifacies, fluviatilis, maculatus, splendidus, subpictus and perhaps aconitus, jamesii, jeyporiensis, pallidus and tesselatus. These species have either been incriminated as vectors of malaria in Nepal or elsewhere and/or have been taken biting man during the present study. When possible, species should be compared concurrently, but if sufficient specimens are not available, species should be tested using the Hetauda laboratory strain of An. fluviatilis as a control DURING EACH FEEDING.

Specimen collection - Females should be collected as pupae or late fourth instar larvae and then transported to the Research and Training Center in Hetauda. Alternatively, females could be collected resting or biting, induced to oviposit in the laboratory, and the progeny reared to adults under insectary conditions in Hetauda. Newly emerged females should be offered a sugar meal and held for three to five days prior to blood feeding to ensure that their ovaries have matured to Stage IIa, the stage at which a replete blood meal is usually imbibed. Feeding newly emerged females at Stage I may result in a large number of partial, pregravid meals which would seriously underestimate vector competence.

If collection sizes are sufficiently large, 50 females of each candidate Anopheles species should be fed on three P. falciparum and three P. vivax gametocyte carriers, pairwise using the Hetauda laboratory strain of An. fluviatilis as a control. Females should be sorted by abdominal condition into fully fed and partially fed or empty categories immediately after feeding is completed. Only fully fed females should be saved for incubation and later dissection. About 14 days after feeding, females should be dissected to determine salivary gland infection. The gut of negative females should be examined for oocysts to determine if the female was infected, but could not develop a sporozoite infection in the salivary glands.

Research extension - Although the research is progressing fairly well, initial problems in staffing, supervision and protocol alterations indicate that it may be useful to extend the research program for an additional four to six months to replicate sampling during the epidemiologically critical spring/ early summer period. NMEO NHQ has advised that funds for salary and per diem are available, and thus additional costs would be restricted to minimal supply and allowance costs. I think the

extra information which could be gathered would be well worth these minimal additional expenditures.

Table 1. Mean abundance of Anopheles females (♀/man hour or bait night) collected by 7 methods at 4 sites in Sindhuli District (August 1987 to April 1988)

Collection Method[1]	Effort (MH/BN)	Anopheles species[2] acon.	annu.	culi.	fluv.	jame.	jeyp.	karw.	macu.	pall.	phil.	rams.	sple.	subp.	tess.	vagu.	varu.	barb.	giga.	nige.	sine.
TALLO RANIBAS																					
Resting																					
Human	85.2	-	.04	.01	.06	-	-	-	.07	.01	-	-	.14	.04	-	.94	-	-	-	-	-
Mixed	39.0	-	.41	.08	.08	.08	-	-	.74	.03	-	-	2.10	.05	.10	.59	-	-	-	-	-
Animal	45.1	-	.44	.09	.67	.18	-	.02	2.13	.09	-	-	4.79	.38	.09	1.06	.02	.02	-	-	.08
N.O.S.	108.0	-	.02	-	.26	.14	.01	-	5.00	.20	-	-	.18	-	.73	.15	-	.04	.01	.04	.08
Pit	48.0	.02	-	-	.38	.02	-	-	.44	-	-	-	.25	-	.02	.04	-	-	-	-	.02
Biting																					
Cattle	20.0	-	2.30	.10	.20	2.95	.50	.05	15.25	2.85	-	-	6.00	.20	1.55	3.05	.10	.05	-	.40	1.45
Human	72.0	-	.07	-	.11	.01	.06	-	.44	.11	-	-	.08	-	-	-	-	-	-	-	-
TALLO KHUTEPANI																					
Resting																					
Human	82.6	.02	.07	-	.04	.02	.02	-	.05	-	-	-	.07	.04	.02	.06	-	-	-	-	-
Mixed	17.1	-	.70	.12	.70	-	.05	-	.88	.05	-	-	.82	-	.05	.10	-	-	-	-	-
Animal	64.6	.05	.68	.08	.59	.12	.06	-	2.12	.09	.02	-	1.18	.02	.04	.29	-	.06	-	-	.06
N.O.S.	108.0	.08	.01	-	.17	.05	.12	-	1.14	.14	-	-	.13	-	.52	.07	-	.02			.09
Pit	64.0	.05	-	-	.19	.02	-	-	.19	.09	-	-	.08	-	.09	.09	.02	-	-	-	-
Biting																					
Cattle	18.0	.06	2.89	.12	1.11	3.28	1.33	.17	24.83	3.17	-	.12	8.61	-	2.06	1.44	.17	-	-	.12	1.50
Man	72	.01	-	-	.04	-	.07	-	.64	.29	-	-	.01	-	-	.03	-	-	-	-	.03
BARDIA GOTH																					
Resting																					
Mixed	5.5	.18	.36	8.00	7.8	1.46	-	-	30.00	-	-	-	8.00	-	-	1.64	-	-	-	-	.55
N.O.S.	18.0	-	.06	-	.50	.06	-	-	4.06	.06	-	-	.50	-	.22	.22	-	-	-	-	.11
Biting																					
Man	12.0	-	-	-	11.08	-	-	-	6.92	.08	-	-	.50	-	-	-	-	-	-	.08	-
DOVAN GOTH																					
Resting																					
Mixed	3.67	-	.55	1.36	1.09	-	-	-	7.08	-	-	-	2.73	-	1.64	.27	-	-	-	-	.27
N.O.S.	18.0	-	-	.11	.17	.11	-	-	.94	.11	-	-	-	-		.17	-		-	-	.17
Biting																					
Human	12.0	-	-	.08	1.17	-	-	-	14.33	.17	-	-	.17	-	.08	.17	-	--	-	-	.08

[1]Methods: Resting catches were 2 men collecting inside 12 houses, 6 mixed animal/human dwellings and 6 animal sheds on 2 occasions each month in each village; an additional 2-3 structures of each were selected randomly and have been combined. N.O.S. (natural outdoor shelters) were searched for 6 man hours on each of 2 occasions per month. Pits were 4 Muirhead-Thompson style pit shelters with canopy and were sampled twice per month. Biting catches were 2 teams of 2 men (1 indoors and 1 outdoors or on the veranda) at 2 fixed stations on 2 occasions per month. Cattle biting collections were made by 2 men at different sites working for 30 minutes per hour on 2 occasions per month.

[2]Species names were: An. (Cellia) aconitus, annularis, culicifacies, fluviatilis, jamesii, jeyporiensis, karwari, maculatus, pallidus, philippinensis, ramseyi, splendidus, subpictus, vagus and varuna, and An. (Anopheles) barbirostris, gigas, nigerrimus and sinensis.

Table 2. Total number of anopheline females dissected for salivary gland sporzoite infections in Sindhuli and Kanchanpur Districts (August 1987 - April 1988).

	Anopheles species[1]								
Village	acon.	ann.	culi.	fluv.	macu.	pall.	sple.	subp.	nige.
Sindhuli District									
T. Ranibas	1	90	10	90	787	1	17	-	-
T. Khutepani	8	53	1	79	352	-	-	-	-
Bardia Goth	-	1	31	174*	221	-	-	-	-
Dovan Goth	-	2	7	19	207	-	-	-	-
Total:	9	146	49	362	1567	1	17	0	0
Kanchanpur District									
Badaipur	-	156	490	38	425	-	3	71	116
Musepani	-	81	1240	127	175	-	3	138	9
Amthala Goth	-	57	9	10	311	-	-	39	1
Total:	0	294	1739	175	911	0	6	248	126

[1]Species abbreviations follow Table 1. * = 4 females positive for sporozoites in April 1988 (see text).

Table 3. Parity rates (number dissected in parathenses) of some common anopheline mosquitoes collected in Sindhuli and Kanchanpur Districts (August 1987 - April 1988).

	Anopheles species					
Village	annular.	culicifac.	fluviatil.	maculatus	nigerrim.	subpictus
Sindhuli District						
Ranibas	.78(72)	-	.68(79)	.49(798)	-	-
Khutepani	.56(87)	-	.65(90)	.58(498)	-	-
Bardia	-	.67(12)	.55(147)	.61(166)	-	-
Dovan	-	-	-	.64(112)	-	-
Kanchanpur District						
Badaipur	.75(119)	.63(312)	.43(28)	.38(233)	.54(98)	.85(39)
Musepani	.71(63)	.58(547)	.52(87)	.46(142)	.00(3)	.62(13)
Amthala	-	-	.43(7)	.55(165)	-	-

Table 4. Mean abundance of female anophelines (♀/man hour or bait night) collected by 6 methods at 3 sites in Kanchanpur District (August 1987 to April 1988)

Collection[1] Method	Effort MH-BN	Anopheles species[2] annu.	culi.	fluv.	macu.	sple.	subp.	vagu.	varu.	giga.	nige.	sine.
					BADAIPUR							
Resting												
Human	58.8	-	1.48	-	.37	-	7.35	.23	-	-	-	-
Mixed	14.2	-	4.45	-	.57	-	9.18	.06	-	-	-	-
Animal	29.4	.75	9.45	.92	3.50	.03	2.75	.09	-	-	-	-
N.O.S.	63.3	.03	.03	.03	4.30	-	.25	.02	-	-	-	-
Biting												
Animal	18	13.61	4.78	.61	1.78	.11	6.06	.39	-	-	18.30	-
Man	72	-	-	-	.03	-	.03	-	-	-	.01	-
					MUSEPANI							
Resting												
Human	52.2	.17	9.82	.48	.08	.02	1.46	.04	-	-	-	-
Mixed	11.0	.09	10.77	-	-	-	2.01	-	-	-	-	-
Animal	37.3	.51	14.60	1.34	1.00	-	.86	-	-	.03	-	-
N.O.S.	50.8	.02	.32	.78	1.08	-	.10	.04	-	.02	-	-
Biting												
Cattle	17	3.82	10.88	1.59	4.94	.24	2.53	.19	.06	-	.65	.06
Man	64	-	.06	.02	.03	-	-	-	-	-	-	-
					AMTHALA GOTH							
Resting												
Human	6.1	-	.98	-	1.31	-	5.06	-	-	-	-	-
Cattle	2.0	-	1.5	-	-	.50	5.00	-	-	-	-	-
N.O.S.	4.0	-	-	-	1.00	-	-	-	-	-	-	-
Biting												
Cattle	6.0	9.3	-	1.67	49.83	-	2.83	.17	-	-	.67	-
Man	11.0	-	-	-	.46	-	.18	-		-	.18	-

Footnotes and abbreviations follow Table 1 except that only 8 houses, 4 mixed dwellings and 4 cattle sheds were sampled routinely and pit shelters were not constructed.

VILLAGE:- T. RANEBAS

50 Step = 1"
Total House :- 149
Total Population :- 965
Total Structure :- 134

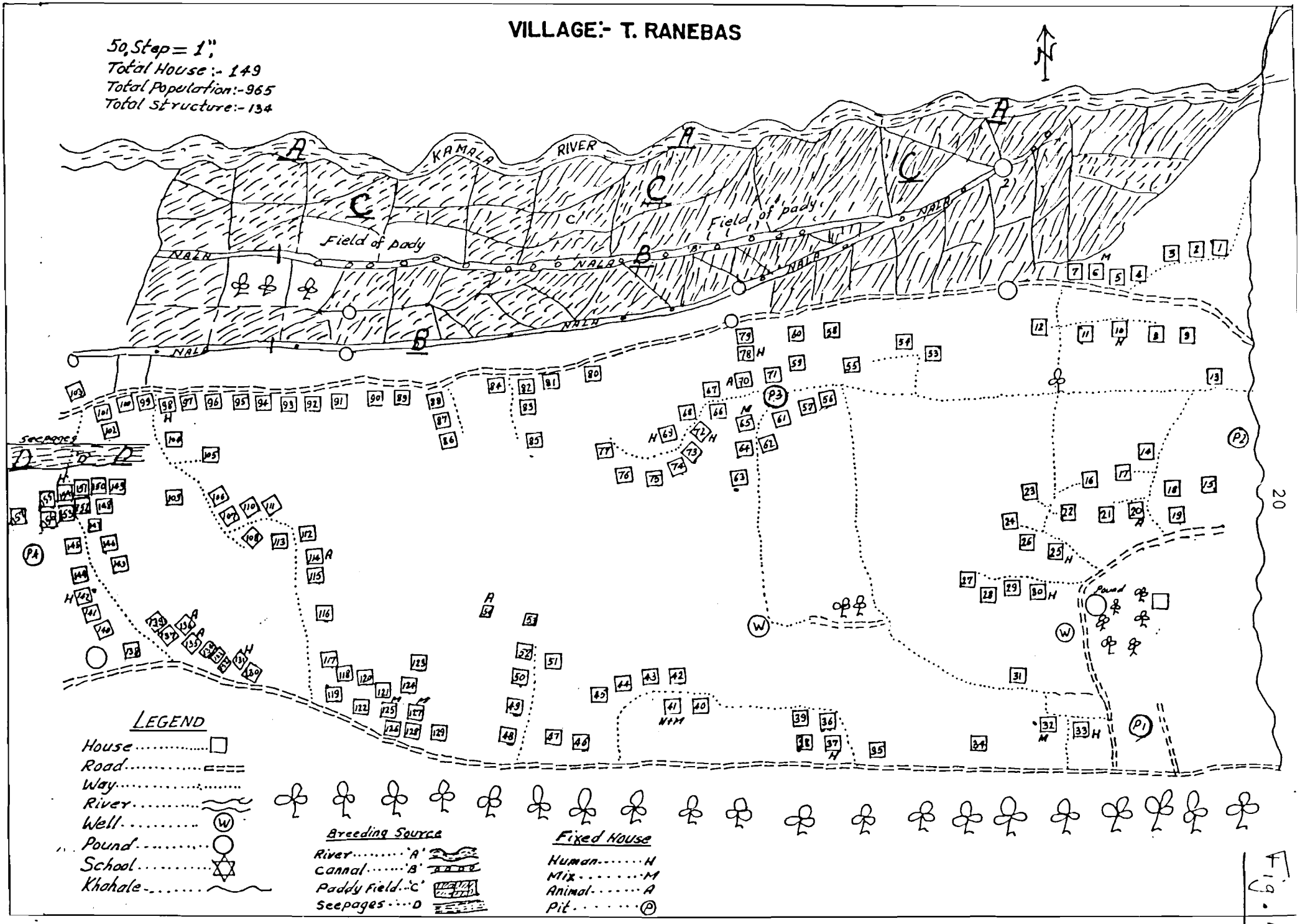


Fig. 1

BEST AVAILABLE COPY

VILLAGE:- T. KHUTEPANI

50. Step = 1"
Total Houses:- 42
Total Population:- 262
Total Structure:- 34.

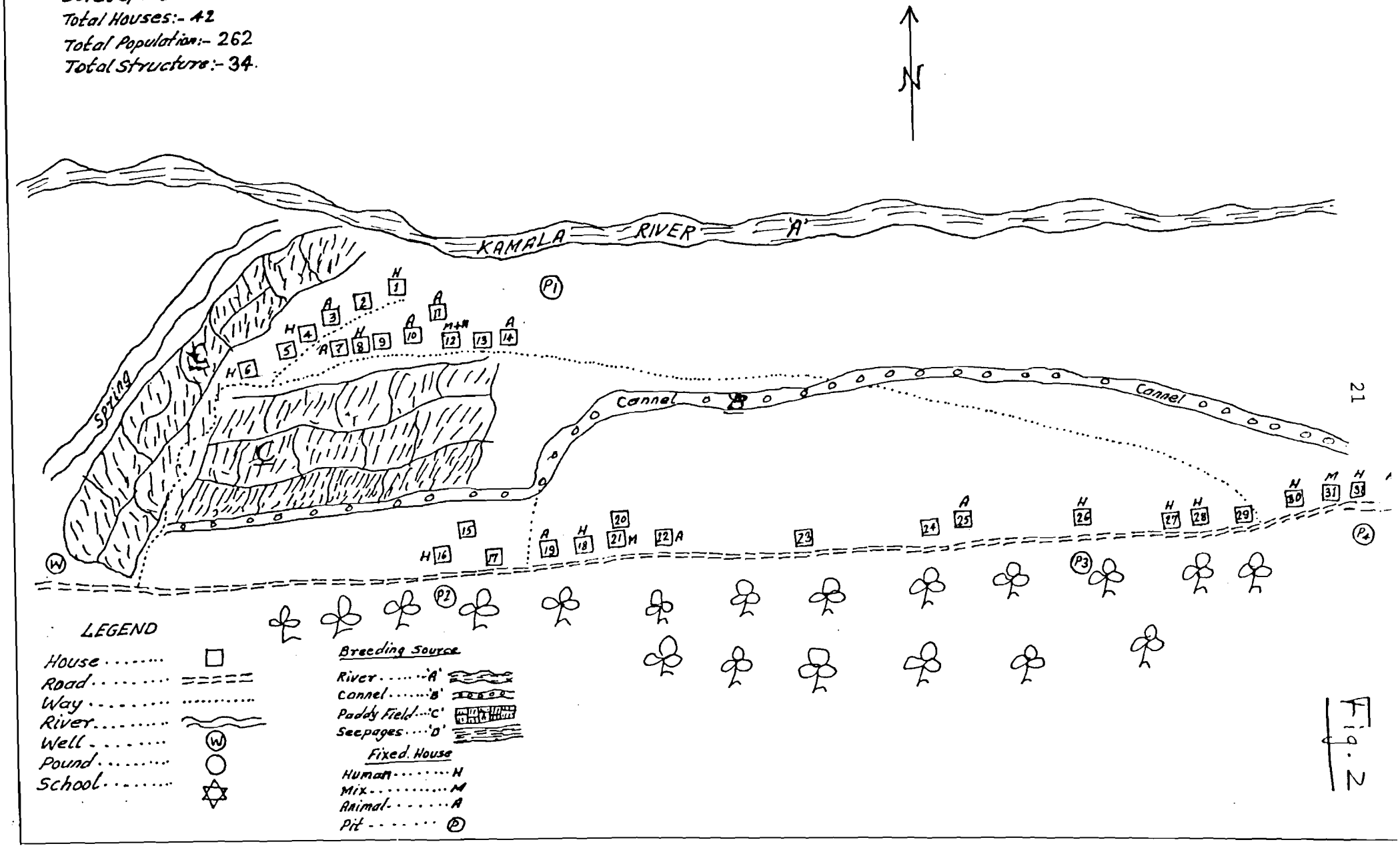


Fig. 2

BEST AVAILABLE COPY

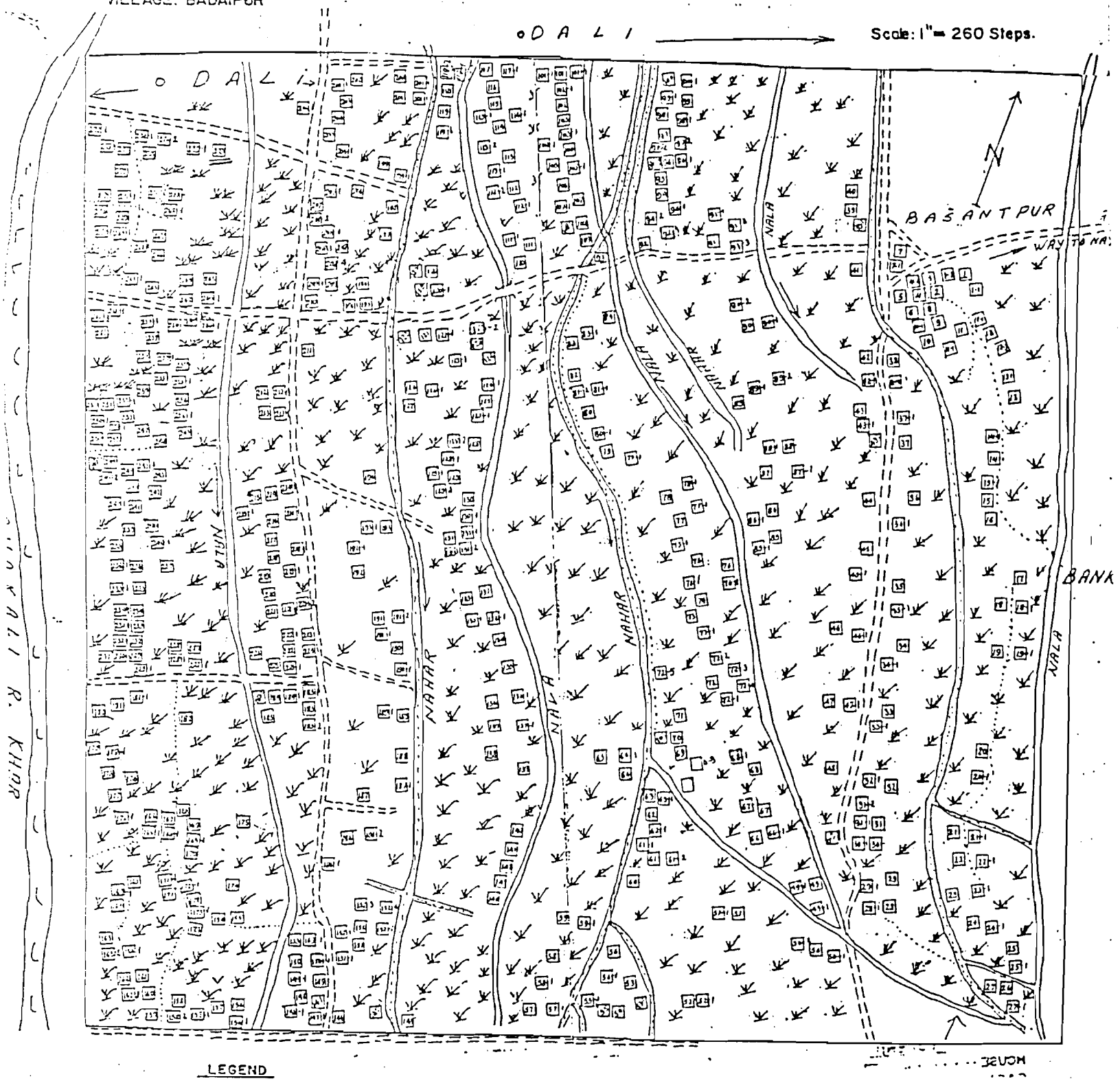
Fig 3

REGION. FAR WEST
UNIT. MAHENDRANAGAR II
VILLAGE. BADAIPUR

N M E O
KANCHANPUR

T. H.- 275
T. Str. 225

Scale: 1" = 260 Steps.



LEGEND

HOUSE
CART TRACK
FOOT PATH
PADDY FIELD
NAHAR
NALA

BEST AVAILABLE COPY

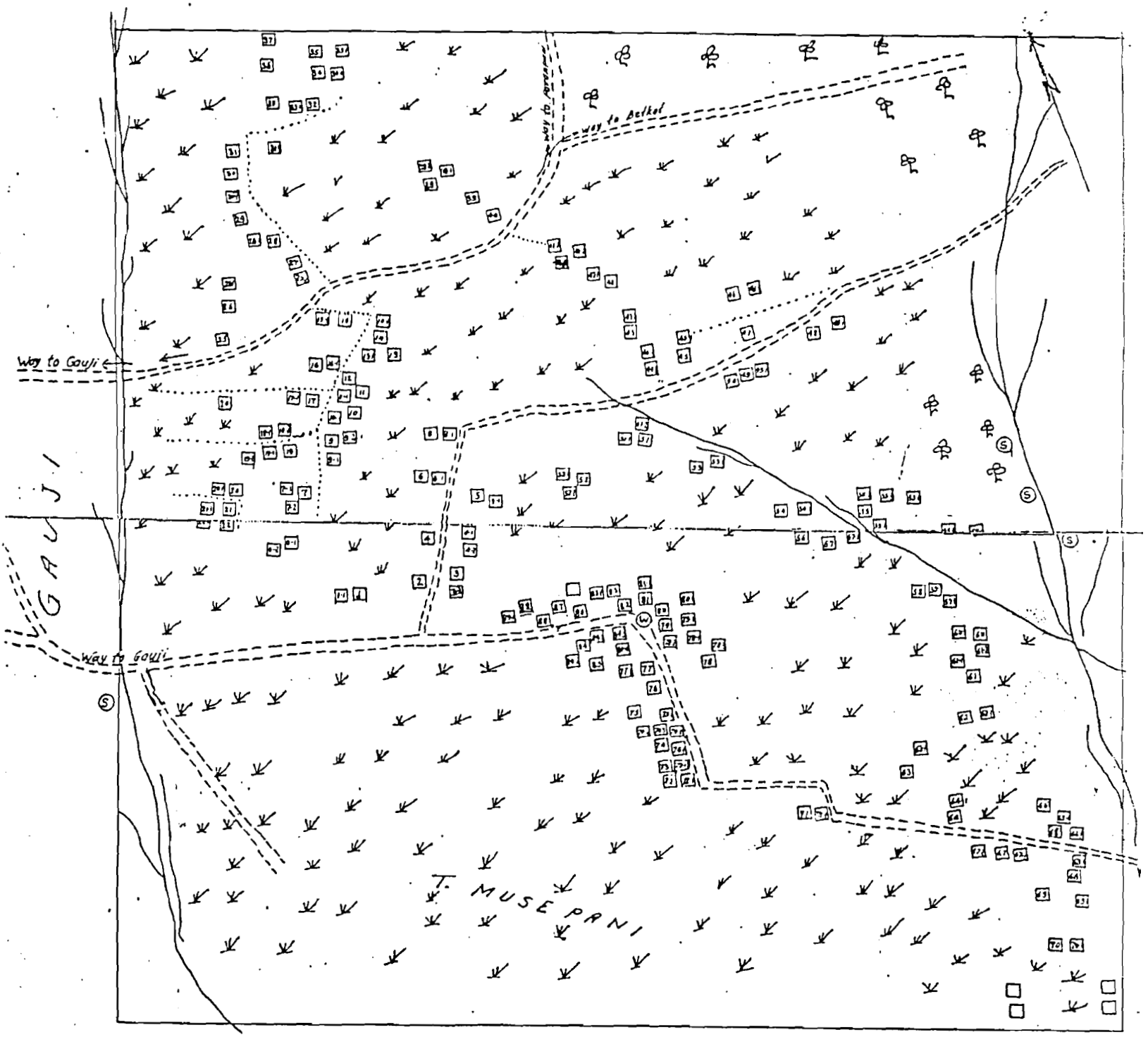
Fig. 4

REGION:- FAR WEST
UNIT :- IV BHUDA
LOC.No.:- 1
VILLAGE:- MALLOMUSEPANI

N M E O
. KANCHANPUR

T. H. — 89
T. Str. — 92

Scale 1" = 220 S.



LEGEND

HOUSE.....
CART. TRACK.....
FOOT PATH.....
FOREST.....
PADDY FIELD.....
SMALL RIVER OR STREAM---
WATER SPRING.....
WELL.....

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Fig 5

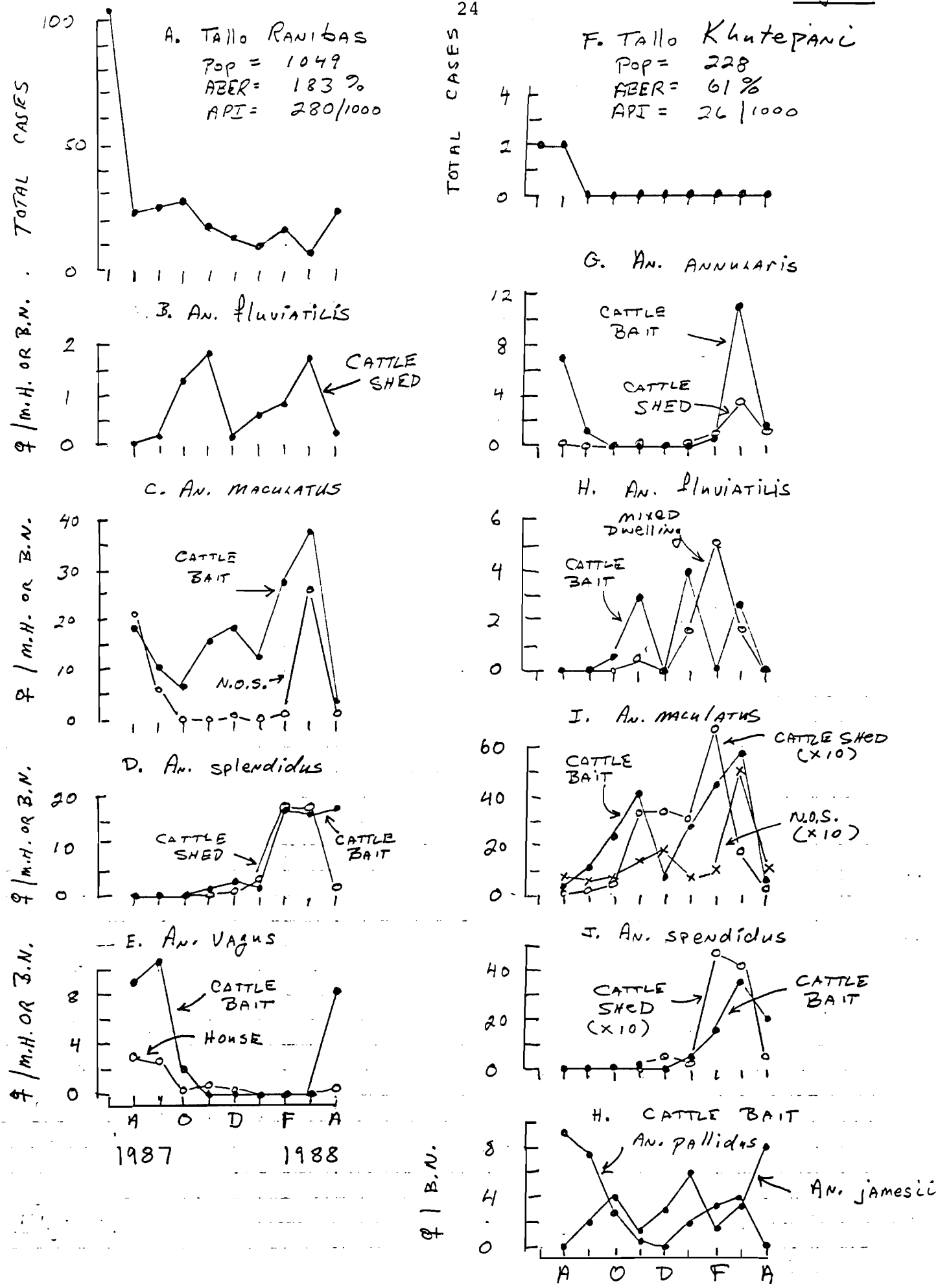
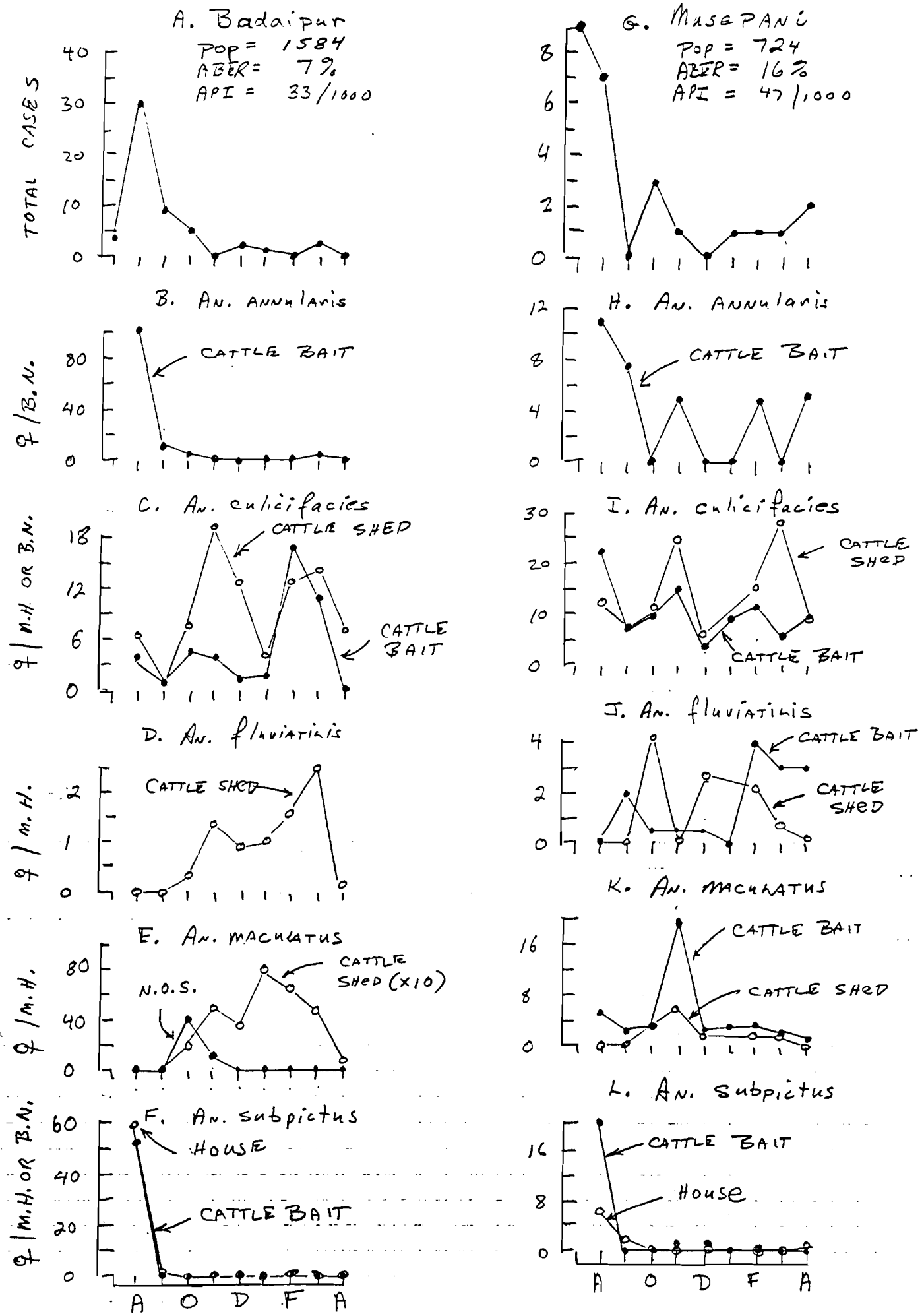

A. Tallo Ranibas
Pop = 1049
ABER = 183 %
API = 280/1000
TOTAL CASES
B. An. fluviatilis
♀/m.H. or B.N.
CATTLE SHED
C. An. maculatus
CATTLE BAIT
N.O.S.
D. An. splendidus
CATTLE SHED
CATTLE BAIT
E. An. vagus
CATTLE BAIT
HOUSE
A O D F A
1987 1988
F. Tallo Khutepani
Pop = 228
ABER = 61 %
API = 26/1000
G. An. annularis
CATTLE BAIT
CATTLE SHED
H. An. fluviatilis
mixed Dwelling
CATTLE BAIT
I. An. maculatus
CATTLE SHED (x10)
CATTLE BAIT
N.O.S. (x10)
J. An. spendidus
CATTLE SHED (x10)
CATTLE BAIT
H. CATTLE BAIT
An. pallidus
An. jamesii
♀/B.N.
A O D F A

Fig. 6


A. Badaipur
Pop = 1584
ABER = 7%
API = 33/1000
TOTAL CASES
G. Musepani
Pop = 724
ABER = 16%
API = 47/1000
B. An. annularis
♀/B.N.
CATTLE BAIT
H. An. annularis
CATTLE BAIT
C. An. culicifacies
♀/M.H. OR B.N.
CATTLE SHED
CATTLE BAIT
I. An. culicifacies
CATTLE SHED
CATTLE BAIT
D. An. fluviatilis
♀/M.H.
CATTLE SHED
J. An. fluviatilis
CATTLE BAIT
CATTLE SHED
E. An. maculatus
♀/M.H.
N.O.S.
CATTLE SHED (x10)
K. An. maculatus
CATTLE BAIT
CATTLE SHED
F. An. subpictus
♀/M.H. OR B.N.
HOUSE
CATTLE BAIT
L. An. subpictus
CATTLE BAIT
House
A O D F A

ANNEX 1

ITINERARY FOR WKR TRIP TO NEPAL

April - May 1988

April 13	Arrived in Kathmandu and reviewed project data collated by USAID Malaria Advisor.
April 14-15	Visited USAID to discuss trip itinerary. Visited NMEO and discussed status of research with Interim Chief Officer. Met with NMEO parasitology staff and set up ELISA assay. Reviewed NMEO blood meal assay methods.
Arpil 16	Reviewed project field data.
April 17-22	Visited study villages in Sindhuli District with USAID staff. Observed research activities by the NMEO entomological team and assisted with mosquito collection and processing.
April 24-29	Visited study villages in Kanchanpur District with USAID staff. Observed research activities by the NMEO entomological field team and assisted with mosquito collection and processing.
April 30 - May 2	Summarized field collection records and wrote report.
May 3	Debriefed NMEO and USAID staff and departed for USA.

ANNEX 2

PERSONS CONTACTED

Kathmandu

Dr. D.H. Calder, Chief, Health and Family Planning, USAID
Mr. J.L. Anderson, Population Specialist, USAID
Mr. S.P. Pradhan, Malaria Advisor, USAID
Mr. J.P.B. Shrestha, Interim Chief Officer, NMEO
Ms. N. Palikae, Parasitologist, NMEO
Mr. S.N. Jha, Parasitologist, NMEO
Dr. A.N.A. Arayasundere, Malaria Advisor, WHO

Sindhuli District

Mr. S.B. Shrestha, Malaria Inspector and Team Leader, NMEO
Mr. H.N. Jha, Malaria Inspector, NMEO
Mr. R.S. Shah, Malaria Inspector, NMEO
Mr. B. Mahato, Insect Collector, NMEO
Mr. M. Singh, Insect Collector, NMEO
Mr. L.K. Ghale, Insect Collector, NMEO
Mr. N. Bartaula, Insect Collector, NMEO

Far West Region

Dr. S.S. Tulachand, Regional Director, Far Western Region
Mr. G.P. Shrestha, Malaria Officer, Kailali District, NMEO
Mr. C. Shah, Acting Regional Malaria Officer, NMEO
Mr. G.R. Sharma, Malaria Inspector and Team Leader, NMEO
Mr. L.P. Chaudari, Insect Collector, NMEO
Mr. H.N. Jha, Insect Collector, NMEO
Mr. U.R. Sharma, Insect Collector, NMEO
Mr. A.K. Shrestha, Insect Collector, NMEO

ANNEX 3

ELISA KITs FOR DETECTION OF PLASMODIUM FALCIPARUM AND P. VIVAX SPOROZOITES IN INFECTED MOSQUITOES

30 October 1987

Enzyme-linked immunosorbent assays (ELISAs) were developed to detect and monitor *Plasmodium falciparum* and *P. vivax* circumsporozoite (CS) proteins in infected mosquitoes. The sensitivity and specificity of the ELISAs are based on the use of monoclonal antibodies (MABs) produced against sporozoites isolated from the salivary glands of infected mosquitoes.

The *P. falciparum* and *P. vivax* sporozoite ELISAs are currently being field tested. It must be stressed that ELISA results are not synonymous with salivary gland sporozoite rates, as the ELISAs detect CS protein present in the developing oocysts and on sporozoites present in the haemocoel as well as that present on salivary gland sporozoites. **A positive ELISA on a mosquito is not proof that the species is a vector.** Much better correlation with salivary gland rates can be achieved by cutting the mosquito and assaying only the head-thorax. There is still the possibility of detecting haemocoel sporozoites which do not enter the salivary glands. **It is therefore essential that all results be reported and interpreted in respect to these limitations.**

The ELISAs can be performed on fresh, frozen, or dried specimens. It is much easier to cut fresh or frozen mosquitoes. Dried insects become brittle and must be broken using forceps. **If dried, specimens must be processed quickly and kept dry until testing.** The presence of a blood meal in the mosquito gut does not interfere with the assay. Before collection of the mosquitoes is begun, consideration should be given to the possibility of conducting other assays, e.g. blood meal ELISA, speciation by electrophoresis or DNA probe, etc., which may determine storage and grinding buffer requirements.

The "sandwich" ELISA is begun by adsorption of the *P. falciparum* or *P. vivax* capture MAB to the wells of a microtitration plate (Fig. 1.A). After overnight incubation at room temperature the well contents are aspirated and the remaining active binding sites on the plate are blocked with blocking buffer. Mosquitoes to be tested are ground in blocking buffer containing Nonidet P-40, diluted with blocking buffer and aliquots added to the wells. Positive and negative controls are also added to specific wells at this time; recombinate proteins are used for the quantitative positive controls. If CS antigen is present (depicted as a double arrow in Figure 1.B) it will form an antigen-antibody complex with the capture MAB. After 2 hours incubation, the mosquito triturate is aspirated and the wells are washed. The respective peroxidase-linked MAB is then added to the wells, completing the formation of the "sandwich" (Fig. 1.C). After 1 hour incubation the well contents are aspirated, the plate is washed and the clear peroxidase substrate solution is added. As the peroxidase enzyme reacts with the substrate a dark green product is formed (Fig 1.D), the intensity of the color being relative to the amount of CS antigen present in the test sample. Results are read visually or

at 414 nm using an ELISA plate reader 30 or 60 minutes after the substrate has been added.

Positive mosquitoes must be retested to: a) confirm the positives and b) determine the number of sporozoites per mosquito, if this information is desired.

Using the expendable contents of a single kit, approximately 2000 mosquitoes can be tested for *P. falciparum* or *P. vivax* sporozoites. We hope to have sporozoite ELISAs for *P. malariae* and *P. ovale*, using MABs developed at the Centers for Disease Control (CDC), available in the near future. All items except the positive control materials, supplied by Smith Klein and French, and the MABs are available commercially. Once development and field evaluation are complete plans are for the distribution of ELISA kits and reagents through the World Health Organization (WHO). Development of these assays has been a cooperative effort among the Board on Science and Technology for International Development, CDC, Naval Medical Research Institute, New York University, WHO and the Walter Reed Army Institute of Research.

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Collins, F. H. et al. 1984. First field trial of an immunoradiometric assay for the detection of malaria sporozoites in mosquitoes. Am. J. Trop. Med. Hyg. 33: 538-543.

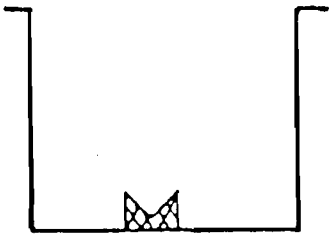
Wirtz et al. 1986. Identification of *Plasmodium vivax* sporozoites in mosquitoes using an enzyme-linked immunosorbent assay. Am. J. Trop. Med. Hyg.34: 1048-1054.

Wirtz et al. 1987. Comparative testing of *Plasmodium falciparum* sporozoite monoclonal antibodies for ELISA development. Bull. WHO 65:39-45.

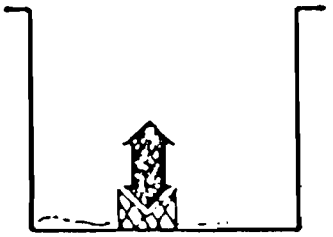
(WIRTZ*4; "SP.ELISA.DIRECTIONS.4 ")

Figure 1. The sandwich ELISA for detection of *Plasmodium falciparum* or *P. vivax* sporozoites.

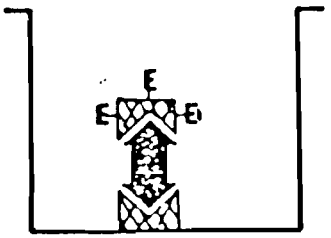
A. Anti-sporozoite monoclonal antibody adsorbed to the plate



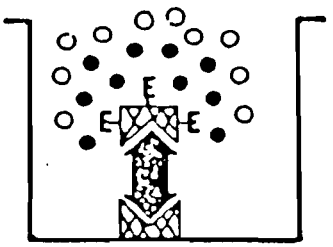
B. Mosquito triturate added to the well



C. Peroxidase linked anti-sporozoite monoclonal antibody added



D. Peroxidase substrate added



1 µl = 0.001 ml

SPOROZOITE ELISA SOLUTIONS

1. PHOSPHATE BUFFERED SALINE (PBS), pH 7.0-7.4: Use laboratory PBS OR add 1 bottle Dulbecco's PBS to 1 L distilled water (dH_2O), mix and adjust pH if necessary. Add 0.01 gm phenol red or 100 ul of phenol red stock solution (1 gm/10 ml dH_2O) per L PBS. Store 4o C. Shelf life - 2 weeks.

2. BLOCKING BUFFER (BB): Dissolve preweighed tube contents or mix as follows.

BSA (1.0%)	5.00 gm	10.00 gm
Casein (0.5%)	2.50 gm	5.00 gm
PBS, pH 7.4	500.00 ml	1000.00 ml
Thimerosal*		
1 gm/10 ml dH_2O	0.50 ml	1.00 ml
OR powder	0.05 gm	0.10 gm
Phenol red*		
1 gm/10 ml dH_2O	0.10 ml	0.20 ml
OR powder	0.01 gm	0.02 gm

(1) Suspend bovine serum albumin (BSA) and casein in PBS and mix for 2 hours or until dissolved. Some casein may not dissolve.
(2) Add the thimerosal and phenol red.

Shelf life at 4o C is 1 week. Solution maybe frozen for later use.

*Stock solutions of thimerosal and phenol red eliminate the need to weigh out small amounts of these materials.

3. BLOCKING BUFFER : NONIDET P-40 (BB:NP-40):
This is the mosquito grinding solution.
To: 1 ml BB add 5 ul NP-40.
5 ml BB add 25 ul NP-40.
Mix well to dissolve the NP-40 in the BB.
Shelf life at 4o C is 1 week.

For Pastel wash

4. WASH SOLUTION (PBS-Tw): PBS plus 0.05% Tween 20. Add 0.5 ml of Tween 20 to 1 L of PBS. MIX WELL. Store at 4o C. Shelf life - 2 weeks.

5. SUBSTRATE SOLUTION: Mix ABTS (Solution A) and hydrogen peroxide (Solution B) 1:1 immediately before use. Add 100 ul/well.

SPOROZOITE ELISA DIRECTIONS

(See following notes for details)

1. Place 50 ul of MAB solution in each well of the ELISA plate:
   P. falciparum = 0.10 ug/50 ul PBS
   P. vivax = 0.025 ug/50 ul PBS
   Use a separate plate for each sporozoite species.
   Cover plate and incubate overnight at room temperature.

OVERNIGHT

make sure to fill wells

2. Aspirate well contents and fill with BB (ca. 200 ul/well).
3. Place the mosquito head-thorax in a labeled 1.5 ml micro centrifuge tube and grind in 50 ul BB:NP-40 (5 ul NP-40/ml BB, mix well before use). Rinse pestel with 2 - 125 ul volumes of BB, catching the rinses in the tube (Total = 300 ul). Test sample or store frozen. Rinse pestel in PBS-Tw twice and dry to prevent contamination between mosquitoes.

1 HOUR

4. Aspirate well contents and add 50 ul mosquito triturate/well.
   Add positive and negative controls.

2 HOUR

5. Wash wells twice with PBS-Tw.
6. Mix MAB-peroxidase conjugate in blocking buffer:
   0.05 ug/50 ul BB for both *P. falciparum* or *P. vivax*
   Check enzyme activity by mixing 2-5 ul with 100 ul substrate. There should be a rapid color change within 1-2 minutes indicating that the peroxidase enzyme and the substrate are functional.

1 HOUR

7. Wash wells ~~3~~ 4 times with PBS-Tw.
8. Add 100 ul substrate solution per well.
9. Read visually, or at 414 nm, 30 or 60 minutes after adding substrate.

NOTES FOR SPOROZOITE ELISAs

1. Dissolve the lyophilized MAB and the peroxidase-conjugated MAB in diluent (1:1 mixture of distilled water and glycerine) to give stock solutions of 0.5 mg/ml (0.5 ug/ul). Store stock solutions at 4o or -20o C. The water: glycerine diluent allows routine storage at -20o C without freeze-thawing. Refrigerator / freezer —— Dilute as on vial ——

2. Do not add sodium azide to ELISA solutions as it is a peroxidase inhibitor.

3. To fill each of the 96 wells on a plate with 50 ul/well requires 4.8 ml. It is convenient to make up 5.0 ml of each MAB solution and 10.0 ml of substrate (100 ul/well) per plate.

SPECIES	MAB	UG/50 UL/WELL	UG/5 ML	UL STOCK/5 ML
P. f.	capt	0.100 ug/50 ul	10.0 ug	20 ul stock/5 ml PBS
P. v.	capt	0.025 ug/50 ul	2.5 ug	5 ul stock/5 ml PBS
P. f.	perox	0.050 ug/50 ul	5.0 ug	10 ul stock/5 ml BB
P. v.	perox	0.050 ug/50 ul	5.0 ug	10 ul stock/5 ml BB

4. Cover plate with a second plate during incubations to prevent evaporation.

5. Place 1 mosquito head-thorax in each tube and grind in 50 ul of BB:NP-40. Rinse pestle with 2 - 125 ul washes BB, catching the rinses in the tube containing the mosquito triturate (total volume = 300), and test or freeze for later use. Rinse pestel in water twice and dry to prevent contamination between mosquitoes.

6. **Negative controls**: Triturate laboratory reared, known uninfected female mosquitoes (same as test species if possible) or field collected male anophelines (same as test species if possible) in 50 ul BB:NP-40, dilute with 250 ul BB (total volume 300 ul) and place 50 ul from each into negative control wells 1B-1H for initial testing or wells 1A-1H for confirmational testing. To determine the negative cut off value for initial testing calculate mean and standard deviation on these 7 negative controls and retest any mosquito with a value of the mean plus 5 standard deviations. Calculate a mean and standard deviation for the 8 negative control mosquitoes for confirmational testing and use the mean plus 5 standard deviations as the negative cutoff value.

500ul = 0.5ml

7. Positive controls:

P. falciparum: Dissolve lyophilized positive control stock solution in 50 ul of distilled water for 1 mg/1 ml (1 ug/1 ul).

1:100 Vial I = Transfer 5 ul (5 ug) of positive control stock solution (1.0 mg/ml) to 500 ul BB to for 100 ng/10 ul.

1:100 Vial II = Transfer 10 ul (100 ng) Vial I to 1000 ul BB to for 1000 pg/10 ul BB.

1:50 Vial III = Transfer 10 ul (1000 pg) Vial II to 500 ul BB to yield 100 pg/50 ul BB (Use 50 ul in well 1A). Dilute 1:1 with BB (250 ul Vial III plus 250 ul BB, etc.) for positive control curve for quantitative confirmational retesting (Fig. 2) of 100 (Vial III), 50, 25, 12, 6, 3 and 1.5 pg/50 ul BB. Run standard curve in triplicate (wells 2A-4A to 2H-4H) as well as 8 negative control mosquitoes (wells 1A-1H) when retesting. Freeze the stock solution and Vials I and II for continued use.

P. vivax: Dissolve lyophilized positive control stock solution in 25 ul of distilled water to for 1 mg/1 ml (1 ug/1 ul).

1:100 Vial A = Transfer 5 ul (5 ug) of positive control stock solution (1.0 mg/ml) to 500 ul BB to for 100 ng/10 ul.

1:100 Vial B = Transfer 10 ul (100 ng) Vial A to 1000 ul BB to for 2000 pg/20 ul BB.

1:25 Vial C = Transfer 20 ul (2000 pg) Vial B to 500 ul BB to yield 800 pg/200 ul BB.

1:4 Vial D = Transfer 200 ul (800 pg) Vial C to 800 ul BB to yield 40 pg/50 ul BB (Use 50 ul in well 1A). Dilute 1:1 with BB (250 ul Vial D plus 250 ul BB, etc.) for positive control curve for quantitative confirmational retesting (Fig. 3) of 40 (Vial C), 20, 10, 5, 2.5, 1.25 and 0.6 pg/50 ul BB. Run standard curve in triplicate (wells 2A-4A, 2H-4H) as well as 8 negative control mosquitoes (wells 1A-1H) when retesting. Freeze the stock solution and Vials A, B and C for continued use.

8. Retesting for quantitation: Dilute strong positives (30 min abs > 2.00) by adding 250 ul BB before retesting (Volume would then be 500 ul). Repeat if necessary to insure that the absorbance value for the test sample is in the linear portion of the positive control curve.

9. If you order supplies insure that they are identical to those in the kit. Different 1.5 ml tubes have different internal dimensions and may not work with the pestles, microtiter plates have different binding qualities, different caseins are less efficient in blocking, etc. *

10. We need your suggestions, corrections and questions to improve this assay. Please send them to: Robert A. Wirtz, Dept. of Entomology, WRAIR, Washington, DC 20307-5100; telephone (202) 576-3275/3719.

LTC Richard G. Andre
Chief, Entomology

*SPOROZOITE ELISA NO:*_____
(INITIAL TESTING)

DATES: Coat:_____ Test:_____

	1	2	3	4	5	6	7	8	9	10	11	12
A+Pos+												
B-Neg-												
C-Neg-												
D-Neg-												
E-Neg-												
F-Neg-												
G-Neg-												
H-Neg-												
	1	2	3	4	5	6	7	8	9	10	11	12

_____ 1) Coat PVC flex plate with capture MAB (OVERNIGHT)
0.025 ug P.v.= _____ul stock; 0.5 ug/ul; lot#
0.10 ug P.f.= _____ul stock; 0.5 ug/ul; lot #
50 ul PBS ml PBS

_____ 2) Aspirate; fill wells with blocking buffer (BB) (1 hr)
_____ 3) Add mosquito triturate (2 hr). (Pos cont lot#_____)
Total volume/mosquito: _____ ul

_____ 4) Aspirate and wash twice with PBS- 0.05% Tween 20
_____ 5) Add Pf or Pv peroxidase MABs (Lot #s:_____) (1 hr)
0.05 ug = _____ ul stock (0.5 ug/ul)
50 ul BB ml BB

_____ 6) Aspirate and wash 3 times with PBS - 0.05% Tween 20
_____ 7) Mix substrate (1:1): _____ a) Enzyme check; _____ b) 100 ul/well
_____ 8) Read absorbance (414 nm) at 30 and/or 60 minutes.

*SPOROZOITE ELISA NO:*_____
(CONFIRMATIONAL TESTING)

DATES: Coat:_____ Test:_____

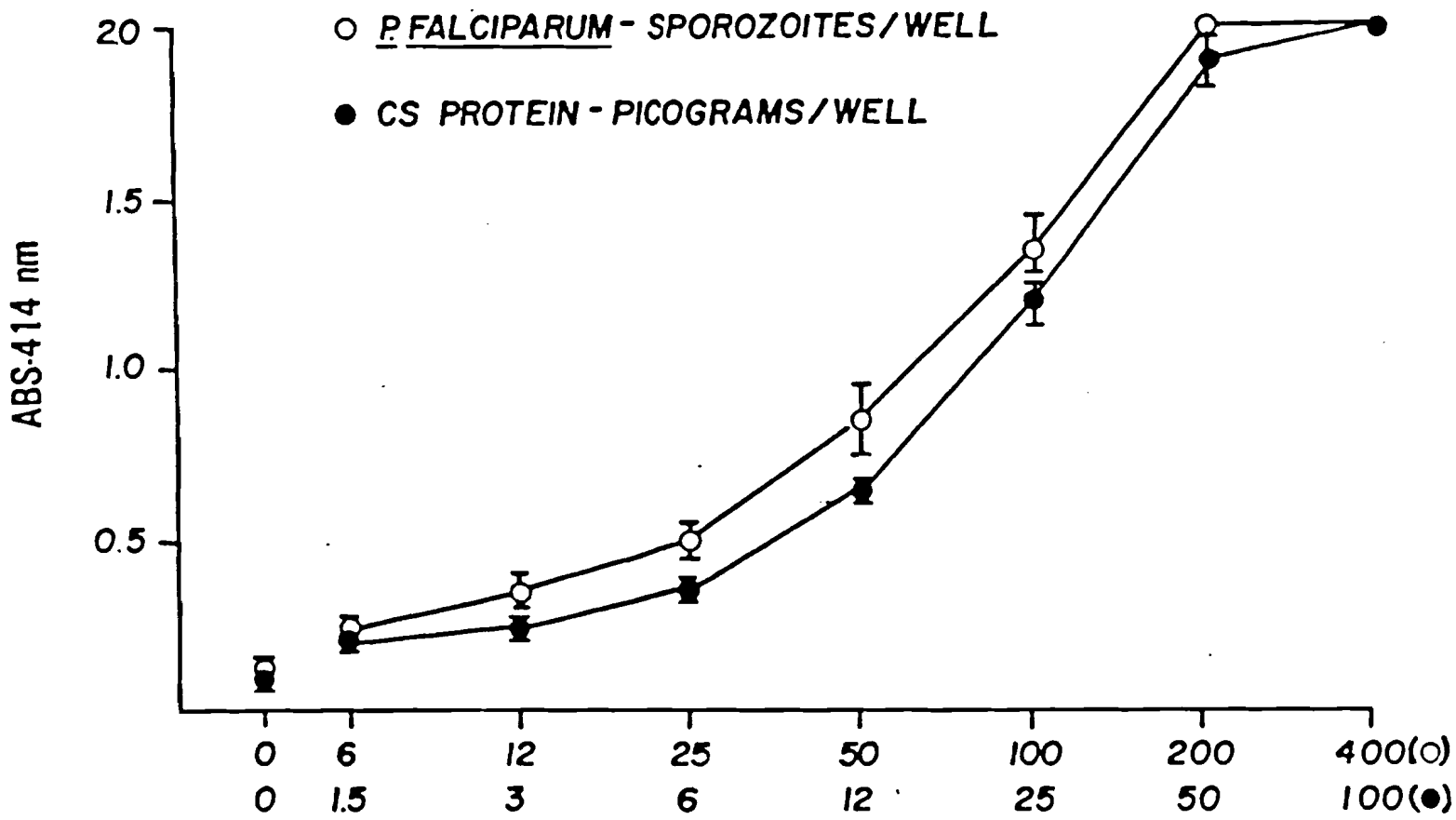
	NEG	POSITIVE CONTROL										
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
E												
G												
H												
	1	2	3	4	5	6	7	8	9	10	11	12

_____ 1) Coat PVC flex plate with capture MAB (OVERNIGHT)
0.025 ug P.v. = _____ul stock; 0.5 ug/ul; lot#
0.10 ug P.f. = _____ ul stock; 0.5 ug/ul; lot #
50 ul PBS ml PBS

_____ 2) Aspirate; fill wells with blocking buffer (BB) (1 hr)
_____ 3) Add mosquito triturate (2 hr). (Pos cont lot#_____)
Total volume/mosquito: _____ ul

_____ 4) Aspirate and wash twice with PBS- 0.05% Tween 20
_____ 5) Add Pf or Pv peroxidase MABs (Lot #s:_____) (1 hr)
0.05 ug = _____ ul stock (0.5 ug/ul)
50 ul BB ml BB

_____ 6) Aspirate and wash 3 times with PBS - 0.05% Tween 20
_____ 7) Mix substrate (1:1): _____ a) Enzyme check; _____ b) 100 ul/well
_____ 8) Read absorbance (414 nm) at 30 and/or 60 minutes.



PLASMODIUM FALCIPARUM SPOROZOITES(○) AND CIRCUMSPOROZOITE PROTEIN(●)

ANNEX 4

RESULTS OF SPOROZOITE ELISA ASSAY RUN AT NMEO NHQ

O = Positive wells

P. FALCIP

	1 (+ CONT)	2	3	4	5	6	7	8	9	10	11	12
+++ A	(VIAL)	–	(1)	9	17	25	(33)	–	–	(VIAL)		
+++ B	(50)	–	2	10	18	(26)	34	–	–	(50)		
++ C	(25)	–	3	11	19	27	(35)	–	–	(25)		
++ D	(12)	–	4	12	20	(28)	(36)	–	–	(12)		
+ E	(6)	–	(5)	(13)	(21)	(29)	–	–	–	(6)		
F	–	–	6	14	22	30	–	–	–	–		
G	–	–	7	15	23	31	–	–	–	–		
H	–	–	8	16	24	32	–	–	–	–		

color intensity

P. VIVAX

	1	2	3	4	5	6	7	8	9	10	11	12
A	(D)	–	1	9	17	25	33	–	–	(D)		
B	(50)	–	2	18	18	26	34	–	–	(50)		
C	(25)	–	3	11	19	27	35	–	–	(25)		
D	(12)	–	4	12	20	28	36	–	–	(12)		
E	(6)	–	5	13	21	29	–	–	–	(6)		
F	–	–	6	14	22	30	–	–	–	–		
G	–	–	7	15	23	31	–	–	–	–		
H	–	–	8	16	24	32	–	–	–	–		

1-15 An. maculatus colony } fed 15+16 days before
16-36 ~~An. fluviatilis colony~~ } ~~on patient~~ gametocemia. (1-2/fie
$\gtrsim$ 150/mm³?

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ANNEX 5

SAMPLES FOR ELISA TESTING

Tube No.	Village	Date	Collection Method	Anopheles species	Pool Size	ELISA Test Pre.	Conf.