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EPIDEMIOLOGY AND CONTROL OF MALARIA, LEISHMANIASIS AND SCHISTOSOMIASIS IN BRAZIL

FINAL REPORT

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Human malaria in Brazil is caused by Plasmodium falciparum and Plasmodium vivax. The incidence of malaria cases reported from the Amazon Basin is increasing yearly due, in part, to the influx of colonists and miners from other regions of Brazil. Control measures currently in use are ineffective and little is known about the biology and infectivity of potential vectors. This study shows that Anopheles darlingi is the primary vector of both species of malaria in the Costa Marques, Rondonia area, and other anophelines serve to augment the seasonal transmission. Circulating gametocytes in the blood of some patients were infective to mosquitoes 21 days after treatment with quinine.

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FOREWORD

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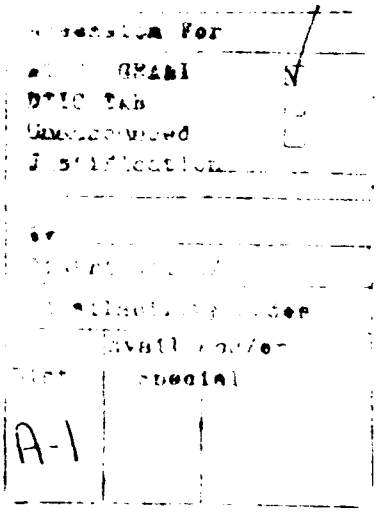

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MALARIA

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1. BACKGROUND

The Costa Marques area (Lat. 12 26'S, Long. 64 14'W) is located on the east bank of the Guapore River which forms the boundary between Bolivia and Brazil. The topography is relatively flat and the vegetation consists of tropical forest in areas which have not been cleared for agriculture. Rainfall is distributed in a dry season, from May to September, and a wet season, from October to April. Immigration has been facilitated by the construction of a paved airport for small aircraft and a 320 km dirt road which is impassable for short periods during the rainy season.

Houses are of variable construction from concrete block, wood frame to bamboo with thatch roof. Only a few hosues have screened windows. In general, the poorer constructed houses are on the periphery of the city and along the road. Temporary open shelters of bamboo with thatch roof are common.

2. OBJECTIVES

A. To determine the seasonal and diurnal biting behavior of Anopheles mosquitoes and their infectivity rates

B. To determine the susceptibility of anopheline mosquitoes to human malaria and effects of anti-malarial drugs on sporogeny.

C. To define the taxonomy of mosquitoes in Costa Marques with emphasis on potential vectors of malaria.

D. To determine the insecticide resistance/susceptibility of anopheline mosquitoes in the Costa Marques area. [No progress]

A. Seasonal and Diurnal Biting Behavior of Anopheles Mosquitoes and Their Infectivity Rates

This study, conducted in the Costa Marques, Rondonia area of the Brazilian Amazon, provides baseline data necessary for a future malaria vaccine trial and also provides military commanders with an insight of the potential malaria threat for similar areas of the Amazon basin. It compares the

susceptibility of anopheline species to infection with P. vivax and P. falciparum. Susceptibility was based on the proportion of mosquitoes with oocysts and sporozoites, the mean number of oocysts and the number of sporozoites per infected mosquito.

The seasonal and diurnal biting behavior portion of this study was completed prior to the initiation of the grant.

MATERIALS AND METHODS

Anopheline mosquitoes to provide progeny for this study were collected from human and bovine bait in and near the town of Costa Marques, Rondonia, Brazil. Mosquitoes were provided blood meals on human volunteers, identified, placed in screen topped 215 cm^3 (pint) cartons and provided a 5% sucrose solution. On day 3 post-feed, gravid females of all species were traumatized by removing one wing with forceps to induce oviposition. Each female was then placed in a small plastic cup (5 x 3.5 cm) with filtered tap water. Eggs were placed in larval rearing pans with filtered tap water and surrounded by a plastic drinking straw to prevent them from sticking to and desiccating on the sides of the tray. Larvae were fed one part finely ground wheat germ and two parts baby fish food. Pupae were put in finger bowls with water and placed in screened cages, 32 cm on each side, where they emerged. The insectary was maintained at 26 $\pm$ 2(o)C and 65 $\pm$ 20% RH. A combination of natural and fluorescent lighting was used. The light cycle was maintained at 12 $\pm$ 2 hrs daylight.

Adult volunteer patients infected with P. vivax or with P. falciparum were screened by a medical doctor at the malaria clinic in Costa Marques who admitted them to the study following the provisions of a human use protocol. The patients were treated by a M.D. after the mosquitoes were fed on them.

Anopheles darlingi was selected as the control species because preliminary studies showed it was susceptible to both P. vivax and and P. falciparum, and because wild mosquitoes were present in Costa Marques throughout the year. The susceptibility of An. darlingi to malaria parasites was compared to each of the seven test species. Mosquitoes were placed in separate screened pint cartons (30/carton), starved for 4-6 hours to enhance blood feeding and allowed to feed for 30 minutes on the patients. A maximum of 60 An. darlingi and, depending on the number of species fed, 60 or 120 test mosquitoes were fed simultaneously on the same malaria patient. All blood fed mosquitoes were maintained under the same environmental conditions in the insectary at Costa Marques. The number of feedings for each species ranged from 2 to 33. Seasonal availability of some mosquitoes limited the number of feeds.

One-third to one-half of each group of mosquitoes were dissected on day 8 or day 9 post-feed for oocysts. Midguts were removed, placed in a drop of phosphate buffered solution (PBS) with coverslip and examined with a compound microscope (400X). Oocysts were counted and the oocyst infection rate and mean number of oocysts per mosquito determined. Oocyst counts that exceeded 200 were recorded as 200 for calculations.

The salivary glands were removed from the surviving mosquitoes on days 16-20 post-feed, transferred to a drop of PBS, gently squashed to release sporozoites and examined at 400X. The sporozoite infection rate and the sporozoite frequency was determined.

RESULTS

Anoopheles darlingi, An. deaneorum and An. mediopunctatus readily fed on the malaria patients in the laboratory. Anopheles triannulatus, An. oswaldoi, An. albitarsis, An. braziliensis and An. benarrochi fed less readily and generally required a 30 minute period to obtain sufficient numbers of engorged females for comparative studies. The number of mosquitoes available for examination of the midguts and salivary glands depended on the numbers that blood fed and subsequent mortality.

P. vivax

The oocyst infection rate and mean numbers of oocysts for An. darlingi and each of the test species is shown in Table 1. The percentage of An. darlingi and An. deaneorum infected with oocysts were similar. Individual oocyst counts for An. darlingi and An. deaneorum were frequently greater than 100. The mean numbers of oocysts and the percentage of mosquitoes with oocysts for the other mosquito species were much lower.

The size of P. vivax oocysts were similar for An. deaneorum, An. triannulatus, An. albitarsis, An. mediopunctatus and An. darlingi. The oocysts of An. oswaldoi, An. benarrochi and An. braziliensis on day 8 post-feed developed fewer sporozoites and were generally 1/3 or less than the diameter of oocysts seen in An. darlingi.

Table 1. Percent of mosquitoes infected with oocysts of Plasmodium vivax and mean numbers of oocysts in Anopheles darlingi compared to seven other Anopheles species examined 8 days after feeding on gametocytic patients.

Anopheles Species	Number Trials	Number Mosquitoes Dissected	Oocyst Infection Rate	Mean* (Range) of Oocysts
darlingi	29	394	89	46 (3-137)
deaneorum		426	82	37 (<1-132)
darlingi	7	94	94	55 (7-162)
albitarsis		74	52	10 (<1- 19)
darlingi	5	65	80	57 (<1-162)
mediopunctatus		55	38	6 (0- 24)
darlingi	33	518	85	41 (<1-165)
triannulatus		417	60	7 (0- 30)
darlingi	22	338	92	55 (5-165)
oswaldoi		272	53	15 (0- 56)
darlingi	2	25	73	54 (3-149)
braziliensis		22	54	15 (0- 20)
darlingi	2	39	97	37 (7- 66)
benarrochi		40	22	1 (<1- 2)

* Number of oocysts counted / number mosquitoes dissected.

The sporozoite infection rate and the numbers of sporozoites observed in the salivary glands of An. darlingi and An. deaneorum were similar (Table 2). When compared to An. darlingi, only about two-thirds and one-half as many of An. albitarsis and An. mediopunctatus, respectively, had sporozoites in the salivary glands. The percentage of An. triannulatus and An. oswaldoi with salivary gland infections was less than 10% and 5%, respectively. In limited trials, all An. braziliensis and An. benarrochi were negative for sporozoites in the salivary glands while more than 70% of An. darlingi fed at the same time had sporozoites in the salivary glands.

Table 2. Numbers of Plasmodium vivax sporozoites observed in the salivary glands of Anopheles darlingi compared to seven other Anopheles species on days 14-16 post-feed.

Anopheles Species	No. Trials	No. Dissected	% Mosquitoes by Sporozoite Counts 0	1-10	11-100	101-1000 >1000
darlingi	23	309	40	7	15	24	14
deaneorum		342	45	6	19	20	10
darlingi	7	95	22	8	8	24	38
albitarsis		87	49	2	4	22	23
darlingi	4	49	49	12	8	17	14
mediopunctatus		69	75	3	3	6	13
darlingi	29	500	38	7	17	25	13
triannulatus		479	91	4	3	1	1
darlingi	21	290	27	7	13	32	21
oswaldoi		292	98	1	<1	<1	<1
darlingi	2	12	25	0	0	42	33
braziliensis		15	100	0	0	0	0
darlingi	2	20	25	0	25	35	15
benarrochi		19	100	0	0	0	0

The observed number of mosquitoes infected with P. vivax parasites on days 14-16 post-feed was compared to the estimated number of infected mosquitoes. The estimated number of infected mosquitoes was determined by multiplying the total number of mosquitoes dissected on days 14-16 post-feed by the oocyst infection rate on day 8 post-feed (Table 3). We assumed that there was no differential in mortality between the infected and non-infected populations.

There were little differences in the observed and estimated number of infected An. deaneorum and An. albitarsis when compared to An. darlingi. The estimated number of both An. oswaldoi and An. triannulatus was approximately 10 fold greater when compared to An. darlingi fed at the same time.

Table 3. Number of P. vivax infected mosquitoes observed on days 14-16 compared to the number estimated from the oocyst infection rate on day 8 post-feed.

Anopheles Species	Number Trials	Number Dissected	Number Observed Infected	Number Estimated Infected *	Percent Difference **
darlingi	23	309	258	275	-9
deaneorum		342	251	280	-10
darlingi	7	95	91	89	+2
albitarsis		87	47	45	+4
darlingi	4	49	27	39	-31
mediopunctatus		69	19	26	-27
darlingi	29	500	409	425	-4
triannulatus		479	163	287	-43
darlingi	21	290	257	267	-4
oswaldoi		292	131	155	-15
darlingi	2	12	9	9	0
braziliensis		15	10	8	+25
darlingi	2	20	18	19	-5
benarrochi		19	0	4	N/D

* Number of mosquitoes examined on days 14-16 post-feed X Oocyst infection rate on day 8 postfeed.
** (Observed infected / Estimated infected) 100.

An estimate of the potential of sporozoites to migrate from the oocysts and invade the salivary glands of infected mosquitoes was determined by dividing the observed sporozoite infection rate on days 14-16 by the oocyst infection rate of mosquitoes dissected on day 8 post-feed. The potential sporozoite infection rate for An. deaneorum, An. Albitarsis and An. mediopunctatus were similar to An. darlingi fed at the same time. However, only 15% of the An. triannulatus and 4% of the An. oswaldoi with oocysts on day 8 would be expected to produce sporozoite infected glands on days 14-16.

P. falciparum

The oocyst infection rates for mosquitoes dissected on day 9 post-feed for comparative feeds of An. darlingi and the anopheline test species are shown in Table 4. Except for An. mediopunctatus, An. darlingi had higher mean numbers of oocysts than any of the test species. In limited trials where the mosquito infection rates were low, An. mediopunctatus demonstrated a higher oocyst infection rate and mean number of oocysts than An. darlingi. The oocyst infection rate and mean number of oocysts of An. deaneorum were similar to compared feedings of An. darlingi. The oocyst infection rate and mean number of oocysts for An. triannulatus, An. oswaldoi and An. albitarsis were much lower than compared feeds of An. darlingi.

Table 4. Percent of mosquitoes infected with Plasmodium falciparum oocysts and mean number of oocysts in Anopheles darlingi compared to five other Anopheles species 9 days after feeding on gametocytic patients.

Anopheles Species	Number Trials	Number Mosquitoes Dissected	Oocyst Infection Rate	Mean* (Range) of Oocysts
darlingi	17	312	35	9 (<1-90)
deaneorum		283	34	7 (<1-74)
darlingi	11	196	37	13 (<1-90)
triannulatus		155	8	<1 (0- 2)
darlingi	7	100	59	7 (2-25)
oswaldoi		104	13	<1 (0- 1)
darlingi	3	41	7	<1 (0-<1)
mediopunctatus		37	35	1 (<1- 3)
darlingi	3	70	13	1 (<1- 2)
albitarsis		36	6	1 (0-<1)

* Number of oocysts counted/number of mosquitoes dissected.

The percent of mosquitoes infected with oocysts for most groups was relatively low. In only two patients did individual oocyst counts for An. darlingi exceed 200 for individual mosquitoes. Sporogony appeared to be normal and sporozoites were observed in the haemocoel of mosquitoes for all groups.

The sporozite infection rates for An. darlingi, An. deaneorum and An. mediopunctatus were similar (Table 5). An triannulatus and An. oswaldoi had fewer oocysts and fewer mosquitoes with infected salivary glands than An. darlingi. Sporozoites were rarely observed in An. oswaldoi and most of them

only had a few sporozoites in the salivary glands (Table 5). An albitarsis, although developing oocysts and with sporozoites observed in the haemocoel, were never observed with sporozoites in the salivary glands.

Table 5. Number of Plasmodium falciparum sporozoites observed in the salivary glands of Anopheles darlingi compared to five other Anopheles species 15-17 days after feeding on gametocytic patients.

Anopheles Species	Number Trials	Number Dissected	% Mosquitoes by Sporozoite Counts				
			Neg	1-10	11-100	101-1000	>1000
darlingi	11	163	63	2	10	13	12
deaneorum		150	67	7	5	15	6
darlingi	11	148	62	1	6	14	17
triannulatus		136	93	1	3	3	0
darlingi	8	102	63	0	6	19	12
oswaldoi		111	97	2	1	0	0
darlingi	3	45	91	0	0	9	0
mediopunctatus		67	91	1	0	8	0
darlingi	2	39	79	0	18	3	0
albitarsis		21	100	0	0	0	0

CONCLUSIONS

Anopheles darlingi, the most commonly collected anopheline mosquito, is the primary vector of P. falciparum and P. vivax in Costa Marques, Rondonia, based on its seasonal distribution, relative abundance, peridomiciliary and biting behaviors, host contact and high natural infection rate. Anopheles deaneorum is nearly equally susceptible to P. falciparum and P. vivax, but was less frequently collected. In Costa Marques, An. deaneorum is only seasonally important in malaria transmission, whereas, in other areas it may be a vector throughout the year. Anopheles mediopunctatus is a potential vector of P. falciparum. It readily bites man, but its distribution is seasonal and it is only collected in and near forests in Costa Marques. Because it is susceptible to both P. falciparum and P. vivax, An. mediopunctatus may be an important vector in immigrant populations living in or near forested areas or indigenous natives living in the Amazon forest. Because of their seasonal distribution in Costa Marques, both An. deaneorum and An. mediopunctatus may serve to augment transmission of malaria. Although both An. triannulatus

and An. oswaldoi developed oocysts of P. falciparum and P. vivax, sporozites were infrequently observed in the salivary glands. Based on their exophilic and zoophilic behavior, low sporozoite infection rate and low numbers of sporozoites in the salivary glands, neither species is considered important in falciparum or vivax malaria transmission.
Anopheles albitarsis is a secondary vector of P. vivax, but is not a vector of P. falciparum since sporozoites were never observed in the salivary glands.

B. EFFECTS OF ANTIMALARIALS ON MOSQUITO INFECTIVITY

This study was designed to determine if quinine or quinine + tetracycline have sporontocidal or gametocytocidal activity on immature and mature gametocytes.

MATERIAL AND METHODS

Sixty 3-6 day old laboratory reared F_1 An. darlingi were placed in screen topped 215 cm^3 (pint) cartons and allowed to feed for 20-30 minutes on falciparum patients prior to receiving quinine or quinine + tetracycline. Additional mosquito feeds were done at 1-5 day intervals for up to 21 days following treatment. Bloodfed females were provided a 10% sucrose solution throughout the study. A portion (2-5) of the mosquitoes were examined for oocysts commencing on day 5 post-feed. In addition, from days 9-16, a portion of the mosquitoes were dissected and examined by light microscopy (400X) and/or enzyme linked immunosorbent assay (ELISA) for sporozoites. Infection rates determined by light microscopy were recorded as negative, 1-10, 11-100, 101-1,000 and >1,000 sporozoites.

RESULTS

The duration patients were infective to mosquitoes after treatment was variable. Some patients with gametocytes did not infect mosquitoes 5 days after initial treatment. Other patients demonstrated gametocytes in blood films and were infective to mosquitoes at 21 days post treatment with quinine or quinine + tetracycline when they were treated with 45 mg primaquine. Twenty-four h after treatment with primaquine, patients were negative for circulating gametocytes or had abnormal gametocytes not infective to mosquitoes. Because some patients were still infective when treated with primaquine, the potential duration that patients remain infective to mosquitoes following quinine treatment was not determined.

Although in general low numbers of circulating gametocytes resulted in few infected mosquitoes, no correlation was found.

C. Taxonomy of Mosquitoes in Costa Marques with Emphasis on Potential Vectors of Malaria.

1. BACKGROUND

This was a continuation of the collaborative effort with entomology personnel at the Biosystematics Unit, Smithsonian Institution which began in the mid 1980's. This effort was primarily concerned with general surveys for determining the mosquito fauna of the Brazilian Amazon, but also includes a reexamination of certain groups of Anopheles mosquitoes.

2. MATERIAL AND METHODS

Blood engorged mosquitoes collected during the malaria studies were placed in ovoposition tubes. The eggs were transferred to small plastic rearing pans partially filled with water. After hatching, the larvae and pupae were placed in individual plastic rearing vials containing tap water. Associated larval and pupal skins were placed in 1/4 dr glass vials containing 70% ethyl alcohol and later mounted on slides for microscopic examination. The adults were killed with ethyl acetate, mounted and labeled. The adults and corresponding larval and pupal skins were sent to the Biosystematics Unit, Smithsonoan Institution for taxonomic study.

3. RESULTS

The taxonomic studies are continuing and the results will be presented later. These studies have confirmed the biological evidence gathered during mosquito collections that the species which has been called An. albitarsis is actually two distinct species, An. albitarsis and An. deaneroum.

4. CONCLUSIONS

Malaria is a disease of principal importance and the vector populations must be correctly identified to provide an estimate of the disease potential in diverse endemic areas. Little progress has been made in the elucidation of species complexes and longitudinal surveys in the Amazon Basin. Serious identification problems, involving suspected and known arboviruses and malaria mosquito vectors still exist. Taxonomic studies must be continued to help solve these problems.

LEISHMANIASIS

1. BACKGROUND.

Visceral leishmaniasis is present throughout Brazil, and is common in the lower income populations in the central and northeastern states. The diagnosis is usually based on clinical history, symptoms and the presence of amastigotes in Giemsa stained smears prepared from bone marrow aspirates. Serological determinations are available in central hospitals which have laboratorial support, but medical personnel in poor rural communities who treat the majority of cases have no access to this method of diagnosis. An inexpensive and technically simple diagnostic test which can be performed quickly by paramedics without electricity or special equipment would be very useful.

American cutaneous leishmaniasis is a disease of rural subsistence farmers. In endemic areas of Brazil the diagnosis is usually made by a paramedic based on the clinical appearance of the lesion without laboratory support. While the initial injection is usually given by medical personnel, subsequent injections are given intramuscularly at home by a non-skilled neighbor. Compliance depends on the treatment schedule, availability of the drug, side effects, and cost. Given these practical constraints, knowledge of the optimal schedule of each drug is fundamental.

2. OBJECTIVES.

a. To develop a simple DOT-ELISA and/or Direct Agglutination Test for use at the technical level in the field for the diagnosis of human and canine visceral leishmaniasis.

b. To develop immunologic tests to detect leishmanial antigen in the urine of patients with kala azar and to determine their value in diagnosis and prognosis. [No progress]

c. Determine the diagnostic value of the Direct Flourescent Antibody Test using Leishmania specific monoclonal antibodies for the detection of amastigotes in mucotaneous lesions caused by Leishmania (Viannia) braziliensis. [No progress]

d. To determine the optimal treatment schedule of Glucantime for the treatment of simple cutaneous leishmaniasis caused by L. (V.) braziliensis in Brazil using a double blind protocol with strict compliance.

A. A simple DOT-ELISA for the diagnosis of canine and human visceral leishmaniasis.

METHODS.

The DOT-ELISA was modified from the methods of Pappas et al. To prepare antigen, promastigotes of L. chagasi (LUT/87/EO345 Jacobina) were cultured in Schneider's Drosophila Medium supplemented with 20% heat inactivated Fetal Bovine Serum. Log phase promastigotes were washed X3 in PBS and fixed with 1.5% formalin (Scientific Products, 37% Formaldehyde, 12-15% Methanol) diluted in PBS. After washing X3 by centrifugation in PBS, 50-60,000 promastigotes were dropped onto 0.45 um pore nitrocellulose membranes in one ul volumes. After drying overnight at room temperature, the membranes were blocked in a 0.5% solution (w/v) of non-fat milk diluted in PBS, dried, and stored at -4 or -20 C. Before use the sensitized nitrocellulose was cut into small disks and placed individually into wells of a microtiter plate.

The ELISA was conducted at ambient temperature. To minimize the effects of prozoning, two dilutions of each dog serum were screened. Serum dilutions of 1:32 and 1:512 were used to screen for anti-leishmania IgG while dilutions of 1:8 and 1:32 were used to screen for IgM. The antigen sensitized disks were incubated with 50 ul of sera for 15 minutes and washed X3 using 100 ul volumes of PBS. The disks were then incubated for 15 minutes with 50 ul of affinity purified peroxidase conjugated goat sera specific for the heavy and light chains of dog IgG or IgM (Kirkegaard & Perry, Bethesda, MD) diluted in 0.1% non-fat milk in PBS, Ph 7.4. After washing X3 in PBS as described above, the disks were treated with 50 ul of substrate (4-chloro-1-naphthol and 1 part hydrogen peroxide from Kirkegaard & Perry with 2 parts PBS). The reaction was stopped after 15 minutes by washing the antigen disks X3 with 0.05% NP-40 diluted in PBS. A positive reaction was visualized as a purple dot on a white background. Each test included negative control sera from dogs living in Maryland which were diluted 1:32. Sera from dogs infected with L. chagasi diluted to 1:1024 in PBS were used as positive controls.

The indirect immunofluorescent antibody test modified from Shaw and Lainson[2] was used for comparison. Antigen amastigotes of Leishmania amazonensis were isolated from hamsters, purified, fixed with 2% formalin (Merk, Sao Paulo) and diluted with Phosphate Buffered Glucose. They were aliquoted onto glass slides using a dilution that approximated 50 parasites per microscopic field at 400 X. The sensitized slides were air dried and stored at -20 C until used.

Sera were screened at a dilution of 1:20 in phosphate buffered saline at Ph 7.4 (PBS) and the titers of reactive sera were determined from doubling dilutions. Fifty ul of diluted sera were incubated with the amastigotes for 30 minutes at room temperature in a humidity chamber. After washing with PBS, the amastigotes were incubated in 10 ul of fluorescene conjugated goat sera specific for the heavy and light chains of canine IgG (Miles-Yeda Laboratories, USA) diluted 1:50 in PBS for 30 minutes. After washing again in PBS the amastigotes were overlaid with buffered glycerine and examined at 400 diameters under a flourescent compound microscope.

Blood was collected from dogs that were sacrificed at the Centro de Zoonoses in Teresina, Piaui. Some dogs were strays, but most of them were selected for extermination because they were seropositive in a routine indirect immunofluorescent test used by SUCAM in a program to control the canine reservoir of human kala azar. All dogs were examined clinically to determine if they had signs of kala azar and parasitologically for infection with Leishmania.

Parasitologic techniques to detect Leishmania included stained smears, cultures and hamster inoculation. The organs sampled included the spleen, liver, bone marrow, blood, skin of the nose, urethral secretions of males and vaginal secretions of females. The types of parasitologic techniques and the tissues sampled for Leishmania varied among three groups of dogs.

Impression smears were stained with Giemsa and examined for amastigotes by light microscopy at 400 X. Cultures were prepared from tissues aspirated into a sterile 5 ml syringe using a 22 gauge needle. The aspirates were inoculated onto NNN media overlaid with 0.9% NaCl with Gentamyacin and Penicillin/Streptomyosin, and incubated of 30 days at 24 C. The cultures were examined at frequent intervals under an inverted microscope for promastagotes. Positive cultures were identified by isoenzime electrophoresis[3] and cryopreserved. Tissue aspirates were also inoculated into hamsters which were examined for signs of infection at weekly intervals for 6 months. They were examined for Leishmania using impression smear and culture techniques.

Sera from healthy dogs living in Silver Spring, Maryland were used for testing specificity.

Sera from dogs experimentally infected with Trypanosoma cruzi at the Schoool of Veterinary Medicine, Louisiana State University in Baton Rouge were used for testing cross reactivity.

RESULTS

Sera taken from 98 dogs in Teresina were examined microscopically for Leishmania amastigotes. The efficiency of DOT-ELISA, and IFA tests conducted at the Universidade de Brasilia Parasitology laboratory were compared using these sera. The results are as follows:

Serological test	True positive	False positive	True negative	False negative
IFA-UnB	62%	38%	90%	10%
DOT-ELISA	79%	21%	55%	45%

The proportion of false positives is too high for serological tests. These false positives may indicate that:

a) The dogs may indeed be infected, but the culture and impression smear techniques failed to detect Leishmania in the liver and spleen.

b) Other organs such as skin and medula were infected with Leishmaniasis.

c) The dogs were infected with L. chagasi or L. brasiliensis at one time and cured spontaneously.

d) The dogs were never infected with Leishmania and the serological test cross-reacted with T. cruzi or other similar antigen.

The proportion of false negatives was unacceptably high for the DOT-ELISA. Some reasons for parasite positive-IgG results negative are:

a) These dogs were recently infected and no secondary IgG response has been iniciated.

b) These dogs have high titers for anti-Leishmania IgM and low titer for IgG. In this situation the serological reaction between IgM and the Leishmania antigen may block the IgG-Leishmania reaction. If the IgM was not detected by the IgG-Leishmania conjugate, these sera were judged not reactive in both IFA and DOT-ELISA.

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D. The optimal Glucantime schedule for treatment of Leishmania (Viannia) braziliensis

Recommended schedules for the treatment of American cutaneous leishmaniasis are largely based on case reports and clinical experience. There is only one double blind study with strict compliance which concluded that Pentostam, a pentavalent antimonial, given at 20 mg Sb^{v}/kg body weight/day intravenously for 20 days heals all patients with cutaneous ulcers caused by Leishmania (Viannia) panamensis with minimal, reversible side effects[1]. The extrapolation of this treatment schedule to Brazil is risky because (1) Pentostam is not available and (2) L. (V.) panamensis is not endemic. The optimal treatment schedule of Glucantime, a polyvalent antimonial with a different chemical structure, for the treatment of simple cutaneous leishmaniasis caused by L. (V.) braziliensis in Brazil using a double blind protocol with strict compliance was determined.

METHODS

The study was conducted at the Health Clinic in Corte de Pedra, Bahia, Brazil, approximately 150 km southeast of Salvador (Lat. 13 S, Long. 39 W).

Patients were considered for admission to the study if they had cutaneous ulcers consistent with leishmaniasis, either they or their parents gave informed consent to participate, and they lived within driving distance of the clinic, a radius of 32 Km. The patients were admitted to the study if they fulfilled two of the following criteria: Parasitologic evidence of Leishmania infection; a positive Montenegro skin test; presence of Leishmania-specific antibody; and a histopathologic response consistent with leishmaniasis.

To make a parasitologic diagnosis, tissue samples from the raised edge of the lesions were examined for Leishmania by: (1) Giemsa stained smears prepared from punch biopsies; (2) hematoxylin-eosin stained histologic sections of punch biopsies; (3) parasite isolation in blood agar cultures from tissue aspirates; and (4) parasitic infection of hamsters with triturates of punch biopsies[3-5].

Immunodiagnosis included the Montenegro skin test[6] for delayed hypersensitivity to soluble leishmanial antigens and the indirect immunofluorescence antibody (IFA) technique for serum antibodies to leishmanial amastigotes[7,8]. IFA titers of 1:20 or higher were considered positive.

Histopathological sections were used to characterize the type of inflammatory infiltrate[4].

Isolates of Leishmania were identified using monoclonal antibodies specific to L. (V.) braziliensis, L. (V.) guyanensis, and L. (Mexicana) amazonensis[9,10].

Patients were excluded from the study if they had completed a previous course of Glucantime. Three patients with persistent lesions were admitted even though they had taken partial treatment - a mean of 10 mg Sb^{v}/Kg/day for 5 days. Pregnant women and people with diabetes, varicose veins, arterial hypertension, and cardiac, liver, and renal diseases were excluded.

The patients were randomized into four Glucantime treatment groups: (1) 20 mg Sb^{v}/kg body weight/day for 20 consecutive days (20 mg/20 days); (2) 10 mg Sb^{v}/kg/day for 20 days (10 mg/20 days); (3) 20 mg Sb^{v}/kg/day for 10 days (20 mg/10 days); (4) 10 mg Sb^{v}/kg/day for 10 days (10 mg/10 days). The Glucantime was produced by Rhodia, São Paulo, Brazil. It was diluted in 5% dextrose solution up to a volume of 20 ml by a technician who was unaware of the patients' histories. To guarantee compliance, the physican or the nurse visited each patient daily in their homes to inject the Glucantime solution intravenously.

Clinical examinations were conducted before treatment, on day 10 for all patients, and on day 20 for patients given 20 days of treatment. Follow-up examinations were performed 1 1/2, 3, 6 and 12 months after the beginning of treatment. Although the attending physician knew which patients received the 10 and 20 day treatment schedules, he did not know their dosages until after the 3 month follow up examination.

At each clinical examination, the lesions were classified as follows: **Active lesion** - peripheral border raised with infiltrate without re-epithelization of the ulcer; **Healing (equivocal) lesion** - reduction or absence of the raised border

with vascularization, granulation and partial deposition of new skin over the ulcer; **Healed lesion** - Absence of the raised border with new skin covering the entire ulcer, frequently with hypo- or hyperchromia.

Parasitological follow-ups were performed on all patients on the final day of treatment or 1 day later by examining aspirations[5] and biopsies of the lesion margin for Leishmania as described above. Parasitologic examinations were limited to those patients with active lesions during follow-up examinations conducted at 1 1/2, 3 and 6 months.

During treatment the patients were questioned daily to detect side effects from Glucantime. Serum levels of transaminases, alkaline phosphates, bilirubin, urea, and creatinine were performed on day 10 and/or day 20 of treatment, at the 1 1/2 month follow-up visit and, in one group, at the 3 month visit. Standard calorimetric techniques were used[11].

Serology was performed on sera collected before, during, and after treatment on days 0, 10, 20, and 1 1/2, 3, 6 and 12 months.

The number of patients entered into the study was limited to the minimum required to detect differences of 35% in the frequency of lesion heal and side effects among the treatment groups at a power of 80% with a significance of 95%. Mean differences in the frequency of side effects and healing among the groups were evaluated by Fisher's Exact Test, One Way ANOVA Test and Student's T Test. Multi-variant analysis was used to evaluate the influence of the following factors on healing: age, sex, nutritional status, and the size, duration, location and number of lesions. A probability level of 0.05 was considered significant.

RESULTS

Eighty one patients were admitted to the study. The ages of the patients ranged from 5 to 72 years with a median of 16 years. The male to female ratio was 2:1.

The leishmanial ulcers were distributed over all parts of the body, but more than half of them (59%), were on or below the knee. Forty nine patients had single ulcers while the remaining 32 had 2 to 8 ulcers. Multiple ulcers were grouped closely together in the same anatomical location in 16 patients, but distributed at distant anatomical locations in the remaining 16. The diameter of the ulcers ranged from 6 to 60 mm with a median of 16 mm (Table 1). The width of the raised edge of the lesions ranged from 2 to 15 mm with a mean of 5 mm. The age of the lesions was estimated from patient interviews to be 15 - 119 days with a median of 30 days (Table 2). Although the patients were

assigned randomly, there were clinical differences in lesion characteristics among the treatment groups (Table 3).

TABLE 1. Diameter of pretreatment ulcers measured from the outer edges of raised borders

Diameter (mm)	Number (%) of Patients
<10	20 (25%)
10-19	45 (56%)
20-29	10 (12%)
30-39	3 (4%)
>40	3 (4%)

TABLE 2. Age of ulcers at initial examination.

Days	Number (%) of patients
<29	11 (14%)
30-59	43 (53%)
60-89	14 (17%)
90-119	13 (16%)

TABLE 3. Comparison of the four Glucantime schedules against seven variables.

Variable	20 days		10 days		Statistical test (P value)
	20 mg	10 mg	20 mg	10 mg	
Sex (m/f)	14/7	16/8	14/6	11/5	Fisher's (0.5-0.6)
$\bar{X}$ age (years)	19.7	17.8	24.0	25.8	Student's (0.1-0.8)
$\bar{X}$ number of lesions	2.2	1.5	1.6	2.3	Anova (0.3)
Site of primary lesion (knee & below/above knee)	11/10	12/12	13/7	10/6	Fisher's (0.4-0.6)
$\bar{X}$ diameter of lesion (mm)	18	14	21	16	Anova (0.1)
$\bar{X}$ width of raised border (mm)	6.6	4.5	4.4	4.4	Anova (0.03-0.9)
Age of lesion (days)	52	48	33	31	Anova (0.006-0.7)

Parasites were seen or isolated from 65 patients. Taxonomic studies using species specific monoclonal antibodies were

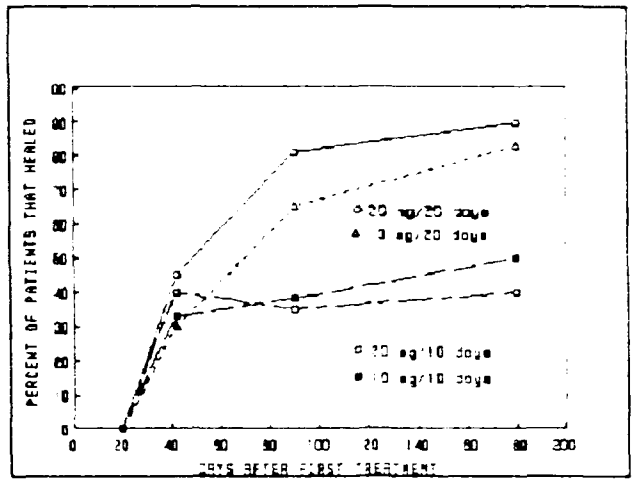
performed on 40 isolates and they were all L. (V.) braziliensis. The remaining 16 patients had positive Montenegro skin tests and histopathologic reactions compatible with cutaneous leishmaniasis. Fourteen of these 16 patients were seropositive at the initial examination and the remaining two seroconverted during treatment.

The best response in terms of the frequency of healed lesions was in the 20 mg/20 day group where 81% of the patients healed by 3 months and 90% by 6 months (Table 4, Fig. 2). In the 10 mg/20 day group 65% of the patients had healed at 3 months and 83% at 6 months. Differences in the proportion healed after treatment with 10 mg Sb^{v}/kg and 20 mg Sb^{v}/kg for 20 days were not significant (Fisher's, $P > 0.4$). The proportions of patients who healed after receiving the 10 day schedules were significantly less (Fisher's $P = 0.001$) than those given the 20 day schedules regardless of dose (Table 4, Fig. 2).

TABLE 4. Follow-up of patients treated with Glucantime

Treatment Groups					
Dose	Days	N	Active	RE & PH	Healed
			1 1/2 months		
20mg	20	20	3 (15%)	8 (40%)	9 (45%)
10mg	20	23	6 (26%)	10 (43%)	7 (30%)
20mg	10	10	1 (10%)	5 (50%)	4 (40%)
10mg	10	12	4 (33%)	4 (33%)	4 (33%)
Total		65	14 (21%)	27 (42%)	24 (37%)
			3 months		
20mg	20	21	2 (10%)	2 (10%)	17 (81%)
10mg	20	23	2 (9%)	6 (26%)	15 (65%)
20mg	10	17	4 (24%)	7 (41%)	6 (35%)
10mg	10	16	2 (12%)	8 (50%)	6 (38%)
Total		77	10 (13%)	23 (29%)	44 (57%)
			6 months		
20mg	20	20	1 (5%)	1 (5%)	18 (90%)
10mg	20	24	1 (4%)	3 (12%)	20 (83%)
20mg	10	20	4 (20%)	8 (40%)	8 (40%)
10mg	10	16	2 (12%)	6 (38%)	8 (50%)
Total		80	8 (10%)	18 (22%)	54 (68%)

RE = re-epithelization
PH = partial healing



There were two types of therapeutic failures when the lesions were judged at 3 months after the start of treatment: 1) healing followed by relapse at the same site and 2) the development of new satellite lesions near the original lesion site. The lesions on 15 patients healed partially or completely and then relapsed within 3 months from the start of treatment. The frequency of relapse was associated with the dose of Glucantime because 44% of the patients in the 10 mg/10 days group relapsed compared to 12% of patients on the other schedules (Fisher's, $P < 0.009$). All of these patients healed after a second treatment with 20 mg/10 days.

Three patients developed new lesions near the site of the original lesion between the 1 1/2 and 3 month examinations. It is likely that these satellite lesions were caused by amastigotes that survived Glucantime treatment in the original lesion rather than from reinfection by vector sand flies. The original lesions were healed at 3 months, and additional treatment with 20 mg/10 days was given to heal the satellite lesions. .

Finally, three patients developed new lesions between the 3 and 6 month examinations. There is a possibility these lesions were caused by reinfection from sand flies rather than surviving amastigotes because they occurred at anatomical locations far from the original ulcer.

Seven patients had negative pretreatment IFA titers, but positive Montenegro tests. Of these seven, three developed titers of 20-80 during the study. Titers decreased during the study and all were $\geq$ 160 by the 6 month examination (Table 5). No differences in titers were found between the four groups.

TABLE 5. The number of patients with indirect IFA titers throughout treatment with Glucantime.

	Titers									
	0	20	40	80	160	320	640	No.	G.M.*	S.I.**
Before treatment	7	5	14	21	13	9	3	72	60.9	1.7
End of treatment	8	8	20	12	13	8	1	70	51.6	0.9
1 1/2 months after	3	3	8	6	2	3	1	26	44.5	0.8
3 months after	9	10	16	10	11	6	1	63	38.3	0.8
6 months after	13	11	8	12	8	0	0	52	20.0	0.6
1 year after	35	15	8	3	0	0	0	61	4.2	0.05

* Geometric mean
** Serological index: titers ≥160/≤80

Systemic side effects of Glucantime treatment such as fever, arthralgia, myalgia and anorexia were more frequent in the group treated with 20 mg/kg for 20 days (Table 6) than in the other groups (Fisher's exact test $P < 0.01-0.02$) No differences in occurrence of head colds and asthenia were found between the groups (Fisher's $P > 0.3$). Two cases of cutaneous eruption were observed and both were in the 20 mg/kg 20 day group. There were no adverse effects from drug injection nor marked changes in the biochemical parameters measured with the exception of alkaline phosphatase. This enzyme level was sometimes raised, but no differences could be detected between the 4 groups (Fisher's, $P = 0.4-0.6$).

CONCLUSIONS

From a practical viewpoint, a daily dose of 20 mg Sb^{v} is unrealistic in rural Brazil because the volume of drug is too large to be injected intramuscularly by a neighbor. A typical 50 Kg person would require a 11.8 ml of Pentostam and 10 ml of Glucantime, volumes which cause unacceptable pain and patient refusal to complete the 20 day course of treatment.

TABLE 6. Side effects in the four treatment groups

	Treatment schedule			
	20 days		10 days	
Side effects	20 mg	10 mg	20 mg	10 mg
Fever	45%	17%	20%	6%
Myalgia &/or Arthralgia	32%	4%	5%	12%
Anorexia	18%	4%	0%	0%
Coryza (head cold)	18%	12%	10%	6%
Indisposition &/or Asthenia	18%	12%	5%	6%
Cutaneous eruption	9%	0%	0%	0%
Cephalea (headache)	0%	2%	1%	2%

The results demonstrate a drug effect which is almost equal between the 2 groups receiving 20 days treatment. Probably the most practical field treatment for cutaneous lesions caused by L. (V.) braziliensis is 10 mg Sb^{v}/kg of body weight/20 days by a single, slow, intravenous injection because there were few side effects, the volume of drug injected is still painful, but acceptable.

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ARBOVIROLOGY

1. BACKGROUND.

In 1986, the Malaria Clinic in Costa Marques, Rondonia, received 20,000 people seeking medical attention of which 30% were slide negative for malaria parasites. It is believed that a major portion of these febrile patients without malaria may be infected with arboviruses.

2. OBJECTIVES.

a. To isolate and characterize viruses from patients with suspected arvboviral disease.

b. To determine the prevalence of arboviral infections and antibodies for non-malarious febrile patients at the Malaria Clinic in Costa Marques.

c. To determine if the pilot project should be expanded into a more comprehensive program for arboviruses in the Amazon Basin.

Arborviral Isolations and Antibodies From Non-malarious Febrile Patients Living in the Costa Marques, Rondonia Area.

METHODS.

Patients with non-malarious fevers were asked to participate in the arboviral survey and to submit a 4 ml sample of venous blood for laboratory studies. The blood was allowed to clot and then centrifuged. The sera were labelled, cryopreserved in liquid nitrogen, and sent to the Instituto Evandro Chagas, in Belem for the following viral studies:

a. Serological tests for IgM antibodies specific for 18 different viruses were made by ELISA (Table 1). In addition, a standard hemaglutination inhibition assay was performed - a test of broad specificity for arboviruses.

b. Attempts to detect, isolate and identify arboviruses were made by inoculating suckling Swiss white mice intracerebraly and Vero cell monolayers. Virus isolates were identified using the plaque neutralization assay. The Belem laboratory has the capability of identifying the 141 arboviruses which exist in the Amazon

Table 1. List of arboviruses tested by ELISA using type specific monoclonal antibodies.

Virus	Abbreviation
Bussuquara	BSQ
Cacipacore	CPC
Caraparu	CAR
Catu	CATU
Dengue 1	DEN - 1
Dengue 4	DEN - 4
Eastern equine encephalitis	EEE
Guaroa	GRO
Ilheus	ILH
Jurona	JUR
Mayaro	MAY
Mucambo	MUC
Oropouche	ORO
Rocio	ROC
St. Louis encephalitis	SLE
Tacaiuma	TCM
Western equine encephalitis	WEE
Yellow fever	YF

4. RESULTS.

During the reporting period sera from 15 patients were assayed for antibodies to the 18 viruses. Follow-up sera taken 2-3 weeks after the first were tested from five of these patients.

Antibody to at least one of the 18 viruses was detected in the sera from 12 of the 15 patients. No antibodies to Oropouche virus or Jurona virus were found. One patient had antibody to 10 and another to nine viruses (Table 2). Antibody to yellow fever was detected in the sera of 11 patients.

TABLE 2. Antibodies to 17 viruses detected in the sera of 15 patients from Costa Marques, Rondonia.

	Patient												
Virus	1	2	3	4	5	6	7	8	9	10	11	12	Total
BSQ		X			X		X						3
CPC		X			X		X			X	X		5
CAR							X					X	2
CATU							X					X	2
DEN 1		X					X						2
DEN 4		X					X						2
EEE					X						X		2
GRO			X										1
ILH		X			X					X	X		4
MAY		X			X				X		X		4
MUC		X			X						X		3
ROC		X			X		X						3
SLE		X											1
TCM							X						1
WEE					X						X		2
YF	X	X	X	X	X	X	X	X	X	X	X		11
Total	1	10	2	1	9	1	9	1	2	3	7	2	48

Guaroa virus was isolated from a logger, patient number 3, on 30 September 1988. This patient had been sick for 1 day with the following symtoms: fever 37 C, heart beat 80, blood pressure 130 x 80, mialgia, arthralgia and exantema. Antibody to GRO virus, titer of 1:160, was detected in sera taken 14 days later. This is the first recorded isolation of GRO virus from man.

5. CONCLUSIONS

The few sera collected during the 7 month grant period yielded an extremely high antibody rate in residents of the Costa Marques area. This preliminary study should be expanded to include the non-malarious febrile patients treated by the Brazilian Army medical personnel at isolated posts in the Amazon. These medics treat the non-military population as well as military personnel. Gold deposits are being discovered in remote sites throughout the Amazon Basin, and are attracting thousands of miners. The miners live in make-shift shelters in the forest, and sleep on the ground or in hammocks slung between trees. These miners, who are treated by army medics, would be superb sentinels for virus isolation attempts.

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