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INFECTIOUS AND COMMUNICABLE DISEASES AFFECTING POPULATIONS INTRODUCED INTO ENDEMIC AREAS

John C. Swartzwelder, et al

Louisiana State University

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John C. Swartzwelder, Víctor M. Villarejos, George A. Thurber, and 30 professional colleagues

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13. ABSTRACT

The title of this contract implies a direct relationship to specific military function and operation. The studies have a direct relationship to medical problems of military importance with particular reference to the tropics. The medical problems which have been studied include the etiologies of diarrheal diseases in the tropics, pathogenesis of amebic lesions, etiology of infectious hepatitis, etiology of acute respiratory disease in Costa Rica, biological control of Chagas' disease vectors, reconnaissance for arbovirus activity in the Mississippi River delta region, taxonomy, distribution, and ecology of phlebotomine flies in Costa Rica, clinical importance of prothrombin time in snake venom poisoning, pathophysiology and treatment of septic shock, skin test for hookworm infection, and enteroviruses in rural families and their animals. In accordance with the stated aims of Project THEMIS, the LSU/THEMIS program has: (1) Conducted multidisciplinary research by groups of faculty members and research associates; (2) Made substantial progress in research projects involving several biomedical disciplines; (3) Involved at least 20 faculty members in research activities; (4) Aided significantly seven graduate students, three received the Doctor of Philosophy and four Master of Science degrees; (5) Published numerous scientific papers; (6) Presented papers at scientific meetings; (7) Collaborated in research with personnel of federal agencies; and (8) Continued our activities in research on problems in tropical medicine of importance to the military.

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Black flies, Costa Rica						
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Septic shock, pathogenesis and treatment						
Hookworm infection, intradermal test						
Enteroviruses, man and animals						

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REPORT NUMBER 6

INFECTIOUS AND COMMUNICABLE DISEASES AFFECTING POPULATIONS INTRODUCED INTO ENDEMIC AREAS (U)

FINAL REPORT

J. Clyde Swartzwelder, Ph.D.
Víctor M. Villarejos, M.D., Dr. P.H.
George A. Thurber, M.S.

and

Stanley H. Abadie, Ph.D.
Jorge A. Arguedas, M.D., M.P.H.
Joseph H. Bellina, M.D.
Charles B. Carson, Jr.
George E. Childs, Jr., Ph.D.
Harry E. Dascomb, M.D.
Jane E. Deas, M.S.
Robert H. Gilman, M.D.
Robert S. Gohd, Ph.D.
Nathaniel Grew, D.V.M., M.S.
Julio Jiron, M.S.
James I. Kato, B.S.
Chin Chiu Lee, Ph.D.
William B. Lushbaugh, Ph.D.
James A. Miller, M.S.
Joseph H. Miller, Ph.D.
Margaret I. Nickle, M.S.
William Pelon, Ph.D.
A. Peña Chavarría, M.D., Dr. P.H.
Danny B. Pence, Ph.D.
Michael Roy, M.S.
Harold Trapido, Ph.D.
Bernard V. Travis, Ph.D.
Leon Troper, M.D.
Gilberto Vargas, M.D.
Mario Vargas, Ph.D.
Jorge E. Velasco, M.S.
Lionel G. Warren, Sc.D.
Rodrigo Zeledón, Sc.D.
Moisés Zomer, M.D.

30 September 1974

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Louisiana State University
New Orleans, Louisiana 70112

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Approved for public release; distribution unlimited.

SUMMARY

1. Studies under this contract have a direct and apparent relationship to specific military function and operation. This is apparent from the project title "Infectious and Communicable Diseases Affecting Populations Introduced into Endemic Areas." Our studies have been conducted in tropical Central America, the Caribbean islands, Bolivia, and in Louisiana, which is a subtropical area of this country.

2. The main problems which have been studied include the etiologies of diarrheal diseases in the tropics, pathogenesis of amebic lesions, etiology of infectious hepatitis, etiology of acute respiratory diseases, biological control of Chagas' disease vectors, reconnaissance for arbovirus activity in the Mississippi Delta region, and the taxonomy, distribution, and ecology of phlebotomine flies in Bolivia and of black flies in Costa Rica. Data from studies on other subjects are presented in annual reports for prior years.

3. The publications, dissertations and theses which have resulted at least in part through project THEMIS are listed in this final report.

4. Seven graduate students who have obtained degrees were aided significantly by this project. Three received Doctor of Philosophy and four received Master of Science degrees.

5. During the project at least 20 faculty members from several departments of the University participated in the studies.

6. Personnel from a large number of other institutions in several geographic areas, which are listed in this report, collaborated in the research.

7. An enterovirus-like agent, designated CR69(076) and possessing properties unlike the known enteroviruses, as well as an unusual growth requirement, was isolated from a fecal sample collected from a 7-year-old male Costa Rican experiencing clinical hepatitis. The agent, isolated in cultures of WI-38 cells, replicates best within a stable and relatively narrow pH range provided by the maintenance medium. Poor or no replication is observed when a medium having a hydrogen-ion concentration above pH 7.0, or one which is excessively low, is employed. The agent is not a rhinovirus, being acid stable and having an optimum temperature of 36°C for its replication. The usual spectrum of cells found to be susceptible to infection by many enterovirus types was not infected by CR69(076). In addition to a limited cell spectrum, the agent is relatively unstable

to a temperature of -70°C under the storage conditions employed. CR69(076) has a particle size of approximately 29 nm and possesses an icosahedral morphology. It is ether-resistant and possesses a ribonucleic acid core. Although some antigenic crossing was noted with antiserum to ECHO virus type 25, no inhibition was noted with antisera to other enteroviruses tested. In addition to its isolation from a HeA case, paired sera from nine cases of HeA occurring in Costa Rica also showed increments in antibody levels. Furthermore, many of the properties of CR69(076) were found to be similar to those described for the hepatitis A agent isolated in marmosets and observed in the feces of a volunteer with hepatitis A by investigators at the National Institutes of Health.

8. A prospective study of acute respiratory disease was conducted in three different climatic areas of Costa Rica. A copy of the manuscript for publication of Part I of the study is included in the body of the report. Forty families comprising 364 persons were under surveillance for 1 year. The annual attack rate per person was significangly lower in the temperate San José (central plateau) than in the semi-arid hot city of Puntarenas (Pacific coast) or in the humid tropical Puerto Limon (Atlantic coast), which had the highest rate. The period of peak incidence coincided with the onset and early months of the rainy season, May through July. As a whole, the incidence of acute respiratory illnesses in these three areas of uniform year-round climate was much lower than reported from places in northern United States with marked seasonal changes of climate. However, the age distribution was similar, with highest attack rates among children under 10 years. The prevailing moderate or warm temperatures, with slight seasonal variations, could well be the determinating factor for the lower incidence of acute respiratory illness.

The virological findings are summarized in detail below since the manuscript for publication of Part II of the study on acute respiratory diseases in Costa Rica is in preparation.

A total of 1532 throat washings and swab suspensions was obtained for virus isolation. From these 563 viral agents were isolated; 81 percent were identified (details below). Of 1511 swabs cultured for bacteria, there were 128 isolations of beta-hemolytic streptococci scattered throughout the year and 26 of Hemophilus influenzae, the majority during April, May and June 1969.

Poliovirus was the most frequently isolated, but only type 1 appeared in significant numbers. Of the Coxsackie A viruses, 20 and 24 were the common types and second only to poliovirus in number of isolations. Rhinoviruses were

isolated only 42 times, and ECHO and adenoviruses were found only in negligible numbers. No Coxsackie B viruses were identified.

The only influenza isolates were obtained during the last 2 months of the study in the San José area, after the other two area surveys had been terminated. All 15 were isolated only from chick embryos and were identified as A2 (Hong Kong). MK cultures failed to yield hemadsorbing agents such as other influenza or parainfluenza viruses.

Although the rhinovirus-like isolates fulfilled the biological requisites for identification, none was neutralized by any of the 35 reference typing antisera.

Because of the large number of isolates of poliovirus, it was of interest to know if their origin was vaccine or if they were wild strains. Twenty isolates were tested for growth at 40°C and all were positive, suggesting that they were not vaccine strains.

The highest incidence of poliovirus occurred in Puntarenas in April and May, whereas in Puerto Limón and San José, the peak was not reached until several months later. On the other hand, Coxsackie A20 isolates were almost all made during July, August and September, in all three cities. The highest number of isolates of both polio 1 and Coxsackie A20 were from San José and the lowest from Puntarenas.

The isolation rate of all these viruses was highest in samples from children under 5 years, but they were also found in substantial numbers up to 15 years. From persons over 40, the Coxsackie viruses were rarely isolated, but poliovirus occurred in approximately 2 percent.

The complement-fixation screen of serum samples at 1:5 dilution indicated that most of the people, even those under 5 years, had some antibody to all of the eight viruses tested. This was true for all agents in all three locations with the exception of parainfluenza, type 1, which appeared only twice in San José but occurred in 22 to 35 percent of the study groups in Limón and Puntarenas, respectively. The other two parainfluenza types were higher in San José and Limón but also frequent in Puntarenas.

Also lower in San José were psittacosis and Q fever and these two were much higher in Puntarenas.

Adenovirus antibody was almost universal in San José and Limón and present in 50 percent of Puntarenas subjects. Respiratory syncytial (RS) virus was also common, especially in San José. The incidence of mycoplasma antibody was substantial in all locations but greater in San José.

The data on persons showing a four-fold rise in CF titer during the study indicate a high incidence of parainfluenza, types 1 and 3, in Puntarenas, and an equally high frequency of psittacosis infections. RS virus occurred in San José and Limón at a rate of 18 and 15 percent respectively, and adenovirus was high in Limón.

The age distribution of serologically identified infections shows that parainfluenza type 1 occurred more frequently in older children and adults especially in Puntarenas where the total incidence was higher. The few cases of type 2 occurred in adults in Puntarenas and in children in San José. Type 3 was divided among the lowest and highest age brackets in all these locations. Adenovirus was limited to young children in San José, but the six cases in Puntarenas were scattered below age 25, and those in Limón occurred at all ages even though the majority were under 5 years. RS virus was also limited to children and young adults in Puntarenas; in the other two locations it was scattered, although most frequent under 5 years. Of the 12 cases of mycoplasma infection only three occurred below 10 years. Psittacosis and Q fever were also found more often in adults.

Sera tested by HI against four influenza antigens representing previous epidemics showed that a few people over 30 years have antibodies to A-PR8 (1933); none of these were Limón residents. Many more have immunity to A1-FMI (1947), from age 15 up, but four younger children were also positive. Antibodies to B-GL (1954) and A2 Jap (1957) were found in high percentage in all three cities and all age groups although B-GL was less prevalent in San José; highest rates occurred in persons over 10. Rises in titer during the study did not occur against PR8 or FMI in any location nor against B-GL in San José or Limón. However, in Puntarenas there was an incidence rate of 38 percent for B-GL.

Data on influenza A2 (1509), the Hong Kong strain, are available only for San José. There was a high incidence of immunity in all age groups and a rising titer in 21 percent of the population under 50.

9. A study has been made of diarrheal diseases occurring in travelers introduced into a tropical environment to reside for periods ranging from 3 to 11 months. Among 145 persons on whom fairly complete data are available, 101 diarrheal episodes were observed. Pathogenic bacteria or parasites were found in 20 of 74 diarrheic feces (27%) and 17 out of 404 nondiarrheic feces (4.2%). Enteroviruses were isolated from 38 diarrheic (51%) and 222 nondiarrheic feces (55%); in both groups, Coxsackie B1 was the prevalent type (37% of diarrheic and 29% of nondiarrheic isolates). However, Coxsackie B group viruses were isolated in a significantly higher proportion of persons having had diarrhea

episodes during the observation period than in those who did not suffer (or report) diarrhea during their stay.

An additional group of 171 persons provided 304 fecal specimens but diarrheal histories of these are incomplete. From them 128 pairs of sera, collected at the beginning and end of their stay, were tested against Coxsackie B viruses. Antibody to types 2-6 were present in up to 50 percent in the first sample. Only a small percentage (1-7) of persons showed increases in titer of antibody to types 1, 2, 5 and 6 during their stay.

Diarrhea episodes observed were of short duration and not incapacitating. The common bacterial and parasitic enteropathogens were found in only a small proportion of such diarrheas. Although no conclusive evidence was found that viruses are the cause of the syndrome, there is a strong suggestion of etiologic relationship of Coxsackie B viruses with diarrhea. Further analysis of the data, which is being carried out, may clarify this possible relationship.

10. Two facets of the study of the pathogenesis of amebic lesions have received continued investigation. Both Entamoeba histolytica, the etiologic agent of amebiasis, and Hartmannella culbertsoni, an ameba associated with meningoencephalitis, have been studied. Entamoeba histolytica seems to possess a specific mechanism, the surface lysosome and its elongate branched extensions, for the release of lytic enzymes. In addition, there may be also a more general release at the plasmalemma which may be held in the glycocalyx. The glycocalyx is morphologically and physically differentiated in in vivo trophozoites which may be related to invasiveness. The free living sometimes pathogenic ameba H. (=Acanthamoeba) culbertsoni seems to have a general release mechanism for lytic enzymes via an apocrine type secretory process along the entire plasmalemma. This is associated with the endoplasmic reticulum and primary lysosomes. The results of these studies formed the basis for several publications, including dissertations and a thesis. Additional details are available in a more extensive summary in the body of the report.

11. Laboratory and field studies were conducted to explore the possibility of a method of biological control of Triatoma dimidiata, the main vector of Chagas' disease in Costa Rica and other countries. The work was conducted in response to the need to control domiciliary triatomids that are showing signs in some countries of resistance to insecticides, and also due to the high degree of contamination by these chemicals in or around human dwellings. Experiments were carried out: a) with spiders found in houses in an endemic area of Chagas' disease (San Rafael de Ojo de Agua, Province of Alajuela, Costa Rica); b) with

a microhymenopteran, Telenomus fariai, found in the same area parasitizing the eggs of T. dimidiata; c) another parasitic microhymenopteran of the genus Gryon; and d) with Pimeliaphilus zeledoni, an ectoparasitic mite of T. dimidiata. Finally, an interesting phenomenon with epidemiological and biological implications, the camouflage instinct of T. dimidiata, was also studied in detail and evaluated as a predator-escape mechanism. An extensive final report of this study appeared in Report Number 5, Annual Progress Report.

12. As part of a reconnaissance for possible Venezuelan equine encephalitis (VEE) enzootic focus and other arbovirus activity in the Mississippi delta region, mosquito larva populations were sampled in water lettuce (Pistia) within a 50-mile radius of New Orleans. The principal species found associated with Pistia were Anopheles quadrimaculatus, Anopheles crucians and Culex (Melanoconion) erraticus. Culex (M.) aikenii, the principal vector of VEE in its enzootic cycle in small mammals in Panama, was not found. It may now be assumed that its distribution does not extend this far north. However, this does not exclude the possibility that there may be an enzootic focus of VEE in the area since the virus has been isolated from Culex (Melanoconion) species other than C. (M.) aikenii in the Everglades of Florida. The mosquitoes collected were identified and sorted into species pools for virus isolation attempts.

Small mammals and incidentally encountered reptiles were collected in two regions of Louisiana, where either a natural cycle of VEE virus, on epidemiologic grounds, seemed likely or where a vaccine strain of VEE had been isolated from mosquitoes. Serological study of a selected sample of domestic animals and small wild mammals of the Basile area and of small wild mammals and turtles from the swamp area near New Orleans gave no evidence of the presence of VEE virus. However, reactors to western (WEE) and eastern (EEE) equine encephalitis antigen were found among the turtle sera in haemagglutination inhibition (HI) tests. An enlarged sample of reptile and some amphibian sera was obtained, including box turtles, red-eared turtles, water snakes, and bull frogs. Additional HI-reactors were found to WEE and EEE and also to St. Louis encephalitis (SLE). Neutralization tests (NT) of the SLE HI-positive sera did not confirm the presence of SLE antibody and suggest the HI-positives may represent infection with another group B arbovirus, possibly Cowbone Ridge.

13. The phlebotomine sandflies of the Los Yungas region of Bolivia have been studied. Sandflies were collected through a transect from 400 m to 2300 m in the Los Yungas region of Bolivia during the period June to September 1971. Leishmaniasis is prevalent in this region. Data were

obtained on the species composition, altitudinal distribution and ecology of the sandflies of the region. Twenty species of phlebotomines were found including 11 previously known: Brumptomyia brumpti, Lutzomyia auraensis, L. campbelli, L. dendrophila, L. longipalpis, L. nevesi, L. punctigeniculata, L. sallesi, L. serrana, L. shannoni and L. trinidadensis. The remaining 9 are either new or of uncertain status; the two new species are described as Lutzomyia boliviana and Warileya yungasi. Also found were the previously undescribed females of L. auraensis, L. dentrophila, and L. nevesi. Fifty-four percent of all phlebotomines were L. longipalpis, a known vector of visceral leishmaniasis in Brazil. Of these, 82 percent were caught in association with chicken coops.

14. The planned observations and collections on black flies made on 100 streams in Costa Rica have been completed. Immature stages, reared adults and biting adults were preserved from each of the streams. The data included (a) monthly observations on 53 streams; (b) bimonthly observations on 47 streams; and (c) bimonthly observations on 28 of the 53 streams. Five different man-biting species of Costa Rica have been identified from the collections. The above observations extended over a period of 2 years and provide a base for some critical studies on the relationships between the flies and disease organisms which they may transmit.

15. A study of prothrombin time as a therapeutic indicator of the effectiveness of antivenin in envenomation by Bothrops atrox, the fer-de-lance, was undertaken. Results indicate that the prothrombin time is a reliable index to measure the envenomation process in response to antivenin therapy. It indicates also the time at which antivenin therapy may be terminated. When the prothrombin level remains at 80 per cent for 24 hours, there is no need for further administration of antivenin. Beside the clinical observation of the patient, periodic determination of the prothrombin time is the only reliable clinical laboratory procedure to follow the evolution of the envenomation and to determine the duration of antivenin therapy.

16. Fifty patients who had sepsis and shock were studied. Therapy consisted of central venous pressure monitoring, restoring blood volume, vasodilating, selecting antibiotics according to the organism suspected by clinical findings and direct smears of exudate, promptly and aggressively draining or excising infected areas, vena caval ligation in suspected pulmonary embolization, and revising subsequent antibiotic therapy depending on clinical course and laboratory findings. Five of the 50 patients (10%) died. This is in marked contrast to a mortality rate of 40 per cent of

such cases at Charity Hospital during 1959 to 1963. Aerobic enteric bacteria predominated as etiologies. These were frequently associated with anaerobic Bacteriodes and streptococci. This finding together with the fact that the anaerobes appeared alone in several instances suggests the need to make anaerobic cultures a routine procedure if specific therapy is to be attained. The results suggest the importance of first managing the shock, promptly determining etiology, administering maximal doses of specific antibiotics parenterally, and aggressively excising or incising and draining the local infection. In most cases shock could have been prevented by earlier bacteriological studies and specific antibiotic therapy.

17. The standard techniques for stool examination to detect and quantitate hookworm infections are too cumbersome and not economically practical for use in hookworm control programs in areas where this infection constitutes a major public health program. Therefore, the usefulness of a skin test with larval N. americanus antigen for assessment of hookworm prevalence was evaluated in an endemic area of Costa Rica. In comparison with standard coprologic techniques employed to survey the population, the skin test detected 83 per cent of infections, showing a fairly satisfactory sensitivity. The overall specificity of the test was 50 per cent i.e. random. No correlation was found between skin reactivity and hookworm burden. The sensitivity of the test increased moderately with age, but its specificity decreased significantly at the same time. False-positive reactions were more numerous among persons formerly infected with hookworm who had been negative for as long as 5 years. There was an indication of cross reactivity with intestinal nematodes other than hookworm. On the basis of the results obtained it was concluded that the skin test evaluated for hookworm is not sufficiently sensitive or specific at the present to be used as a reliable means of diagnostic screening of infection in populations of endemic zones, especially if it is done for the purpose of selecting heavily infected persons for treatment as recommended by Stoll.

18. In Costa Rica during 1968, the prevalence of human enteroviruses in rural families and their domestic animals was studied. In 9 households, 56 humans and 46 animals were sampled. Twenty-five virus isolates were recovered from human stools, 30 from animal stools, and 3 from animal nasal washings. Of the 25 isolates from humans, 12 were identified as coxsackievirus A-20, 3 as poliovirus type 1, 2 as adenovirus type 1, 1 as adenovirus type 2 and 1 as coxsackievirus A-17. Of the 33 isolates from animals, those identified included 3 coxsackievirus A-20, 21 poliovirus type 1 and 1 coxsackievirus A-9. Six of the

isolates from humans and 8 from the animals remain to be identified. Both poliovirus and type A-20 coxsackievirus were reisolated from animals during 25- to 67-day periods. By means of cross-neutralization tests, the type 1 poliovirus isolates were shown to be antigenically similar to each other and to a human vaccine strain (LSC 2 ab). Coxsackievirus A-20 isolates from a child and a dog were antigenically similar to each other but varied from the prototype strain (I.H.-35). Almost all the animals tested had neutralizing substances against type 1 poliovirus and 4 had significant rises in titer. Except for one calf, none of the animals or humans tested had coxsackie A-20 neutralizing substances in their sera.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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The Phlebotomine Sandflies of the Los Yungas Region of Bolivia

BODY OF REPORT

INTRODUCTION

The title of this contract "Infectious and Communicable Diseases Affecting Populations Introduced into the Tropics" implies a direct relationship to specific military function and operation. Diarrheal diseases and infectious hepatitis are major problems of the military. A wide array of arbovirus infections in the tropics represent hazards to newcomers especially in rural, swamp, and forested areas. The prevalence of amebiasis in some communities in Latin America may exceed 50 percent and this poses a problem for civilian and military personnel entering such areas. Chagas' disease, with its complication of myocarditis and the absence of suitable therapeutics, is endemic in wide areas of Latin America. Biting flies, particularly black flies (Simuliidae), produce bites with serious reactions, in addition to being vectors of diseases of man and animals. Phlebotomine flies (sandflies) are vectors of cutaneous, mucocutaneous, and visceral leishmaniasis which are endemic in many areas of Latin America. Often leishmaniasis is a major hazard for persons entering sylvan areas for agricultural or military endeavor. They also transmit bartonellosis.

In accordance with the stated aims of Project THEMIS (DOD Brochure, November 1968), the LSU/THEMIS program has:

(1) Continued multidisciplinary research by groups of faculty members and research associates in the field of tropical medicine;

(2) Conducted and made substantial progress on research projects involving several biomedical disciplines;

(3) Involved at least 20 faculty members from several departments, who represent competence in several disciplines, in the research and training activities;

(4) Included young and developing staff members in the program;

(5) Aided significantly in the training of seven graduate students who have now received their degrees (Doctor of Philosophy, 3; Master of Science, 4);

(6) Published scientific papers and presented results of research at scientific meetings;

(7) Collaborated in research with personnel of Federal Agencies in several geographic areas;

(8) Collaborated with and obtained assistance from scientists of numerous additional institutions;

(9) Provided training in tropical medicine for medical officers of the Armed Forces.

FACILITIES AND COLLABORATIVE INSTITUTIONS

Research and training activities of the THEMIS program of the Louisiana State University Medical Center have been conducted at the School of Medicine and William Pitcher Plaza (Florida Avenue) in New Orleans and at the offshore facility in San José, Costa Rica. These facilities complement each other and provide unusual opportunities for research and training and field studies on infectious diseases of the tropics and subtropics. Excellent research, training, and library facilities are available at the New Orleans and offshore facilities.

Scientists from several institutions, listed below, have collaborated with the staff of the LSU School of Medicine in the research and training projects of this program.

Louisiana State University Medical Center, New Orleans
University of Costa Rica School of Medicine and School of Microbiology, San José, Costa Rica
Hospital San Juan de Dios, San José, Costa Rica
Ministry of Health of Costa Rica
National Children's Hospital, San José, Costa Rica
NASA Lunar Receiving Laboratory (Dr. Cyril J. Hodapp), Houston, Texas
Peace Corps Training Center, Ponce, Puerto Rico
Peace Corps Training Centers, St. Thomas and St. Croix, Virgin Islands
Peace Corps Volunteers, Tegucigalpa, Honduras
Peace Corps Volunteers, San José, Costa Rica
Goshen College (Indiana) Volunteers in Costa Rica
Hammond State School (Dr. Carlos Santos), Hammond, Louisiana
Pinecrest State School, Pineville, Louisiana (to a small degree)
Ministry of Health of Bolivia (Authorization for phlebotomine studies)
University of Florida (Dr. G. B. Fairchild and Mr. D. Young advising on taxonomy of Bolivian phlebotomines)
Delta Regional Primate Center (Drs. A. Felsenfeld and R. Lowrie, collaborating on VEE studies)
Centenary College of Louisiana (Dr. A. B. McPherson, collaborating mammalogist on VEE studies)
Louisiana State University at Eunice (Laboratory facilities made available by Dr. W. J. Lembeck, Head of Science Division)
Louisiana State University Museum of Natural Science (Dr. G. H. Lowery, advising on distribution and ecology of mammals on VEE studies)

PERSONNEL RECEIVING CONTRACT SUPPORT

AND GRADUATE DEGREES RECEIVED

The following professional personnel had various degrees of support under contract THEMIS. In some cases it involved only support through materials provided for the respective research endeavor related to the THEMIS project. In others, salary support was received.

Research Support

Arguedas, J.
Bellina, J. H.
Childs, G. E., Jr.
Dascomb, H. E.
Deas, J. E.
Echt, E.
Gohd, R. S.
Grew, N.
Kato, J. I.
Kotcher, E.
Lushbaugh, W. B.
Miller, J. A.
Miller, J. H.
Nickle, M. I.
Pelon, W.
Peña Chavarría, A.
Pence, D. B.
Roy, M.
Russell, A.
Smith, S.
Swartzwelder, J. C.
Thurber, G. A.
Trapido, H.
Travis, B. V.
Velasco, J. E.
Villarejos, V. M.
Warren, L. G.
Zeledón, R.

Graduate Degrees Received

Danny B. Pence	Doctor of Philosophy	August 1970
Michael Roy	Master of Science	June 1971
George E. Childs, Jr.	Doctor of Philosophy	June 1972
Jane E. Deas	Master of Science	June 1972
Jorge E. Velasco	Master of Science	June 1973
William B. Lushbaugh	Doctor of Philosophy	August 1973
James A. Miller	Master of Science	August 1973

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A number of manuscripts are also in preparation, principally on acute respiratory disease, bionomics of black flies, and etiology of diarrheal disease in Costa Rica.

STUDIES ON THE ETIOLOGY OF INFECTIOUS HEPATITIS (IH)

William Pelon

The investigations which have been conducted have resulted in the isolation of an enterovirus-like agent from a case of IH whose cultural requirements, as well as other characteristics, are unlike those of the majority of the enteroviruses. Serologic responses from cases of IH, properties similar in many respects to the marmoset IH agent, together with a particle size and morphology consistent with the findings reported for the virus-like antigen reported in the feces of a human volunteer with IH, all suggest a possible etiologic relationship between CR69(076) agent and the disease known as hepatitis A (see accompanying manuscript). It is obvious that additional studies are necessary before any definite association may be claimed.

The methodology developed has permitted rapid and consistent replication of the CR69(076) stock virus. The technique has resulted in repeated isolation of this virus from the original fecal sample. However, the data also suggest that the virus content of the specimen is extremely low, possibly due to reduced virus excretion by the time jaundice was apparent and the specimen collected. In addition, the data also suggest that an inhibitor of virus proliferation may be produced in fecal extract-inoculated cultures. If such an inhibitor is present, it appears to be unlike interferon.

Two additional agents have been recovered from specimens of IH cases. These have been designated as CR69(006) and CR69 (052). Although first believed to be adenoviruses on the basis of cytopathic effects, proliferation in cultures pretreated with 5-bromodeoxy-uridine suggests an RNA composition rather than the characteristic DNA attributed to adenovirus types. Similar agents were not obtained from the specimens from control individuals tested. Further studies of these agents are necessary before any conclusions may be drawn.

A manuscript entitled "Hepatitis A: In vitro isolation of an agent with unusual growth requirements from a clinical case occurring in Costa Rica" has been accepted for publication in both Spanish and English editions of the journal Boletín de la Oficina Sanitaria Panamericana (OSP). A copy of the manuscript written in collaboration with J.H. Miller and Jane E. Deas, follows.

HEPATITIS A: IN VITRO ISOLATION OF AN AGENT WITH UNUSUAL GROWTH REQUIREMENTS FROM A CLINICAL CASE OCCURRING IN COSTA RICA[1]

W. Pelon, Ph.D., Associate Professor, Departments of Tropical Medicine & Medical Parasitology and of Microbiology, Louisiana State University Medical Center; J. H. Miller, Ph.D., Professor, Department of Tropical Medicine & Medical Parasitology, Louisiana State University Medical Center; and J. E. Deas, M.S., Medical Research Specialist, Department of Tropical Medicine & Medical Parasitology, Louisiana State University Medical Center.

An enterovirus-like agent having properties unlike the enteric viruses was isolated in vitro. Evidence of infection in paired sera from hepatitis cases was noted. Many properties are consistent with those described for hepatitis agents isolated in marmosets and observed in human feces.

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Introduction

This communication describes a viral agent isolated in cell cultures inoculated with a fecal specimen from a case of clinical hepatitis in Costa Rica and maintained under special conditions. Investigations have indicated that this agent possesses several characteristics which distinguish it from the known enterovirus types.

Picornaviruses are small (15-30 nm) ribonucleic acid-containing viruses, which are further subgrouped as rhinoviruses and enteroviruses (1). The former are differentiated from the latter on the basis of significant reductions in infectious virus titer following exposure to pH 3.0 for 1-3 hours (2). Despite one unconfirmed report of rhinovirus isolation from the feces, attempts at isolation from fecal samples collected from infected patients and volunteers have not been successful (3). In addition, all rhinovirus types tested have been found to be acid-labile (2).

To date, despite persistent attempts by investigators, the in vitro isolation of the hepatitis A (HeA) virus(es) has not been successful. However, an agent having picornavirus-like characteristics has been transmitted to marmosets from cases of HeA (4,5). A spherical virus-like antigen (diameter 27 nm) was reported recently in the feces of a human volunteer with HeA (6).

Current isolation methods for enteroviruses (7) have been unsuccessful in detecting the HeA agent. Since these employ culture media containing 0.11 to 0.22 percent $NaHCO_3$, the use of a more stable acid environment for HeA virus isolation was explored and is reported here.

Methods and Results

Fecal Samples Tested. Fecal samples were collected as a double blind study by the staff of the Louisiana State University International Center for Medical Research & Training (LSU-ICMRT) located in San José, Costa Rica. Specimens were obtained from 35 HeA cases occurring in a goegraphic area found to be endemic for the disease (8,9). Fifty-five specimens were collected from control individuals living outside of the endemic area. All specimens were prepared as 10 percent extracts.

Virus Isolation Studies. All fecal samples were inoculated into primary cultures of Rhesus monkey renal cells and into cultures of HEp_2 cells for enterovirus isolation (7). Those specimens from which virus isolations were obtained were eliminated from further study. The remaining specimens were tested in cultures of the human embryonic diploid lung cell

WI-38. Confluent cultures of cells were maintained in Eagle's basal medium (EBM), prepared in Earle's balanced salt solution (Earle's BSS), with 5 percent agamma calf serum (ACS) and 0.11 percent $NaHCO_3$ (final concentrations) (Medium I), incubated at 36°C, and observed for cytopathic effects (CPE). The pH of the medium ranged from 7.4 at the time of inoculation to 6.6 following 3 days of incubation. In a parallel study, cell cultures were maintained in a serum-free EBM, prepared in a glucose-free Hanks' BSS, gassed with CO_2 (Medium II), and inoculated. Following incubation at 36°C, this medium stabilized at approximately pH 6.8 and remained unchanged during the period of incubation. Fecal extracts frequently were toxic to cells maintained in the serum-free acidic environment, necessitating the passage of fluids from degenerated cultures into others maintained under similar conditions.

CPE usually were noted in cultures held either in the conventional medium alone or in those sustained under both conditions. In one instance, however, CPE were observed only in cultures maintained under the stable glucose-free acid environment and inoculated with a fecal extract designated as CR69(076) (10). This specimen was determined later to have been collected from a 7-year-old male, clinically diagnosed as experiencing HeA. It should be mentioned that this agent was successfully reisolated from the same specimen utilizing the procedures to be described.

Replication of CR69(076) virus. Difficulties in propagating the CR69(076) agent resulted in the following methodology, which permitted rapid and consistent replication. Confluent monolayer cultures of WI-38 cells were maintained in an EBM medium prepared from 100 X concentrates of vitamin and amino acid mixtures and from a 200 mM solution of glutamine in modified Hanks' BSS, which contained an equivalent quantity of galactose in lieu of glucose (11,12). In addition, the medium included 0.035 percent $NaHCO_3$, 0.6 percent fetal calf serum (FCS), sodium pyruvate (55 mg/liter) (11), alanine (45 mg/liter) (11), and 5-bromodeoxy-uridine (BrDUR) (20 µg/ml), and was gassed with CO_2 (Medium III). Following gassing, the pH of the medium was 6.0. Following incubation, the medium reached an equilibrium at a ph of 6.7 to 6.8. Two ml of the medium were added to each culture and allowed to incubate at 36°C for 24 hours prior to inoculation.

With this method, infectious virus titers of CR69(076) were found to range from $10^{6.9}$ to $10^{7.4}$ tissue culture infectious doses ($TCID_{50}$) per ml. Maximum titers usually were reached by the 4th or 5th day of incubation.

Studies have indicated that an acid environment, as provided by Medium III, was advantageous to the replication of CR69(076). Yet, as mentioned earlier, the use of Medium

I resulted in poor replication of this agent even though the hydrogen ion concentrations of inoculated cultures ranged from pH 7.4 at the time of inoculation of pH 6.6 following incubation. To clarify this apparent inconsistency, parallel infectious virus titrations were conducted in cells maintained in Medium I and in those maintained in Medium III, described above. Because of the excessive acid pH developed in the course of incubation, Medium I was changed on the 3rd and 6th day of incubation. The results of these titrations are presented in Table 1. It can be noted that the maximum infectious virus titer was almost attained after the 3rd day of incubation in Medium III, while the amount of virus detected in the presence of Medium I was substantially less. When this medium was changed after 3 days and the virus titer determined at day 6, the amount of infectious virus detected had doubled while only a slight increase was noted in the Medium III-containing cultures during the same period. A second change of Medium I at day 6 resulted in an additional increase in infectious virus titer; to a lesser degree by day 9. It should be mentioned here that, if medium changes were not made, the CPE would regress; infected cells would detach from the glass into the medium, leaving a normal appearing cell sheet.

These findings suggest that the CR69(076) agent not only requires an acid environment for its replication but replicates best within a narrow pH range within the acid environment. In Medium I, cellular metabolism results in a rapid shift in the hydrogen-ion concentration from the alkaline side of neutrality to the relatively acid side, quickly passing through that range optimal for viral replication and permitting only limited infection by CR69(076) to occur.

Since the replication of rhinovirus types not only requires an acid environment but also is enhanced by an incubation temperature of 33°C as compared with 36°C, studies were conducted to determine whether a similar situation existed with CR69(076). Simultaneous duplicate titrations were carried with CR69(076) virus stock. One titration was incubated at 33°C; the other at 36°C. Similar titrations were carried out with ECHO 25 and incubated in the same manner. In both instances, higher infectious virus titers and more rapid virus proliferation were observed at 36°C than at 33°C.

Efforts to detect viral hemagglutinins with human "O" erythrocytes at various temperatures (4°, 20°, 37°C), utilizing normal saline and phosphate buffers (pH 5.7, 6.1, 6.5, 7.1, 7.5, 8.0) as diluents, were unsuccessful.

Table 1

Influence of different maintenance media upon CR69(076) infectious virus titer

Days of incubation	Medium I *	Medium III **
3	2.5***	6.27
6	4.95	6.7
9	5.35	6.87

* EBM in Earle's BSS, 10% FCS, 0.11% $NaHCO_3$. Medium changed in inoculated cultures on day 3 and 6 of incubation.

** EBM in Hanks' BSS (galactose), 0.6% FCS, 0.035% $NaHCO_3$, BrDUR (20 µg/ml), Na pyruvate (55 mg/l), alanine (45 mg/l) and gassed with CO_2. Medium not changed during incubation.

*** $TCID_{50}$/ml, log 10, 10 WI-38 tube cultures/dilution.

CR69(076)CPE. The CPE produced by CR69(076) in cultures of WI-38 cells were similar to those produced by the enteroviruses. Infected cells, stained with Giemsa and with hematoxylin and eosin, often showed swollen nuclei, which later became pyknotic. Although neither cytoplasmic nor nuclear inclusions were apparent, the cytoplasm of some cells contained eosinophilic masses as seen in enterovirus-infected cells.

Limited investigations of the host cell spectrum indicated that CR69(076) was unlike the majority of the enteroviruses. There was no evidence of CPE in primary cultures of Rhesus monkey renal cells either under culture conditions normally employed for enterovirus isolation or under those found to be optimal for this virus. Similar results were obtained with VERO, HEp-2, HeLa, and Chang liver cells. However, CPE were noted with a diploid human foreskin fibroblast cell line (HuFS-6*), utilizing the methodology described.

Physico-Chemical Characteristics of CR69(076). Unlike the enterovirus types, with CR69(076) there was a significant loss of infectious virus titer following storage at -70°C. Infected culture fluid, clarified by centrifugation, was distributed in screw-capped vials in 1 ml aliquots, stored in a mechanical deep freezer at -70°C, then removed periodically and the contents titrated. As shown in Table 2, a substantial loss of infectious virus titer was observed following storage for 3 months. Subsequent lowering of the storage temperature to -90°C reduced the rate of loss.

The stability of infectious CR69(076) virus following exposure to 60°C for 1 hour was investigated. Dilutions of CR69(076) and ECHO 25 were made in sterile deionized water, incubated in a water bath at 60°C for 1 hour, placed in an ice bath, then inoculated into cultures of WI-38 cells, utilizing the medium most suitable to each virus type. No evidence of CPE was observed in cultures inoculated with dilutions of ECHO 25 exposed to 60°C even though CPE were observed with similar dilutions maintained at 20°C. In contrast, in one of four cultures of WI-38 cells inoculated with a 10^{-2} dilution of CR69(076) CPE were demonstrated (confirmed by passage) following exposure to 60°C for 1 hour. CPE were not observed with other dilutions. Similarly, a lack of complete inactivation following exposure to 60°C for 1 hour has been reported with the marmoset HeA agent (5).

CR69(076) showed no loss in infectivity titer following overnight exposure to 20 percent diethyl ether at 4°C or following a 3-hour exposure to pH 3.0 at 20°C when compared with untreated controls. Proliferation in cells previously exposed to BrDUR (20 µg/ml) confirmed its ribonucleic acid composition.

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*North American Biologicals, Inc.

Table 2

Stability of CR69(076) stock
stored at -70°C

Months of storage	$TCID_{50}$/ml (Log_{10})*
0	7.15
1	5.7
3	1.99

*10 WI-38 Tube cultures/dilution

Electron micrographs of CR69(076)-concentrated infected cell culture fluids revealed icosahedral particles having diameters of approximately 29 nm, within the picornavirus size range and consistent with the virus-like antigen previously reported (6) (Figures 1 & 2).

CR69(076) Relationship to Known Enteroviruses. CR69(076), tested against type specific enterovirus antisera by neutralization, produced no inhibition with antisera to ECHO virus types 1-24, 26, 27, 29-32; Coxsackie group A, types 1-3, 9; Coxsackie group B, types 1-6; and poliovirus, types 1-3. ECHO virus type 25 rabbit antiserum was found to neutralize 100 $TCID_{50}$ of CR69(076) at a dilution of 1:4000. The neutralizing antibody titer of this same antiserum was determined to be 1:10,000 versus 1,000 $TCID_{50}$ of the homologous virus (JV-4).

The significance of this observation is uncertain since ECHO virus type 25 antiserum has been reported to be inhibitory to ECHO virus type 12 at a dilution of 1:100 (13). Also, it should be mentioned that cross relationships have been reported between Coxsackie group A, types 3 and 8, 11 and 15, 13 and 18; ECHO virus types 1 and 8, 12 and 29, 6 and 30; and, to a minor degree, poliovirus types 1 and 2 (7). Cross neutralization studies are in progress.

Relationship of CR69(076) to HeA. Paired sera collected from cases occurring during epidemics of hepatitis B (1964) and A (1967) in Costa Rica (8,9) were examined by neutralization and complement fixation procedures with CR69(076) as antigen (Table 3). Of 20 paired sera collected during the 1964 epidemic, only one individual demonstrated a significant increment in the antibody level of the convalescent serum. In contrast, when paired sera from the 1967 epidemic were investigated, 8 out of 24 serum pairs (33 percent) showed significant increases in the antibody levels of the convalescent sera. It is of interest to note that subsequent investigations revealed the nature of the epidemics (9) and that the majority of antibody responses occurred in sera collected during the HeA epidemic.

Discussion

The enterovirus-like nature of the HeA agent isolated in marmosets (5), the demonstration of an enterovirus-like antigen in feces of a human volunteer with HeA by electron microscopic techniques (6), together with the absence of any in vitro isolation of etiologic agent, indicate that current enterovirus isolation procedures have limitations despite the large number of enterovirus types isolated. Furthermore, the need for newer approaches to virus isolation also has been emphasized by electron microscopic

Figures 1 & 2. Representative samples of virus particles obtained from frozen/thawed cell cultures centrifuged to settle cellular debris. The supernatant was then centrifuged at 150,000 g x 3 hr, and the resulting pellet suspended in 2 ml of 10% formalin and negatively stained with 2% phosphotungstic acid. The bar in each figure represents 100 nm (= 0.1 μm).

Figure 1

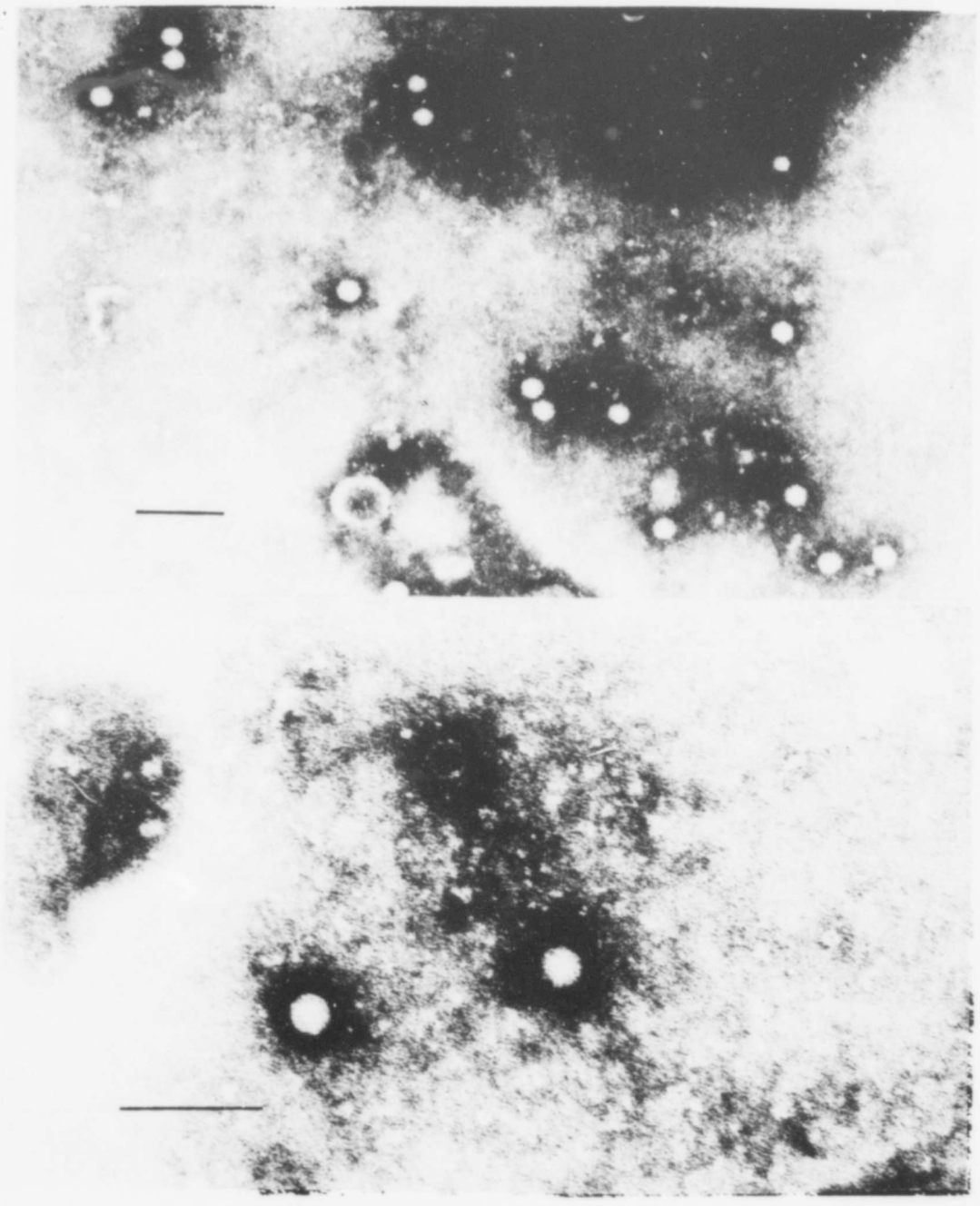

Figure 2

Table 3

Antibody responses to CR69(076) in paired sera from cases of hepatitis (Costa Rica)

Year	Hepatitis epidemic type	No. paired sera tested	No. with significant antibody increases*
1964	B	20	1 (5%)
1967	A	24	8 (33%)

*Fourfold or greater in the convalescent serum

demonstration of orbivirus-like particles in fecal samples from infants with diarrhea and the failure to isolate such agents from virus positive-appearing samples by conventional techniques (14,15).

This report presents data regarding an agent whose presence would have remained undetected if only conventional isolation methods were employed. Regardless of the role of CR69(076) in disease, these observations are felt to be significant and should be given further consideration in those instances where the isolation of an etiologic agent of a disease has been unsuccessful.

The limited hydrogen-ion concentration within which the CR69(076) agent best replicates is a unique characteristic, unlike that attributed to the known enterovirus types. Conceivably, it may be applied in principle to many of the Coxsackie A virus types which infect man but which, to date, have been isolated only in newborn mice, as well as to those newly found virus-like agents such as the orbiviruses.

From our investigations, it appears that extreme acidity does not affect adsorption of the virus to the cell surface but rather interferes with virus penetration. Although the mechanism by which this is accomplished is uncertain, enzymatic activity has been considered as a possible mechanism. This would be influenced by an existing hydrogen ion concentration. Once penetration is accomplished, virus replication does not appear to be affected by existing extracellular environment. It is presumed that a similar situation exists when the extracellular environment is within the alkaline range of neutrality.

Our investigations indicate that CR69(076) is an enteric virus on the basis of its size and particle morphology, nucleic acid composition, ether stability, optimal replication at 36°C, resistance to an acid pH, and by the CPE produced. It also possesses characteristics unlike those of known enteric virus types. This is evidenced by its limited host cell spectrum and its limited replication within a narrow pH range within the acid side of neutrality, its failure to replicate either under alkaline or extremely acid conditions, and its relative instability under the storage conditions described. On the basis of these observations, it is felt that CR69(076) may be the prototype strain of a new virus group.

CR69(076) is markedly similar in its physical-chemical properties to those described for the HeA virus isolated in marmosets (5). Filtration studies of the latter show it to have a particle size less than 50 nm but greater than 25 nm. Further support for the enterovirus-like nature of the HeA

virus was obtained with immune electron microscopy studies of fecal samples from a volunteer with HeA(6). These detected a viral-like agent having an icosahedral morphology and an average particle size of 27 nm. Electron micrographs of CR69(076) (Fig. 1,2) also revealed an icosahedral morphology and an average particle size of 29 nm, similar to that of enteroviruses and indistinguishable from that of the reported virus-like antigen (5). These data, together with considerations as to the sources from which isolations were made, indicate that further studies of the relationship between these agents are necessary even though an antigenic similarity may not be demonstrated.

At the present time, it is not possible to claim an etiologic relationship between CR69(076) and HeA on the basis of a single virus isolation from a case of HeA and nine immunologic responses from cases of the disease. It is obvious that additional investigations along the lines presented are necessary.

Considerations should be given to the existence of multiple HeA virus types in the evaluation of relationships between the various reported HeA isolates as well as to the disease itself. One only needs to recall the search for the virus of the common cold, its initial isolation by the senior author and colleagues (16), and the existence today of at least 100 distinct serologic types. Conceivably, CR69(076) may not have been the prevailing virus type involved in the 1967 epidemic; and the serologic responses obtained in 33 percent of the paired sera from cases of clinical hepatitis may represent infections by a second virus type occurring at a low level within a geographic area known to be endemic for the disease (9). In addition, the very nature of hepatitis itself may make serologic diagnosis difficult, particularly if the infected individual experienced a prolonged acute or preicteric phase of the syndrome. Under such conditions, it is reasonable to assume that the immune response already has been elicited by the time jaundice becomes apparent, medical attention is sought, and appropriate serum samples are collected for study. It is possible that, under such conditions, maximum levels of antibody already may have been attained; or, if increments were detected, these may not be within the range to be considered significant. It is obvious, though perhaps not realistic, that initial or acute serum samples be collected early in the preicteric stages of the disease in order to permit a true serologic evaluation.

The problem of isolating additional strains of the CR69(076) virus also should be considered. It is possible that a loss of infectious virus titer, as the result of prolonged storage of specimen material, may have occurred

during our investigations. This aspect would be most critical, particularly if the virus were excreted at a low level at the time the specimen was obtained and if the specimen were collected in the later stages of the disease. It is felt that the investigation of fresh fecal samples, collected as close to the onset of jaundice as possible, would be more productive. Volunteer studies have indicated that virus excretion ceases about 1 week after the onset of jaundice (17). It has been our experience that patients often experience jaundice for several days before seeking medical attention. Because of its clinical course, the collection of a proper specimen appears to be an even more critical problem in etiologic investigations of HeA than in the investigations of other enterovirus-associated illnesses. The need for further investigations in this area is obvious.

Summary

An enterovirus-like agent, designated CR69(076) and possessing properties unlike the known enteroviruses, as well as an unusual growth requirement, was isolated from a fecal sample collected from a 7-year-old male Costa Rican experiencing clinical hepatitis. The agent, isolated in cultures of WI-38 cells, replicates best within a stable and relatively narrow pH range provided by the maintenance medium. Poor or no replication is observed when a medium having a hydrogen ion concentration above pH 7.0, or one which is excessively low, is employed. The agent is not a rhinovirus, being acid stable and having an optimum temperature of 36°C for its replication. The usual spectrum of cells found to be susceptible to infection by many enterovirus types was not infected by CR69(076). In addition to a limited cell spectrum, the agent is relatively unstable to a temperature of -70°C under the storage conditions employed. CR69(076) has a particle size of approximately 29 nm and possesses an icosahedral morphology. It is ether-resistant and possesses a ribonucleic acid core. Although some antigenic crossing was noted with antiserum to ECHO virus type 25, no inhibition was noted with antisera to other enteroviruses tested. In addition to its isolation from a HeA case, paired sera from nine cases of HeA occurring in Costa Rica also showed increments in antibody levels. Furthermore, many of the properties of CR69(076) were found to be similar to those described for the hepatitis A agent isolated in marmosets and observed in the feces of a volunteer with hepatitis A by investigators at the National Institutes of Health.

Addendum

In an effort to obtain additional virus isolates, 160 specimens of feces, urine, and liver biopsies, collected from cases of clinical hepatitis occurring in Costa Rica and in the United States from 1963 to the present, were inoculated into WI-38 cell cultures, utilizing the methodologies described. Although studies are still in progress, virus isolations have been obtained from one liver biopsy, one urine, and two fecal specimens thus far. Past efforts to isolate agents from these same specimens were unsuccessful when inoculated into cultures of WI-38 and Rhesus monkey cells maintained under the usual cultural conditions employed for enteric virus isolation. The relationship of these recent isolates to CR69(076) as yet has not been determined.

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A PROSPECTIVE STUDY OF ACUTE RESPIRATORY DISEASE IN COSTA RICA

I. Incidence of Acute Respiratory Disease

J. Arguedas, R.S. Gohd, V.M. Villarejos, M.I. Nickle, J.C. Swartzwelder, J.I. Kato, and J. Jiron

Acute respiratory illness is by far the most common cause of short-term disability in industrial societies. However, there is little information regarding its importance in developing countries, where the incidence of respiratory illness may surpass even that of diarrheal disease[1].

To amplify our information regarding the incidence and prevalence of respiratory disease and the infective agents prevalent in a developing tropical country, a year-long prospective study was conducted in Costa Rica. Epidemiological data on the relation of geographic location, age and seasonal differences to frequency of attacks of respiratory illness are present in this report. The results of the study of etiologic agents are reported in a companion paper.

Costa Rica is a Central American republic of 32,000 square miles with 1.5 million people (Fig. 1), bordered on the north by Nicaragua and on the south by Panama. Two mountain chains running northwest-southwest enclose a 4000 foot high plateau between the Pacific and Atlantic lowlands. These three main zones have marked differences in temperature and climate. Seasonal variations are limited to a rainy and dry period throughout the country; the rains usually begin during the middle of May and cease towards the end of November. The western slopes of the mountains and the Pacific coast are hot and relatively dry; there is virtually no rainfall from December through June. The eastern coast is truly tropical, the heat and humidity remaining high during the entire year. In the mountain plateau, the temperature is mild and relatively constant. Climatological data for the three areas of study are presented in Table 1.

Materials and Methods

Selection of sites for study

In temperate zones, especially in the United States, epidemics of acute respiratory disease and pneumonia have occurred during the winter months, implying that season and climate play a role in respiratory disease. To assess the

Figure 1. Location of three sites in Costa Rica of the acute respiratory disease study

impact of these factors on respiratory illness in Costa Rica a representative site was selected in each of three climatic settings. Puntarenas, a major seaport on the Pacific coast, was selected to represent the semi-arid region. Puerto Limón, sole seaport on the Caribbean Sea, was chosen to represent an area of high rainfall and high humidity, with precipitation more than twice that of Puntarenas, San José, capital of the country and site of its only international airport, represented the temperate zone, the "land of eternal spring", with intermediate rainfall (Figure 1).

Since the three cities are business centers for their respective provinces, the residents are exposed to infections introduced by international travelers, as well as being in contact with people from the adjacent areas. Epidemics of acute respiratory disease arising within the country could be expected to find expression in the population of these cities. In addition, these populations would act as sentinels for infective agents of foreign origin introduced through the seaports of Puntarenas and Limón and by air into San José.

Table 1

Meteorological data of the study sites
(10-year averages)

	Puntarenas	Limón	San José
Rainfall (in.)	59	134	79
Mean yearly temperature (°F)	82.0	78.4	68
Mean relative humidity %	76	90	82

Selection of the population

Family studies in Cleveland[2], New York[3] and Michigan[4] showed that children experience acute respiratory disease more frequently than their parents. In the present investigation, the entire family was studied in order to obtain comparative data.

Socioeconomic conditions within the cities studied differed considerably from one section to another, ranging from extreme poverty to comfortable middle class living

conditions. To insure that all economic groups would be represented, the cities of Puerto Limón and Puntarenas were divided geographically into two sections; San José, a larger city, was divided into four. Families were selected in each of the divisions by the following methods:

Each block in the city was numbered and five blocks in each geographic division were selected by a statistically valid random method. The third house from a corner of the block was visited by a member of the investigating team. If the family contained at least two children, one of which was of school age, the investigator explained the nature of the project and attempted to enlist their cooperation in the study. If the family was unsuitable, then the house on the right was approached; if that proved unsuitable, then the house on the left of that originally selected was visited next. In this manner, five families in each of two sections of the city were enlisted in Puerto Limón and Puntarenas, and 20 families in the four sections of San José. The clinical surveillance of this group of 40 families included collection of samples for isolation of viruses and bacteria as well as for serologic examinations. An additional 20 families were randomly selected in each of the three areas, and were studied by serologic methods only. These families, plus the other described above, brought the total under investigation to 100 families.

Criteria for determination of an attack of acute respiratory disease

The following were considered cardinal signs of acute respiratory infection: cough, sore throat, rhinorrhea, and laryngitis. The occurrence of any one of these in conjunction with fever, headache, conjunctivitis, or malaise, was considered as a respiratory infection.

Frequently cough and rhinorrhea persisted intermittently for prolonged periods and it was sometimes difficult to determine if an infection was new or persisting. It was decided arbitrarily that following an infection an individual should be free of signs and symptoms of respiratory illness for at least 2 weeks before being considered as a new attack.

A public health nurse visited each household at least twice each week and assessed the health status of each person in the family. A physician accompanied the nurse once every 2 weeks or when especially requested by her. When the development of an acute respiratory infection was detected in a member of the household, a physical examination was performed and the nurse obtained specimens for virologic and bacteriologic study. Two throat specimens were taken for each acute illness, the second 48 hours after the first. Blood specimens for serology were collected at regular intervals during the study.

The study started in March 1968 in Puntarenas, followed by Puerto Limón in May and San José in June 1968, and continuing for one full year in each place.

Composition of the study group

The total number of participants was 364, 179 males and 184 females. The composition of the study groups arranged by age, sex, and geographic area is shown in Table 2.

The household units averaged 7.8 members in both Puntarenas and Puerto Limón and 10 in San José, with a range from 5 to 15. The larger households represented more a clan than a family, being composed of a husband and wife, their single and married sons and daughters, and the grandchildren.

As a whole, the study population was young, with 70 per cent being less than 20 years; almost 40 per cent were children under 10 years of age.

Results

Incidence of acute respiratory disease

The age-specific attack rates of acute respiratory disease encountered in the three locations are shown in Table 3 with the standard deviation included as an indication of the variability within each group. Of the 737 attacks observed, 182 occurred in Puntarenas, 222 in Puerto Limón and 333 in San José. The yearly attack rate per person was similar in both tropical environs, 2.9 in Limón and 2.3 in Puntarenas, but notably lower (1.6) in the temperate San José. The difference between the latter and the two tropical settings is statistically highly significant ($p < .001$).

The brunt of respiratory disease was borne by the younger age groups. Children under 5 were particularly prone to infection, followed by those 5 to 9 years old who experienced somewhat lower rates. In Puntarenas, the 10 children in the 1-4 years bracket experienced 61 attacks of respiratory disease, averaging 6 per individual; the rate for the same age group was 4.6 in Puerto Limón and 4.0 in San José. For ages over 15 the attack rates decreased sensibly in San José and Puerto Limón, but remained relatively high in Puntarenas.

The overall incidence of acute respiratory disease was slightly higher in females than in males (Table 4). However, there were noticeable differences in the age distribution between sexes. While children up to 9 years of both sexes suffered a similar number of disease attacks during the year, the attack rates of females from 10 years up through adulthood were two and three times higher than among males in the same age groups.

Table 2

Composition of the respiratory disease study group by age, sex and geographic area

Age	Puntarenas			Limón			San José			Total		
	*M	F	T	M	F	T	M	F	T	M	F	T
<1	1	-	1	-	1	1	3	1	4	4	2	6
1 - 4	7	3	10	7	7	14	12	15	27	26	25	51
5 - 9	9	7	16	12	9	21	25	22	47	46	38	84
10 - 14	8	5	13	6	7	13	18	25	43	32	37	69
15 - 19	5	6	11	4	2	6	10	11	21	19	19	38
20 - 29	5	5	10	1	3	4	6	11	17	12	19	31
30 - 49	7	6	13	8	7	15	17	19	36	32	32	64
50+	2	2	4	3	1	4	5	8	13	10	11	21
Totals	44	34	78	41	37	78	96	112	208	181	183	364

*M = male, F = female, T = total

Table 3

Yearly incidence of respiratory disease by age and geographic area

Age group	Puntarenas			Puerto Limón			San José		
	No. of persons	Attacks person/ year	S.D.	No. of persons	Attacks person/ year	S.D.	No. of persons	Attacks person/ year	S.D.
<1	1	13.0	-	1	1.0	-	4	2.3	2.1
1 - 4	10	6.1	3.6	14	4.6	4.4	27	4.0	2.6
5 - 9	16	2.4	2.3	21	3.9	3.7	47	1.9	1.8
10 - 14	13	1.8	1.9	13	2.2	2.2	43	1.6	1.9
15 - 19	11	1.4	1.7	6	0.3	0.5	21	0.6	0.8
20 - 29	10	1.2	1.7	4	0.5	1.0	17	0.5	1.0
30 - 49	13	1.4	1.7	15	2.5	2.6	36	0.8	1.0
50+	4	0.5	0.6	4	0.8	1.5	13	0.9	1.1
Totals	78	2.3	2.9	78	2.9	3.2	208	1.6	2.3

Table 4

Incidence of respiratory disease by sex and age

	Males		Females		Total	
Age group	No. in group	Mean incidence person/year	No. in group	Mean incidence person/year	No. in group	Mean incidence person/year
<1	4	4.3	2	3.0	6	3.8
1 - 4	26	4.6	25	4.6	51	4.6
5 - 9	46	2.5	38	2.4	84	2.5
10 - 14	32	1.3	37	2.1	69	1.7
15 - 19	19	0.4	19	1.1	38	0.8
20 - 29	12	0.5	19	1.1	31	0.7
30 - 49	32	0.8	32	1.8	64	1.3
50+	10	0.5	11	1.0	21	0.8
Totals	181	1.9	183	2.1	364	2.0

Throughout the year there was consistently more respiratory disease in Limón than in Puntarenas or San José, and in certain months the incidence rate in Limón was double that observed in the other two sites (Figure 2).

San José experienced two peaks of occurrence of respiratory disease, in July when the rainy season began and again when it ended in November. During the dry period this temperate zone had far less disease than either Puntarenas or Puerto Limón; low infection rates prevailed from December through February.

Clinical observations

Disease episodes in both adults and children were generally mild and involved the upper respiratory tract. Acute lower respiratory illness was infrequent and occurred almost exclusively in children under 5 years of age, However, none was severe enough to warrant hospitalization, although fever occurred in 43 per cent of cases. Pneumonia or other serious complications were not seen in this population during the limited span of the study.

The average duration of the episodes observed in the three settings was widely different (Table 5). While the average duration of attacks was 8 days in the semi-arid Puntarenas, they lasted almost twice as long (14 days) in the rainy Puerto Limón, with an in-between 10 day average duration in San José. There were no marked differences in duration of the illness between children, adolescents and adults in either of the three settings.

Discussion

The purpose of this study was to obtain basic information on the incidence of acute respiratory disease in tropical settings. The family was chosen as the epidemiologic unit for investigation because it represents a suitable sentinel population, sensitive to diverse infectious agents which are introduced into the home environment through contacts at work, school, play, in social interchange and daily shopping trips to the crowded market places. Therefore, close surveillance of the family would be expected to reveal both epidemic and endemic occurrence of respiratory disease.

In the present investigation the overall attack rate per person per year varied from 2.8 for Puerto Limón to 1.6 for San José. For all three sites the mean rate was 2.0. These values are remarkably lower than those observed in the family studies in Cleveland[2], the Virus Watch Program in New York City[3], and the Tecumseh study in Michigan[4]. The overall attack rate in Cleveland was 6.2 or almost four times higher than in San José; in New York City and Tecumseh the attack rates were also significantly higher than in any of the sites in Costa Rica.

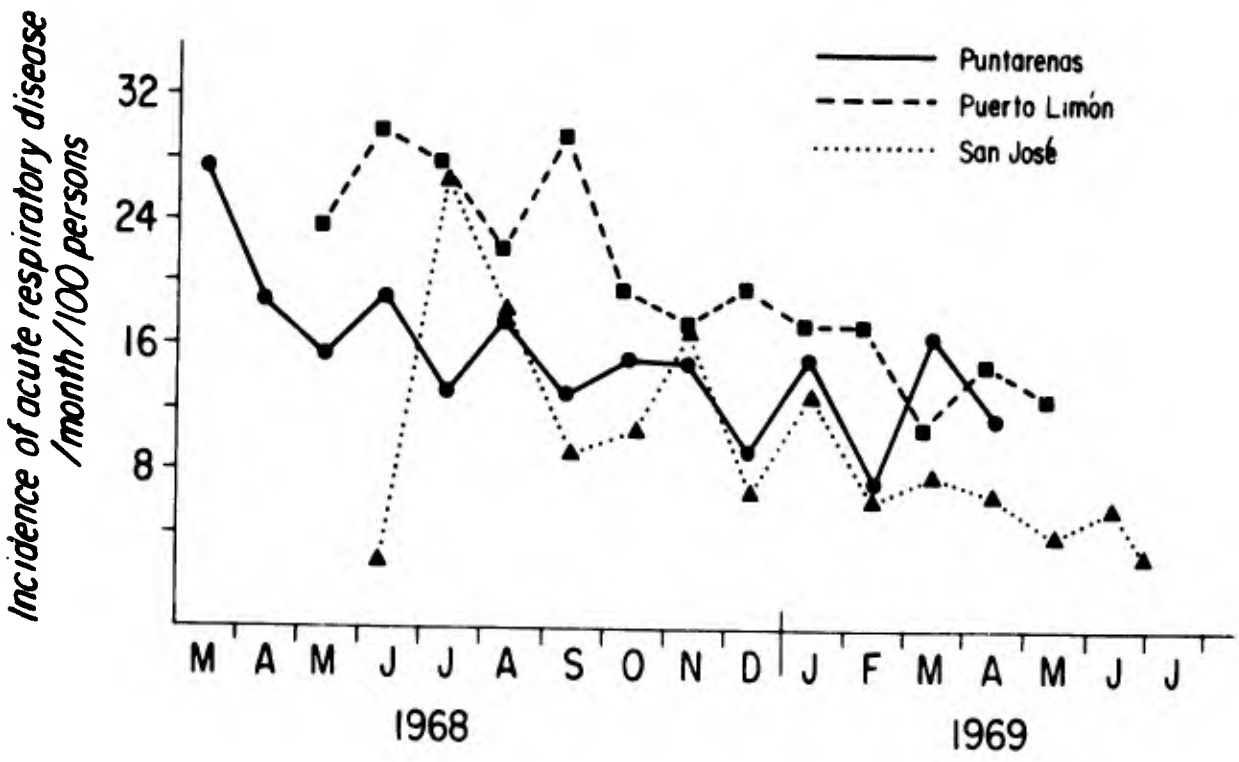

Figure 2. Incidence of acute respiratory disease in three sites in Costa Rica

Table 5

Duration of respiratory disease in three study populations by age group

Age group	Puntarenas		Puerto Limón		San José		Total	
	No. of attacks	Mean duration (days)	No. of attacks	Mean duration (days)	No. of attacks	Mean duration (days)	No. of attacks	Mean duration (days)
<1	13	12	1	11	9	7	23	10
1 - 4	61	7	65	13	109	10	235	10
5 - 9	38	7	82	14	89	11	209	11
10 - 19	38	8	31	13	79	8	148	9
20 - 29	12	8	4	14	9	9	25	9
30 - 49	18	8	36	15	27	9	81	11
50+	2	4	3	10	11	9	16	9
Totals	182	8	222	14	333	10	737	10

The curve of incidence of acute respiratory disease for Puntarenas suggested that the start of the study had coincided with the final phase of an epidemic. Serologic studies performed later indicated that an influenza outbreak had occurred at that time, with relatively high incidence among adults. From then on, the disease occurred evenly throughout the whole year.

The distribution of acute respiratory disease by age groups followed in a general way the observations in the above studies with the highest age-specific rates in the age groups under 10 years. In Costa Rica the rates for children 1-4 years and 5-9 years were 4.6 and 2.5 respectively, significantly less than the 5.4 and 4.9 values in New York City and the almost identical rates in Tecumseh; they were ostensibly lower than the 8.5 and 6.0 rates of the Cleveland families. Overall rates dropped considerably among adolescents and adults, probably due in part to some degree of age-related immunity. The distinctly higher attack rates of females, starting in adolescence and sustained through adulthood, suggest that the homebound female members of the family are more prone to acquire infections from young children in the household environment; a similar observation was reported from Panama[5].

There are many reports of seasonal differences in the incidence of respiratory disease in temperate zones; in the family studies in the United States, highest incidence occurred in the winter months, November through March. In the tropics the seasonal variation is related to rainfall rather than temperature. Increase in the incidence of respiratory disease in Costa Rica coincided with the start of the rainy season in May and June, and it dropped markedly with the arrival of the dry season between December and January. These observations are in agreement with studies conducted in Paraiso, in the Panama Canal Zone[1]. Uniformity of temperature could well be a factor determining the lower incidence of respiratory disease found in this study, especially when moderate temperatures prevail throughout the year, as is the case in San José. Quantitative differences in frequency of attacks were more pronounced between the temperate San José and Puerto Limón which, although warmer, has frequent bouts of prolonged rainy and stormy weather. Rainfall and the sea cold wind, causing sudden changes in temperature, may make the latter population more prone to respiratory infection. Such climatic influences are also evident from the distinctly longer average duration of attacks in the humid Puerto Limón than in the semi-arid Puntarenas or the temperate San José.

The data presented here support the concept that acute respiratory disease in the tropics occurs less frequently and is less severe than in places with great variations in climate.

Summary

A prospective study of acute respiratory disease was conducted in three different climatic areas of Costa Rica. Forty families comprising 364 persons were under surveillance for 1 year. The yearly attack rate per person was significantly lower in the temperate San José (central plateau) than in the semi-arid hot city of Puntarenas (Pacific coast) or in the humid tropical Puerto Limón (Atlantic coast), which had the highest rate. The period of peak incidence coincided with the onset and early months of the rainy season, May through July.

As a whole, the incidence of acute respiratory illnesses in these three areas of uniform year-around climate was much lower than reported from places in northern U.S.A. with marked seasonal changes of climate. However, the age distribution was similar, with highest attack rates among children under 10 years.

The prevailing moderate or warm temperatures, with slight seasonal variations, could well be the determinating factor for the lower incidence of acute respiratory illness.

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ETIOLOGY OF DIARRHEAL DISEASE IN PERSONS INTRODUCED INTO A TROPICAL ENVIRONMENT

Victor M. Villarejos, Margaret I. Nickle and William Pelon

Introduction

It is well-known that persons coming from a sanitary environment suffer frequent gastrointestinal disturbances when entering tropical areas where infectious diarrhea is endemic. Those disturbances can be severe and incapacitating, and constitute an important military as well as civilian health problem. Studies of the etiology of such gastrointestinal disturbances conducted in Mexico, Puerto Rico and other countries have not yielded clear results. On the other hand studies of endemic diarrheas performed by the LSU-ICMRT disclosed the significant association of Coxsackie B viruses with diarrhea. An opportunity for further investigation of that association, and of the etiology of diarrheas in newcomers to the tropics in general, appeared to be available through a study of North Americans visiting Central America for short periods of time, as well as of members of the Peace Corps assigned to Costa Rica and Honduras, where the LSU-ICMRT has investigational facilities.

Objective

An attempt was made to determine the infectious etiology of the diarrheas of newcomers to the tropics through virological, bacteriological, parasitological, and serological examinations.

Methods

Peace Corps volunteers and North American professors and students participating in various training programs of LSU and other institutions in Central America were requested to participate voluntarily in the study. Stool specimens and blood samples were obtained within the first 3 to 7 days after arrival in the country. The participants were instructed to provide stool specimens at the onset of any gastrointestinal disturbance during their stay. The ICMRT offered free medical consultation and drugs to the participants, requesting that the stool specimens be submitted at the time of consultation concerning gastrointestinal disorders. Final blood samples were obtained from these persons at the end of their stay in the country, as well as a final stool sample, together with a history of diarrhea episodes experienced and use of antidiarrheal drugs. Sera were separated promptly from blood samples and frozen at -20°C.

The stool specimens were divided in three portions for virological, parasitological, and bacteriological examinations. The portion for virology was frozen and stored at -70°C. The samples for bacteriological and parasitological studies were processed immediately through standard methods for detection of bacterial and protozoan enteropathogens. In addition, stools were preserved in formalin and later examined by the Ritchie concentration method. One group of samples (from Honduras) was also preserved in MIF for confirmation of protozoa identification, especially Entamoeba histolytica.

Fecal extracts were made and inoculated into newborn mice and tissue cultures of HEp-2 and WI-38 cell lines. Extracts from positive mice were passed to the two types of cell cultures. All cultures showing CPE were screened by neutralization for poliovirus; positives were absorbed with poliovirus antisera and retested against antiserum pools of Coxsackie and ECHO viruses. Those not so identified or showing typical CPE were tested with adenovirus antisera.

The various populations studied were the following:

1. Peace Corps volunteers receiving training and orientation in Puerto Rico and St. Thomas, V.I., before being assigned to posts in various Latin American countries. The blood and fecal specimens were collected there and shipped to the Central Laboratory in San José. Although feces for bacteriology were sent in Stuart's transport medium, examination were subject to the limitations of unpredictable delays in delivery. This group was followed for only 6 to 7 weeks and it was impossible to obtain a complete record of diarrhea incidents or collection of diarrheal feces. It was intended that members of this group who were later stationed in Costa Rica would be followed there, but soon after arrival the volunteers were sent to distant locations in the country and it was impossible to monitor them.

2. A Peace Corps group in Honduras. Sera were collected when this group first arrived in Puerto Rico for training and again, together with fecal samples, when they arrived in Honduras 2 months later. Although some were stationed in rural areas, they kept in close contact with the headquarters in Tegucigalpa. Some were permanently stationed in the city and added to these was a group consisting of Peace Corps staff, American Embassy personnel and American School teachers who came directly form the United States. These could be followed closely during the study period which lasted nearly a year. Samples of normal feces were collected monthly and it was possible to obtain diarrheic samples for most of the episodes.

3. Exchange students from Goshen College, Indiana, who came to San José for 3 months. Although 5 to 6 weeks were spent in rural areas in Peace Corps type activities, a fairly complete study could be made. Four such groups were followed during their stay.

A fifth group of students, sponsored by the Associated Colleges of the Midwest, was also included. However, the directors were less wholehearted in their cooperation, the students were not as well disciplined and the small number of volunteers were not highly motivated to complete participation in the program, although it is valuable to include the data that were obtained.

Results

Specimens studied in all groups are summarized in Table 1.

Puerto Rico and Virgin Islands

Only two pathogenic organisms, a Salmonella and a Trichuris trichiura were encountered, both from diarrheal specimens.

Although the virology has not been completed yet on this group, 60 isolates have been obtained to date. Only two of these are from diarrheal specimens. They are among 29 poliovirus positives which are yet to be absorbed before it can be determined if other viruses are present.

This is the only group whose serum have been examined. Paired samples obtained from 128 persons were tested only against Coxsackie B, types 1-6. Many of the initial sera indicated an immunity already established, and increase in titer was minimal throughout the group. Significant titers in the first samples and increments indicated in the second samples were distributed as follows:

Number examined: 128

Coxsackie type	% with antibodies in First sample	% with increase in Ab titer in Second sample
B1		1
B2	38	7
B3	53	
B4	16	
B5	25	5
B6	26	5

Honduras

Three diarrhea cases were probably caused by Shigella and a fourth by Staphylococcus aureus. Shigella dysentery was contracted by one Peace Corps volunteer working in a rural area near the border of Guatemala where there was a severe epidemic of Shigella A1, a situation rarely encountered by a traveler. No pathogenic bacteria were isolated from normal stools.

E. histolytica was seen in two cases of diarrhea and one normal; Ascaris lumbricoides appeared in one diarrhea and two normal; Giardia lamblia occurred in five non-diarrheic samples. Two of the E. histolytica infections occurred in roommates a month after arrival. One patient went exploring in a cave and developed an illness eventually diagnosed as histoplasmosis. The early diarrhea specimen contained A. lumbricoides.

Of 67 specimens positive for virus, only seven were from diarrheas, and of 82 isolates (from any or all of the three systems, including double infections), only eight were from diarrheas. Of these, seven were in the Coxsackie B group and 5 of these were type B1.

Eight people had viruses when they arrived. Three were Coxsackie B6 and there was one each of Coxsackie B1, A15 and A20, and ECHO 3 and Adeno 1. Two had double infections with poliovirus. None of these developed viral diarrhea.

In only three instances was the same virus isolated from the same person after a lapse of time: one persisted 5 months after diarrhea, one was found 2 months before diarrhea, and the third was isolated three times in 3 months with no relation to a diarrheal episode. All were Coxsackie B1.

Double infections occurred in ten instances, only one of which was during diarrhea. Five of these were infections with poliovirus.

Four cases of diarrhea were completely negative.

Goshen College and ACM

From diarrheic samples, three Salmonella and one Shigella were isolated; from non-diarrheic, two Salmonella and a Shigella. In the Goshen group, three diarrhea specimens and four normal contained G. lamblia. The ACM diagnoses were made in other cities and reported to us verbally by the students; there were five G. lamblia from diarrhea, and in two cases without diarrhea; diagnosis was "amebas" and "hookworm".

A larger number of viruses was isolated from these people than from the Honduras group, in particular from the first Goshen contingent. In all, there were 30 isolates associated with diarrhea and 178 that were not; there were 21 undiagnosed diarrheas.

In the first samples, 25 were positive, including 10 Coxsackie B1, 9 B3 and 2 B6; 5 of these also had poliovirus. Coxsackie A1, ECHO 8 and 11 and Adeno 1 and 4 also were in one each. Only one of these appeared later in connection with diarrhea, a Coxsackie B1 that was in all four samples from one patient, whose diarrhea occurred during the first week.

In the ten instances where the same virus was found in more than one specimen, seven were B1. Poliovirus is not included in these data but it occurred frequently as a repetition, both alone and in combination with other viruses in one or both samples.

Of the viruses isolated from diarrhea, ten were B1 and nine polio. There were 35 multiple infections, only six of which were in diarrheas.

Because of the notable differences in the virological data from the four Goshen groups, they were tabulated separately in Table 2. The difference cannot be entirely explained by the season, since groups 1 and 4 came during the same months; other parameters, such as weather might reveal some difference, between the two years.

The virological data are still incomplete so the final compilation will be slightly different. There is also the possibility of some errors because the present data were hand-tabulated. It will be interesting to see how the serological data to be obtained will affect the picture. However, some general conclusions can be drawn for the data presented.

Conclusions

1. Diarrheas that occur in travelers are rarely severe enough to incapacitate the individual and they are of short duration.

2. There was not as high a percentage of bacterial and parasitological infection as is commonly believed. The potential pathogens, *Salmonella*, *Shigella*, *E. histolytica*, *G. lamblia*, *A. lumbricoides* and *T. trichiura* do occur, although not always accompanied by illness.

3. Viruses are often brought by people into the area and infections may be acquired enroute or very early after

arrival, since they were found essentially as often in the first specimens collected after arrival as in those obtained later during the stay in the country.

4. Although no conclusive evidence was found that viruses are the cause of the syndrome, there is a strong suggestion of etiologic relationship of Coxsackie B1 virus with diarrhea. Further analysis of the data being carried out will clarify this relationship.

5. Neither the diarrheal incidents nor the isolation of agents were correlated with length of stay in the country. Infectious agents appeared at random throughout the period. Their presence was transitory, rarely persisting even when apparently related to a clinical syndrome.

A summary of the data for the three most nearly completed groups (Goshen, ACM and Honduras) is found in Table 3.

Table 1

Specimens studied in all groups

Group	No. persons	No. samples		No. diarrheal episodes	No. diarrheal feces
		Blood	Feces		
Honduras	49	115	197	19	23
Puerto Rico	171	164	304	14	9
Goshen	72	143	238	62	41
ACM	24	48	43	20	10

Table 2

Data on four Goshen groups

Goshen group	No. persons	No. samples Feces	No. diarrheal episodes	No. diarrheal feces
1 (May - July 1971)	18	65	23	15
2 (Sept.-Dec. 1971)	14	46	11	7
3 (Jan. -Mar. 1972)	21	66	14	9
4 (May - July 1971)	19	61	14	10

Isolates

Group	Bacteria Total	Bacteria Diarrhea	Parasites Total	Parasites Diarrhea	Viruses Total	Viruses Diarrhea	Base-line
1	1	3	6	3	81	17	13
2	1	1	1	0	24	2	2
3	0	0	0	0	30	2	1
4	0	0	0	0	14	4	0

Table 3

Summary of data, three groups - Goshen College, Associated Colleges of the Midwest, and Peace Corps in Honduras

Persons studied		145	
Specimens collected:	Blood	306	
	Normal feces	404	
	Diarrheal feces	74	(478 feces)
Diarrheal episodes		101	

	Normal	Diarrheic
Pathogenic bacteria	3	8
Pathogenic parasites	14	12
Viruses	222	38
Most prevalent viruses:		
Poliovirus	60	8
Coxsackie	124	19
ECHO	21	1
Adenovirus	10	2
Coxsackie B viruses:		
B1	65	14
B2	19	0
B3	13	3

COXSACKIE GROUP B VIRUS-ASSOCIATED DIARRHEAS IN PRIMATES

William Pelon

In the course of investigations of diarrheal disease in Costa Rica, it was found that it was possible to induce diarrheas in spider monkeys by adding diarrhea-associated strains of Coxsackie Group B virus types to their drinking water. Symptoms included the loss of appetite, apparent malaise (deviation from the normal behavior), followed by a watery diarrhea usually about 7 to 10 days following ingestion of the virus.

Attempts were made to study this phenomenon in New Orleans. Spider monkeys were received from Costa Rica, examined for the presence of parasites, treated specifically for such organisms, then given prophylactic doses of tetracycline in the drinking water in an effort to eliminate possible intestinal bacterial pathogens.

Following this regimen, the animals were challenged with the identical strains of virus employed in Costa Rica, with no diarrhea being evidenced either among the virus inoculated animals or among uninoculated controls. Repeated attempts with other virus types of the diarrhea strains of Coxsackie B also were unsuccessful.

Studies by an Iowa group showed that diarrhea among calves occurred only when a bovine enterovirus and an Escherichia coli strain were infecting the animal simultaneously, with no diarrhea being observed in the absence of either organism. This gave rise to speculation that prophylactic tetracycline therapy may have altered the intestinal flora, possibly eliminating an ordinarily comsensal bacterial organism, whose presence was essential at the time of virus infection in order to produce the diarrhea syndrome.

A second group of spider monkeys were received from Costa Rica. These were examined for parasites and specifically treated for those found. No tetracycline was administered as prophylaxis. Such animals were held in an area separated from the first group of monkeys for a 3-week period, during which time no symptoms of diarrhea were observed. The animals other than those designated as controls were challenged with the diarrhea virus strains in their water. As in Costa Rica, the the virus-fed animals developed diarrhea after about 7 to 10 days; however, the diarrhea experienced appeared to be milder, with less discomfort as based upon the observed behavior of the animals.

A suspension was prepared from a diarrheal fecal specimen and added to the drinking water of several of the monkeys in the first group. These subsequently developed diarrhea. Following recovery, these same animals were challenged once more with a different type of a Coxsackie B diarrhea strain. Diarrhea was observed once more. It was concluded that the observations in our experiments paralleled those observed with calves, and that both Coxsackie Group B virus and an unknown bacterial type were essential for the induction of diarrhea in primates. It is speculated also that the differences in the severity of the diarrheas observed in Costa Rica and in New Orleans may have been dependent upon diets the animals were receiving, together with the increased opportunities to consume fecally contaminated foods in Costa Rica which could contribute towards establishing an appropriate bacterial flora.

An effort was made to determine the nature of the tetracycline-sensitive flora in the gastrointestinal tracts of the monkeys. Since such organisms would include mostly nonpathogens, this endeavor became logarithmic in scope and, because of reduced funding, had to be terminated.

It is presently felt that this type of investigation should be continued but in the area where the diarrhea problem exists so as to provide further insight into the bacterial organism or organisms which may be involved. With such data, it may be possible to control diarrheas during the critical times of infancy, if similar bacterial species were detected in the gastrointestinal tracts of infants through the use either of appropriate narrow spectrum antibiotics or specific chemotherapy, since the development of an attenuated strain of the Coxsackie Group B types as vaccine would not be readily accomplished.

PATHOGENESIS OF AMEBIC LESIONS

Joseph H. Miller, Victor M. Villarejos, Leon Troper,
Robert H. Gilman, Chin Chiu Lee, Danny B. Pence,
George E. Childs, Jr., William B. Lushbaugh,
Jane E. Deas and James A. Miller

The purpose of this project was to elucidate the mechanism of tissue invasion by Entamoeba histolytica through the electron microscopic study of biopsy and necropsy specimens from patients with the disease. In addition, experimental infections were maintained in guinea pigs for cytochemical study by transmission electron microscopy. In order to aid the study, species of Hartmannella (=Acanthamoeba), free living organisms that can produce pathology in man and animals by rapid invasion of tissue, were studied by electron microscopy. It was thought that the elucidation of the invasive mechanism of free living amebae could contribute to the understanding of the process in parasitic species.

Studies on Entamoeba histolytica

Striking differences in the vacuolar system were noted when trophozoites of E. histolytica obtained in vivo were compared with those grown in culture. There was a remarkable increase in the smooth endoplasmic reticulum, and numerous small vesicles appeared to congregate at the plasmalemma. Frequently, there was stacking of elongate slightly dumbbell-shaped cisternae with smooth membranes reminiscent of a Golgi apparatus. Autophagic and phagocytic vacuoles associated with primary lysosomes were frequently observed. This immediately suggested that E. histolytica contained all the potentials for extracellular secretion. The process of secretion seems to be via elongate branched extensions of the plasmalemma. Lytic enzymes move from vesicles (primary lysosomes) located at the base of the extensions into the interior of the extensions. After discharge, the vesicle collapses and its membranes undergo disorganization. The extension may persist as a semi-permanent organelle for future secretory use. The enzymes are probably released from the extension via an apocrine type of budding distally at the extremities of the extension. This process may be selective since tube-like processes from an ameba have been found in contact with other amebae without the apparent discharge of associated vesicles. Acid phosphatase reactions in experimental infections were localized in the lysosomes and branched extensions. Small blebs from the plasmalemma were also noted to have some activity as did the glycocalyx. The former may be an additional method of release of lytic enzyme beside the specialized process mentioned earlier.

Dying and dead amebae found in our material also lend support to the theory of Villarejos (1961, Tulane University Dr. P.H. thesis) that, in any colony of amebae, endoenzymes liberated from those which die may cause lysis of host tissue and contribute to the overall invasive process.

A study of the glycocalyx of E. histolytica trophozoites under cultural and in vivo conditions demonstrated a marked difference between in vivo and in vitro axenic trophozoites. Both types of trophozoites have the same basal layer to the glycocalyx; however, in the in vivo organisms, the base coat is overlayed by irregular peaks of fibrillar material. This outer layer is a mosaic of high electron-negative charge densities probably composed of acid mucopolysaccharides, glycoproteins or glycolipids. The fibrillar outer layer, with the majority of charging in the in vivo cells, may be correlated with an increased resistance to host defenses and virulence. No evidence was obtained to support a change in surface charging at surface lysosomes with subsequent rupture of their membranes and discharge of lytic enzymes to the exterior which has been proposed by some workers.

Studies on Hartmannella (=Acanthamoeba) culbertsoni

In vivo and in vitro trophozoites were compared and acid phosphatase distribution was studied. There is a marked difference in the vacuolar system between the two types of trophozoites studied. The in vivo trophozoites have a much greater number of primary and secondary lysosomes. No secretory mechanism such as the extensions of surface lysosomes in E. histolytica were demonstrable. However, many small plasmalemmal outpocketings, appearing as buds, were noted in the in vivo trophozoites. Acid phosphatase was localized in these structures as well as in the endoplasmic reticulum at the plasmalemma. It is, therefore, concluded that release of lytic enzyme is not restricted as in E. histolytica trophozoites, but takes place generally at the plasmalemma by an apocrine process.

Through density gradient and biochemical studies it was demonstrated that acid phosphatase activity was associated with membranes. In addition, peroxisomes, seen in electron micrographs as dense bodies, were confirmed in the catalase-rich fractions of the gradient. This was the first demonstration of this organelle in a protozoan.

Conclusion

Entamoeba histolytica seems to possess a specific mechanism, the surface lysosome and its elongate branched extensions, for the release of lytic enzymes. In addition, there may be also a more general release at the plasmalemma which may be held in the glycocalyx. The glycocalyx is morphologically and physically differentiated in in vivo trophozoites which may be related to invasiveness.

The free-living sometimes pathogenic ameba H. (=Acanthamoeba) culbertsoni seems to have a general release mechanism for lytic enzymes via an apocrine type secretory process along the entire plasmalemma. This is associated with the endoplasmic reticulum and primary lysosomes.

This study provided training and research experience for graduate students. Three Doctor of Philosophy and one Master of Science degrees were awarded on the basis of original research done under this contract.

ADDENDUM TO FINAL REPORT ON

BIOLOGICAL CONTROL OF CHAGAS' DISEASE VECTORS*

Rodrigo Zeledón

Six new treatments with Telenomus were effected in house 44 which brings the total number to 44 in the entire period of 3-1/2 years. (See Table 11, Ann. Prog. Rept. No. 5, Oct. 1972.) During the first four treatments there was a mean population of nine insects in house 44, with no first or second instars present, and of 17 in house 27 (control) with all instars present. During the last two treatments a mean of 14 insects was found in both houses and first and second instars were present in house 44. The explanation for both the increase in number and the presence of small nymphs in house 44 is probably that after a period in which the inhabitants were using kerosene for cooking, they went back to use of firewood; thus a new population of insects was introduced in the infested wood harboring hidden triatomines.

New experiments to study the persistence of Telenomus between treatments in house 44 showed high parasitism of the eggs offered in a group of flasks throughout the whole interval. No Telenomus were detected during the same period in house 27 (control). In an experiment in which three groups of 30 triatomid eggs each were offered (covered with dust, just dusted, and clean) to a small number of Telenomus (born from two eggs), they were able to detect and parasitize only seven of the clean eggs.

A few experiments were performed with a new microhymenopteran of the genus Gryon. The parasitoid was sent to us from India where it parasitizes the eggs of Triatoma rubrofasciata and of Linshcosteus sp. Eggs from several species of triatomines were exposed to wasps obtained from Linshcosteus eggs. A few eggs of Triatoma infestans, T. dimidiata, T. maculata and Panstrongylus megistus were parasitized, giving rise to wasps that were maintained for a second generation only in the respective species, after which the strain was lost. T. lenti and T. vitticeps were parasitized, but wasps were not born. T. platensis was not parasitized.

With wasps obtained originally from T. rubrofasciata and later from T. dimidiata eggs, new experiments with more species of triatomines were performed (Table 1). We tried to breed wasps in eggs of several species for a few generations with negative results. (Some went to the second generation only.) Only those in T. dimidiata eggs bred indefinitely.

* An extensive final report of this study appeared in Report Number 5, Annual Progress Report. These additional observations were obtained through July 31, 1973.

Wasps from a heterologous host were less able to parasitize the same host species than when originating either from T. rubrofasciata or T. dimidiata eggs. In T. dimidiata there has been no tendency to diminish the ability to parasitize new eggs as in the case of other species. We are maintaining the strain without problems in T. dimidiata eggs and were able to raise eight generations (at time of report). In T. dimidiata it was possible to study the number of eggs a single female is able to parasitize. Virgin females could parasitize the maximum number of eggs exposed, i.e. 20, and the descendents were all males. Mated females could parasitize up to 28 eggs from 40 exposed to a single individual. It took from 25 to 28 days for a wasp to hatch at 26°C and they were able to live from 3 to 12 days without food.

Three experiments were performed to observe the ability of the wasps to parasitize eggs of T. dimidiata of different ages at 26°C (hatching time of T. dimidiata nymphs at this temperature: 23-24 days). Groups of 20 eggs from 0 to 22 days were exposed to 20 couples of Gryon at the same time. Although parasitism and hatching occurred in eggs up to 20 or 21 days old, hatching decreased drastically in those older than 16 or 17 days.

Table 1

Parasitism of triatomine eggs by the wasp Gryon sp. originally from T. rubrofasciata eggs*

Species	No. eggs exposed/ No. eggs parasitized	No. wasps born
T. rubrofasciata	19/17	17
T. dimidiata	50/35	34
T. vitticeps	160/71	22
T. lenti	65/19	1
T. infestans	100/39	19
T. platensis	53/3	1
T. gerstaeckeri	20/11	0
T. protracta navajoensis	15/0	0
Panstrongylus megistus	50/15	2
P. lignarius	48/20	11
Rhodnius prolixus	40/0	0
R. neivai	7/0	0
Dipetalogaster maximus	16/0	0

* Results are from three to five separate experiments

RECONNAISSANCE FOR ARBOVIRUS ACTIVITY IN THE MISSISSIPPI RIVER DELTA REGION

Harold Trapido and Charles B. Carson, Jr.

These studies at first concentrated on the attempt to determine if an enzootic focus of Venezuelan equine encephalitis (VEE) might exist in permanent swamps near New Orleans, and the exploration of the possible establishment of VEE in the vicinity of Basile, Louisiana, where a vaccine strain of the virus was isolated from mosquitoes by a United States Army team during the Texas epizootic and epidemic of the summer of 1971. Serological study of a selected sample of domestic animals and small wild mammals of the Basile area and of small wild mammals and turtles from the swamp area near New Orleans, collected during the summer of 1972, gave no evidence of the presence of VEE virus. However, reactors to western (WEE) and eastern (EEE) equine encephalitis antigen were found among the turtle sera in haemagglutination inhibition (HI) tests. An enlarged sample of reptile and some amphibian sera was obtained during the summer of 1973, including box turtles, red-eared turtles, water snakes, and bull frogs. Additional HI reactors were found to WEE and EEE and also to St. Louis encephalitis (SLE). Neutralization tests (NT) of the SLE HI positive sera did not confirm the presence of SLE antibody and suggest the HI-positives may represent infection with another group B arbovirus, possibly Cowbone Ridge. NTs with Cowbone Ridge antigen are being performed.

THE PHLEBOTOMINE SANDFLIES OF THE LOS YUNGAS REGION OF BOLIVIA

Jorge E. Velasco and Harold Trapido

Introduction

There are no published studies on the phlebotomine sandflies of Bolivia. This investigation was undertaken to provide information on the species composition, ecology, distribution, and disease relationships of phlebotomines in this country.

The region of Los Yungas, in the Department of La Paz, was chosen for the present study because it was known to have favorable climatological conditions for phlebotomines and because the presence of leishmaniasis in the area indicated that information on the sandflies, which are the only known transmitters of this disease, would be of epidemiological value. Adding to the attractiveness of the region as a study area was the fact that it occupies the Andean foothills between the low plains of the Amazonian Basin and the high peaks of the Andes, providing a diversity of natural life zones from 400 to 4600 meters in altitude.

The region in which the study was made includes the provinces of Nor Yungas, Sud Yungas, and portions of Murillo and Larecaja in the Department of La Paz. This primarily subtropical region occupies the eastern slope and foothills of the Andes mountains. It lies between the tropical Amazonian lowlands and the high, cool, temperate region of the Bolivian Altiplano which has an average altitude of 3700 m above sea level.

The region of Los Yungas encompasses the drainages of two adjacent river systems. One, the Coroico, joins the Tipuani to become the Kaka, which flows into the Beni. The other, the Unduavi, joins the Taquesi to become the Tamampaya which, after receiving the waters of the Solacama, enters the Bopi; together with the Santa Elena, they form the Beni. These rivers originate in the highland province of Murillo, enter the provinces of Nor Yungas and Sud Yungas, and pass into the lowland areas of the province of Larecaja. The mountain range which divides the river systems originates in the "Cordillera Oriental" and gradually decreases in altitude to the northeast.

The Los Yungas region has a rugged topography with precipitous mountains dissected by torrential rivers in the highlands and subtropical regions. In the lower, more tropical areas, the valleys are wider, the mountain slopes less steep, and the rivers less rapid.

Long term temperature and rainfall data are available from the Servicio Nacional de Meteorologia e Hidrologia of Bolivia for two representative localities, Chulumani (1800 m) and Caranavi (600 m). At Chulumani, the mean annual temperature is 20°C with a mean minimum of 18°C in July and a mean maximum of 22°C in the summer months, October through January. At the lower elevation of Caranavi it is warmer, with a mean annual temperature of 24.6°C and a mean minimum of 21°C in July and a mean maximum of 26°C from October through February. The mean annual rainfall at Chulumani is 1235 mm and at Caranavi 1661 mm. Variation in monthly rainfall is very pronounced at both localities. During the warmer months, December through February, rainfall is high, of the order of 160 to 230 mm at Chulumani and 230 to 280 mm at Caranavi. The rainfall gradually declines after February, to only about 20 mm in July at both localities, after which it gradually increases during the remainder of the year.

The distribution of leishmaniasis in Bolivia includes the departments of Beni and Pando, where it is very prevalent; La Paz, especially the provinces of Nor and Sud Yungas, and the province of Caupolican; Santa Cruz, provinces of Chiquitos and Nuflo de Chavez; Cochabamba, province of Chapare; and Chuquisaca, province of Acero.

Data on the incidence of cutaneous leishmaniasis are not readily available because cases rarely are reported to public health officials and usually run their natural course.

Persons with mucocutaneous involvement may have yaws or tertiary syphilis rather than leishmaniasis. On biopsy, some cases in the Yungas have been shown to represent concomitant infections of both Leishmania sp. and Treponema pertenue (Dr. R. Mercado, personal communication, 1971).

The region of highest prevalence of mucocutaneous leishmaniasis appears to be around Yolosa (1200 m) and Suapi (1400 m). All of the cases in these localities are Negroes. Negroes comprise about 30 percent of the population of Yolosa and Suapi and are seldom seen in other parts of Los Yungas.

Information provided by local health officials and health personnel may be summarized as follows: Yolosa, six cases confirmed by biopsy (Dr. Luis Valverde-Chinel); Suapi, three clinical cases (Elena Escobar, Sanitary Post); Irupana,

two clinically diagnosed of 194 examined (Dr. Ramiro Maldonado-Cuenca); Chulumani, one new case diagnosed of about 200 patients seen per month (Dr. Rene Villareal).

Materials and Methods

Collection attempts were made in all microhabitats thought suitable for phlebotomines. These included tree buttresses, tree holes, under loose tree bark, between exposed roots, crevices in the ground, under fallen trees, under fallen leaves, animal burrows, domestic animal shelters, inside and outside houses, masonry, caves, mine shafts, and any other sheltered place which might afford some protection from wind and other unfavorable atmospheric conditions.

In the highlands, where there is little vegetation, the available hiding places most encountered were crevices in the ground, houses, masonry, caves, mine shafts, animal burrows, and domestic animal shelters. At the middle altitudes the same habitats were available as well as small tree buttresses and tree holes. In the lowlands, which are sparsely inhabited, the most frequent candidate resting places were tree holes and buttresses, under loose bark, and under exposed roots. In this region there were fewer crevices in the ground and fewer houses than in the highlands. Animal shelters usually proved to be too open and well ventilated to afford favorable resting sites.

Resting phlebotomines were activated by blowing cigarette smoke into presumed hiding places. Occasionally insect repellent spray proved to be more useful. At times a piece of cloth was placed in front of the opening of the presumed microhabitat to provide a convenient place for the phlebotomines to alight. Also, human bait collections were made during the evening and night.

Sandflies were collected by aspiration into a collecting tube and killed with chloroform. They were transferred into cardboard pill boxes lined with soft tissue paper and protected from attack by insects and mold by the addition of a pea-sized grain of paradichlorobenzene. Each pillbox was labeled with a field number; and, on a correspondingly numbered field sheet, data were entered recording temperature, relative humidity, altitude, date, time, location of the collection site, topography, vegetation, approximate height above the floor of the valley, proximity to human habitations, height above ground at which the specimen was collected, and whether or not the area had been sprayed with insecticides.

Two minature CDC light traps powered by 6-volt motorcycle batteries were used to collect night-flying arthropods attracted to light. These were run throughout the night and examined at daybreak. The detachable nets containing the collections were placed in a plastic bag together with a piece of cotton soaked in chloroform. After all the insects were dead, the collection was placed on a flat surface; the Lutzomyia were picked out and placed in cardboard pillboxes; and the remaining insects were preserved in 70% alcohol for possible study by other specialists.

Results

Species collected. During the months of June, July, August, and part of September, 1971, in which the collections of sandflies were made in Los Yungas, a total of 404 specimens was captured. These were of 20 species representing three of the four genera of Phlebotominae known in the New World. They are listed in Table 1. Eleven are known species while the remaining nine are either new or of uncertain taxonomic status.

Of the known species, Lutzomyia dendrophila, L. auraensis, and L. nevesi have been described from the male only. It is believed that their female counterparts have been found in the present study.

Of the nine remaining species, two are clearly new and are being described as L. boliviana n. sp. (Fig. 1) and Warileya yungasi n. sp. (Fig. 2). The others are most probably new but are either of taxonomically difficult groups in which the described species are closely related, or the present material is insufficient to warrant description at this time.

Numbers of specimens by species and sex. Table 2 lists the number of specimens of each sex by species. Sixty-five percent of the total collection was L. longipalpis while the next most numerous species, L. nevesi, made up only eight percent of the collection. Almost twice as many males (260 or 64%) as females (144 or 36%) were caught. The predominance of males was largely determined by the disproportionate sex ratio of L. longipalpis (203 or 78% males and 59 or 22% females).

Of the species with both sexes represented, L. sp. "E", L. longipalpis, L. punctigeniculata, L. serrana, and L. trinidadensis also showed a substantial predominance of males, while for Brumptomyia brumpti, L. boliviana, L. dendrophila, and L. sallesi the sex ratios were equal or nearly

Fig. 1. Lutzomyia boliviana n. sp. Holotype male and allotype female. (A) male, head; (B) male, antennal segment 4; (C) female, head; (D) female, antennal segment 4; (E) female, cibarium and pharynx; (F) male, genitalia; (G) female, cibarium; (H) male, genital pump and filaments; (I) female, wing; (J) male, wing; (K) female, spermatheca (in phenol before mounting); (L) female, spermathecae (in balsam after mounting). Scale in mm. Figures drawn with a camera lucida except heads and wings which were projected and traced.

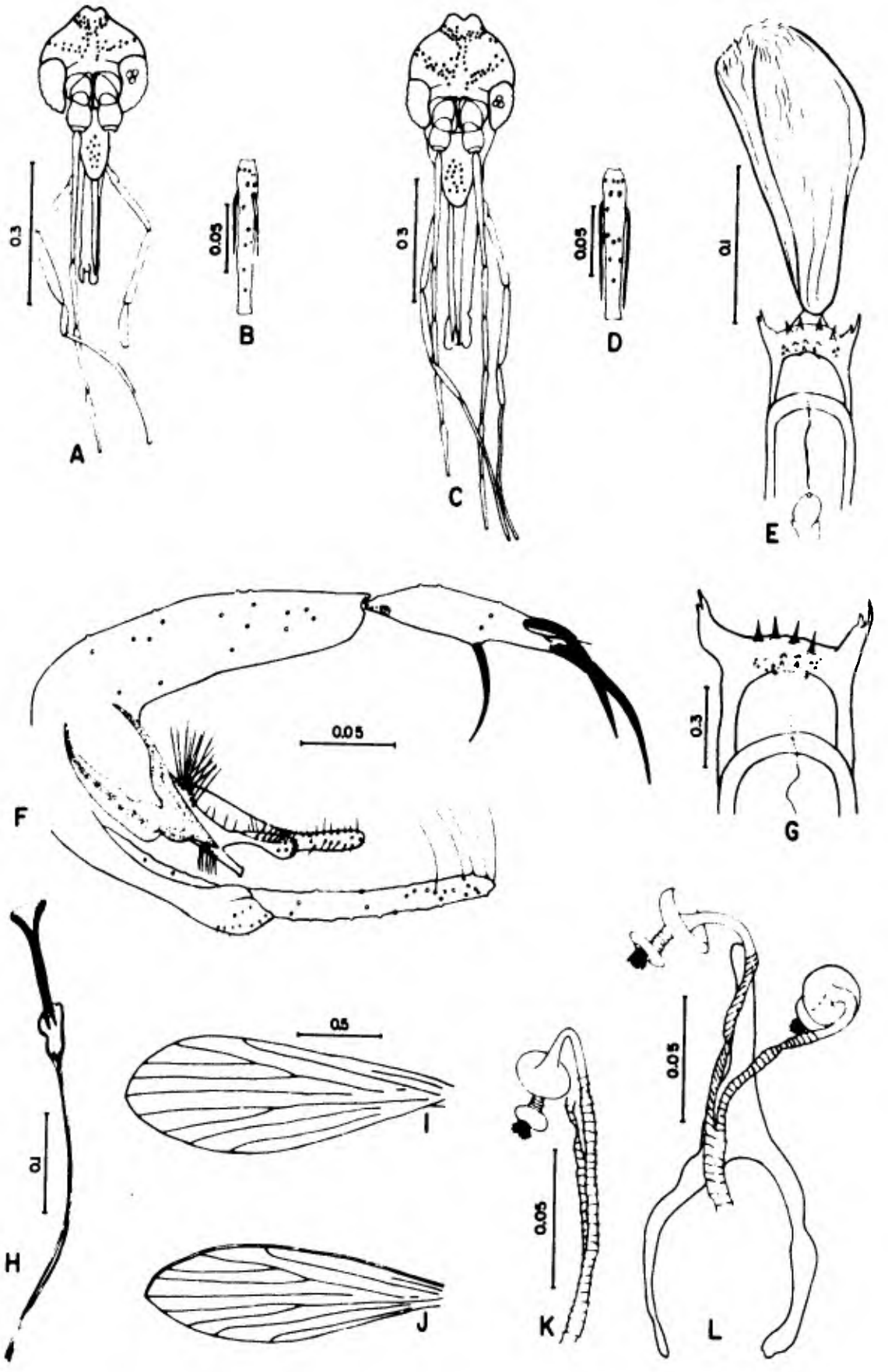

Figure 1

Fig. 2. Warileya yungasi n. sp. Holotype male. (A) head; (B) antennal segment 4; (C) terminal portion of style; (D) wing; (E) detail of tufts of setae on coxa of paratype specimen JVW-054-1; (F) genital pump and filaments with adjacent intra-abdominal rods; (G) genitalia. Scale in mm. Figures drawn with a camera lucida except head and wing which were projected and traced.

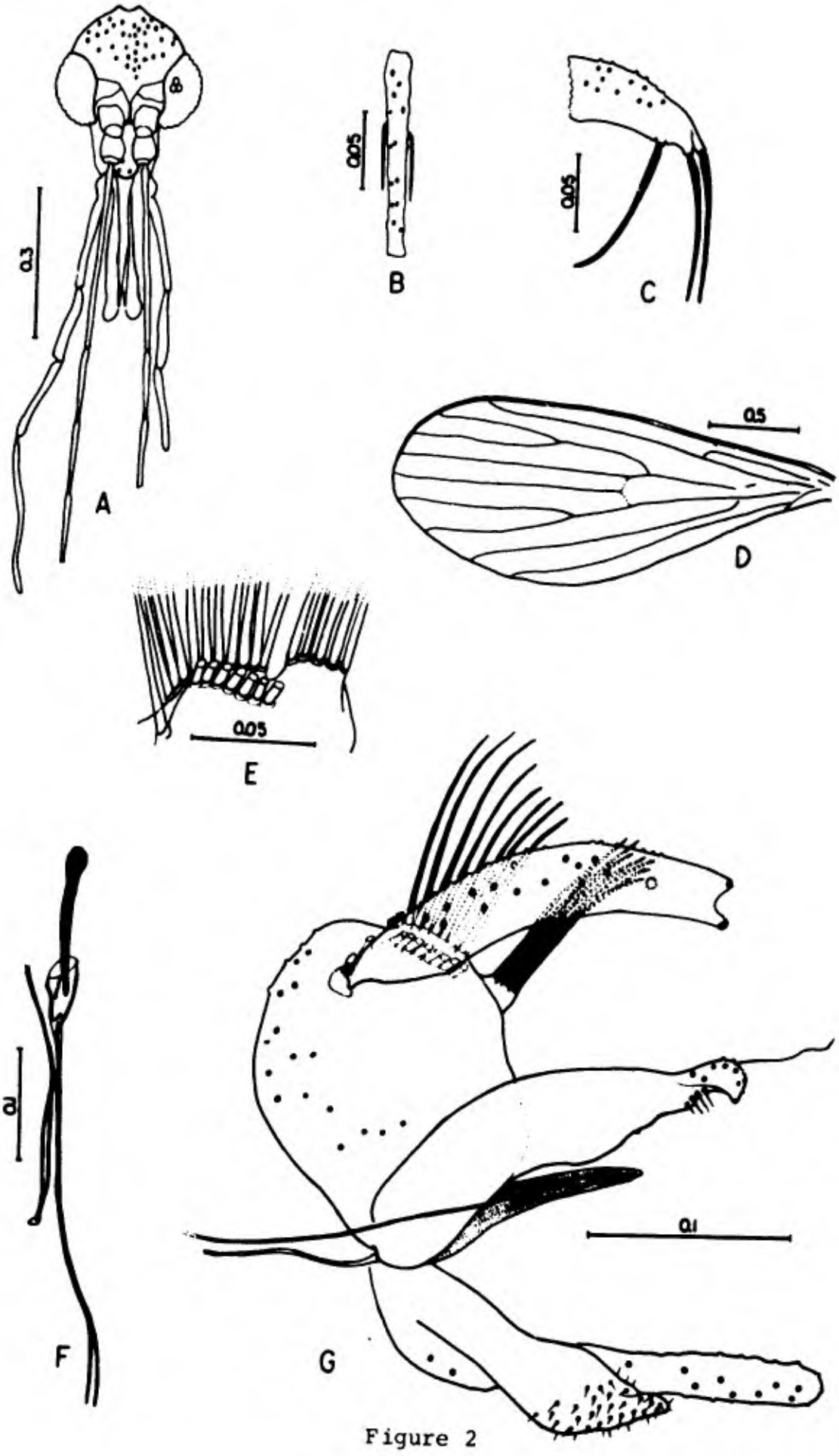

Figure 2

Table 1. List of phlebotomine species collected in Los Yungas

Genus Warileya
Warileya yungasi n. sp. (Male and female)

Genus Brumptomyia
Brumptomyia brumpti (Male and female)

Genus Lutzomyia

Subgenus Lutzomyia
Lutzomyia longipalpis (Male and female)

Migonei group
L. sallesi (Male and female)

Verrucarum group
L. nevesi (Male and female)
L. serrana (Male and female)
L. sp. "I" (Female; probably a new species)

Vexatrix group
L. sp. "H" (Female; possibly L. imperatrix)

Intermedia group
L. sp. "F" (Male; probably a new species)

Auraensis group
L. auraensis (Male)
L. sp. "G" (Male; near L. auraensis: probably a new species
L. auraensis or L. sp. "G" (Female; conspecific with L. auraensis or L. sp. "G")

Subgenus Psychodopygus
L. sp. "C" (Female; probably a new species)

Shannoni group
L. dendrophila (Male and tentatively associated female, as female previously undescribed)
L. punctigeniculata (Male and female)
L. shannoni (Male)

Subgenus Pressatia
L. sp. "D" (Male; probably a new species)
L. sp. "E" (Male and female tentatively associated as this species is probably new)

Castanheirai group
L. campbelli (Male)

Ungrouped species
L. boliviana n. sp. (Male and female)
L. trinidadensis (Male and female)

Table 2. Numbers of males and females of each species

Species	Total	Males	Females
Brumptomyia brumpti	19	10	9
Lutzomyia auraensis	2	2	-
L. sp. "G"	4	4	-
L. auraensis or L. sp. "G"	5	-	5
L. boliviana n. sp.	2	1	1
L. campbelli	1	1	-
L. dendrophila	4	2	2
L. sp. "C"	1	-	1
L. sp. "D"	1	1	-
L. sp. "E"	10	9	1
L. sp. "F"	1	1	-
L. sp. "H"	16	-	16
L. sp. "I"	13	-	13
L. longipalpis	262	203	59
L. nevesi	32	1	31
L. punctigeniculata	7	6	1
L. sallesi	5	2	3
L. serrana	5	4	1
L. shannoni	3	3	-
L. trinidadensis	8	7	1
Warileya yungasi n. sp.	3	3	-
TOTAL	404	260	144

Table 3. Distribution of phlebotomine species by habitat or collection method

Habitat or collection method	Species found	Total no. of specimens
Chicken coop	Lutzomyia longipalpis (215), L. sallesi (1), L. shannoni (3), Brumptomyia brumpti (1),	220
Sheep pen	L. longipalpis (15), L. sp. "I" (2), L. dendrophilia (1), L. trinidadensis (1), L. punctigeniculata (4)	23
Pig pen	L. longipalpis (10), L. sp. "I" (6), L. sallesi (2)	13
Human bait	L. longipalpis (12), L. sp. "I" (6), L. sp. "H" (15), L. nevesi (31)	64
Tree holes, buttresses, or hollow trees	L. dendrophila (3), L. sp. "I" (1), L. boliviana n. sp. (1), L. punctigeniculata (3), L. trinidadensis (7), L. nevesi (1), L. campbelli (1), L. serrana (5), L. sp. "C" (1), L. sp. "D" (1), L. sp. "E" (4)	31
Light trap	L. longipalpis (8), L. sp. "I" (2), L. auraensis (2), B. brumpti (15), L. sp. "H" (1), L. sp. "E" (4), L. sp. "F" (1), L. sp. "G" (4), L. sp. "G" or L. auraensis (5)	42
Masonry	L. longipalpis (1), L. sp. "I" (1), B. brumpti (3), L. sallesi (2)	7
Mine shaft	Warileya yungasi n. sp. (3)	3
Thatched roof	L. longipalpis (1)	1
	TOTAL	404

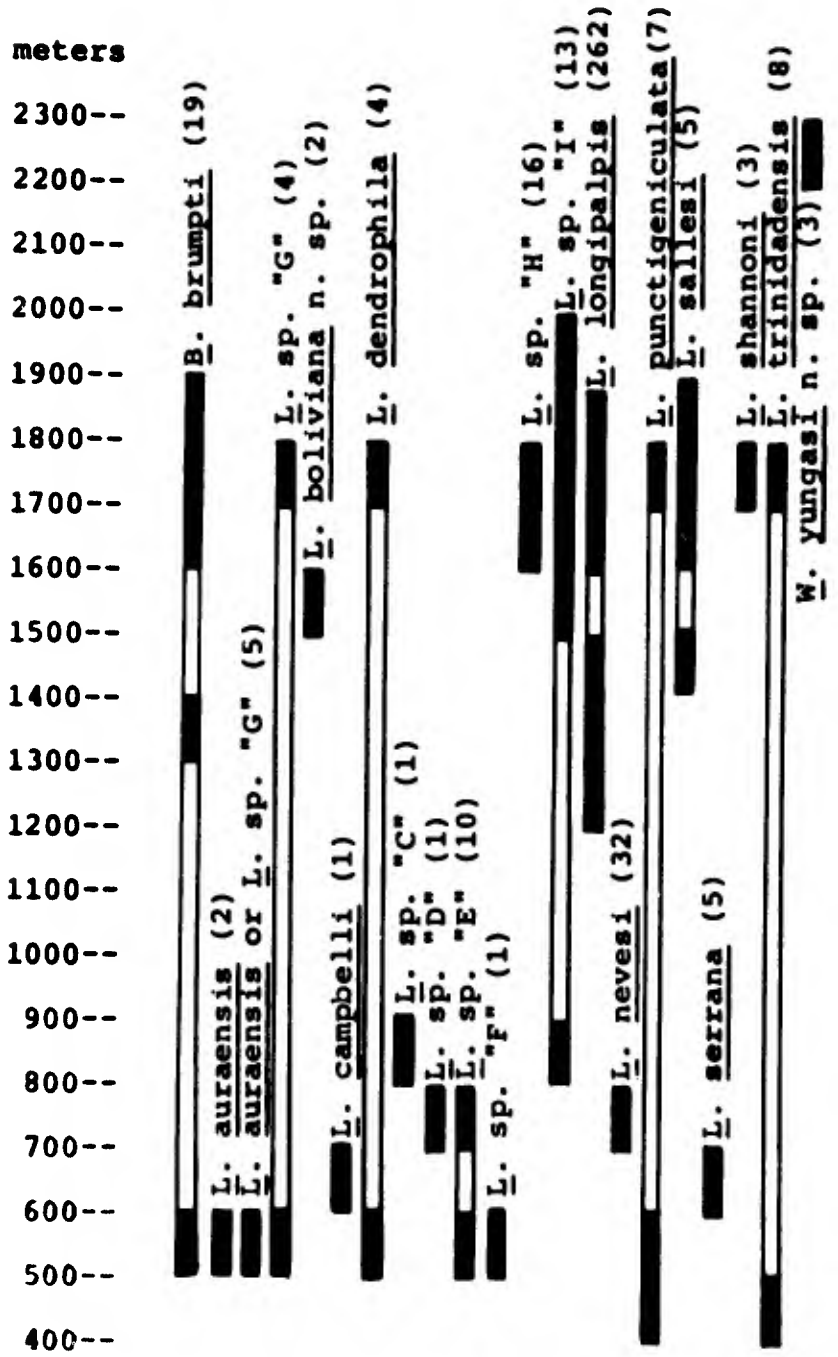

Figure 3. Altitudinal distribution by species of phlebotomine sandflies in 100 m increments. The number of specimens is shown in parentheses. Darkened areas indicate altitudes at which specimens were taken.

equal. In the case of L. nevesi there was an overwhelming predominance of females; the one male taken was aspirated from a resting site during the day, while night human-bait collections at the same site yielded the females.

Distribution of species by habitat and collecting method. Data on the number of specimens of each species taken are summarized by habitat or collection method in Table 3.

Chicken coops were the habitat where more than half of the total collection was obtained. Of phlebotomines in chicken coops, about 98% were Lutzomyia longipalpis.

Sheep pens provided five species of which about 65% were Lutzomyia longipalpis.

Pig pens did not yield many sandflies; yet of those present, about 77% were Lutzomyia longipalpis.

Human bait collections were mostly made at sites previously known to be positive for phlebotomines during daytime collections. Only one of the four positive collections was from an open field; the other three were made inside either a chicken coop, a sheep pen, or a hollow tree.

Tree holes and tree buttresses provided a variety of species. More than half of the 20 species collected were found in these habitats, although in no case was the number of specimens of any one species greater than seven.

Light trap collections produced eight species. The number of sandflies of each species, however, was relatively low.

Masonry, a mine shaft, and a thatched roof constituted the remaining collection sites. The combined numbers of sandflies in these three habitats amounted to less that 3% of the total number of specimens caught.

Distribution of species by altitude. Sandflies were collected at altitudes ranging from 400 to 2300 m above sea level. The altitudinal distribution of the species captured is shown by 100 meter increments in Figure 3.

Some of the species, notably Brumptomyia brumpti, L. sp. "G", L. dendrophila, L. punctigeniculata, and L. trinidadensis, have a very great altitudinal range, which in some cases is as much as 1400 m. While L. shannoni is shown only at the 1700 to 1800 m altitude, if the females tentatively attributed to L. dendrophila later prove to be L. shannoni,

this species would have as extensive an altitudinal range as L. dendrophila. In all these cases, the numbers collected were small, and often a single specimen considerably extended the altitudinal range of a particular species.

Lutzomyia longipalpis, which was by far the most common species, was restricted to the subtropical zone between 1200 m and 1900 m. Lutzomyia sallesi, despite the small numbers taken, had an altitudinal range of 500 m.

ARTIFICIAL KEY TO THE GENERA AND SPECIES OF THE PHLEBOTOMINE SANDFLIES OF THE REGION OF LOS YUNGAS, BOLIVIA

In the original manuscript, males and females of all species are figured in the form included here to illustrate the new species Lutzomyia boliviana and Warileya yungasi (see Figures 1 and 2). In the present condensed version the following key will serve to identify the species encountered.

Males

1. Wings broad and rounded at tip, R2,3 and 4 forking slightly before the R-M crossvein; pleura without setae; coxa rounded and shorter than style; palp 3 shorter than palp 4 .... Warileya yungasi n. sp. (Plate 7)

   Wings somewhat narrow, pointed at tip, R2,3 and 4 usually forking some distance beyond R-M crossvein; pleura with setae; coxa elongate and longer than style; palp 3 longer than palp 4 2

2(1). Interocular suture complete; style with 5 large spines with one pair arising distally and one pair arising from a common tubercle; coxa with a basal tuft of more than 10 short, bladelike setae, distal setae, long, 4 to 6 in number, arising in a straight line; genital filaments long, coiled .... Brumptomyia brumpti (Plate 8)

   Interocular suture incomplete; style with 3 to 6 spines but never in combination as above; coxa with basal tuft, if present, having less than 10 stout setae which are long and somewhat curved, distal setae, if present, variable in number, usually irregularly aligned; genital filaments of varied lengths, never coiled .....(Lutzomyia)... 3

3(2). Coxa with basal tuft of setae; style with a subterminal seta, with 3 or 4 spines; ascoids simple 4

   Coxa without basal tuft of setae; style with or without a subterminal seta, with 3 to 5 spines; ascoids simple or with spurs 8

4(3). Coxa with a basal tuft of more than 10 fine, long setae and less than 10 thicker and longer setae borne on a separate tubercle; style with 3 spines; parameres distally with an elongate, rounded dorsal projection and a pointed ventral projection; lateral lobes indented distally; genital pump to filament ratio less than 3 5

Coxa with a basal tuft of about 4 stout, long setae; style with 3 or 4 spines; parameres not as above; lateral lobes not indented distally; genital pump to filament ratio usually greater than 3 6

5(4). Basal tuft of coxa with fine setae borne on triangular shaped tubercle, stout setae arising beneath, long and separated; paramere with distal setae short and straight .....L. sp. "E" (Plate 24)

Basal tuft of coxa with fine setae borne on a knob-like tubercle; stout setae arising beneath, bladelike, clustered together; paramere with distal setae long and curled .....L. sp. "D" (Plate 23)

6(4). Parameres not truncate, without pointed projections and without ordered row of setae on the proximal ventral margin; head rounded 7

Parameres truncate, with 2 small pointed projections and with a straight row of about 9 setae on the proximal ventral margin; head somewhat elongate .... L. sallesi (Plate 10)

7(6). Style with 4 spines; paramere with 2 stout, recurved dorsal setae; lateral lobe long and rounded; length of palp 5=2 x palp 4, < palp 1+2, < palp 3+4 ...L. longipalpis (Plate 9)

Style with 3 spines; paramere without stout, recurved dorsal setae; lateral lobe short and somewhat pointed; length of palp 5=3 x palp 4, > palp 1+2, > palp 3+4 .... L. serrana (Plate 12)

8(3). Paramere with a medial branch having a ventral projection; style with 3 spines and a subterminal seta; head elongated, eyes very small .....L. boliviana n. sp. (Plate 26)

Paramere without a medial branch; style with 4 or 5 spines, with or without a subterminal seta; head rounded; eyes large 9

9(8). Paramere with a basal tuft of curved setae, borne on a tubercle, distally with a pointed recurved projection; tips of genital filaments with teeth .....L. campbelli (Plate 25)

Paramere without a basal tuft of hairs, lacking pointed projection; tips of genital filaments without teeth 10

10(9). Style with 5 spines; ascoids simple; palp 3 usually longer than palp 1+2 .... L. trinidadensis (Plate 27)

Style with 4 spines; ascoids simple or complex; palp 3 usually shorter than palp 1+2 11

11(10). Paramere distally with a sharply delimited patch of setae; genital filaments at least 6 x length of pump; coxa with many nondeciduous setae; palp 3+4 < 5; δ < .30 mm 12

Paramere with setae not as above; genital filaments usually less than 5 x length of pump; coxa with few, if any, nondeciduous setae; palp 3+4 usually < palp 5; δ < .30 mm ... 13

12(11). Paramere with a distal setose, rounded, cap-like structure; coxa with scattered nondeciduous setae ..... L. auraensis (Plate 16)

Paramere with a distal, concave, pointed, setose, cap-like structure; coxa with a patch of nondeciduous setae..L. sp. "G" (Plate 17)

13(11). Paramere pointed apically; style with a subterminal seta; ascoid simple, extending 1/2 length of antennal segment.L. nevesi (Plate 11)

Paramere rounded apically; style without a subterminal seta; ascoids, if simple, extending almost to distal end of antennal segment 14

14(13). Lateral lobe equal or only slightly exceeding paramere; ascoids simple; genital filaments less than 3 x length of pump .... L. sp. "F" (Plate 15)

Lateral lobe extending some distance beyond paramere; ascoids with posterior spurs; genital filaments more than 3 x length of pump.. 15

15(14). Paramere abruplty constricted distally; setae stout and short, recurved apically .... L. dendrophila (Plate 20)

Paramere wide proximally, tapering gradually distally; setae thin, straight 16

16(15). Large species; ascoids with long, pointed posterior spurs; δ > .15 mm..L. shannoni (Plate 22)

Small species; ascoids with characteristic short posterior spurs; δ < .15 mm .... L. punctigeniculata (Plate 21)

Females

1. Interocular suture complete; spermathecae segmented; ducts more than 6 x length of body; cibarium with only horizontal teeth ..... B. brumpti (Plate 8)

   Interocular suture incomplete, spermatheca segmented or not; if segmented, ducts less than 6 x length of head; cibarium with horizontal and vertical teeth ...(Lutzomyia)..... 2

2(1). Spermathecae and ducts not segmented; ascoids simple or complex 3

   Spermathecae or ducts segmented; ascoids simple 7

3(2). Spermathecae cylindrical; common duct wrinkled, diameter irregular..L. sp. "E" (Plate 24)

   Spermathecae round or sausage-shaped; common duct smooth, diameter uniform 4

4(3). Spermathecae round with narrow protruding terminal knob; ducts more than 5 x length of spermatheca; head, elongate; eyes small ..... L. sallesi (Plate 10)

   Spermathecae sausage-shaped with a short terminal knob; ducts less than 5 x length of spermathecae; head rounded; eyes large 5

5(4). Ducts more than 1/3 width of spermathecae; cibarium with horizontal teeth parallel; pharynx unarmed; ascoids reaching tip of antennal segment and having posterior spurs; palp 3 < 1+2, palp 3+4 > 5 6

   Ducts about 1/4 width of spermathecae; cibarium with horizontal teeth directed medially; pharynx armed; ascoids simple, not reaching the tip of antennal segment; palp 3 > 1+2, palp 3+4 < 5 .... L. trinidadensis (Plate 27)

6(5). Cibarium with about 8 short, vertical teeth; ascoids with a characteristic short posterior spur; spermathecae with angular terminal knob; $\gamma < \delta$, $\zeta > \beta$ .... L. punctigeniculata (Plate 21)

Cibarium with about 4 long, vertical teeth; ascoids with posterior spurs reaching to the proximal end of the antennal segment; spermathecae with rounded terminal knob; $\gamma > \delta$, $\delta > \beta$ .... L. dendrophila or L. shannoni (Plate 20)

7(2). Spermatheca imbricated; individual ducts not apparent; palp 5 short almost length of palp 4; $\beta > \gamma$ ..... L. sp. "C" (Plate 19)

Spermathecae not imbricated, individual ducts evident; palp 5 usually more than 2 x length of palp 4; $\beta < \gamma$ 8

8(7). Spermathecae with large rounded proximal portion and smaller rounded distal portion joined by segmented constriction; head elongate; eyes small .... L. boliviana n. sp. (Plate 26)

Spermathecae not as above; head rounded; eyes large 9

9(8). Spermathecae at least 3 x width, segmented except for the distal 1/4; palp 3+4 > palp 5 ...10

Spermathecae of various dimensions, completely segmented; palp 3+4 < palp 5 11

10(9). Spermathecal ducts readily visible, not segmented; cibarium with 4 horizontal teeth; palp 3 < palp 5; $\delta < \beta$. L. sp. "H" (Plate 14)

Spermathecal ducts, hardly visible, segmented; cibarium with about 11 horizontal teeth; palp 3 > palp 5; $\delta > \beta$ .... L. auraensis or L. sp. "G" (Plate 18)

11(9). Cibarium with about 11 horizontal teeth; spermatheca cylindrical; ducts 5 x length of spermathecae ..... L. longipalpis (Plate 9)

Cibarium with about 4 vertical teeth; spermathecae gradually tapering into ducts which are not more than 2 x length of spermathecae 12

12(11). Spermatheca with terminal knob rounded and lobulate; boundary between spermathecae and ducts not well defined... L. nevesi (Plate 11)

Spermathecae having cylindrical terminal knobs without lobules; boundary between spermathecae and ducts well defined 13

13(12). Spermathecae carrot-shaped, with elongate terminal knobs bearing long bristles; the most distal segment of spermatheca head as wide as rest of segments .... L. serrana (Plate 12)

Spermathecae usually cylindrical, tapering abruptly to form the duct, which has terminal knobs broad, bearing short bristles; the most distal segment of spermathecae conspicuous and wider than the others ..... L. sp. "I" (Plate 13)

BIONOMICS OF BLACK FLIES IN COSTA RICA

Bernard V. Travis and Mario Vargas V.

Summary

The black fly observations and collections made on 100 streams in Costa Rica have been completed. Immature stages, reared adults and biting adults were preserved from each of the streams. The data included (1) monthly observations on 53 streams; (2) bimonthly observations on 47 streams; and (3) bimonthly observations on 28 of the 53 streams. The observations extended over a period of 24 months and provided a base for some critical studies on the relationships between the flies and disease organisms which they may transmit.

Publications

Each publication will have the general title "Bionomics of black flies in Costa Rica," with appropriate subtitles which are listed below.

Travis, B.V., and Vargas V., M. 1972. Bionomics of black flies in Costa Rica. II. An ecological consideration. Proc. 57th Annual Meeting New Jersey Mosquito Extermination Assoc.: 111-112.

Vargas V., M., and Travis, B.V. 1973. Bionomía de los simúlidos (Diptera: Simuliidae) IV. Localización y descripción de los lugares de recolección. Rev. Biol. Trop. 21: 143-175.

Travis, B.V., Vargas, M., and Swartzwelder, J.C. 1974. Bionomics of black flies (Diptera: Simuliidae) in Costa Rica. I. Species biting man, with an epidemiological summary for the Western Hemisphere. Rev. Biol. Trop. 22: 187-200.

List of proposed publications

All with the major title as shown above. All except taxonomic papers are in final state of preparation.

I. Biting species and epidemiology
III. Weekly population fluctuations
V. Techniques for making black fly surveys
VI. Ecological factors
Other: Taxonomic papers based on subgenera

CLINICAL IMPORTANCE OF PROTHROMBIN TIME DETERMINATION IN SNAKE-VENOM POISONING

A. Peña Chavarría, V. M. Villarejos, and M. Zomer

Summary

A study of the prothrombin time of patients bitten by Bothrops snakes, principally Bothrops atrox, the fer-de-lance, was undertaken for possible use as a guide to the severity of envenomation and the effectiveness of antivenin medication. Twenty-six cases of snake-bite poisoning, in which prothrombin-time determinations (Quick) were made every 8 hours, were studied. The patients represented two groups: 1) 20 who had been bitten by a snake 8 to 10 hours before admission, some of whom had already received one or two ampules of antivenin, and 2) six patients seen within 3 hours of the bite. The patients received antivenin every 6 to 8 hours. In the first group, the prothrombin time was 5 per cent of normal on admission and reached a peak of 80 per cent at about 40 hours. In the second group, the prothrombin time was 20 per cent of normal on admission, dropped within 8 hours to less than 5 per cent despite intensive antivenin therapy, and then followed the pattern of the larger group. Clinical improvement paralleled the return of prothrombin time to normal. In none of the patients studied did gangrene requiring surgical treatment develop.

PATHOPHYSIOLOGY AND TREATMENT OF SEPTIC SHOCK

H. E. Dascomb and J. H. Bellina

Summary

Fifty cases, all females, who had sepsis and shock, were studied. The patients varied from 12 to 77 years; most were under 45 years of age. The younger patients had pelvic infection resulting from pregnancy or lower genital and urinary tract infection. The pathology in five older patients included degenerating fibroids, diabetes and renal disease, chronic lung disease, liver abscesses with ascending cholangitis, and carcinoma of the cervix.

After clinical evaluation of the patients, specimens were collected. These consisted of blood, urine, biopsy of cervix uteri, colpotomy aspirate or pelvic exudate collected during surgery. They were promptly cultured aerobically, anaerobically, and for cell-wall defective bacteria in appropriate cases. Therapy with antibiotics was initiated based on clinical data and results of Gram's stain when available. Antimicrobial revision depended upon clinical response and laboratory findings.

Blood cultures were accomplished using blood samples collected in a syringe containing 100 to 200 mgm of heparin. Portions of this were inoculated into beef-heart infusion broth, agar for poured plates, and hypertonic media for *Mycoplasma*. Two poured plates of each sample were incubated anerobically using a BBL Gaspak anaerobic jar. Organisms recovered were identified morphologically, biochemically, and occasionally serologically. Coagulase test of *Staphylococcus aureus* was done using human plasma and emulsion of colonial growth on a glass slide. Aerobic enteric organisms predominated, but anaerobes such as *Bacteroides* and streptococci were found frequently.

Therapy consisted of central venous pressure monitoring, restoring blood volume, vasodilating, selecting antibiotics according to the organism suspected by clinical findings and direct smears of exudate, promptly and aggressively draining or excising infected areas, vena caval ligation in suspected pulmonary embolization, and revising subsequent antibiotic therapy depending on clinical course and laboratory findings.

Details of the methodologies of this study were presented in Annual Report No. 1 (1968).

Five of the 50 patients (10%) died. This is in marked contrast to the rate of 40 per cent in such cases at Charity Hospital during 1959-1963. The remarkably low mortality in this series (10%), in part, reflects prompt aggressive therapy directed first against the shock, then for the infection. The former was accomplished by employing vasodilators and blood volume expansion to tolerance as determined by central venous pressure monitoring. For the latter, specific intensive antibiotic administration and, above all, radical debridement and drainage were employed. Furthermore, the complicating embolization from pelvic veins was minimized by appropriate vena cava ligation and anticoagulants. Microvascular thromboses were demonstrated in four cases in which patients died, suggesting that intravascular coagulation of blood may be one of the most important causes or irreversible shock.

Also, most of the patients were young and had minimal degenerative disease which, no doubt, accounts greatly for the low mortality of the series. We believe that continued study in such patients may define more clearly the pathogenesis of bacteremic shock than in older persons.

During the 2 years this study progressed, it was possible to anticipate to some extent those patients who may develop severe bacteremia. For this reason many cases with premature rupture of membranes, criminal abortions, pelvic inflammations, and patients with bacteriuria were studied bacteriologically and treated intensively prior to onset of high fever, manipulation, or surgical procedures. As a result, progressively fewer cases developed bacteremia and hypotension and were thus unavailable for study. But many patients at risk from a highly mortal disease were spared. We feel that prevention is, as in other infections, not only desirable but possible. The experience gained in this study is applicable not only to obstetrics and gynecology, but to the other clinical disciplines.

EVALUATION OF THE SKIN TEST FOR HOOKWORM AS AN EPIDEMIOLOGICAL TOOL

V. M. Villarejos, J. C. Swartzwelder,
J. A. Arguedas, G. Vargas, and
A. Peña Chavarria

Summary

Efforts toward eradication of hookworm infection in the tropical and subtropical parts of the world through sanitary education and improvement of environmental conditions have had only modest success. With the advent of new broad spectrum anthelmintics, the idea of a therapeutic shortcut to accelerate the process of control of helmintic infections received renewed attention. The fact that mass treatment may reduce the prevalence of hookworm to negligible levels, sustained over periods of many years, was shown recently by Swartzwelder et al (1). Stoll (2) and others have proposed also that gradual control of hookworm in an endemic population could be achieved through treatment of only the severe infections present in the community, a procedure which should gradually diminish soil infestation.

In order to define the populations to be treated in mass deparasitization programs, extensive epidemiological studies are needed. The standard coprologic techniques used in those studies for detecting helminthic infections are often cumbersome, time consuming, and demand specialized personnel and equipment, not always available where intestinal parasitism, specially hookworm infection, constitutes a major public health problem. Furthermore, the parasitological examination of a few localities may not reflect the true prevalence of parasites for the whole area, which has to be considered when planning comprehensive deparasitization campaigns. Thus, the search for a rapid screening method of large populations for assessment of prevalence and degree of helminthic infection, particularly with hookworm, seems well justified.

Skin testing with helminth extracts was proposed first by Brunner in 1928 as an alternate to stool examination for the diagnosis of intestinal parasitosis (3). Since then, special attention has been directed to the investigation of skin hypersensitivity responses to hookworm

antigens (4,5,6), with often conflicting results. Recently two groups of workers (7,8) using third stage larval hookworm antigens for intradermal tests both achieved similar high percentages of sensitivity and specificity, showing a positive correlation between hookworm burden and skin reactivity. Therefore these authors, as most others reporting on the subject, recommend the use of the skin test procedure as a rapid screening technique for detection of hookworm infection.

The usefulness of a skin test with larval N. americanus antigen for assessment of hookworm prevalence was evaluated in an endemic area of Costa Rica. In comparison with standard coprologic techniques employed to survey the population, the skin test detected 83 per cent of infections, showing a fairly satisfactory sensitivity. The overall specificity of the test was 50 per cent, i.e. random. No correlation was found between skin reactivity and hookworm burden. The sensitivity of the test increased moderately with age, but its specificity decreased significantly at the same time. False-positive reactions were more numerous among persons formerly infected with hookworm who had been negative for as long as 5 years. There was an indication of cross reactivity with intestinal nematodes other than hookworm.

The test was used to detect hookworm infected persons in the community for selective treatment, in comparison with mass treatment of all the people in another village. The selective administration of an anthelminthic drug to skin test positive persons only did not achieve the same high cure rate of hookworm as the indiscriminate treatment of the whole population.

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Manuscript in press: Am. J. Trop. Med. & Hyg.

ENTEROVIRUSES IN RURAL FAMILIES AND THEIR DOMESTIC ANIMALS

N. Grew, R. S. Gohd, J. Arguedas, and J. I. Kato

Summary

A study of viruses in animals and humans was conducted to define the occurrence, extent and significance of human respiratory virus and enterovirus infections in domestic animals. A rural area in Costa Rica was the site of this study on zoonoses. Nine families were chosen by random sampling. The family members totaled 78 and their domestic animals numbered 77. Of the 56 family members sampled, 13 had signs of severe conjunctivitis, eight had signs of respiratory disease, and four had diarrhea.

The prevalence of human enteroviruses in rural families and their domestic animals was studied in 9 households, 56 humans and 46 animals were sampled. Twenty-five virus isolates were recovered from human stools, 30 from animal stools, and 3 from animal nasal washings. Of the 25 isolates from humans, 12 were identified as coxsackievirus A-20, 3 as poliovirus type 1, 2 as adenovirus type 1, 1 as adenovirus type 2 and 1 as coxsackievirus A-17. Of the 33 isolates from animals, those identified included 3 coxsackievirus A-20, 21 poliovirus type 1 and 1 coxsackievirus A-9. Six of the isolates from humans and 8 from the animals remain to be identified. Both poliovirus and type A-20 coxsackievirus were reisolated from animals during 25- to 67-day periods. By means of cross-neutralization tests, the type 1 poliovirus isolates were shown to be antigenically similar to each other and to a human vaccine strain (LSC 2 ab). Coxsackievirus A-20 isolates from a child and a dog were antigenically similar to each other but varied from the prototype strain (I.H.-35). Almost all the animals tested had neutralizing substances against type 1 poliovirus and 4 had significant rises in titer. Except for one calf, none of the animals or humans tested had coxsackie A-20 neutralizing substances in their sera. In view of their importance, identification of the pertinent isolates was double-checked by a reference laboratory.

FEASIBILITY STUDIES

I. Systemic and Dermal Mycoses

Preliminary exploration of this subject indicated that a project on these infections was not feasible with the existing budget.

II. Filariasis Vectors

Preliminary study by Dr. R. Zeledón and Dr. A. Peña Chavarría showed that a study of filariasis vectors in Limón, Costa Rica would require a broader epidemiological project. This could not be supported with funding available due to the overall phasing out of Project Themis.

III. Leishmaniasis Epidemiology

Preliminary work was initiated and a decision was reached to attempt to make this a comprehensive and independent study. We submitted a contract proposal to that effect with Dr. Rodrigo Zeledón as the principal investigator. This was funded as DADA 17-73-C-3086 and is now in full operation.