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FACTORS CONTROLLING TROPISMS IN LEISHMANIASIS

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Executive Summary

Classically, Leishmania chagasi, a New World equivalent of Old World L. infantum, causes visceral leishmaniasis (VL). It, atypically, caused a sudden outbreak of cutaneous leismaniasis (CL) in a new suburb of Liberai, in Northern Costa Rica. One case of the many was VL. We aimed to shoe why cases were cutaneous rather than visceral. A computerized database was set up and the population at risk and their livestock were surveyed. Nutritional status of children under the age of 6, those most at risk was examined. It was high and considered the main reason for self-curing CL rather than debilitating death-threatening VL occurring. Holding chickens, then pigs and dogs are main risk factors; they attract the vectors. Sero-diagnosis by an ELISA and a PCR facility were set up in Costa Rica for diagnosis and surveillance. This created an increased and sustainable capacity to monitor CL and VL. We revealed continued presence of the parasite in new foci, a continuing threat. Parasites from cases of CL and VL were characterized, compared and shown to be the same organism, *L. infantum*. It is difficult to determine the impact of the project as the sudden out break had subsided before the project began. No further outbreaks have occurred. The project did instigate greater awareness and interest in this disease by the local medical and public health authorities and the population itself.

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Preface

This preface is intended to give readers of this report a general background to the leishmaniases caused by the parasite named *Leishmania donovani chagasi* (=*L. d. chagasi*), which appears to be the New World equivalent of the Old World parasite *L. donovani infantum* (=*L. d. infantum*) and, probably, the same type of organism or a very close variant of it. Israel is one of the many Old World countries where *L. d. infantum* occurs and where it causes typical human infantile visceral leishmaniasis (VL). Though the parasite and the disease have been well studied in Israel over many years and, more recently, also in the neighbouring Palestinian Authority, no human cases of cutaneous leishmaniasis (CL) without concomitant signs and symptoms of VL have ever been recorded. However, the main reason for undertaking the project being reported here was because all the cases in Costa Rica recorded prior to the onset of the project that were shown to be caused by what was considered to be *L. d. chagasi* were human cases of cutaneous leishmaniasis (CL) without typical signs and symptoms of VL and no human cases of just VL had been encountered. At the time of the onset of the project, there appeared to be recrudescence of VL in Israel (Baneth *et al.*, 1998) and the neighbouring territories (Abdeen *et al.*, 2002). This allowed for a comparison between the two situations, one Old World the other New World. All the VL caused by *L. d. infantum* is zoonotic with infected domestic dogs serving as the medically important reservoir of the disease in man and feral canids, *e. g.*, foxes, jackels and wolves, are the putative natural animal reservoir of the disease in domestic dogs. Domestic dogs with infections of *L. d. infantum* have been found in most Old World foci and domestic dogs with infections of *L. d. chagasi* have been found in some New World foci but this does not include Costa Rica.

All leishmaniases, are vector-borne diseases as well as being zoonoses. Female sand flies of different species of the genus *Phlebotomus* are their vectors in the Old World and those of the genus *Lutzomyia* serve as such in the New World.

Human cutaneous (CL) and visceral (VL) leishmaniases were first reported in the Old World. Two types of VL were described: one occurring chiefly as epidemics in India and, later, Sudan and East Africa that affected mainly young adults and was caused by *Leishmania donovani donovani* (= *L. donovani*); the other distributed much more widely throughout most of the tropics and sub-tropics of, both, the Old and New World, that affected mainly infants and young children and was caused by *L. d. infantum*. *L. d. infantum* has not been recorded in those parts of India where *L. d. donovani* does.

A few years after CL and these two types of VL were described in the Old World, both, CL (Lindenberg, 1909) and VL (Migone, 1913) were also reported in the New World: the former firstly in Brazil; the latter firstly in Paraguay. The leishmanial parasite responsible for that CL was named "*L. brasiliensis*" (= *L. braziliensis*) (Vianna, 1911), suggesting that it originated in and was indigenous to the New World. Since then, a number of different species and subspecies of *Leishmania* have been described to cover the various types of leishmanial parasite isolated from the different types of CL encountered in the New World, all of which do appear to have originated in and are specific to the New World (Lainson and Shaw, 1987; Schnur and Greenblatt, 1995). The leishmanial parasite responsible for VL encountered in the New World was named, though much later, "*L. Chagasi*" (= *L. donovani chagasi*) (Marques da Cunha and Chagas, 1937), which suggested that it also originated in and was indigenous to the New World. However, with regard to the case of VL described by Migone (Migone, 1913) and its cause, the patient, then 47-years old, while having been admitted to and treated in a hospital in Asuncion, Paraguay, had been born in Padua, Italy, and had grown up and travelled widely in Italy as young man. He had immigrated to Brazil when in his thirties, had lived in Santo for many years and then moved to Sao Paulo and had worked on the railway running between Sao Paulo and the city of Corumba, the capital city of Matto Grosso State before displaying patent VL. This case history poses the possibility of this case of VL having been one of Old World origin caused by *L. d. infantum* that had remained asymptomatic for many years and had became patent owing to immuno-suppression acquired much later. This possibility is supported by more recent reports describing what are considered to be

asymptomatic cases with cryptic infections, some of which do become, with time, clinically obvious cases with patent infections.

Human VL is a sporadic disease in Latin America. The technical report of a WHO Expert Committee cites 14 countries where cases had been recorded (WHO, 1990). It inferred that in Bolivia, Brazil, Colombia, Honduras and Panama the causative agent has definitely been shown to be *L. d. chagasi* and in Argentina, Ecuador, El Salvador, Guatamala, Mexico, Nicaragua, Paraguay, Suriname and Venezuela it is the putative causative agent. It also inferred that in Brazil, El Salvador and Venezuela domestic dogs are the proven medically important reservoir host and in Brazil New World foxes, *i. e.*, *Cerdocyon thous* and *Lycalopex vetulus*, have been found infected and accredited with being the feral animal reservoir hosts (Lainson and Shaw, 1987), and a few opossums, *i. e.*, *Didelphis* sp. have also been found infected with *L. d. chagasi*. During the tenure of this project, Costa Rica was added to this list of countries where human VL has been recorded. This was after the first and only autochthonous case of VL from Costa Rica was reported (Carrillo *et al.*, 1999), the cause of which was shown to be *L. d. chagasi* (=*L. d. infantum*).

The classical portrayal of *L. d. donovani* and *L. d. infantum* as the cause of human VL and all the other species of *Leishmania* as the cause of CL was changed somewhat when increasing numbers of cases of CL without concomitant signs and symptoms of VL that were shown to have been caused by *L. d. infantum* were reported from several Old World countries. These included: France (Rioux *et al.*, 1986); Spain (Callego *et al.*, 2001); Italy (Gramiccia *et al.*, 1987); Greece (Frank *et al.*); Azabaijan (Gasan-zade *et al.*, 1990); and Pakistan (Rab *et al.*, 1992). Though, the dermatotropic behaviour of, both, *L. d. infantum* (Benhamou and Foures, 1935; Benhamou *et al.*, 1935) and *L. d. donovani* (Cahill, 1964) had been recorded much earlier. Soon after the relatively recent re-discovery of CL caused by *L. d. infantum* in the Old World, CL, without concomitant signs of VL, caused by *L. d. chagasi* was discovered in the New World, in Brazil (Oliveira Neto *et al.*, 1986), Costa Rica (Zeledon *et al.*, 1989), Honduras (Ponce *et al.*, 1991), and Nicaragua (Belli *et al.*, 1999). In fact, in Costa Rica, the first cases of CL caused by *L. d. chagasi* were discovered before the first and only case of VL caused by it was.

Indian VL is considered to be anthroponotic. No animal reservoir has ever been exposed and the infected human population is assumed to be the source of further

transmission and infection. In Sudan and East Africa, VL is, possibly, zoonotic as parasites identified as *L. d. donovani* (Schnur *et al.*, 1981) have been isolated from a number of different types of wild animal, *e. g.*, *Genetta genetta*, *Felis serval*, *Arvicanthis niloticus*, *Acomys albigena*, and *Rattus rattus*, (Hoogstraal and Heyneman, 1969; Ashford and Bettini, 1987) and also from domestic dogs (Mutinga *et al.*, 1980). All the VL caused by *L. d. infantum* is zoonotic with infected domestic dogs serving as the medically important reservoir and feral canids, *e. g.*, foxes, jackels and wolves, being the putative natural animal reservoir. Central and South American VL is also zoonotic, again, with infected domestic dogs serving as the medically important reservoir and feral canids , *e. g.*, New World foxes, *Cerdocyon thous* and *Lycalopex vetulus*, being the suggested natural reservoir (Lainsonand Shaw, 1987; Schnur and Greenblatt, 1995). However, more recent research and thinking propose that *L. d. chagasi* is, actually, Old World *L. d. infantum* imported into Latin America from the Old World, possibly, on many occasions (Momen *et al.*, 1993; Mauricio *et al.*, 2000). If this is so, then Latin American feral canids, supposedly, got their infections from either imported infected domestic dogs and/or their infected masters. The reverse situation is thought to be the case in the Old World with domestic dogs having acquired their infections from feral canids and humans getting their infections from the infected domestic dogs.

In addition to *L. d. chagasi* (=*L. d. infantum*), two other species of *Leishmania* occur in Costa Rica, both of which are associated with New World CL: *L. brazilensis panamensis* (=*L. panamensis*, the cause of most cases of simple CL in Costa Rica; and *L. brazilensis brazilensis* (=*L. brazilensis*), which also has only been the cause of simple CL in Costa Rica but can cause muco-cutaneous leishmaniasis (MCL), none of which has been recorded in Costa Rica so far. (Peraza *et al.*.). Since most cases of leishmaniasis caused by *L. d. chagasi* in Costa Rica are cases of CL and since *L. b. panamensis*, the cause of most cases of simple CL in Costa Rica, and *L. b. brazilensis* have also been isolated from domestic dogs, albeit, in Brazil (Pirmez *et al.*, 1988; Falqueto *et al.*, 1991; Reithinger and Davies, 1999), one cannot affirm the identity of the causative agents of Costa Rican CL and would be cases of canine leishmaniasis without characterizing and identifying the parasites. No cases of canine leishmaniasis have been reported in Costa Rica. There is no firm evidence for the existence of species of the '*L. mexicana*' complex in Costa Rica (Zeledon *et al.*, 1981).

Purpose of the Project

The origin and reasons for this project are as follows. From 1986 to 1987, there was an outbreak of an "unusual cutaneous disease" in Costa Rica. This occurred in some of the neighbourhoods of the town Liberia, which is in the Province of Guanacaste, a semi-arid region of north-west Costa Rica. About 200 individuals were affected, who were mainly children. This was shown to have been an atypical form of CL. Most of the cases were from Nazareth, a neighbourhood developed in 1978, with a population of 1200 inhabitants, about five percent of who were Nicaraguan immigrants. Scattered cases had been seen since 1976 but these were thought to have been cases of nodular infantile tuberculoid leprosy. The lesions were chronic small round nodules or papules or erythematous plaques, some of which healed spontaneously. Thirty-nine individuals with skin lesions were skin tested with leishmanin, 28(71.8%) of who reacted positively, as did 5 (22.7%) out of 22 control subjects who had no signs or histories of skin lesions. Forty-three individuals were examined, two of who had amastigotes in their stained dermal tissue smears and four of who yielded positive cultures. The promastigotes proved difficult to cultivate in Senekjie's medium. One strain was sent to the Oswaldo Cruz Institute on Rio de Janeiro, Brazil, where it was identified as *L. d. infantum*. Inoculation of either aspirates from patients' lesions or cultured promastigotes into hamsters' noses and feet did not produce lesions. The disease was thought to have been imported by the Nicaraguan immigrants (Zeledon *et al.*, 1989). Published reports of Nicaraguan VL (Duarte *et al.*,) and CL (Belli *et al.*, 1999) only appeared after this supposition was expressed. The relative suddenness of the outbreak described above lends credence to the idea of importation but a subliminal level of infection may always have been present in Costa Rica as portrayed by the scattered cases seen since 1976, which was prior to the outbreak of 1986 to 1987. One of these possibilities does not exclude the other and both may have been in effect in Costa Rica at that time. The fact that Nazareth had been developed not long before the outbreak might have been the reason for the outbreak. In Israel, outbreaks of leishmaniasis appear to follow agricultural and suburban development owing to the attendant turmoil (Jacobson *et al.*, 2003; Schnur, *et al.*). As mentioned, no cases of VL were encountered in Costa Rica at that time and only one infantile case has been recorded since then. Sand flies collected in latrines close to houses with animal bait and CDC traps included *Lu. longipalpis*, which is anthropophilic and has been proven to be the vector of *L. d. chagasi* (=*L. d. infantum*) in Bolivia, Brazil and Colombia (WHO, 1990). *Lu. evansi*, which is also anthropophilic, and *Lu.*

cayennensis and *Lu. chipanensis* that are zoophilic. None of the sandflies carried infections of promastigotes.

Four points mentioned in the above account of Costa Rican CL caused by *L. d. chagasi* (=*L. d. infantum*) caught our interest: the relative suddenness of the outbreak; the quite large number of individuals involved; the fact that the causative agent of this CL was *L. d. chagasi* (=*L. d. infantum*); and, at the time, there had not been and were no known cases of VL in Costa Rica. As stated, the only case reported so far (Carrillo *et al.*, 1999) occurred during the tenure of this project and none have been recorded since then. On comparing this situation with the classical one associated with of *L. d. infantum* in the Old World, as described above, we assumed that the circumstances in Costa Rica were exceptional. Our stated aims at the time of writing our project proposal were: "To study the epidemiology of the human leishmaniases: asymptomatic infections, cutaneous lesions (CL) and visceral disease (VL), caused by *Leishmania infantum* (=*L.chagasi*) in Costa Rica, a region where the parasite has recently been discovered, to discern whether human host, parasitic or sand fly vectorial factors govern the dermatotropic and viscerotropic nature of the clinical spectrum. In doing so, to transfer technical methodology for this to Costa Rican colleagues and train them in its use. The following subsidiary objectives would be undertaken to achieve this.1. The human population would be surveyed for signs and symptoms of leishmaniasis. The nutritional and immunological status of normal, possibly asymptomatic and clinically obvious cases would be determined and correlated with disease manifestations. 2. Leishmanial strains would be isolated from human cases and sand flies and characterized by as many parameters as possible to determine whether parasite variation parallels the different human clinical conditions. 3. To determine, experimentally in laboratory animal models, whether the distinct composition of saliva from different types of sand flies that is injected with the infecting parasites at transmission influences and is related to the clinical and pathological situation seen in human cases." However, as described in the preface, similar situations were also beginning arise in some of the neighbouring Central American and South American countries and, also, many Old World countries where *L. d. infantum* is endemic. This would mean that our findings would be more widely significant. In fact, while doing fieldwork in Costa Rica for the first time in 1997, we travelled to Managua and Leon in Nicaragua and met with Nicaraguan doctors, public health workers and research scientists working on leishmaniasis to try and form a network of people working on this type of leishmaniasis. Since this network could not be covered by this USAID-CDR project, Dr Schnur contacted colleagues at the

WHO concerning funding for this but they, while showing an interest, were unable to assist this.) Since there were no recorded cases of VL in Costa Rica and hence no Costa Rican visceralizing strains and there was, supposedly, the possibility of both in Nicaragua (Duarte *et al.*, 1994 ; Belli *et al.*, 1999) , involvement Nicaraguan researchers would also help facilitate our studied. We also planned to visit Dr Carlos Ponce's group working in Tegucigalpa (Ponce *et al.*, 1991) after the Israeli-Costa Rican fieldwork done in 1998 but this was delayed because of grant 'cash flow problems' and when planned in connection with the Israeli-Costa Rican fieldwork done in 1998 was prevented because of the devastation caused by Hurricane George. Drs Harry Noyes and Michael Chance of the Liverpool School of Tropical Medicine had worked together with Dr Carlos Ponce and his team in Hondurus and had amassed a number of strains of *L. d. chagasi* (=*L. d. infantum*) that had been stored in Liverpool (Noyes *et al.*, 1997) On one occasion on returning to Israel after doing fieldwork in Costa, Dr Schnur visited Dr Noyes and Dr Chance, intending to get some of the those strains, especially visceralizing ones, but by then they had had a cold storage failure and all their Honduran strains of *L. d. chagasi* (=*L. d. infantum*) had been lost.

In retrospect, our stated aims were too ambitious and depended on further and continuing outbreaks of this type of leishmaniasis. This is what was assumed would happen at that time but did not. The proposal was submitted in August, 1994, was accepted after two and a half years and was officially started in January, 1997. Our first visit to and fieldwork done in Costa Rica took place in late Sept.- early Oct.,1997, ten to eleven years after the outbreak in Liberia, Guanacaste, had occurred. No further outbreaks have occurred since then. This meant that there were too few cases of CL and, essentially, no cases of VL to carry out our full commitments regarding studying the human population. The paucity of cases also led to a scarcity of strains for the comparative studies between dermatotropic and viscerotropic strains that were undertaken.

The leishmaniases were first described from the patent cases showing the obvious signs and symptoms of either CL or VL. All the other individuals in the populations were generally considered to be normal, healthy and ununfected. However, with the advent of the leishmanin skin test and, later, serological testing for anti-leishmanial antibodies and the general surveying of populations in places where leishmaniases are endemic, numbers of individuals without current or previous clinical signs and symptoms of leishmaniasis were found to be positive by these tests. They were assumed to be asymptomatic cases that had had

and might still have subliminal infections. Surveying in this way, also showed that there were usually significantly more asymptomatic cases in a populations than patent ones. This raised the questions of why do some individuals remain asymptomatic while others develop patent disease and why do some of those that develop patent disease get CL without patent signs and symptoms of VL and vice versa? This could be owing to differences among various parasite populations within a leishmanial species like *L. d. chagasi* (=*L. d. infantum*) or differences in general health and immuno-competence among various human hosts. Variation in the delivery of the parasites by the sand fly vectors has also been propounded. This whole aspect is more of an academic and medical interest than one that needs consideration in terms of development.

All leishmaniases seem to circulate in nature at a subliminal cryptic level with 'sudden' outbreaks and, in some cases, even large epidemics, *e. g.*, India and Sudan, occurring from time to time. These outbreaks seem to be linked to and follow, quite quickly, after upheavals and the turmoil they cause to the environment, climatic changes, some leading to famine and poor health and immunological resistance, destructive earthquakes, land preparation for agriculture and suburban development and forest clearance for timber, and the building of roads and railways, etc. and, of course, war. By studying leishmaniases in such places and understanding what actually occurs to the existing natural equilibrium that triggers and causes an outbreak, one might be able to develop ways to prevent some of those conditions from occurring or avoid them if and when they do begin to occur in future instances. Once an outbreak has occurred the main problem is to manage it medically by treatment of the cases, if necessary, and by measures to contain the outbreak like spraying against the sand fly vectors and using repellents and bed nets. With large epidemics as seen in India and Sudan this is not easy. In the case of the outbreak at Liberia in Cost Rica, it was essentially over before this project began and did not cause significant and dangerous VL. The CL encountered did cure, pass and leave residual protection to re-infection. However it could have developed into something more threatening to the population of and surrounding Liberia and did deserved attention and surveillance until it was clear that there would be no re-occurrence. Though it looked exceptional at its onset, it did fit into the general pattern seen elsewhere with regard to *L. d. chagasi* (=*L. d. infantum*) in Latin America, once cases of CL caused by *L. d. chagasi* (=*L. d. infantum*) were found to occur in other Central and South American countries and the first case of VL in Costa Rica was reported (refer to preface above).

Methods and Results

I. Field surveys in foci of leishmaniasis caused by *L. d. chagasi* (= *L. d. infantum*)

A computerized database was set up at the Veterinay Department of the National University in Costa Rica at the start of the project, using a computer acquired through the project. This was used extensively in surveying the human population at risk in the region where leishmaniasis caused by *L. d. chagasi* (=*L. d. infantum*) was endemic.

Two main surveys were carried out that included the evaluation of the nutritional status within communities where cases of leishmaniasis had occurred. Evaluation of nutritional status was done in collaboration with Dr Mario Calvo, a dematologist employed at the hospital in Liberia. The first was done in 1997. Data collection cards were prepared for recording epidemiological, anthropometric and clinical information. These were in Spanish and not presented here. A census of the total human population and inventory of its primary domestic animals was carried out in Nazareth, the suburb of the city Liberia in the Province of Guanacasta, northern Costa Rica, where the outbreak of CL caused by *L. d. chagasi* (=*L. d. infantum*) occurred originally. Liberia is 70 km from the Nicaraguan border. A map of the suburb was drawn up, showing the distribution of the residences from which the 70 recorded cases of CL came. Sixty-four of the cases came from among 773 inhabitants belonging to 142 households situated in the northern half of the suburb (OR=19.3; CI=5.5-80.9). Six cases came from among 1,005 inhabitants belonging to 187 households situated in the southern half (Map and Tables 1 and 2). Analysis of the data, indicated that the mean number of households with chickens was greater in the north of the suburb than in its south ($p < 0.05$), with, numerically, more chickens in most northern households ($p < 0.05$) (Table 3). The presence of pigs appeared to be significantly associated with the presence of cases (OR=4.29; CI=1.0-16.9), as was the presence of dogs (OR=2.3; CI=1.0-5.2) (Table 4). Collection of sand flies, actively by aspiration and passively using CDC light traps, showed that the species *Lu. longipalpis*, the proven vector of *L. d. chagasi* (=*L. d. infantum*) in other parts of Latin America, was abundant in three different areas of the northern half of the suburb but was not found in six collecting sites set up in the southern half (Map). The data suggested an association between houses

with 10 or more chickens and the presence of the putative vector. Chicken coops with more than 10 chickens seem to be attractive to *Lu. longipalpis*, apparently, playing a role in the epidemiology of the disease. In three households where chicken coops were removed the sand flies disappeared and in two more the sand fly population fell considerably. Larvae of *Lu. longipalpis* were found in a soil sample collected from under a chicken coop on one occasion,. This was in itself interesting as the breeding sites of *Lu. longipalpis*, like all other species of sand fly, have yet to be definitely determined. We hypothesized that the vector is associated with households that have chicken coops with sufficient numbers of chickens, as was more common in the northern part of the suburb, and that keeping chickens is a risk factor (Figs 1, 2 and Table 5) as are dogs and, supposedly, pigs (Table 2). Other animals, *e.g.*, cows and goats also attracted *Lu. longipalpis* but they were not present in the area in significant numbers (Table 2). Households with six or more persons and persons having lived in the area more than five years were also considered a risk factor, increasing the chances of acquiring the disease (Table 4). The presence of latrines did not appear to be a risk factor (Table 4) even though *Lu. longipalpis* was sometimes found resting in them. The vector population increased considerably after the end of the rainy season. This was clearly shown, later, by two yearlong studies done the year 2000: one in the suburb of Nazareth (Fig. 3); the other in the suburb of Pueblo Nuevo (Fig. 4). A specific residence was selected in each suburb for consistency throught the year. The over-all constitution of several captures of *Lu. longipalpis* in short periods of time during the period 1999-2000 is given in Table 6. Female sand flies were usually far fewer than male sand flies in collections, for *Lu. longipalpis* and the much smaller numbers of some other species of sand fly caught together with *Lu. longipalpis*, *i. e.*, *Lu. cayennensis*, *Lu. chiapanensis* and *Lu. shannoni* (Table 7). None of the female sand flies caught were infected with promastigotes and no isolations of leishmanial parasites could be made. This fitted with the general reduction and disappearance disease in the suburb of Nazareth prior to and during the project and its almost non-existence in the suburb of Pueblo Nuevo, Liberia.

Evaluation of nutritional status was carried out in children under the age of six. This was the age group considered most at risk as cases of VL in Nicaragua tend to be in this age group. 469 homes were visited and 375 children under the age of six were weighed and their heights were measured. Their nutritional status was determined using the weight and height for age parameters, according to the National Center for Health

Statistics reference standard (Table 8A). This evaluation showed that the nutritional status of the children up to six years of age in Nazareth was generally good.

The second similar survey was done in 1998. This time a census of the total human population and inventory of its primary domestic animals was carried out in Pueblo Nuevo, another the suburb of the city Liberia, the one where the first and only case of VL reported in Costa Rica had occurred. 352 homes, encompassing 1,496 inhabitants, were visited and A 224 children under the age of six were weighed and measured to determine their nutritional status. Again, weight and height for age parameters and the National Center for Health Statistics reference standard were used for assessment (Table 8B). This evaluation showed that the nutritional status of the children up to six years of age in Pueblo Nuevo was also generally good.

It is thought that the general good nutritional status of the children and other citizens of Costa Rica accounts for the almost absolute non-existence of VL and the occurrence of only self-curing CL when leishmaniasis caused by *L. chagasi* (=*L. infantum*) did occur in Costa Rica. Even the cases of CL were relatively few and had dwindled since the time of the original outbreak. This contrasts with other Latin American countries like neighbouring Nicaragua where nutritional status is generally lower and VL has occurred more frequently (Belli *et al.*, 1999). Having said this, it is pointed out that the first human case of VL seen in Costa Rica was recorded as being a 15-month old eutrophic and immuno-competent child with classical signs of the disease (Carrillo *et al.*, 1999). The strain of *Leishmania* isolated from that case, known as the LVCR or Dixon strain, has been now been fully characterized and is without doubt a strain of *L. d. chagasi* (=*L. d. infantum*) (see Section III).

II. New foci of leishmaniases caused by *L. d. chagasi* (=*L. d. infantum*).

In the absence of new cases of either CL or VL caused by *L. d. chagasi* (=*L. d. infantum*) occurring in the immediate vicinity of Liberia, an active search for cases in areas more distant from the city was begun. Field studies done in 1998 and 1999, during further visits by Drs Schnur and Warburg, exposed new active cases in new foci, indicating that *L. d. chagasi* (=*L. d. infantum*) was still circulating at that time and posing a risk to the human population. While CL is not a life-threatening disease as is

VL, it is a chronic disfiguring disease that can cause substantial distress, and there is always the risk of its progressing to become fulminating VL under failing immunity.

In 1998, one definite human case of CL was diagnosed at El Salto, which is near but not part of Liberia. This was an 18-year old woman with a large lesion on her left cheek. Numerous amastigotes were seen in ther stained smears of her dermal tissue aspirate. Usually the parasites are very scanty and only detected by culture. We failed to isolate the strain because her cultures had become contaminated. Since the 18-year old woman was positive by her smears, we could have returned for a second attempt. However, she had been greatly bothered by her lesion and had even tried to treat the lesion by applying battery acid to it. To alleviate her problem, Dr Zeledon treated her by injecting Glucantime into the lesion. A second suspect case was seen at El Salto. This was a schoolgirl with a very diffuse lesion on her nose. No amastigotes were seen in the stained smears of her dermal tissue aspirate. Her cultures were also contaminated. Four suspect cases of CL were encountered in Pueblo Nuevo. Amastigotes were not seen in their stained preparations and their cultures were also contaminated.

In 1999, two more definite cases were diagnosed among five suspect cases seen in two more new foci, La Arena de Liberia and La Carana de Liberia, Guanacaste Province. The definite case from Arena de Liberia was a 41-year old male with a nodular lesion on his shoulder. His smears were negative but the cultures were positive (the Diego strain). The definite case from La Carana de Liberia was a 19-year old male with a nodular lesion on his aural pinna. His smears and cultures were positive (the Luismi strain). This provided us with our first Costa Rican isolates from cases of CL for comparison with the one from the infantile VL case from Pueblo Nuevo, Liberia. Multifarious characterization and comparison of the strains from the cases of CL and VL was carried out (see Section III). The strains from the cases of CL and the case of VL were injected into Syrian hamsters kept at the Veterinary Institute in Heredia, Costa Rica, to maintain their infectivity and observe and compare their specific tissue tropisms in experimental animals. They were also sent to Dr Schnur in Israel and deposited in the leishmanial strain bank of the WHO's Jerusalem Reference Centre for the Leishmaniases housed in the Department of Parasitology of the Hebrew University–Hadassah Medical School. Dr Zeledon's group saw another four suspect cases of CL

after this but they were not confirmed to have been definite cases. No definite or suspect cases of leishmaniasis were seen during the field studies undertaken in 2000.

III. Leishmanial strain characterization

Various methods, encompassing biological [infectivity and behaviour in laboratory animals (Schnur and Zuckerman, 1977)], serological [antigenic analysis by Excreted Factor (EF) serotyping (Schnur and Zuckerman, 1977)], biochemical [enzyme analysis (Rioux *et al.*, 1990)] and molecular biological [DNA analysis Eisenberger and Jaffe, 1999)] parameters were used to characterize, identify and compare the strains isolated from Costa Rican human cases of CL and VL. At the start of the project, no molecular biological technology was available in either the Costa Rican group's or the Israeli group's laboratories. Several different polymerase chain reaction (PCR) techniques were incorporated into the Israeli groups leishmanial strain typing capability (Jacobson *et al.*, 2003; Schnur, *et al.*). The permissively primed intergenic polymorphic (PPIP)-PCR, which was developed in the Israeli groups laboratory, was the most useful one as it was able to distinguish the different species of *Leishmania* and differentiate PPIP-PCR sub-types within them (Fig. 5).

Before the two Costa Rican strains from human cases of CL, *i. e.* the Diego and Luismi strains, were available, a Honduran strain from a human case of CL, strain HN194 (WHO code =MHOM/HN/1993/HN194), was compared with the strain isolated from the Costa Rican case of VL, *i. e*, the LVCR or Dixon strain. This strain and the strain HN194 were identical by EF serotyping and both belonged to the EF sub-serotype B_2. Later, the Diego and Luismi strains were also shown to belong to EF subserotype B_2. In fact, all the strains of *L. d. infantum and L. d. chagasi* (=*L.infantum*) type in this way have belonged to the EF sub-serotype B_2.

Enzyme electrophoretic analysis was done in starch gel and 15 enzymes were examined to construct enzyme files: malate dehydrogenase, MDH, EC 1.1.1.37 ; malic enzyme, ME, EC 1.1.1.40 ; isocitrate dehydrogenase, ICD, EC 1.1.1.42 ; 6-phosphogluconate dehydrogenase, PGD, EC 1.1.1.44 ; glucose-6-phosphate dehydrogenase, G6PD, EC 1.1.1.49 ; glutamate dehydrogenase, GLUD, EC 1.4.1.3 , NADH diaphorase, DIA, EC 1.6.2.2 ; purine nucleoside phosphorylase, NP1, EC

2.4.2.1 ; purine nucleoside phosphorylase, NP2, EC 2.4.2.* ; glutamate-oxaloacetate transaminases, GOT1 and GOT2, EC 2.6.1.1 ; phosphoglucomutase, PGM, EC 5.4.2.2 ; fumarate hydratase, FH, EC 4.2.1.2 ; mannose phosphate isomerase, MPI, EC 5.3.1.8 ; glucose phosphate isomerase, GPI, EC 5.3.1.9. The enzyme profiles of the LVCR or Dixon strain from the Costa Rican human case of VL and the Luismi strain from a Costa Rican human case of CL, were identical to one another and to that of the WHO reference strain of the Old World species *L. infantum*, *i.e.*, strain MHOM/TN/1980/IPT1, and, therefore belonged to the zymodeme MON 1. The enzyme profile of the Diego strain, also from a Costa Rican human case of CL, was identical to that of the other three strains in the electrophoretic mobilities of 14 of the 15 enzymes examined but differed in the electrophoretic mobility of its malate dehydrogenase (MDH). This put it into the zymodeme MON-34. Leishmanial strains of both zymodemes have been isolated in the Old World from, both, human cases of VL and CL, and also cases of canine leishmaniasis. However, strains from the zymodeme MON-34 have mainly been isolated from human cases of CL, All the Latin American strains of *L. d. chagasi* (=*L.infantum*) examined in this way until now have belonged to the zymodeme MON-1. The Diego strain is the first exception.

For PPIP-PCR analysis, strains were mass cultured as promastigotes in Schneider's *Drosophila* medium supplemented with 10% FCS, washed and collected by centrifugation and had their DNA extracted from the pellets according to Eisenberger and Jaffe (1999). The PCR reactions were performed also as described by Eisenberger and Jaffe, 1999) with minor changes, *i.e.*, using 20 ng/ul of genomic DNA and amplified with 5 uM of the single leishmanial-specific primer, 2B (5' –CAG GAG CGC GCA CAC GCA CAC ACG), and 2 U of recombinant *Taq* DNA polymerase (MBI Fermentas, Amherst, NY). The PPIP-PCR results are given and compared in Fig. 5. The WHO reference strain of the Old World species *L. infantum*, *i.e.*, strain MHOM/TN/1980/IPT1, was PPIP-PCR type *Li*A1. In fact all the Old World strains of *L. infantum* examined by this method have been this PPIP-PCR type. Strain HN194 (MHOM/HN/1987/HN194) from a Honduran human case of CL PPIP-PCR displayed the same PPIP-PCR profile as strain IPT1 but had an exrta band of approx. 500bp in its profile, indicating and different and new PPIP-PCR type, *Li*A^{X} (= temporary designation). However, the LVCR or Dixon strain from the Costa Rican human case of VL and the Diego strain from a Costa Rican human case of CL, while sharing the same

PPIP-PCR profile and therefore having the same PPIP-PCR type, *Li*A^{Y} (= temporary designation), were different because their profile lacked the faster-running component of approximately 420bp of the doublet present in the profiles of the other strains examined. The PPIP-PCR type *Li*A^{Y} might be specific to the New World and even to Costa Rica. for the time being it is, but more strains need to be checked from othe New and Old World foci. However, it is, apparently, not an indicator of a strain's predisposition to dermato- or viscero-tropism.

IV. Leishmanial strain tropisms

A. Development of an experimental model to study the of tropisms

Syrian hamsters were used for this. The effect of cortisone acetate on the bodyweight of hamsters was assessed during its twice weekly subcutaneous injection over 10 weeks. One experimental group got doses of 1mg/100gm bodyweight; another got doses of 2mg/100g bodyweight. A moderate loss of weight was observed in the treated groups compared with an untreated the control group. All the treated hamsters survived this immuno-suppression. The dose of 1mg/100gm bodyweight was used on animals in observing and comparing experimental infections caused by strains of *L. d chagasi* (=*L. infantum*) isolated from human cases of either CL or VL. Thus, 32 hamsters were treated with cortisone acetate at the dosage of 1mg/100g bodyweight. Another 32 were left untreated. Four groups of 16 hamsters were arranged, 8 of which were treated and 8 of which were not. The following four strains of *L. d. chagasi* (=*L. infantum*) isolated from human cases were studied: MHOM/HN/1987/HN-029 from a Honduran case of VL; MHOM/HN/1993/HN-194 from a Honduran case of CL; MHOM/CR/1998/Dixon from a Costa Rican case of VL; and MHOM/CR/1999/Luismi from a Costa Rican case of CL. One strain was injected into one group of 16 hamsters. Each hamster received 10^{7} promastigotes that were injected intradermally into its nose. Two hamsters treated with cortisone acetate and two left untreated from each group were sacrificed at 4 days and at 4, 8 and 10 weeks post-infection. Smears and cultures were made of tissues from the nasal skin at the site if infection, the spleens, and the livers of the animals. Three samples were taken from each of these organs for culture in Senekjie's biphasic medium to increase the chance of isolating the parasites. Duplicate cultures of saline aspirates from the noses were also made in Senekjie's biphasic

medium but no smears were made from these. Four samples were taken from each of the organs and duplicate samples of nasal aspirates were collected and stored at -20°C for PCR in the event of over-all negative results. The smears were stained with Giemsa's stain and checked microscopically for parasites.

All four strains were able to infect hamsters. Treatment with cortisone acetate seemed to have little effect on the course and degree of infecton. The infections were somewhat patchy and there was some contamination of cultures, leaving gaps in the general picture. The results are summarized in Tables 9 and 10. From this study, Strain MHOM/HN/1993/HN-194 from a Honduran case of CL appeared to be only dermato-tropic, causing a patent infection with parasites visible in stained smears at the site of infection by 4 days. This became cryptic and was detectaed only by culture at 4 to 8 weeks and had, possibly, disappeared by 10 weeks. Prior to its employment in the study this study of tropisms, it was checked for its infectivity in Syrian hamsters in Costa Rica and Israel. In both cases, viscralization did occur but only after a very prolonged period of many months to a year. The other three strains did visceralize in the time period of this study (Table 9B). Strain MHOM/HN/1987/HN-029 from a Honduran case of VL did cause a patent infection with parasites visible in stained smears at the site of infection by 4 days but, owing to contamination it is difficult to know if it became cryptic or just disappeared. It also caused a patent visceral infection in the spleen and liver by 4 days that was still patent at 8 and 10 weeks (Table 9A). Strain MHOM/CR/1998/Dixon from the Costa Rican case of VL caused a patent infection with parasites visible in stained smears at the site of infection by 4 days that continued as a patent infection throughout the 10 week period. It also caused a patent visceral infection in the spleen by 4 days that remained patent for all 10 weeks and also in the liver from the eighth week that continued into the tenth week (Table 10A). Strain MHOM/CR/1999/Luismi from a Costa Rican case of CL caused a patent infection with parasites visible in stained smears at the site of infection by 4 days. This became cryptic and detected only by culture by 4 weeks and had, possibly, disappeared by 10 weeks. It also caused a patent visceral infection in the spleen and liver by 4 days that was still patent at 8 and 10 weeks (Table 10B). The Costa Rican strains from the case of VL, MHOM/CR/1998/Dixon, and the case of CL, MHOM/CR/1999/Luismi, behaved quite similarly in hamsters and, if anything, the former strain, which visceralized in its human

host, appeared to be more dermato-tropic in hamsters than the latter strain, which was dermato-tropic in its human host (Table 10A and B).

B. Leishmanial strain dissemination patterns in Syrian hamsters

During the early part of the project when Central American strains of *L. d. chagasi* (= *L. d. infantum*) from human cases of either CL or VL were unavailable, some pertinent studies in line with the overall aims of this project were done using other types of strain. The biochemical structural differences seen among excreted factor (EF) - surface lipophosphoglycan (LPG) from different species of *Leishmania* that confer EF serotypes might be involved in determining the variation seen in leishmanial parasite tissue tropisms in humans and animals and, through this, the different clinical conditions with which the different species of *Leishmania* are associated. In an old dissemination study predating this project (Schnur and Zuckerman, 1977), two EF sub-serotype A_1 strains of *L. major* from Israel disseminated in Syrian hamsters after intrasplenic (IS) injection of recently isolated, infective culture promastigotes, causing patent infection in the skin of their body extremities (nose, lips, paws, tail and also testes and epididymides). Under the same conditions, two EF sub-serotype B_2 strains of *L. donovani*, one from India the other from Ethiopia, did not. They disseminated only in the viscera (spleen, liver, bone marrow, lymph glands, testes, epididymides, kidneys, adrenal glands, lungs, diaphragm). This suggested that there might be a link between EF serotype and amastigote dissemination pattern. Within the project, the following experimental study was done to try and verify this. Some strains typing as *L. major* by enzyme and DNA analysis are EF sub-serotype B_2 and, therefore, serologically like the strains of *L. donovani* mentioned. One of these from Kenya was also injected IS into hamsters in the same manner to observe its dissemination pattern, which was very similar to that of the EF sub-serotype A_1 strains of *L. major* studied previously, despite its B_2 EF sub-serotype. However, it took longer, 18 instead of 12 weeks, to cause patent cutaneous disease in the body extremities. Also, bone marrow infection was only cryptic and heart blood was infected. All of these strains initiated infection in the spleen, an unnatural site. This and the incongruity between EF/LPG serotypes and subsequent dissemination patterns of the amastigotes should be considered when speculating on the role of EFs and LPGs in infection, dissemination and clinical outcome of infection. It should be noted that the EF sub-serotypes of two Central American strains, the strain

HN-194 from a Honduran case of CL and the strain LVCR or DIXON from the Costa Rican case of VL had the same EF sub-serotype, B_2, but their tropisms in humans were quite different. When these strains were brought to Jerusalem they were injected intra-peritoneally into hamsters to check and maintain their infectivity. The examination of the spleens of the hamsters many months after showed heavy infections of amastigotes in the spleens of the animals injected with the Costa Rican strain from the human case of VL and mild infections of amastigotes in the spleens of animals infected with the Honduran strain from the human case of CL. There were no obvious signs of cutaneous infections in any of the hamsters.

V. Studies on domestic dogs in relation to human and canine leishmaniasis

The role of domestic dogs as the medically important reservoir of VL and CL caused by *L. d. chagasi* (= *L. d. infantum*) was described in the preface but the study of leishmaniasis in dogs was not written into the project proposal. However, since this was pertinent to the project, some work was carried out on dogs, mainly in Israel but also in Cost Rica. Dr. Luis Araya visited the Hebrew University-Hadassah Medical School from September 1st-30th, 1997, and was trained in the immuno- and sero-diagnosis of human and canine VL. On his return to Costa Rica, he surveyed dogs from the regions of Costa Rica where leishmaniasis endemic. In the absence of Costa Rican strains of *L. d. chagasi* (= *L. d. infantum*), he prepared antigen by culturing a Honduran strain of *L. d. chagasi* (= *L. d. infantum*). He collected and examined 158 sera from dogs from Nazareth, Guanacaste Province, 15 (10%) of which were positive, using the ELISA developed in Israel (Rachamim *et al.*, 1991). A further 120 sera were collected dogs from Nazareth that were to have been be tested for anti-leishmanial antibodies but no more has been reported on them. In endemic foci, the numbers of sero-positive dogs tend to be much greater than the numbers of infected people and people with significant titres of ani-leishmanial antibodies. Thus, the presence and numbers of positive dogs are good indictors of the presence and degree of *L. d. chagasi* (= *L. d. infantum*) in actual and suspected foci of the disease.

As stated, *L. d. chagasi* (= *L. d. infantum*) from the New World leishmanial is essentially the same as *L. d. infantum* from the Old World and studies done in Israel on the leishmaniases it causes in humans and canines can be compared to what was seen in

Costa Rica. A major difference is that in Costa Rica all the human cases caused by *L. d. chagasi* (= *L. d. infantum*) but one have been CL without concomitant VL. No cases of human CL without concomitant VL caused by Old World *L infantum* have been recorded in Israel. They have been relatively few and have all been cases of infantile VL. Though cases of CL without concomitant VL have been recorded in other Mediterranean countries and other parts of the Old World (see preface). Sero-diagnotic surveys of dogs in endemic foci in Israel showed that a number of them had observable titres of anti-leishmanial antibodies. A few people did also and they are thought to have been asymptomatic cases. The sero-positive dogs usually went on to develop patent canine VL. This might be another interesting difference compared with Costa Rica as no cases of canine leishmaniasis have been reported from there not even from those foci where the human cases were seen. It is not known if any of the sero-positive dogs bled by Dr Araya did become patent cases of canine VL. During the time of the project, 57 strains were collected in Israel from dogs and many more have been collected since. All of them have been EF sub-serotype B_2 by serotyping. Eleven of them were also characterized by enzyme electrophoretic analysis using 15 enzyme systems. They all displayed the same enzyme profile that identified them as strains of *L. d. infantum* belonging to the zymodeme MON-1. In these respects, Costa Rican and Israeli strains are alike. Some of the Israeli were also characterized by various DNA analyses, which confirmed their identity. During the time of the project three Israeli human infantile cases of VL were also recorded. Their leishmanial strains were *L. infantum* and identical in character to the characterized canine strains, and the strains LVCR or Dixon and Luismi isolated from the Costa Rican human cases of VL and CL, being EF sub-serotype B_2 and belonging to the zymodeme MON-1. One strain isolated from a Palestinian infantile case of VL was different. It was EF sub-serotype B_2 but its enzyme profile was different and showed this strain to belong to a new zymodeme of *L infantum*, zymodeme MON-281. The strains examined by the PPIP-PCR were PPIP-PCR type *Li*A like all the other Old World strains of *L. infantum* checked by the PPIP-PCR. The strains LVCR or Dixon and Luismi from Costa Rica, and HN-194 from Honduras (Fig. 5) were not. The situation in Israel remains classical with many more dogs being infected than humans and all cases being very young children with VL. Jackals and foxes are often cited as being one natural animal reservoir hosts of *L infantum* in the Old World. This includes endemic regions in Israel. Sero-diagnosis of sera from 53 jackals and 20 foxes, using the same type of ELISA as that introduced in

Costa Rica, exposed 4 (7.6%) sero-positve jackals and 1 (5%) sero-positive fox but no strains of *L. d. infantum* were isolated.

VI. A symptomatic cases leishmanin (Montenegro) skin testing and serology

At the beginning of the project before we had any Costa Rican or even Honhuran strains, cultures were seeded with skin lesion aspirates from the 10 patients with suspected CL from Nazareth, Liberia. However, they had remained negative, even though nine of them had skin tested positively. Their stained tissue smears had been negative. Generally, in Costa Rican cases of CL caused by *L. d. chagasi* (=*L. d. infantum*), amastigotes tend to be scarce and are rarely seen in stained smears. However, as skin tests were positive in nine cases, it is possible that immunity to infection had also been acquired and the few parasites that might have been there originally had been eliminated. The tenth case, a seven-year old child, had a skin lesion atypical of CL and his skin test was negative so he might not have been a case. This small group of subjects had also been tested to determine the efficacy of the skin test antigen (leishmanin) prior to mass testing in field surveys. Since the original out break in Nazareth had ended and no new cases were appearing there, the search for asymptomatic cases by leishmanin skin testing and serology was abandoned. Even when the new cases mentioned were exposed in other foci, these were very small scattered communities that would not have permitted a meaningful survey.

VII. The effect of sand fly saliva in establishing leishmanial infections

Various studies were carried out on sand fly saliva and some of its effects regarding leishmaniasis.

1. Host macroghage chemotaxis: Experimental inoculation of small numbers of infective metacyclic promastigotes fail to initiate infections in susceptible mouse strains but small numbers inoculated by female sand fly vectors can give flourishing infections. Leishmanial promastigotes are naturally inoculated into vertebrate host skin together with vector saliva that contains pharmacologically active components like vasodilators and platelet aggregation inhibitors. These components facilitate the location of blood in the skin, prevent hemostasis and assure adequate blood for the feeding Sand

fly saliva also exacerbates experimental cutaneous leishmaniasis caused by several different species of *Leishmania*. Different mechanisms, i.e., inhibition of antigen presentation, reduction of nitric oxide (NO) production in macrophages (Mϕ) infected with amastigotes and enhancement of interleukin (IL)-4 secretion, might contribute to this. Another plausible mechanism enhancing of CL by sand fly saliva is its effect on leishmanial promastigotes. Saliva from *Lu. longipalpis* inhibits promastigote division, possibly, promoting metacyclogenesis and enhancing parasite infectivity.

Microporous membranes were used to study the chemotactic effect of saliva on murine Mϕs. *In vitro,* Mϕs gravitated towards increasing concentrations of saliva. Saliva also increased the percentage of culture Mϕs that became infected with promastigotes and increased the amastigote load in these cells. Saliva, probably, reduces the exposure of promastigotes to natural killer cells during natural transmission by attracting Mϕs to the site of inoculation of the parasites, aiding their quicker uptake entry. Saliva might also enhance lesion development by shortening the generation time of dividing intracellular amastigotes.

The vasodilator maxadilan, which is found in the saliva of *Lu. longipalpis*, the vector of New World VL, is similar to the calcitonin gene-related peptide (CGRP) but much more vasoactive. Maxadilan has been cloned and characterized and it is possible to test the pharmacological activities of the recombinant peptide in its pure form. It stimulates IL-6 but inhibits tumor necrosis factor (TNF)-α production in murine peritoneal Mϕs. It also exhibits a dose-dependent inhibition of T-cell proliferation on stimulation with Con-A or αT cell receptor (TCR). It inhibits proliferation induced by alloantigen in a mixed lymphocyte reaction and suppresses delayed-type hypersensitivity in mice. Therefore, maxadilan, which is the in saliva of *Lu. longipalpis*, is considered to be responsible, at least partially, for the exacerbation of CL. However, it is certainly not the only molecule of consequence in exacerbating leishmaniasis. The saliva of Costa Rican *Lu. longipalpis* is very low in maxadilan and the saliva of *Ph. papatasi*, one of the vector of in the Old World, lacks maxadilan all together. Both types of saliva exacerbate leishmaniasis very efficiently, suggesting that there are other factors and mechanisms.

2. Effect of sand fly saliva on phosphorylation of Mφs and leishmanial proteins: We showed that saliva from the Old World species of *Phlebotomus*, some of which are vectors of leishmaniases, and that of *Lu. longipalpis*, the Old World vector of *L. d. chagasi* (=*L. d. infantum*), contained significant levels of endogenous protein phosphatase (PP) activity that was heat labile, inhibitable by okadaic acid and calyculine-A and did not require divalent cations (PP-1/2A-like). Furthermore, *Phlebotomus* saliva was shown to modify the levels of phosphorylation of specific cellular proteins in Mφs and leishmanial promastigotes. Protein phosphorylation and dephosphorylation reactions, mediated by protein kinases (PK) and PPs, respectively, trigger signal transduction events that control diverse cellular responses to both internal and external signals. Our study was the first demonstration of the ability of an insect's saliva to specifically influence protein phosphorylation, a key process in eukaryotic cell-signal transduction.While studying the effect of sand fly saliva on protein phosphorylation, we noted a dose dependent inhibition of exogenous (Mφ and leishmanial parasites) PP1/2A activity by saliva from *Phlebotomus* spp., in which endogenous PP activity had been abrogated by boiling. We also demonstrated that saliva down-regulated the expression of the inducible nitric oxide synthase (iNOS) gene and reduced nitric oxide (NO) production in murine Mφ. Since NO is toxic to intracellular parasites, its down-regulation may be a key mechanism by which the saliva of *Ph. papatasi* exacerbates CL.

3. Sand fly salivary gland adenosine, AMP and protein phosphatase activity: Female phlebotomine sand flies inject saliva into the skin of their mammalian hosts, including humans, when they probe in the skin for blood. This prevents hemostasis. In the case of sand flies serving as vectors of leishmaniases, the saliva also promotes leishmanial infections by suppressing mammalian macrophage immunological function. This is very important for the survival of leishmanial amastigotes, which live intracellularly in mammalian host macrophages. The saliva of *Ph. Papatasi*, a vector transmitting Old World CL, contains adenosine and 5'-AMP. We showed that its saliva and, also, pure adenosine down-regulate the expression of the inducible nitric oxide (NO) synthase gene in activated macrophages. We also showed that saliva from *Ph. papatasi* inhibits the production of NO, whereas that of *Lu. longipiapis*, a vector of *L. d. chagasi* (=*L. d. infantum*), does not. These experimental results, taken together, suggest that salivary adenosine is responsible for down-regulation of NO synthesis. The saliva

of, both, the genus *Phlebotomus* and the genus *Lutzomyia* were shown to possess significant levels of heat labile endogenous protein phosphatase-1 2A- like activity that is inhibited by okadaic acid and calyculine a. and does not need divalent cations.

4. Pharmacologically active quantities of sand fly salivary gland adenosine, and 5'-AMP: By liquid chromotography, UV, and mass spectrophotometry, and by bioassays, we showed that homogenates of salivary glands from *Ph. papatasi* contain large quantities of adenosine and 5'-AMP, *i. e.*, about 1nmol per pair of salivary glands. These purines, many of which, 75-80%, are secreted by the glands after a blood meal, display vasodilatory and anti-platelet activities. This probably assists the sand flies' obtaining blood meals. The salivary 5'-AMP is responsible for the protein phosphatase inhibitor in the salivary glands of *Ph. papatasi* reported earlier. This was shown to be artifactual in nature owing to allosteric modification of the phosphatase substrate employed, *i.e.*, phosphorylase a, by the AMP.

Impact Relevance and Technology Transfer

It is difficult to determine the impact of the project as the sudden out break of leishmaniases caused by *L. d. chagasi* (=*L .d. infantum*) that initiated the project had subsided by the time the project began and no further outbreaks have occurred since then. However, the project did instigate a greater awareness of and interest in this disease by the local medical and public health authorities. The fact that there were almost no cases of VL might have reduced the importance the disease in the community if its potential dangers were not given greater coverage. The project was very useful in this respect. The project also revealed the continued presence of the parasite in hitherto unknown foci and this could have lead to further outbreaks. In fact, this threat remains. Should new outbreaks occur, there is now increased expertise to deal with them and for maintaining good surveillance for early awareness should they occur. In terms of development, as stated earlier, outbreaks of leishmaniasis often appear to follow agricultural and suburban development owing to the attendant turmoil these create. In situations like that in and around Liberia, Guanacaste Province, surveying sites to be developed, beforehand, for their potential to become the sites of outbreaks of leishmaniasis, could lead to applying preventative measures to reduce and even stem the disease before work begins and before a community enters a newly developed area. The outbreak at Liberia, Guanacaste Province, appeared to be unique and unusual because all the human cases were

case of CL rather than VL. At that time, Old World *L. d. infantum* and New World *L. d. chagasi* (=*L. d. infantum*) were generally considered to cause only infantile VL. However, at about the same time of the Costa Rican outbreak, similar situations were occurring elsewhere in Latin America and *L. d. infantum* was found causing CL as well as VL in many Old World foci. Thus, the 'Costa Rican' situation was much more universal and knowledge gained through this project has a much wider significance. The isolation, characterization and identification of strains from, both, Costa Rican human cases of CL and VL have added to the greater pool of information showing that they are caused by the same type of leishmanial parasite and that factors other than the parasites are responsible for whether there is viscero- or dermato- tropism.

Collaboration was established together with Dr Mario Calvo, a dermatologist at the hospital in Liberia, Guanacaste Province, and his colleagues. They constantly surveyed the local population for potential of cases CL and collaborated in the nutritional studies. This also increased ongoing local public awareness. We visited doctors, public health workers and research scientists working on leishmaniasis in Managua and Leon, Nicaraguan and had planned to visit Dr Carlos Ponce's group in Tegucigalpa, Honduras to try and form a network of people working on leishmaniases caused by *L. d. chagasi* (=*L. d. infantum*). This visit was thwarted, initially, by 'cash flow' problem and then by the advent of Hurricaine George. We also contacted colleagues at the WHO concerning funding for this network but they were unable to assist us.

While the focus at Nazareth where the original outbreak had occurred had become devoid of cases, new foci of CL caused by *L. d. chagasi* (=*L. d. infantum*) were exposed at Pueblo Nuevo, El Salto, La Arena de Liberia and La Carana de Liberia. However, very few case were seen and very few strains of *L. d. chagasi* (=*L. d. infantum*) were isolated. However, those that were characterized and identified, confirming that the causative agent is, essentially, like *L. d. infantum* from the Old World and that it is responsible for both CL and VL.

A computerized epidemiological database encompassing all the survey data was established at the National University. Two major technologies were also set up in the Costa Rican laboratories: a system for doing ELISAs and a PCR facility. Prior to the beginning of the project there were no functioning molecular biological methods being used in the Israeli laboratories. During the project, various PCR-base methods were established for diagnosis of

cases and the characterizaton of leishmanial isolates. One of them, the PPIP-PCR was produced there. The project ended before these technologies could be transferred to Costa Rica when Costa Rica was removed from the list of countries able to acquire USAID-CDR funding.

Project Activities and Outputs

Four project joint meetings were held in Costa Rica. These were held in conjunction with the field studies in which Israeli partners participated, which occurred during the years 1997-2000. It was during the field studies of 1997 that our visit to colleagues in neighbouring Nicaragua was made, Dr Zeledon came to Israel on two occasions. They were at times of international meetings held in Europe that he and Israeli partners attended. His first visit co-incided with the First World Congress on Leishmaniases held in Istanbul, Turkey, in 1997. His second visit co-incided with the Third International Symposium on Phlebotomine Sand Flies held in Montpellier, France, in 1999. Dr Schnur and Dr Warburg also attended these two meetings. Results of our Costa Rican field studies were presented at the second meeting (see abstracts at the end of this report). Dr Zeledon also attended the Fifteenth International Congress for Tropical Medicine and Malaria, held in Cartagena, Colombia, in 2000 where he presented more project fieldwork results (see abstracts at the end of this report).

A number of Costa Rican and Israeli students and other personnel received training and some acquired higher degrees. Dr Luis. Araya was trained in Israel in the immuno- and serodiagnosis of human and canine VL and established the ELISA developed in Israel in Costa Rica. Mr Johnny Peraza was trained as a technician to work with leishmanial parasites in most aspects pertaining to the laboratory search of this project. Adaeze Okeke, a medical student under Dr. Greg Lanzaro of the University of Texas, Galveston, spent six weeks, at the Veterinary Institute in Heredia and participated in a survey of past cases of Costa Rican human CL caused by *L. d. chagasi* (=*L. d. infantum*) and in fieldwork in Pueblo Nuevo. Duing his visit to Costa Rica in1998, Dr Schnur demonstrated EF serotyping to Dr Zeledon's laboratory staff and trained them in its use. They then serotyped strains in the Costa Rican bank of leishmanial strains. The following students studied and worked their higher degrees within the context of the project. Ms Andrea Urbina worked for her M. Sc. degree thesis

in Costa Rica. In Israel. Oren Katz completed his M. Sc. and began his Ph. D., Roni Zer completed his M. Sc., Tamar Grossman worked for her Ph. D., Abdelmajeed Nasereddin completed his M.P.H.

Three full papers associated with this project have been published. One paper has been submitted for publication. A further paper on the characterization and comparison of the Costa Rican strains from cases of CL and VL is in preparation. Five abstracts of presentations given at international meetings have also been published (see abstracts of publications at the end of this report).

Project Productivity

The project did achieve many of its goals like the finding new foci of Costa Rican human CL caused by *L .d. chagasi* (=*L. d. infantum*) and isolate strains from the new cases and characterize and compare them, including their dissemination behaviour in laboratory animals. This showed that the same leishmanial entity was responsible for both. It so indicated that nutrition status appears to be a major factor in the outcome of infection. However, because the sudden out break of leishmaniases caused by *L. d chagasi* (=*L. d. infantum*) that initiated the project had subsided by the time the project began and no further outbreaks occurred it was impossible to carry out some of the aims like searching for asymptomatic cases. Also, the earlier lack of Costa Rican leishmanial strains meant that other studies using non-Costa Rican were carried out, but these did produce useful results. Transfer of technology was achieved and many personnel were traind and some student acquired postgraduate degrees. There was no possibility of continuing the project otherwise there would have been further transfer of PCR technology. Inmany restects the project aims were over ambitious and depended on a return and continuance of the disease.

Future Work

Costa Rica was removed from the list of countries able to acquire USAID-CDR funding and we were unable to carry out further collaborative research.

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Abstracts of Publications and Presentations

1. Waitumbi, J. & Warburg, A. 1998. Sand fly saliva inhibits protein phosphatase activity and nitric oxide production by murine macrophages. *Infect Immun* **66**: 1534 –1537.

Abstract:

Leishmania parasites, transmitted by phlebotomine sand flies, are obligate intracellular parasites of macrophages. The sand fly *Phlebotomus papatasi* is the vector of *Leishmania major*, a causative agent of cutaneous leishmaniasis in the Old World and its saliva exacerbates parasite proliferation and lesion growth in experimental cutaneous leishmaniasis. Here we show that *Phlebotomus papatasi* saliva contains a potent inhibitor of protein phosphatase - 1 and protein phosphatase - 2A of murine macrophages. We further demonstrate that *Ph. papatasi* saliva down regulates the expression of the inducible nitric oxide synthase gene and reduces nitric oxide production in murine macrophages. Partial biochemical characterization of the protein phosphatase and nitric oxide inhibitor indicated that it is a small, ethanol-soluble molecule, resistant to boiling, proteolysis, DNAse and RNAse treatments. We suggest that the *Ph. papatasi* salivary protein phosphatase inhibitor interferes with the activated macrophages' ability to transmit signals to the nucleus, thereby preventing the up-regulation of the induced nitric oxide synthase gene and inhibiting the production of nitric oxide. Since nitric oxide is toxic to intracellular parasites, the salivary protein phosphatase inhibitor may be the mechanism by which *Ph. papatasi* saliva exacerbates cutaneous leishmaniasis.

2. Ribeiro, J.M.C., Katz, O., Pannell, L., Waitumbi, J. & Warburg, A. 1999. Salivary glands of the sand fly *Phlebotomus papatasi* contain pharmacological amounts of adenosine and 5'-AMP. *J Exp Biol.* **202**: 1551-1559.

Abstract:

Salivary gland homogenates of the sand fly *Phlebotomus papatasi* contain large amounts of adenosine and 5'-AMP, of the order of 1 nmol per pair of glands, as demonstrated by liquid chromatogrphy, ultraviolet spectrophotometry, mass spectrometry and bioassays. The purines, 75-80%of which are secreted from the glands following a blood meal, have vasodilatory and anti-platelet activities and probably help the fly to obtain a blood meal. Salivary 5'-AMP is also responsible for the previously reported protein phosphatase inhibitor in the salivary glands of *P. papatasi*, which is shown to be artificial in nature as a result of allosteric modification by AMP of th phosphatase substrate used (phosphorylase a).

3. Katz, O., Waitumbi, J., Zer, R. & Warburg A. 2000. Adenosine, AMP and protein phosphatase activity in sand fly saliva. *Am J Trop Med Hyg*, **62**: 145-150.

Abstract:

AS they probe the skin for blood, sand flies inject saliva that prevents hemostasis. Sand fly saliva also promotes leishmaniasis by suppressing immunologic functions of macrophages. Saliva of *Phlebotomus papatasi*, the vector of Old World cutaneous leishmaniasis, contains adenosine and AMP. We show that *Ph. papatasi* saliva as well as pure adenosine down-regulate the expression of the inducible nitric oxide (NO) synthase gene in activated macrophages. In addition, *Ph. papatasi*, but not *Lutzomyia longipalpis*, saliva inhibits the production of NO. Taken together, these data suggest that salivary adenosine is responsible for the down-regulation of NO synthesis. Saliva of both genera, *Phlebotomus* and *Lutzomyia* contain significant levels of endogenous protein phosphatase-1/2A-like activity that is heat labile, inhibitable by okadiac acid and calyculine a, and does require divalent cations.

4. Bader, K. A., Schnur, L. F., Nasereddin, A., Pratlong, F., Dedet, J.-P., Shaheen, L., Yousef, O., & Greenblatt, C. L. Palestinian infantile visceral leishmaniasis: a case caused by a genetic variant of *Leishmania infantum* belonging to a new zymodeme. Full paper submitted to *Trop. Med Intl Hlth.*

Abstract:

A Palestinian case of infantile visceral leishmaniasis (IVL) from the Jenin District is described. The parasite causing it was isolated, characterized and identified as *Leishmania infantum* by excreted factor (EF) serotyping, enzyme variant electrophoresis and by applying various molecular biological techniques that targeted kinetoplast (kDNA) and nuclear (nDNA) DNA. The parasite was identical to the WHO reference strain of *L. infantum* in most respects, including its enzyme profile, except for the faster electrophoretic mobility of its malate dehydrogenase (MDH), which also differed from all the other known electrophoretic variants of MDH found among strains of *L. infantum*. This designated it the reference strain of a new zymodeme of *L. infantum*, zymodeme MON-281. The RFLP profile generated from its kDNA PCR amplification product after digestion with the endonuclease *Rsa*I displayed a unique 200 bp component that made the parasite genetically different also. Three strains isolated from domestic dogs from the Jenin District were also *L. infantum* by serological and genetic analysis, indicating that infected domestic dogs are the medically important reservoir of human IVL in that region.

5. Schnur, L.F. 1998. Relevance of EF and LPG serotypes to infection by and dissemination of leishmanial parasites. *Abstracts, Trypanosomiasis and Leishmaniasis Symposium*, (Arcachon, France) Abstract 102.

Abstract:

Different species of *Leishmania* exhibit distinct capabilities of infecting and disseminating in mammalian hosts like Syrian hamsters. They also have different excreted factor (EF) serotypes that correlate with and are, probably, determined by the biochemical structural differences in the various types of somatic lipophosphoglycans (LPG) associated with different species of *Leishmania*. Two Israeli EF subserotype A1 strains disseminated to cause patent infections in the skin of the body extremities, i.e., nose, lips, ears, paws and tail, of Syrian hamsters, following intrasplenic (IS) injection of recently isolated, infective culture promastigotes: whereas, Indian and Ethiopian EF subserotype B2 strains of *L. donovani* did not under the same conditions. However, some strains shown to be *L. major* by enzyme and DNA analysis are EF subserotype B2 like *L. donovani*. Syrian hamsters were infected IS with one of these from Kenya to see if it would disseminate like EF subserotype A1 strains of *L. major* or like EF subserotype B2 strains of *L. donovani*. Its dissemination pattern was congruent with that of the EF subserotype A1 strains of *L. major*, despite its being EF subserotype B2 like *L. donovani*. These various strains were all able to initiate infection at the spleen, an unnatural site. This ability to initiate infection at non-dermal sites and the lack of congruence between EF/LPG types and amastigote tropisms should be accounted for when speculating on the role played by LPGs and Efs in initiating leishmanial infections in mammalian hosts and on the style of their dissemination.

6. Katz, O., Waitumbi, J. N. & Warburg, A. 1998. Effect of sand fly saliva on protein phosphatases and the phosphorylation state of *Leishmania* proteins. *Abstracts, 47th Meeting of the American Society of Tropical Medicine and Hygiene*, (San Juan, Puerto Rico). Abstract 166, p. 168.

Abstract:

We have recently shown that salivary gland homogenates (SGH) of the sand fly *Phlebotomus papatasi* contain a potent inhibitor of protein phosphatase (PP) 1 and PP2A activity in murine macrophages. *Leishmania* promastigotes proliferating and differentiating in the alimentary tract of the sand fly are exposed to saliva when Infected females take sugar meals. Therefore we tested for possible PP inhibition in *Leishmania* promastigotes exposed to SGH. Firstly, an *in vitro* phosphorylation assay was employed to quantitate the effect of *Ph. Papatasi* SGH on protein phosphorylation. Autoradiography of promastigote lysates incubated with gamma -ATP revealed two protein bands of approximate size 40 and 42 kD that were phosphorylated to a significantly higher degree in the presence of SGH. Secondly, we measured the effect of SGH on PPI / PP2A-like activity in promastigote lysates *Ph. Papatasi* SGH were found to decrease the PP activity of *L. major* in a dose dependent manner. SGH of other species of Old World sand flies were found to have a similar effect, whereas SGH of *Lutzomyia longipalpis, a New World* sand fly, did not have PP inhibitory activity at all. SGH of all sand fly species tested exhibited endogenous PP1 / PP2A-like activity that was totally abolished by nM calyculin A or 100 nM okadaic acid and inhibited by the *Ph. Papatasi* SGH PP inhibitor. Phosphorylation and dephosphorylation of proteins are crucial processes in cellular sign transduction and their

modulation by sand fly saliva may constitute a key mechanism governing *Leishmania* growth differentiation and infectivity.

7. Schnur, L.F. 1998. Incongruity between leishmanial excreted factor (EF) / lipophosphoglycan (LPG) serotypes and amastigote dissemination patterns. . *Abstracts, 47th Meeting of the American Society of Tropical Medicine and Hygiene*, (San Juan, Puerto Rico). Abstract 632, p328.

Abstract:

The various leishmanial species produce distinct types of excreted factor (EF). These confer serotypes on them that correlate with and are determined by the biochemical structural differences of their diverse lipophosphoglycans (LPG). The species also exhibit distinct capabilities of infecting and disseminating indifferent mammalian species. Two EF subserotype A1 strains of *L. major* from Israel disseminated in hamsters, causing patent infection in the skin of their body extremities (nose, lips, paws, tail) after intrasplenic (IS) injection of recently isolated, infective culture promastigotes. Under the same conditions, two EF subserotype B2 strains of *L. donovani* from India and Ethiopia, did not. They disseminated in the viscera only (spleen, liver, bone marrow, lymph glands, testes, epididymides, kidneys, adrenal glands, lungs, diaphragm). Some strains typing as *L. major* by enzyme and DNA analysis are EF subserotype B2 and serologically like *L.* donovani. One from Kenya was also injected IS into hamsters to observe its dissemination pattern, which was very similar to that of the EF subserotype A1 strains of *L. major*, despite its B2 EF subserotype. However, it took longer, 18 versus 12 weeks, to cause patent cutaneous disease in the body extremities, also, bone marrow infection was only cryptic and heart blood infected. All of these strains initiated infection in the spleen, an unnatural site. This and the incogruity between EF/LPG serotypes and subsequent amastigote dissemination patterns should be considered when speculating on the role of EFs and LPG.

8. Zeledon, R., Rojas, J.C., Castro, R. & Schnur, L.F. 1999. Leishmaniasis caused by *Leishmania infantum* (= *L. chagasi*) in an endemic area of Costa Rica. . *Abstracts, 3rd International Symposium on Phlebotomine Sandflies*, (Montpellier, France). Abstract O42.

Abstract:

Leishmania infantum also named *L. chagasi* in the New World but apparently the same species is associated, classically, with infantile visceral leishmaniasis (VL). More recently and increasingly, it has also been the cause human cutaneous. leishmaniasis (CL) without concomitant patent VL. In Costa Rica, *L. infantum* has been associated, exclusively, with human CL, with one exception that was an infantile case of VL. A survey of the total human population and their primary domestic animals was carried out in the suburb of Nazareth in the northern Costa Rican town of Liberia, where cutaneous leishmaniasis caused by *L. infantum* is endemic. By charting the distribution of the cases, it became evident that most of the cases (64 / 70) were from the northern half of the town (OR=19.3; CI=5.5-80.9). They came from 142 households that totalled 773 individuals. Only 6 cases were from in the southern half. These came from 187 households that totalled 1,005 inhabitants. The mean number of households with chickens was greater in the north than in the south ($p < 0.05$), with numerically more chickens in most northern household ($p < 0.05$). The presence of pigs was significantly associated with the presence of cases (OR=4.9; CI=1.0-16.9), as was the presence of dogs (OR=2.3; CI=1.0-5.2). The vector, *Lutzomiya longipalpis* was abundant in three different areas of the northern half of the town but was not found in six collecting sites in the southern half. The data suggest an association between houses with 10 or more chickens and the presence of the vector. On one occasion, larvae of *Lu. longipalpis* were found in a soil sample collected beneath a chicken coop.

We hypothesize that the vector is associated with households that have chicken coops with sufficient numbers of chickens, as was more common in the northern part of the town.

9. Zeledon, R., Rojas, J.C., Castro, R. & Schnur, L.F. 2000. Biology of *Lutzomyia longipalpis* and its role in the transmission of leishmaniasis caused by cutaneous and visceral leishmaniasis caused by *Leishmania infantum* (= *L. chagasi*) in Central America. *Abstracts. 15th International Congress for Tropical Medicine and Malaria.* (Cartagena, Colombia). Abstract (in press).

Abstract:

Lu. longipalpis is considered to be the most important vector of American visceral leishmaniasis caused by *L. chagasi* in South and Central America. In a few places, such as northwestern Colombia, it seems to be replaced by *Lu. evansi* which could also act as vector. *Lu. longipalpis* occurs primarily in dry, semiarid habitats and has been detected from Mexico to Argentina. It is accepted today that *Lu. longipalpis* is a species complex composed of at least three sibling species. In Central America, *Lu. longipalpis* has been associated with the transmission of two clinical forms of leishmaniasis, produced by the same parasite, a cutaneous non ulcerated papular or nodular type, and a classical visceral form, mainly in small children. The etiological agent has been identified as *L. infantum* MON-1 (J.P.Dedet, personal communication) identical to strains present in the Mediterranean area. Most cases have been reported from Honduras and Nicaragua where both clinical forms are common, and from Costa Rica where there is a predominace of the cutaneous form and only one case of visceral leishmaniasis has been described. Since 1987 we confirmed the presence of this parasite in an endemic area in the suburb of Nazareth in the northern Costa Rican town of Liberia, Province of Guanacaste, at 70 km from the Nicaraguan border. More recently, we carried out a census-inventory of the human population and domestic animals as well as a survey of children's nutritional status. A register of the clinical cases and of their households was also pinpointed in a chart. *Lu. longipalpis* was captured in several strategic points of the town and some aspects of its population dynamics are at present under observation. It was abundant in three different sites of the northern half of the town, both by manual collecting and by using CDC light traps, but was not found in six other collecting places in the southern half. Some other species of sand flies captured, together with *Lu. longipalpis*, but in small numbers were: *Lu. cayennensis*, *Lu. chiapanensis* and *Lu. Shannoni*. The number of infected persons and of households with cases was significantly higher in the northern area. The mean number of households with chickens was greater in the north, with numerically more chickens in most northern households. Chicken coops with more than 10 chickens seems to be most attractive to this sand fly, apparently playing a role in the epidemiology of the disease. In three households where chicken coops were removed the sand flies disappeared and in two more the population fell considerably. The presence of pigs was also significantly associated with the presence of cases as was the presence of dogs when analyzed statistically as risk factors (Odd Ratios). Other animals such as cows and goats also attracted the sand fly but they were not present in significant numbers in the area. Households with six or more persons and persons with more than five years in the area, were also considered as risk factor increasing the chances of acquiring the disease. The presence of latrines was not a risk factor, even though Lu. longipalpis was found sometimes resting within them. The vector population increased considerably after the end of the rainy season. Larvae of this species were found on one occasion in a soil sample collected beneath a chicken coop.

Map

Chart of the town of Nazareth, Liberia, Costa Rica, showing the distribution of houses and places where sand flies were searched

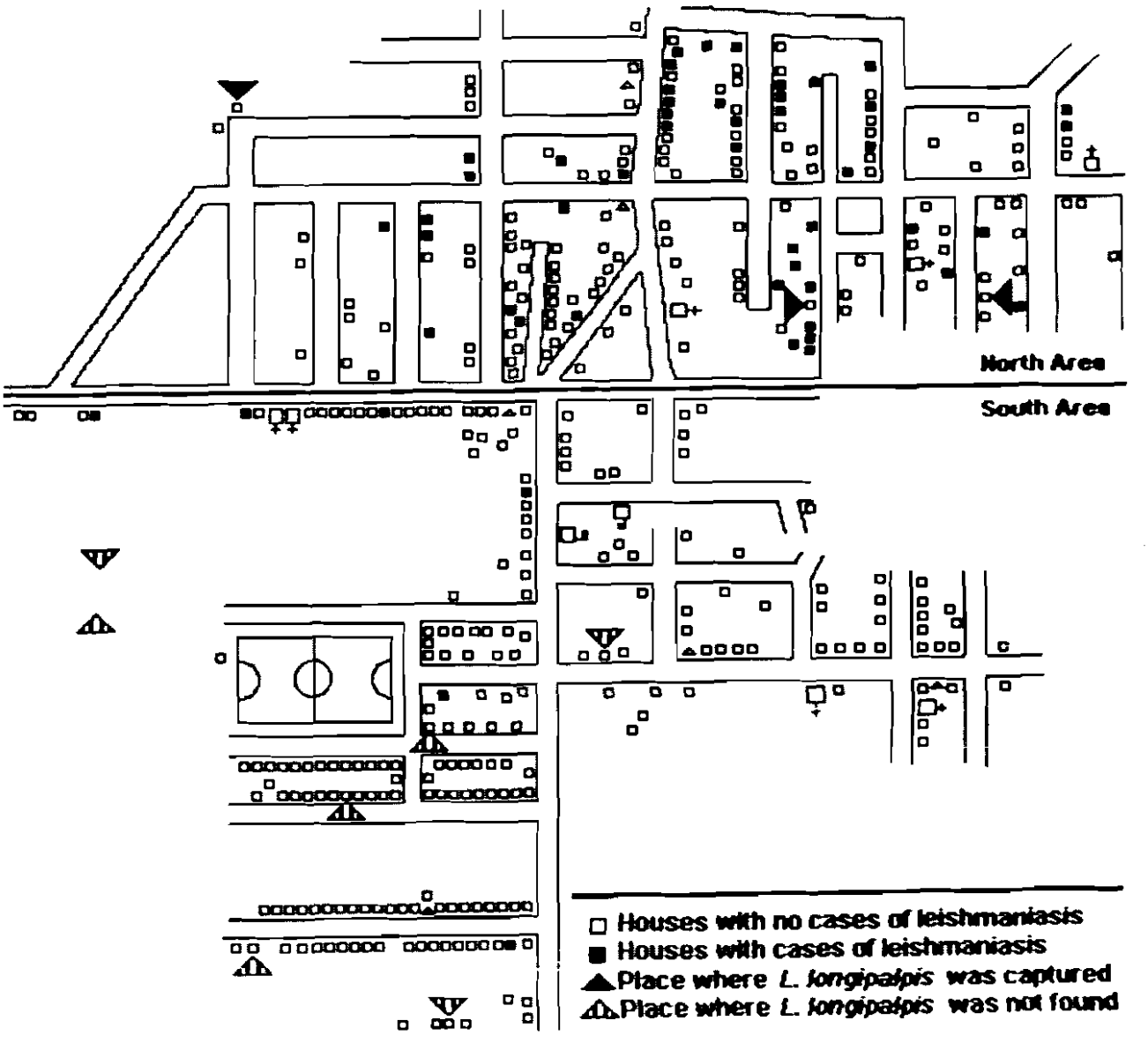


Fig. 1

Chi-Square Test and Fisher's Exact Test for the relationship betwen the presence of leishmaniasis cases and chicken densities in the households

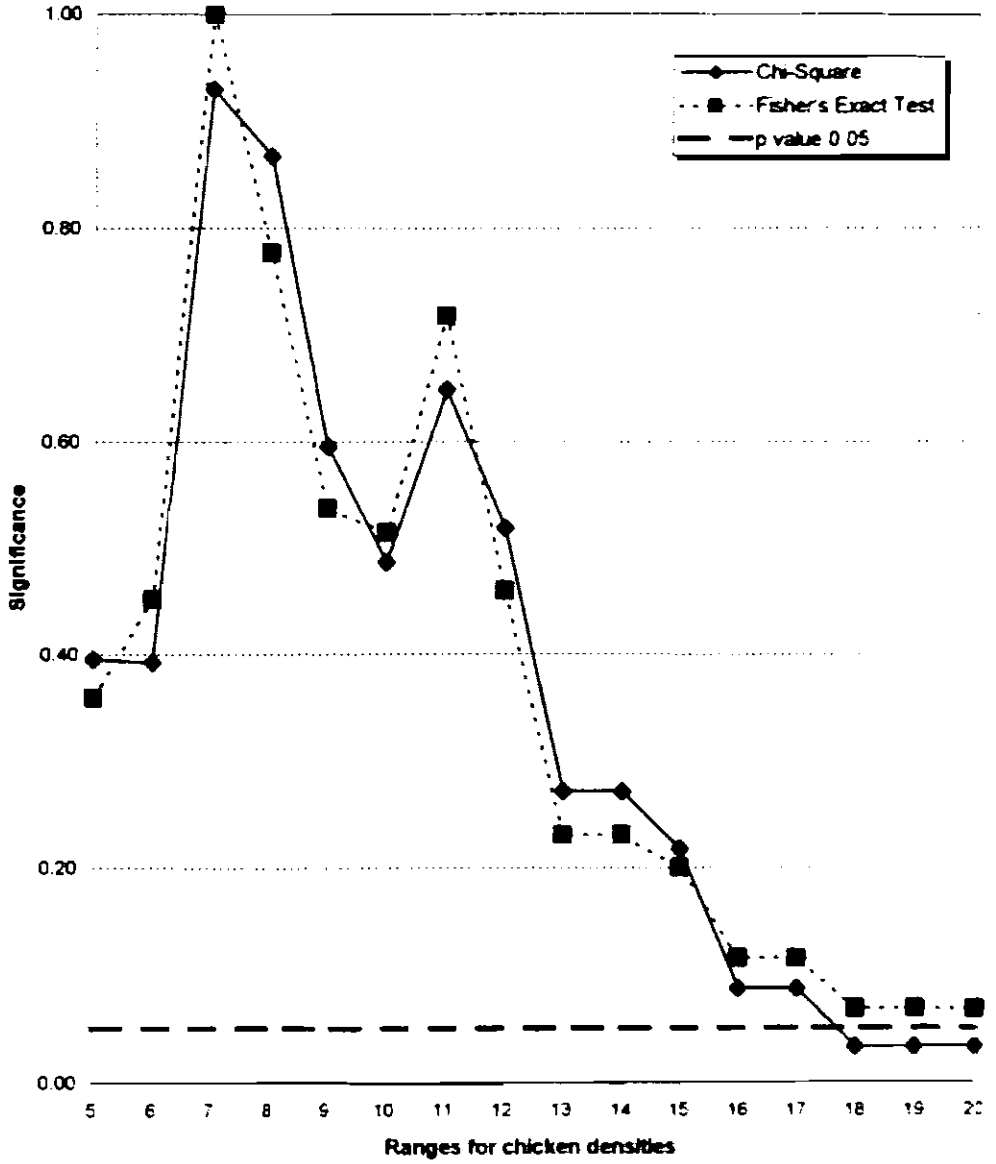


Fig. 2

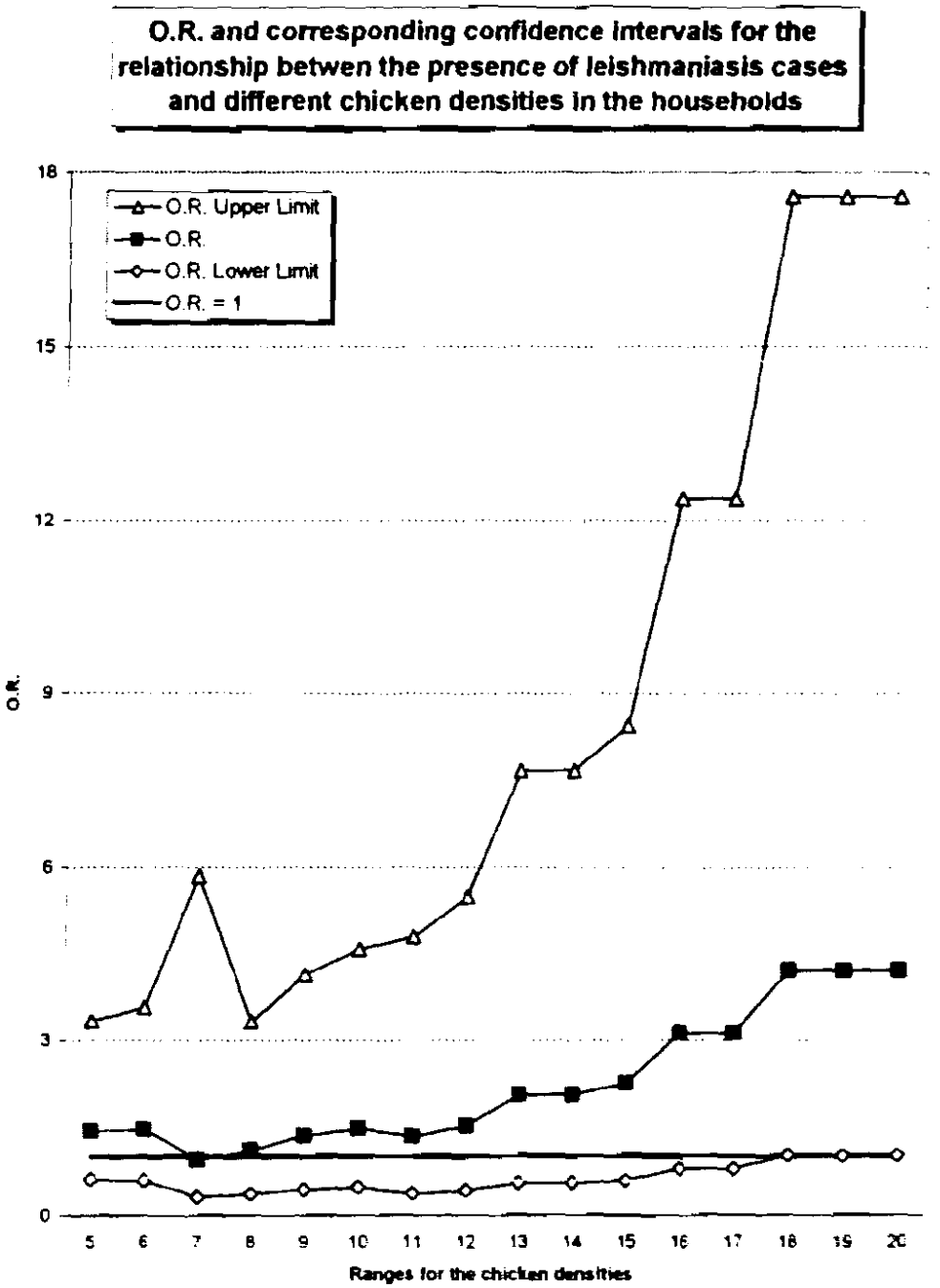

O.R. and corresponding confidence intervals for the relationship betwen the presence of leishmaniasis cases and different chicken densities in the households
O.R. Upper Limit
O.R.
O.R. Lower Limit
O.R. = 1
O.R.
0
3
6
9
12
15
18
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Ranges for the chicken densities

Fig. 3

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
FEMALES	0	0	9	3	24	24	15	3	33	63	12	9
MALES	96	50	96	153	201	255	186	180	172	165	102	84
TOTAL	96	50	105	156	225	279	201	183	205	228	114	93

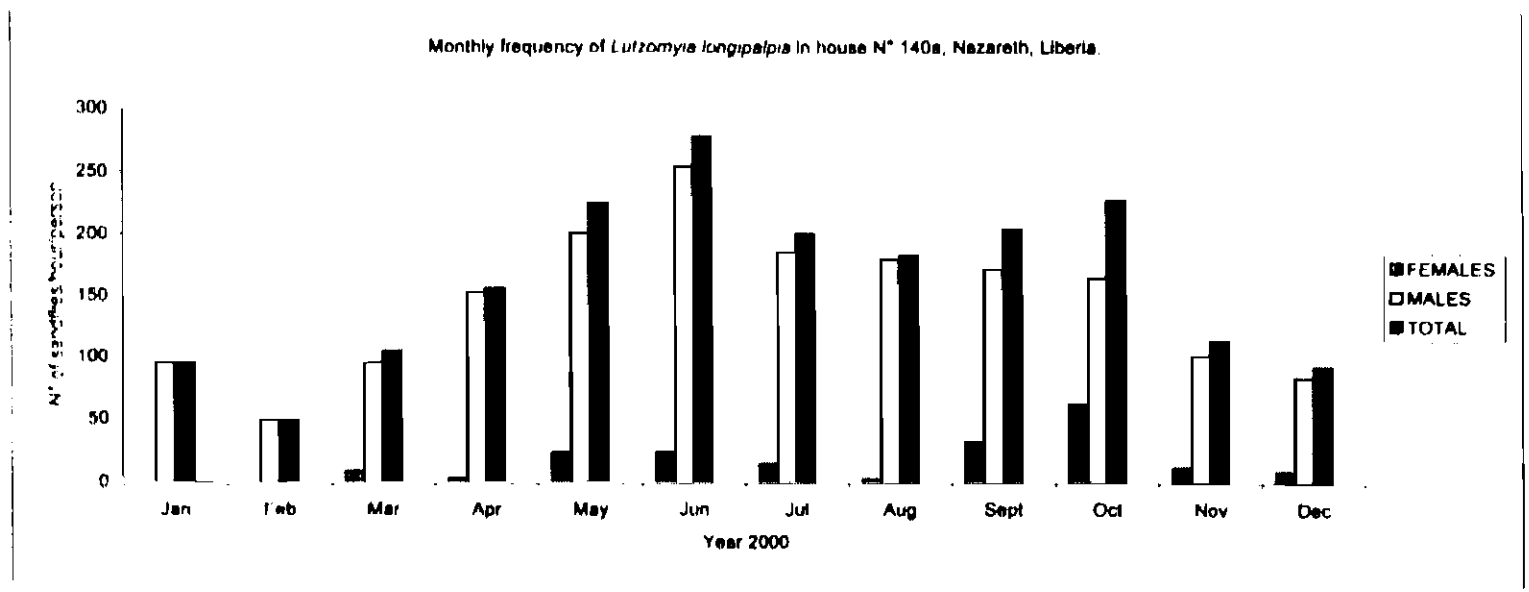


Fig. 4

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
FEMALES	0	14	33	6	30	21	108	66	150	46	69	9
MALES	69	28	111	51	108	138	420	114	66	120	144	72
TOTAL	69	42	144	57	138	159	528	180	216	166	213	81

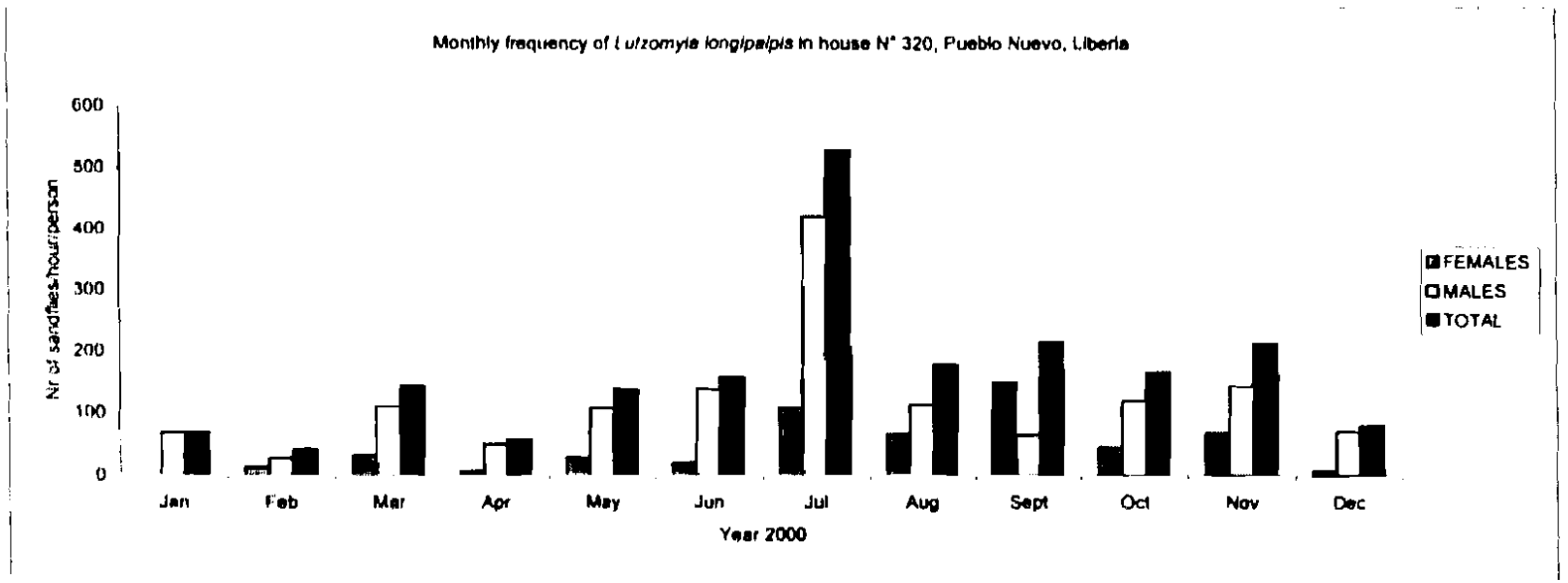
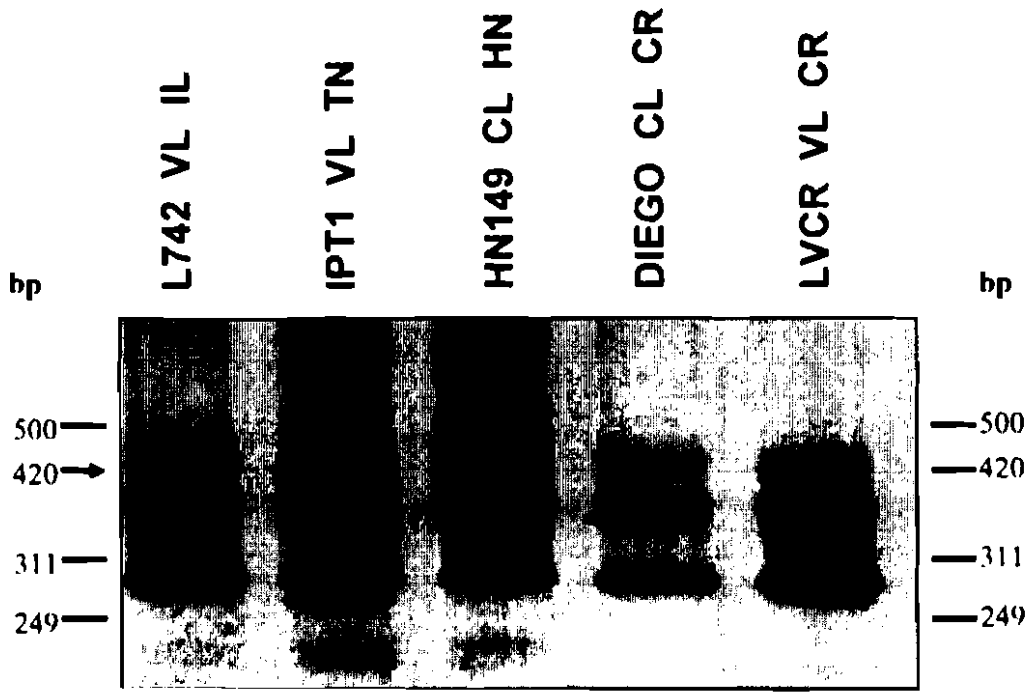


Fig 5.

* PPIP - PCR profiles of Costa Rican strains



* PPIP - Permissively Primed Intergenic Polymorphic PCR

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Table 1: Distribution of cases of cutaneous leishmaniasis caused by *Leishmania infantum* in the town of Nazareth, Liberia, Costa Rica.

Area	Cases (**)		Households		Inhabitants	
	No.	%	No.	%	No.	%
North	64	91.43	142	43.16	773	43.48
South	6	8.57	187	56.84	1,005	56.52
Total	**70**	**100.00**	**329**	**100.00**	**1,778**	**100.00**

(**) significant at 99% level
t-test for equality of means (North vs. South)

Table 2: Distribution of domestic animals in the town of Nazareth, Liberia, Costa Rica, and comparison of the North and South areas of the town

Animal	North Area		South Area		Total		
	No.	Animals / house	No.	Animals / house	No.	Animals / house	
Chickens	411	2.461	310	1.455	721	2.191	(*)
Cats	41	0.246	17	0.080	58	0.176	(**)
Cows	0	0.000	12	0.056	12	0.036	
Dogs	121	0.725	134	0.629	255	0.775	
Ducks	32	0.192	59	0.277	91	0.277	
Goats	1	0.006	4	0.019	5	0.015	
Pigs	17	0.102	13	0.061	30	0.091	

(*) significant at 95% level
(**) significant at 99% level
t-test for equality of means (North vs South)

Table 3: Comparison of the two areas in relation to households with 10 or more chickens and the presence of chicken coops with 10 or more chickens

Condition	North Area			South Area		
	Houses	Chickens	Mean	Houses	Chickens	Mean
Households with 10 or more chickens (*)	14	268	19.14	12	177	14.75
Chicken coops with 10 or more chickens (**)	12	241	20.08	10	132	13.20

(*) Significant at 95% level
(**) Significant at 99% level
t-test for equality of means (North vs. South)

Table 4: O.R. for potential risk factors in relation to the presence of cutaneous leishmaniasis cases caused by *Leishmania infantum* in two areas of the town of Nazareth, Liberia, Costa Rica

Condition	Both Areas		North Area	
	O.R.	C.I.	O.R.	C.I.
Houses with 6 or more persons	2.32	(1.09 - 4.98)*	2.20	(0.97 - 5.13)*
Persons with more than 5 years in the area	3.30	(1.49 - 7.47)**	3.55	(1.46 - 8.78)**
Presence of letrines	0.37	(0.15 - 0.89)*	0.42	(0.15 - 1.16)^{x}
Presence of pigs	4.29	(1.02 - 16.92)*	3.50	(0.67 - 17.80)^{x}
Presence of dogs	2.30	(1.04 - 5.17)*	2.10	(0.87 - 5.30)^{x}

(^{x}) Siginificant at 90% level
(*) Significant at 95% level
(**) Significant at 99% level
Chi-Square test

Table 5: **Resultados de las pruebas Chi-Cuadrado y Exacta de Fisher para la relación entre la presencia de casos y diferentes rangos para la cantidad de gallinas. O.R. y sus límites de confianza al 95%**

Ranks			Significance		O.R.					
			Chi-Square	Fisher Exact	Lower	O.R.	Upper			
Menos de 5	-	5 y más	0.396	0.360	0.619	1.438	3.340	5	0.05	1
Menos de 6	-	6 y más	0.393	0.452	0.605	1.470	3.572	6	0.05	1
Menos de 7	-	7 y más	0.929	1.000	0.317	0.951	5.855	7	0.05	1
Menos de 8	-	8 y más	0.867	0.778	0.364	1.099	3.323	8	0.05	1
Menos de 9	-	9 y más	0.595	0.537	0.442	1.354	4.142	9	0.05	1
Menos de 10	-	10 y más	0.486	0.514	0.483	1.488	4.582	10	0.05	1
Menos de 11	-	11 y más	0.649	0.717	0.376	1.343	4.798	11	0.05	1
Menos de 12	-	12 y más	0.518	0.459	0.422	1.522	5.494	12	0.05	1
Menos de 13	-	13 y más	0.272	0.231	0.553	2.059	7.663	13	0.05	1
Menos de 14	-	14 y más	0.272	0.231	0.553	2.059	7.663	14	0.05	1
Menos de 15	-	15 y más	0.218	0.200	0.599	2.254	8.432	15	0.05	1
Menos de 16	-	16 y más	0.087	0.115	0.793	3.132	12.373	16	0.05	1
Menos de 17	-	17 y más	0.087	0.115	0.793	3.132	12.373	17	0.05	1
Menos de 18	-	18 y más	0.033	0.068	1.006	4.206	17.589	18	0.05	1
Menos de 19	-	19 y más	0.033	0.068	1.006	4.206	17.589	19	0.05	1
Menos de 20	-	20 y más	0.033	0.068	1.006	4.206	17.589	20	0.05	1

Table 6

Total number of *Lutzomyia longipalpis* obtained from several captures in short periods of time during year 2000.

	N°	%
Males	1160	85.5
Females	196	14.5
Fed females	82	41.8
Gravid females	9	4.6
Total	1356	100.0

Table 7

Sand fly species captured during monthly density experiments in House 140a of the town Nazareth, Liberia, Costa Rica, during 1999-2000

Species	N°	%	Males	%	Females	%
Lutzomyia longipalpis	506	92.5	460	90.1	46	9.9
Lutzomyia cayennensis	30	5.5	20	66.6	10	33.3
Lutzomyia chiapanensis	4	0.7	3	75.0	1	25.0
Lutzomyia shannoni	7	1.3	6	85.7	1	14.3
TOTAL	547	100.0	489	89.39	58	10.6

Table 8

A

Nutritional status of 375 children (174 girls and 201 boys) under 6 years of age in Nazareth, Liberia, Costa Rica using the parameter weight/age (w/a).

Status	N° children	%
Normal	177	47.1
Mild malnutrition	125	33.3
Moderate malnutrition	16	4.3
Severe malnutrition	1	0.3
Overweight	53	14.3
Obesity	3	0.8
Total	375	100.0

B

Nutritional status of 224 children (103 girls and 121 boys) under 6 years of age in Pueblo Nuevo, Liberia, Costa Rica, using the parameter weight / age (w/a).

Status	N° of children	%
Normal	117	52.2
Mild malnutrition	60	26.8
Moderate malnutrition	5	2.2
Severe malnutrition	0	0
Over weight	40	17.9
Obesity	2	0.9
Total	224	100.0

TABLE 9

Infectivity and dissemination of:
(A) strain MHOM/HN/1987/HN-029 from a Honduran human case of visceral leishmaniasis;
(B) strain MHOM/HN/1993/HN-194 from a Honduran human case of cutaneous leishmaniasis.

A

Time of sacrifice	**4 days**				**Week 4**				**Week 8**				**Week 10**			
Treatment	C *		NC		C		NC		C		NC		C		NC	
Organ	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm
Spleen	-	+[a]	-	+[a]	-	-	-	-	-	-	-	+/-[c]	-	+[a]	-	-
Liver	-	+[a]	-	+[a]	-	-	-	-	-	-	-	+[a]	-	+/-[a]	-	-
Skin	x	+[a]	x	-	x	-	x	-	x	-	x	-	-	-	x	-
Nasal Aspirate	-	nd	-	nd	-	nd	-	nd	-	nd	-	nd	-	nd	-	-

B

Organ	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm
Spleen	-	-	-	-	-	-	-	-	†		-	-	†		x	-
Liver	-	-	-	-	-	-	-	-			-	-			?	-
Skin	+	+[p]	+	+[p]	+	-	+	-			+	-			?	-
Nasal Aspirate	+	nd	+	nd	+	nd	+	nd			+	nd			?	nd

*Key: C = cortisone; NC = cortisone; cu = culture; sm = smear; [c] = suggestive forms; [a] = amastigotes; [p] = promastigotes; nd = not done; x = contaminated; [c] = parasites did not survive one week; † = hamster died

TABLE 10

Infectivity and dissemination of:
(A) strain MHOM/CR/1998/Dixon from a Costa Rican human case of visceral leishmaniasis;
(B) strain MHOM/CR/1999/Luismi from a Costa Rican human case of cutaneous leishmaniasis.

A

Time of sacrifice	4 Days				Week 4				Week 8				Week 10			
Treatment	C*		NC		C		NC		C		NC		C		NC	
Organ	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm
Spleen	-	-	+	-	-	+/-[c]	-	-	-	+/-	-	-	-	+	-	+[a]
Liver	-	-	-	-	-	-	-	-	-	+	-	+	-	+/-	-	+[a]
Skin	+	3+[p]	2+	+[a]	+	+	4+	-	-	-	-	-	x	+[a]	x	2+[a]
Nasal Aspirate	+	nd	4+	nd	-	nd	2+	nd	-	nd	-	nd	-	nd	-	nd

B

Organ	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm	cu	sm
Spleen	-	+[a]	-	+[a]	-	+/-[c]	-	-	-	-	-	+/-	-	+	-	+/-
Liver	-	+[a]	-	+[a]	-	-	-	-	-	+[a]	-	+[a]	-	2+[a]	-	2+[a]
Skin	x	-	-	+[a]	-	-	-	-	x	-	x	-	-	-	-	-
Nasal Aspirate	- nd		nd		-	+[d]	nd	-	nd	-	nd	-	nd	-	nd	- nd

*Key: C = cortisone; NC = cortisone; cu = culture; sm = smear; [c] = suggestive forms; [a] = amastigotes; [p] = promastigotes; nd = not done; x = contaminated; [c] = parasites did not survive one week; † = hamster died