

**THE EPIDEMIOLOGY OF VISCERAL
LEISHMANIASIS IN TERESINA, PIAUI STATE,
BRAZIL, WITH SPECIAL EMPHASIS ON THE
DIAGNOSIS AND TRANSMISSIBILITY OF
CANINE INFECTION**

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ABSTRACT

Visceral leishmaniasis (VL) is endemic in the semi-arid region of north-eastern Brazil. The causative agent is *Leishmania chagasi* (*infantum*). The domestic, peri-domestic and anthrophilic sandfly vector is *Lutzomyia longipalpis*. The domestic dog may be the principal reservoir of infection in endemic urban regions. The primary aim of this project was to study comparative diagnosis and transmissibility of natural and experimental canine VL in the city of Teresina, Piaui state, Brazil.

Most (67 %) of human cases of VL in Teresina occurred in children under 5 years of age, adult VL predominated in males, and there was a seasonal increase in incidence of cases. No phenotypic diversity was found between *L. chagasi* isolates from dogs and humans, although limited comparisons were performed.

Clinical diagnosis, parasitological diagnosis and serology were compared in a cohort of 209 dogs, comprised of both symptomatic and asymptomatic animals. Presence of clinical symptoms was not sufficiently sensitive to diagnose canine VL: only 42 % of dogs positive by reference standard criteria (RS positive) were symptomatic. Parasitology was less sensitive than serology for the diagnosis of canine VL and no parasitological test showed more than 60 % sensitivity in comparison with RS criteria. The indirect fluorescent antibody test (IFAT) and DOT-enzyme linked immunosorbent assay (DOT-ELISA) were the most sensitive of the serological assays tested. The direct agglutination test (DAT) was highly specific but lacked sensitivity. Serum samples were more sensitive than filter paper blood

spot samples. The Lmet2 DNA probe was generally less sensitive than traditional parasitological and serological methods for diagnosis of canine VL, although the probe was useful for screening sandflies for *L. chagasi* infections (below). The chance of demonstrating parasites in canine VL increased with the serological titre. Nevertheless, parasitologically positive dogs could be found among those that were serologically negative.

Large numbers of *Lu. longipalpis* were found in pigsties and chicken houses in the city of Teresina. The Lmet2 probe was shown to be effective for determining prevalence rates of *L. chagasi* infection in wild caught sandflies. Prevalence of natural infection in sandflies was particularly high when flies were caught in kennels where there were dogs with disseminated cutaneous infections. Experimental studies demonstrated that *Lu. longipalpis* could be very readily infected with *L. chagasi* by feeding on dogs with canine VL and that transmissibility was associated with amastigote infection of the skin. Altered skin of symptomatic dogs was more infective than normal skin of symptomatic animals. Although symptomatic animals were more infective than asymptomatic animals, asymptomatic dogs with normal skin were still infective to large numbers of sandflies and asymptomatic dogs cannot, therefore, be excluded as a significant reservoir of infection.

Transmission of experimental canine VL was demonstrated by a single infective sandfly bite. In a cohort of 25 experimental animals many dogs developed discrete, self-curing, cutaneous lesions, typical of cutaneous leishmaniasis. Seroconversion was the most sensitive test for canine VL, but seronegativity was not a reliable indicator of the absence of infection. Bone

marrow positivity was only seen in dogs that were serologically positive. Apparent recovery from *L. chagasi* infection was seen, with serological reversion.

Aminosidine, dependent on dose, duration of treatment and clinical status of the infected animal, was shown to be capable of producing clinical recovery and clinical cure in a small proportion of infected dogs, but could not be recommended as a systematic method of control.

Single applications of ultra-low volume pyrethroid insecticide to individual animal pens was not effective for controlling *Lu. longipalpis*. Nevertheless, pyrethroid insecticides had a high residual activity against *Lu. longipalpis* when sprayed on to the walls of animal enclosures. Lambda cyhalothrine (ICON) was the most effective of three pyrethroid insecticides tested in the laboratory against *Lu. longipalpis*.

Overall, this project has produced unique observations on canine VL, supports the fundamental role of the dog as a reservoir host, and explains why culling of seropositive dogs is likely to have limited impact as a disease control strategy.

DEDICATION

to you Cássia,

for your perseverance, courage, presence and mainly for

assuming the role of the father in my absence

to

Stephane,

Alessandra,

Isabella,

Andrea Luana,

and

Carina,

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Finally to my parents,

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LIST OF ABBREVIATIONS

4ClIN	4- chloro-1 naphthol
ACA	e-aminocaproic acid
AIDS	acquired immunodeficiency syndrome
BM	bone marrow
BR	Brazil
CaCl ₂	calcium chloride
Cat.	catalogue
CB	coating buffer
CDC	Centre for Disease Control
CFT	complement fixation test
CL	cutaneous leishmaniasis
cm ²	centimetre square
DAB	3,3'-diaminobenzidine
DAT	direct agglutination test
DCL	diffuse cutaneous leishmaniasis
DM	deltamethrin
DNA	deoxyribonucleic acid
dot-ELISA	dot enzyme-linked immunosorbent assay
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
FAST-ELISA	fast enzyme linked immunosorbent assay
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FNS/NHF	National Health Foundation
Gm	giemsa staining
H ₂ O	distilled water
H ₂ SO ₄	sulphuric acid
HDIC	Hospital de Doencas Infecto-contagiosas
hr	hour
ICON	lambda-cyhalothrin
IFAT	indirect fluorescent antibody test
IFN γ	gamma interferon
IHA	indirect haemagglutination test
IgG/M	immunoglobulin G/M
INFP	infectious period
IP	incubation period
K	potassium
KCl	potassium chloride
kDNA	kinetoplast DNA
KH ₂ PO ₄	potassium dihydrogen phosphate
LP	latent period
LSHTM	London School of Hygiene and Tropical Medicine
M	molar
MCL	mucocutaneous leishmaniasis

min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MCL	mucocutaneous leishmaniasis
MPI	mannose phosphate isomerase
N	number
Na ₂ HPO ₄	disodium hydrogen phosphate
Na ₂ N ₃	sodium azide
NaCl	sodium chloride
NaOH	sodium hydroxide
NH ₁	nucleoside hydrolase 1
nm	Nanometre
No.	number
OPD	o-phenylenediamine hydrochloride
PBS	phosphate buffer saline
PBS/M	phosphate buffer saline/milk powder
PBS/T	phosphate buffer saline/0.05 % Tween
PBS/T/M	phosphate buffer saline/0.05 % Tween /2 % milk powder
PCR	polymerase chain reaction
PCR-SHELA	polymerase chain reaction-solution hybridization enzyme linked assay
PE	permethrin
6PGD	6-phosphogluconate dehydrogenase (decarboxylating)
pH	hydrogen potential
PKDL	post kala-azar dermal leishmaniasis
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
rpm	revolutions per minutes
RS	reference standard
S	south
Sb	antimony (pentavalent)
SC	seroconversion
SOD	superoxide dismutase
SSC	sodium saline citrate
TBS	Tris-buffer solution
TBS/M	Tris-buffer Solution/2 % milk
TFN α	tumour necrosis factor alpha
UFP	Federal University of Piaui
ul	microlitre
ULV	ultra low volume
UV	ultraviolet
VL	visceral leishmaniasis
v/v	volume per volume
vs	versus (against)
W	west
w/v	weight per volume
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 History

More than four centuries have elapsed since the first description of clinical leishmaniasis in the nasal cavities of an indigenous inhabitant of the Andean region (Oviedo, 1535; Pizarro, 1571). Cunningham (1885) was the first to observe parasites inside the macrophage and Borovsky (1898) confirmed this discovery. Leishman (1903, 1904) found a similar intracellular parasite in the visceral organs of some fatal cases of kala azar from India, and recognized that the morphology was related to that of trypanosomes. In the same year, Donovan also saw similar organisms in patients from India.

Laveran and Mesnil named these parasites *Piroplasma donovani*, but Ross correctly and justly amended the name to *Leishmania donovani* in 1903 (in Lainson & Shaw, 1987; Killick Kendrick & Rioux, 1991; Elnaiem *et al.*, 1994).

In 1904 Rogers demonstrated the flagellate stage of *L. donovani* *in vitro* and Nicolle in 1908 described leptomonad forms cultured in blood agar medium (NNN).

The first records of leishmaniasis in America were by Lindenberg (1909) and Carini & Paranhos (1909), independently, in the south of Brazil. They found clinical cases of cutaneous leishmaniasis and considered that the infection might be due to *Leishmania tropica*, but Vianna (1911), found the organisms to be different from *L. tropica* and named them *L. braziliensis*.

The first record of autochthonous visceral leishmaniasis of man in the New World was by Migone, in Paraguay in 1913.

1.2 Taxonomy and life cycle

The leishmaniasis are caused by parasitic protozoa of the genus *Leishmania* and sandflies are the bridge between the reservoir and the new hosts: the infection is acquired by the bite of an infected sandfly.

Although the parasites of this genus are similar morphologically, there are several quite distinct species and different pathologies; basically, four clinical forms exist in man, namely cutaneous (CL) diffuse cutaneous (DCL) mucocutaneous (MCL) and visceral leishmaniasis (VL).

The classification can be summarized as follows:

Classification of the genus *Leishmania*

(Lainson and Shaw, 1987)

Kingdom	PROTISTA Haeckel, 1866
Sub-Kingdom	PROTOZOA Goldfuss, 1817
Phylum	SARCOMASTIGOPHORA Honigberg and Balamuth, 1963
Sub-Phylum	MASTIGOPHORA Deising, 1866
Class	ZOOMASTIGOPHOREA Calkins, 1909
Order	KINETOPLASTIDA Honigberg, 1963, emend. Vickerman, 1976
Sub-Order	TRYPANOSOMATINA Kent, 1880
Family	TRYPANOSOMATIDAE Doflein, 1901, emend. Grobden, 1905
Genus	<i>Leishmania</i> Ross, 1903

The taxonomy of *Leishmania* species is summarized in Tables 1.1 and 1.2.

Table 1.1

Taxonomy of the subgenus *Leishmania* Safjanova 1982, as recommended by
Lainson & Shaw (1987)

a. The *Leishmania* (*L.*) *donovani*-complex

<i>L. (L.) donovani</i>	(Laveran and Mesnil, 1903) Ross, 1903 (Old World)
<i>L. (L.) infantum</i>	Nicolle, 1908 (Old World)
<i>L. (L.) chagasi</i>	Cunha and Chagas, 1937 (New World)

Other possible species:

<i>L. (L.) archibaldi</i>	Castellani and Chalmers, 1919
<i>Leishmania (L.)</i> sp.	Kenya
<i>Leishmania (L.)</i> sp.	Eastern Pyrennes
<i>Leishmania (L.)</i> sp.	Italy
<i>Leishmania (L.)</i> sp.	Iraq
<i>Leishmania (L.)</i> sp.	China

Other species, outside the *donovani*-complex:

<i>L. (L.) tropica</i>	(Wright, 1903) Luhe, 1906
<i>L. (L.) aethiopica</i>	Bray, Ashford and Bray, 1973
<i>L. (L.) gerbilli</i>	Wang, Qu and Guan, 1974
<i>L. (L.) major</i>	Yakimoff and Schoklor, 1914 <i>emend.</i> Bray, Ashford and Bray, 1973
<i>Leishmania (L.)</i> sp.	Namibia
<i>Leishmania (L.)</i> sp.	Namibia
<i>Leishmania (L.)</i> sp.	Ethiopia

b. *Leishmania (L.) mexicana*-complex:

<i>L. (L.) mexicana</i>	Biagi 1953 <i>emend.</i> Garnham, 1962
<i>L. (L.) enriettii</i>	Muniz and Medina, 1948
<i>L. (L.) amazonensis</i>	Lainson and Shaw, 1972
<i>L. (L.) aristidesi</i>	Lainson and Shaw, 1979 <i>emend.</i> Lainson and Shaw, 1986
<i>L. (L.) venezuelensis</i>	Bonfante-Garrido, 1980
<i>Leishmania (L.)</i> sp.	Dominican Republic
<i>Leishmania (L.)</i> sp.	Belize, Central America

Other possible members:

<i>L. (L.) pifanoi</i>	Medina and Romero, 1959 <i>emend.</i> Medina and Romero, 1962
<i>L. (L.) garnhami</i>	Scorza <i>et al.</i> , 1979
<i>Leishmania (L.)</i> sp.	Trinidad
<i>Leishmania (L.)</i> sp.	Caratinga, Minas Gerais State, Brazil

c. *Leishmania (L.) hertigi*-complex

<i>L. (L.) hertigi</i>	Herrer, 1971
<i>L. (L.) deanei</i>	Lainson and Shaw, 1977

Table 1.2

**Taxonomy of the subgenus *Viannia*, n. subgen. as recommended by Lainson
& Shaw (1987)**

The *Leishmania (V.) braziliensis*-complex

<i>L. (L.) braziliensis</i>	Vianna, 1911 <i>emend.</i> Matta, 1916
<i>L. (L.) guyanensis</i>	Floch, 1954
<i>L. (L.) panamensis</i>	Lainson and Shaw, 1972
<i>L. (L.) peruviana</i>	Velez, 1913

Unnamed species of the *L. (L.) braziliensis*-complex

<i>Leishmania (V.)</i> sp.	Belize, Central America: in man
<i>Leishmania (V.)</i> sp.	Para State, Brazil (South of the Amazon River) <i>Cholepus didactylus</i>
<i>Leishmania (V.)</i> sp.	Itaituba, Para State, Brazil, in <i>Didelphis marsupialis</i>

Unnamed parasites of the subgenus *Viannia*

<i>Leishmania (V.)</i> sp.	Para State, Brazil, from <i>Lu. tuberculata</i>
<i>Leishmania (V.)</i> sp.	Para State, Brazil, from <i>Psychodopygus</i> sp.
<i>Leishmania (V.)</i> sp.	Para State, Brazil, from <i>Lu. ubiquitalis</i>
<i>Leishmania (V.)</i> sp.	Para State, Brazil, from <i>Dasypus novemcinctus</i>

The genus *Leishmania* has two principal morphological forms, the promastigote and the amastigote.

The promastigotes are found naturally in the sandfly midgut (Killick-Kendrick *et al.*, 1974) and have single free flagellum. These forms can also be found in culture medium.

Killick-Kendrick (1979) recently reported other forms in the sandfly, including the **paramastigote**, in which the kinetoplast is not anterior to the nucleus and appears to be alongside it.

The amastigotes are apparently aflagellate, ovoid forms. Although the amastigotes of *Leishmania* of various species found in mammal hosts are frequently reported to be morphologically identical, different amastigotes morphologies have been described by some authors (Parrot *et al.*, 1932; Kellina, 1962). However, the most significant observations in this context were made by Shaw and Lainson (1976) in which they demonstrated consistent differences between *Leishmania (L.) amazonensis* and *L.(V.) braziliensis*, with the former being larger than the second in giemsa-stained smears. When stained with giemsa, the amastigotes are seen as rounded or oval intracellular bodies or, as extracellular if the cell has ruptured.

The nucleus is apparently round or oval and the kinetoplast is "bacilliform".

When the female sandfly (Diptera, Phlebotomidae, Phlebotominae) takes an infected bloodmeal from a host infected with *Leishmania* after 72 hr the amastigotes elongate and begin to form the flagellar pocket from which the flagellum will emerge. Within 96 hr of infection, the promastigotes are totally formed and in active reproduction by binary fission, and invading different parts of the insect gut. A further transformation, occurs when the parasites migrate to the pharynx 5 to 6 days after infection, where the formation of the infective stage takes place, which is known as the metacyclic form. Some of these forms may

migrate to the lumen of the proboscis. In subsequent bloodmeals these forms are injected into the next vertebrate host.

In the vertebrate, some promastigotes survive the immunological response of the host. They lose their flagellum and transform to amastigotes inside macrophages where they begin a new cycle of multiplication. The sequence of the development of infection in the sandfly and in other mammalian hosts is summarized in **Figure 1.1**.

1.3 The epidemiology of the leishmaniasis

The leishmaniasis are cosmopolitan diseases, which are present on all the continents except Australasia and Antarctica. They, occupy second place of importance in the six tropical diseases selected by the WHO as major public health problems. (Modabber, 1987). Besides the medical importance, the leishmaniasis can also cause serious economic problems, mainly in countries from the third world, which already have several other disease problems.

Parasites of different complexes, with respective vector-reservoir associations, are responsible for four main clinical types of leishmaniasis, distributed geographically as summarized in **Tables 1.3; 1.4, and 1.5**).

1.4 Visceral leishmaniasis

Viscerotropism is characteristic almost exclusively of parasites of the *L.donovani*-complex, although exceptionally *Leishmania (L.) amazonensis* and *Leishmania (V.) braziliensis*, in the New world, may produce visceralization (Barral *et al.*, 1991; Hernandez *et al.*, 1993; Almeida *et al.*, 1996). The geographical distribution of visceral disease is shown in **Figure 1.2**.

Figure 1.1

(1) Delivery of promastigotes into host skin by the bite of the sandfly vector; (2) attachment and engulfment by phagocytosis of promastigotes, by a macrophage; (3) fusion of phagosome containing a promastigote with lysosome in macrophage; (4) differentiation of promastigote into amastigote in the phagolysosome of the infected macrophage; (5,6) multiplication of the amastigote inside the macrophage; (7) rupture of the parasitized macrophage and uptake of amastigotes by a new macrophage; (9) ingestion of parasitized macrophage by a sandfly after blood meal taken from infected human host or reservoir animal; (10-16) development of promastigotes in the sandfly vector; (13) paramastigote forms.

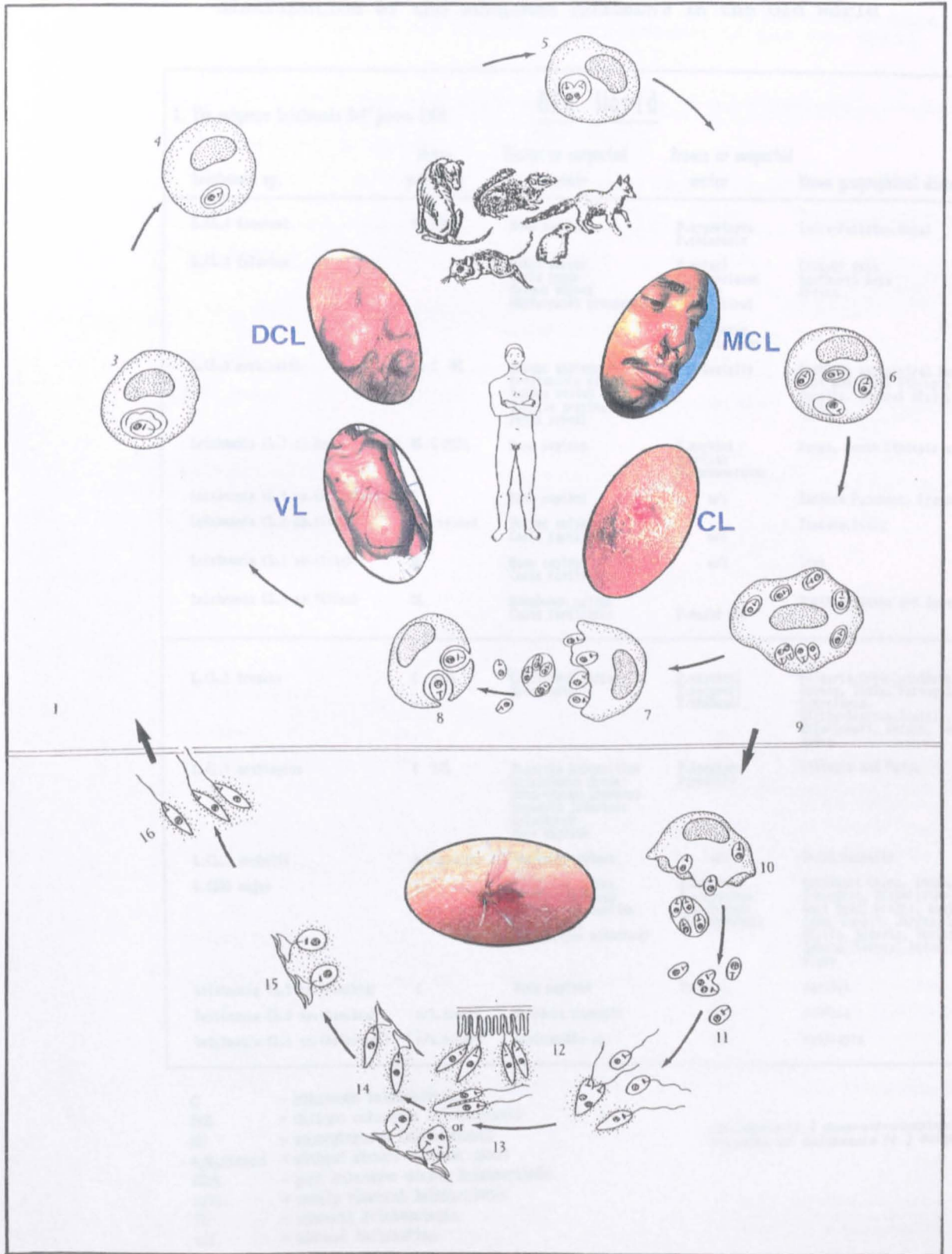


Figure 1.1

Life cycle of *Leishmania*

Table 1.3

Clinical aspects, reservoirs, vectors and known geographical distribution of the subgenus *Leishmania* in the Old World

1. The subgenus <i>Leishmania</i> Saf'janova 1982				
<u>Old World</u>				
<i>Leishmania</i> sp.	Human pathology	Proven or suspected reservoir	Proven or suspected vector	Known geographical distribution
<i>L.</i> (<i>L.</i>) <i>donovani</i>	UL PKDL	<i>Homo sapiens</i>	<i>P. argentipes</i> <i>P. chinensis</i>	India-Pakistan, Nepal
<i>L.</i> (<i>L.</i>) <i>infantum</i>		<i>Canis aureus</i> <i>Canis lupus</i> <i>Vulpes vulpes</i> <i>Nyctereutes procyonoides</i>	<i>P. ariasi</i> <i>P. perniciosus</i> <i>P. major</i> <i>P. perilliewi</i> <i>P. tobii</i> <i>P. chinensis</i> <i>P. perniciosus</i>	Central Asia Southwest Asia Africa
<i>L.</i> (<i>L.</i>) <i>archibaldi</i>	UL C MC	<i>Acomyx albigens</i> <i>Arvicanthus niloticus</i> <i>Rattus rattus</i> <i>Genetta genetta</i> <i>Felis serval</i>	<i>P. orientalis</i>	Southern and central Sudan East and South Ethiopia Somalia, Central Africa Republic Congo
<i>Leishmania</i> (<i>L.</i>) sp. Kenya	UL C PKDL	<i>Homo sapiens</i>	<i>P. martini</i> <i>P. celiae</i> <i>P. vansomerena</i>	Kenya, south Ethiopia and Somalia
<i>Leishmania</i> (<i>L.</i>) sp. (E. Pyrenees)	C	<i>Homo sapiens</i>	w/i	Eastern Pyrenees, France and Spain
<i>Leishmania</i> (<i>L.</i>) sp. (Italy)	n/h. record	<i>Vulpes vulpes</i> <i>Canis familiaris</i>	w/i	Tuscany, Italy
<i>Leishmania</i> (<i>L.</i>) sp. (Iraq)	UL	<i>Homo sapiens</i> <i>Canis familiaris</i>	w/i	Iraq
<i>Leishmania</i> (<i>L.</i>) sp. (China)	UL	<i>Rhombomys opimus</i> <i>Canis familiaris</i>	<i>P. major wui</i>	Northern Gansu and Inner Mongolia
<i>L.</i> (<i>L.</i>) <i>tropica</i>	C r/UL	<i>Canis familiaris</i> <i>Meles meles</i>	<i>P. papatasi</i> <i>P. sergenti</i> <i>P. chabaudi</i>	Bulgaria, Crete, southern France, Greece, Italy, Portugal, Spain, and Yugoslavia. Africa-Algeria, Tunisia, Morocco Asia-Israel, Jordan, Iran, Iraq India
<i>L.</i> (<i>L.</i>) <i>aethiopia</i>	C DCL	<i>Procapra habessinica</i> <i>Heterohyrax brucei</i> <i>Dendrohyrax arboreus</i> <i>Procapra johnstoni</i> <i>Cricetomys</i> <i>Homo sapiens</i>	<i>P. longipes</i> <i>P. pedifer</i>	Ethiopia and Kenya
<i>L.</i> (<i>L.</i>) <i>gerbilli</i>	n/h. record	<i>Rhombomys opimus</i>	w/i	South Mongolia
<i>L.</i> (<i>L.</i>) <i>major</i>	C	<i>Rhombomys opimus</i> <i>Meriones libycus</i> <i>Hemitechnus auritus</i> <i>Lepus tolai</i> <i>Arvicanthus niloticus</i>	<i>P. papatasi</i> <i>P. caucasicus</i> <i>P. andrejevi</i> <i>P. mongolensis</i>	Northwest China, India, Pakistan Turkmenia, Afghanistan, north and east Saudi Arabia, southwest Iran Iraq, Kuwait, Jordan, Libya, Israel Africa, Algeria, Tunisia, Ethiopia Sahara, Senegal, Sudan, Kenya, Mali Niger
<i>Leishmania</i> (<i>L.</i>) sp. (Namibia)	C	<i>Homo sapiens</i>	<i>P. rossi</i>	Namibia
<i>Leishmania</i> (<i>L.</i>) sp. (Namibia)	n/h. record	<i>Procapra capensis</i>	w/i	Namibia
<i>Leishmania</i> (<i>L.</i>) sp. (Ethiopia)	n/h. record	<i>Arvicanthus</i> sp.	w/i	Ethiopia

C = cutaneous leishmaniasis
 DCL = diffuse cutaneous leishmaniasis
 MC = mucocutaneous leishmaniasis
 n/h. record = without record of human cases
 PKDL = post kala-azar dermal leishmaniasis
 r/VL = rarely visceral leishmaniasis
 VL = visceral leishmaniasis
 w/i = without information

Leishmania (*L.*) *donovani*-complex?
 Outside of *Leishmania* (*L.*) *donovani*-complex

Table 1.4

Clinical aspects, reservoirs, vectors and known geographical distribution of the subgenus *Leishmania* in the New World

1. The subgenus <i>Leishmania</i> Saf'janova 1982				
<u>New World</u>				
<i>Leishmania</i> sp.	Human Pathology	Proven or suspect reservoir	Proven or suspected vector	Known geographical distribution
<i>L.(L.) chagasi</i>	VL	<i>Lycalopex vetulus</i> <i>Cercocyon thous</i> <i>Canis familiaris</i> <i>Didelphis marsupialis</i>	<i>Lu.longipalpis</i> <i>Lu. evansi</i>	Argentina, Bolivia, Brazil, Colombia, Ecuador, Paraguay, Venezuela, Guatemala, Guadalupe, Honduras, Martinique, Mexico and El Salvador. Colombia Colombia
<i>L.(L.) mexicana</i>	C DCL r/MC	<i>Ototylomys phyllotis</i> <i>Myotomys sunschristi</i> <i>Signodon hispidus</i>	<i>Lu.olmeca olmeca</i>	Yucatan Peninsula, Belize, Guatemala, southern Texas, and northern Mexico
<i>L.(L.) enrietti</i>	non infec/h	<i>Cavia porcellus</i>	<i>Lu.monticola</i>	Parana State, Brazil
<i>L.(L.) amazonensis</i>	C DCL	<i>Proechimys</i> sp. <i>Oryzomys capito</i> , <i>O.concolor</i> , <i>O.maconelli</i> <i>Neacomys spinosus</i> , <i>Nectomys squamipes</i> , <i>Dasyprocta prymnolopha</i> <i>Marwosa murina</i> , <i>M.cinerea</i> <i>Metachirus nudicaudata</i> , <i>Didelphis marsupialis</i> , <i>Philander opossum</i> and <i>Cercocyon thous</i>	<i>Lu.flaviscutellata</i>	The Amazon Basin, Bahia State, Brazil
<i>L.(L.) aristidesi</i>	n/h.record	<i>Oryzomys capito</i> , <i>Proechimys semispinosus</i> , <i>Dasyprocta punctata</i> and <i>Marwosa robinsoni</i>	w/i	The Sardi, Panama.
<i>L.(L.) venezuelensis</i>	C	<i>Homo sapiens</i>	w/i	Barquisimeto, State of Lara, Venezuela
<i>Leishmania (L.)</i> sp.	DCL	<i>Homo sapiens</i>	w/i	The Dominican Republic
<i>Leishmania (L.)</i> sp.	w/i	<i>Homo sapiens</i>	w/i	Belize, Central America
<i>L.(L.) pifanoi</i>	C DCL	<i>Heteromys anomalus</i>	<i>Lu.flaviscutellata</i>	The State of Yaracuy, Lara and Miranda, Venezuela
<i>L.(L.) garnhami</i>	C	<i>Didelphis marsupialis</i>	<i>Lu.townsendi</i>	The Venezuelan Andes
<i>Leishmania (L.)</i> sp.	C	<i>Oryzomys capito</i> , <i>Proechimys</i> sp., <i>Heteromys anomalus</i> , <i>Caluromys philander</i> , <i>Marwosa fuscata</i> and <i>H.mitis</i>	<i>Lu.flaviscutellata</i>	Trinidad, West Indies
<i>Leishmania (L.)</i> sp.	C	<i>Homo sapiens</i>	w/i	Uale do Ribeiro, Sao Pulo State, southern Brazil.
<i>Leishmania (L.)</i> sp.	n/h.record	<i>Proechimys dimidiatus</i>	w/i	Caratinga, Minas Gerais, Brazil
<i>Leishmania (L.)</i> sp.	w/i	<i>Homo sapiens</i>	w/i	Caratinga, Minas Gerais, Brazil
<i>L.(L.) hertigi</i>	n/h.record	<i>Coendou rothschildi</i>	w/i	Panama and Costa Rica
<i>L.(L.) deanei</i>	n/h.record	<i>Coendou prehensilis</i>	<i>Lu.furcata</i>	Piaui and Para State, Brazil

C = cutaneous leishmaniasis
 DCL = diffuse cutaneous leishmaniasis
 MC = mucocutaneous leishmaniasis
 n/h.record = without record of human cases
 PKDL = post kala-azar dermal leishmaniasis
 r/MC = rarely mucocutaneous leishmaniasis
 w/i = without information

Leishmania (L.) donovani-complex
Leishmania (L.) mexicana-complex
Leishmania (L.) hertigi-complex

Table 1.5

Clinical aspects, reservoirs, vectors and know geographical distribution of the subgenus *Viannia*

<u>New World</u>				
2. The subgenus <i>Viannia</i> n.subgenus				
<i>Leishmania</i> sp.	Human pathology	Proven or suspected Reservoir	Proven or suspected Vector	Known geographical distribution
<i>L.(V.) braziliensis</i>	C CH	<i>Proechinus</i> sp., <i>Thipidomys leucodactylus</i> , <i>Rattus rattus</i> , <i>Bidelphis marsupialis</i> , <i>Choloepus didactylus</i> , <i>Oryzyomys concolor</i> , <i>O. capito</i> , <i>O. nigripes</i> , <i>Alouatta trivulgioides</i> and <i>Canis familiaris</i> .	<i>Lu.wellooni</i> , <i>Lu.intermedia</i> , <i>Lu.whitmani</i> , <i>Lu.nisonei</i> , <i>Lu.pessoal</i>	In Brasil: States of Minas Gerais, Rio de Janeiro, Espirito Santo, Ceara, Bahia, Sao Paulo, Para and Bahia. In Venezuela: State of Lara
<i>L.(V.) guyanensis</i>	C m.lesions	<i>Choloepus didactylus</i> , <i>Tanayana tetradactyla</i> , <i>Proechinus</i> sp. and <i>Bidelphis marsupialis</i>	<i>Lu.uebratilis</i> , <i>Lu.whitmani</i> , <i>Lu.ondzei</i>	north of river Amazonas, Guyana, Surinam, French Guyana, and Brazil in Amapa, north Para, Amazonas State and possibly Roraima
<i>L.(V.) panamensis</i>	C s.lesion	<i>Choloepus hoffmanni</i> , <i>Oryzyomys infescatus</i> , <i>Passaricodon gabilli</i> , <i>Nasua nasua</i> , <i>Potos flavus</i> , <i>Notus trivirgatus</i> and <i>Sanguinus geoffroyi</i>	<i>Lu.trapidoi</i> , <i>Lu.glephiletor</i> , <i>Lu.gomezi</i> and <i>Lu.panamensis</i>	Panama, Costa Rica and Colombia
<i>L.(V.) peruviana</i>	C CH	<i>Canis familiaris</i>	<i>Lu.peruvensis</i> and <i>Lu.verrucarum</i>	Peruvian Andes and north of Argentinian
<i>Leishmania (V.) sp.</i>	C	<i> homo sapiens</i>	w/i	Southern Belize, Central America; Honduras
<i>Leishmania (V.) sp.</i>	n/h.record	<i>Choloepus didactylus</i>	w/i	Para State and south of Amazonas River, Brazil
<i>Leishmania (V.) sp.</i>	n/h.record	<i>Bidelphis marsupialis</i>	w/i	Itaituba-Altamira, Transamazon Highway, Para State, Brazil
<i>Leishmania (V.) sp.</i>	n/h.record	w/i	<i>Lu.tuberculata</i>	Utinga forest, Belem, Para State, north Brazil
<i>Leishmania (V.) sp.</i>	n/h.record	w/i	<i>Lu.sp.</i> , <i>Lu.davisi</i> <i>Lu.panamensis</i>	Serra dos Carajas, Para State, north of Brazil
<i>Leishmania (V.) sp.</i>	n/h.record	w/i	<i>Lu.ubiquitalis</i>	River Parapananema region, Serra dos Carajas, Para, north of Brazil
<i>Leishmania (V.) sp.</i>	n/h.record	<i>Basypus novencinctus</i>	<i>Lu.agrozai</i> , <i>Lu.paraensis</i>	In Para State, Monte Dourado (Rio Jari), north of Amazon River, Island of Karajo and Hoju on the mainland, Amazonas State, Brazil
<i>Leishmania (V.) sp.</i>	C	<i> homo sapiens</i>	w/i	Benevides, near Belem, Para State north Brazil
<i>Leishmania (V.) sp.</i>	C s.lesion	<i> homo sapiens</i>	w/i	Paragominas, Serra dos Carajas, Para State, north of Brazil

C = cutaneous leishmaniasis
 C.m.lesion = cutaneous leishmaniasis, multiple lesions
 C.s.lesion = cutaneous leishmaniasis, single lesion
 MC = mucocutaneous leishmaniasis
 n/h.record = without record of human cases
 w/i = without information

Leishmania (V.) braziliensis-complex

Figure 1.2

Distribution of visceralising *Leishmania* (redrawn from Lainson & Shaw, 1987). *Leishmania (L.) chagasi*: 1, Mexico; 2, Central America; 3, ? Guadeloupe, Martinique; 4, Colombia; 5, Venezuela; 6, Amazonia; 7, Brazilian hyperendemic focus. *Leishmania (L.) infantum*: 8, canine focus Oklahoma (? introduced); 10, Tunisia/Algeria; 11, circum-Mediterranean; 12, Chinese highlands. *Leishmania (L.) ssp.*: 9, Cevennes; 13, Sudan; 14, East African macrofocus; 15, Kenyan epidemic focus; 16, ? Kzyl Orda. *Leishmania (L.) donovani*: 17, Kashmir sporadic focus; 18, Indian epidemic focus; 19, Sinkiang visceral area; 20, Chinese epidemic area; 21, southern Chinese area (said to be extinct). ● , Sporadic human cases; ★ , canine cases only; ○ , presumed limits of endemic zone; ■ , low to moderate endemicity, ■ , High endemicity-endemic.

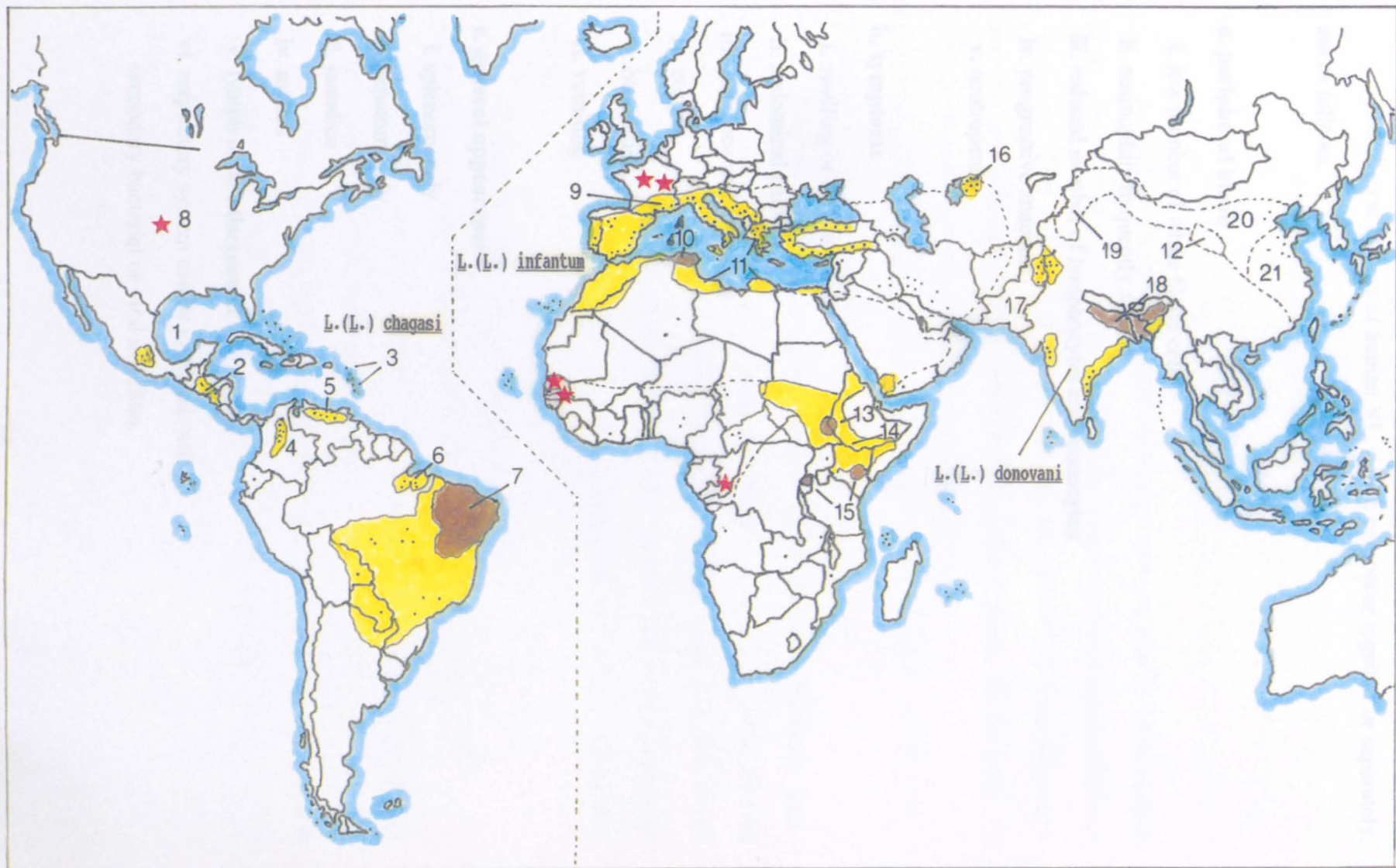


Figure 1.2

The clinical aspects of human VL, which can occur together or separately, are as follows:

a. peripheral blood

- i. low number of white blood cells
- ii. eosinophils frequently absent
- iii. reduced number of lymphocytes and monocytes
- iv. progressive anaemia
- v. neutropenia

b. symptoms

- i. swelling of the abdomen
- ii. abdominal pain
- iii. diarrhoea and dysentery
- iv. cough
- v. headache
- vi. vomiting

c. general appearance

- i. splenomegaly
- ii. hepatomegaly
- iii. jaundice
- iv. ascites
- v. lymph node enlargement
- vi. respiratory system can be involved, with secondary bacterial or viral infection.

vii. cardiovascular system, due to the development of anaemia, is indirectly affected.

viii. nervous system, joints and renal lesions, also can be involved.

Another aspect of VL, in some countries, is classical post-kala azar dermal leishmaniasis, (PKDL). Sometimes this occurs without previous manifestation of clinical VL. This pathology is characterized by the presence of hypo-pigmented macules, erythema and nodules distributed in different places on the body. In some patients the disseminated nodules resemble leprosy.

1.4.1 Visceral leishmaniasis in the Old World

a. Indian region and southwest Asia

Two large outbreaks are summarised in the scientific literature, cases between 1977 and 1982 and another 100,000 cases, with a mortality rate of 28.7%, in 1977 (Thakur *et al.* 1992). On both occasions, more than 60% of the cases were found in people between 10 and 29 years old. The mean age of cases may increase between outbreaks as herd immunity declines (Basu and Mallik, 1995).

VL in these regions has its own characteristics:

- i. the aetiological agent is *Leishmania (L.) donovani*.
- ii. beyond the classical clinical visceral aspect, post kala azar dermal leishmaniasis (PKDL) is found concomitantly.
- iii. the vectors, *P.argentipes* and *P.chinensis*, maintain an active transmission man-sandfly-man when they feed on infected blood. Here, the parasite can be demonstrated in the peripheral blood of man in more than 90% of human cases (Lainson and Shaw, 1987).
- iv. a non-human reservoir is not known.

b. Central Asia, countries of the Mediterranean region and Africa

In these regions the parasites have been isolated and classified as *Leishmania (L.) infantum* and *L. (L.) donovani* (in Africa). Although dogs are the major reservoir for human infection in urban areas, other *Canidae* were also found commonly infected and the disease in man is manifested mainly in children (infantile VL).

In Yugoslavia and Italy, where man is an accidental host, the domestic rodents, *Rattus rattus* and *R. norvegicus*, have epidemiological importance.

In some areas the parasites were isolated only from *Canidae*, without registered human cases, as in Congo, Somalia and Senegal.

Although *P. aiasi* and *P. perniciosus* are the principal vectors, others species have also been incriminated as vectors (**Table 1.1**)

1.4.2 Visceral leishmaniasis in the New world

The aetiological agent of American VL (AVL), is *Leishmania (L.) chagasi* and dogs, in urban areas, are considered to be the principal source of infection for the sandfly vector, *Lu. longipalpis*. This sandfly species has a coincidental distribution with the disease, and has also been found infected with promastigotes in highly endemic areas (Ferro *et al.*, 1995). *Lu. evansi* is a secondary vector in Colombia (Travi *et al.*, 1990).

This disease mainly affects juveniles from slums or poor people and a relationship with malnutrition has been suggested (Cerf *et al.*, 1987; Badaro *et al.*, 1985).

Geographically AVL is distributed in Argentina, Brazil, Bolivia, Colombia, Ecuador, Paraguay, Surinam, Venezuela, Guatemala, Honduras, Nicaragua, Mexico, El Salvador and Guadalupe (Lainson and Shaw, 1987).

1.5 Visceral leishmaniasis in Brazil

In Brazil, the first report of VL was by Penna (1934). He not only found amastigotes in 41 of 47,000 liver fragments, studied while investigating yellow fever, but he also described the first geographical distribution of the disease. Three years later, in 1937, Deane emphasized the presence of VL, describing the clinical aspects of new cases from Para State (Deane, 1938).

In 1938 Chagas *et al.* presented separate reviews on the clinical aspects and treatment of VL. In the same year Ferreira *et al.*, (1938) published the first studies on development of promastigotes in *Lu. longipalpis*.

A review of the scientific literature (Ward, 1977) showed that, of 3,762 human cases registered in South America up to 1976, 3,701, (98,3%), originated from Brazil.

Lu. longipalpis was definitely proven to be a vector only in 1977, when the first experimental transmission in the laboratory was obtained (Lainson *et al.*, 1977).

Today VL is reported mainly from the poorest states of the semi-arid northeast of Brazil (Ceara, Bahia, Piaui, Pernambuco, Rio Grande do Norte) and sporadically from Parnaiba, Alagoas, Sergipe, Goias, Mato Grosso, Espirito Santo and Para. In all the states where VL was or is epidemic, common features were or are, the association with the presence of infected dogs and abundant *Lu. longipalpis*, (Deane & Deane, 1954; Deane, 1956; Alencar, 1956; Alencar *et al.*, 1956; Sherlock & Almeida, 1969; Senra *et al.*, 1985; Alvim *et al.*, 1986 and Costa *et al.*, 1990). More than 90% of the cases of VL in Brazil have been reported from the north eastern region.

Although foxes (*Lycalopex vetulus*, *Cerdocyon thous*) and opossums (*Didelphis albiventris*) are occasionally found infected, infection rates of more than 20% have been recorded for dogs in endemic areas (Silveira *et al.*, 1982;

Lainson *et al.*, 1984). Lainson and Shaw (1987) and Lainson *et al.* (1990) demonstrated that *C. thous* could be infective to sandflies in the absence of any clinical signs of VL.

Leishmania (L.) chagasi, is the causative organism of VL in man, but recently Barral *et al.* (1986) isolated atypical (Almeida *et al.*, 1996) *Leishmania (L.) amazonensis* from the bone marrow of patients with symptoms typical of VL in Jacobina, Bahia State, and Hernandez *et al.*, (1993) isolated *Leishmania (V.) braziliensis* from one other VL patient.

1.5.1 Visceral leishmaniasis in Teresina, Piauí State

VL has been known in Piauí State since 1934 but in 1980 an epidemic occurred throughout the State, in which the principal focus was the urban area of Teresina, the State capital (Costa *et al.*, 1990). This author described the outbreak in 1984, in which the majority of cases affected children less than 10 years old.

1.6 Host immune response

Mitchell (1984) showed clearly that T cells were involved in resistance to infection, working with *L. major*. He used mouse strains resistant to *L. major*; after thymectomy the mice became susceptible. After transfer of small numbers of T cells, from non-infected syngeneic mice, the infection was rejected.

Studies by Wilson *et al.* (1987) with golden hamsters, demonstrated that after the intradermal inoculation of promastigotes, neutrophils and mononuclear phagocytes can ingest the parasites but only neutrophils appeared to eliminate the infection, and this was thought to occur due to production of hydrogen peroxide.

Another factor present in resistant mice, is increased amounts of IFN γ in the lymph nodes and spleen after administration of infective parasites (Locksley *et al.*, 1987; Heinzel *et al.*, 1989). The administration of neutralizing anti-IFN γ during the course of infection, induced appearance of spontaneous lesions in such resistant mice (Muller and Louis, 1989). IFN γ and TNF α act synergistically to

promote the action of nitric oxide synthase (NOS) which induces the production of toxic intracellular nitric oxide. The cytokines IL4 and IL10 have an opposite effect and the intracellular survival of *Leishmania* thus depends on cytokine balance, recovery from infection in mice being linked to γ IFN, IL2, IL12 and a Th1 type immune response.

A newly described factor that may be influence immune response in the leishmaniases is the composition of the saliva of *Lu.longipalpis*, which is said to enhance leishmanial infection in animals (Titus & Ribeiro, 1988). Furthermore substances in saliva can inhibit the ability of the macrophages to produce hydrogen peroxide in response to an activating stimulus from IFN γ (Theodos *et al.*, in press).

A description of infective or "metacyclic" promastigotes of *Leishmania (L.) donovani*, produced *in vitro*, was given by Howard *et al.* (1987). These metacyclic forms, already investigated by Killick-Kendrick (1979) in sandflies have a set of their own characteristics: they are resistant to lysis by complement from normal human and guinea-pig serum, can survive in normal human serum at 37^o C and they are more infective for BALB/c mice than promastigotes of the same strain at the logarithmic-phase.

In patients with active VL and DCL, there is a lack of cell-mediated immune response and for this reason the parasites multiply quickly and there is less production of lymphokines which mediate a range of inflammatory responses, consequently there is no delayed type hypersensitivity (DTH) (Wylter 1982; Haldar *et al.*, 1983; Ho *et al.*, 1983; Sacks *et al.*, 1987). As stated above, in mice, but not necessarily so clearly in patients, protection and recovery are associated with a Th1 type immune response rather than Th2 (Cillari *et al.*, 1995).

There is high production of non-specific immunoglobulin, especially IgG and specific anti *Leishmania* antibody during VL but these antibodies do not

appear to be protective. It is not clear, particularly in dogs infected by sandflies, how soon after infection antibodies are produced and can be detected (see Discussion). Presence of IgG1 in canine VL may be associated with progressive disease (Deplazes *et al.*, 1995).

1.7 Diagnosis

Diagnosis is the process of determining definitely whether or not the suspected disease is present. For VL, the first stage is to perform a differential clinical diagnosis, through which there may be suspicion of the presence of a case of VL but the conclusive and final decision usually depends on the demonstration of the parasite.

Parasitological diagnosis confirms that the parasite is present. In symptomatic patients parasitological methods can be very effective but these methods cannot be applied in the field and some, such as splenic puncture, can be hazardous when performed by persons without experience. Detection of parasite nucleic acids (DNA, RNA) is a new alternative to parasitological diagnosis but residual DNA from dead amastigotes could be mistaken for active infection.

Serological examination detects exposure to the infective agent but not necessarily active disease; it is therefore an indispensable tool, although not always sufficiently specific and sensitive.

1.7.1 Clinical diagnosis

Similar fever, splenomegaly, anaemia and skin alterations, present in symptomatic human VL and PKDL, can be found in several other diseases.

In the presence of a continuous fever, the neutropenia can be one way to exclude the most common diseases of tropical regions such as malaria, trypanosomiasis, brucellosis and tuberculosis. Splenomegaly and anaemia are also characteristic of other tropical diseases such as malaria, schistosomiasis mansoni.

systemic histoplasmosis. Stain and hypopigmentation, can suggest leprosy, syphilis or superficial mycosis (Manson-Bahr, 1987).

In regions where the dog is the principal reservoir symptomatic dogs infected with *Leishmania (L.) infantum* or *L.(L.) chagasi*, present clinically with anaemia, splenomegaly, depilation, cutaneous lesions, and long claws. (Millin *et al.*, 1975; Abranches *et al.*, 1991; Marzochi *et al.*, 1994).

In both situations, symptomatic humans or dogs, the conclusive diagnosis is the detection of the parasite. When suspect dogs are asymptomatic, parasitological or serological diagnosis may be indispensable indicators of which animals must be destroyed (or treated).

1.7.2 Parasitological diagnosis

Parasitological diagnosis of human VL requires traumatic and invasive procedures, in the form of bone marrow puncture and/or spleen or lymph node aspirations, for which hospitalization of the patient is usually recommended.

The samples collected are examined by microscopy of giemsa-stained smears or indirectly by inoculation of infected tissues into semi-solid blood agar or into susceptible animals such as the golden hamster (Evans, 1989a,b). Frequently, by giemsa-staining of spleen aspirates it is easier to find amastigotes than by examining samples from liver, lymph nodes or bone marrow (Ho *et al.*, 1948; Siddig *et al.*, 1988; Zijlstra *et al.*, 1992). By spleen puncture, however, the patients are subjected to a procedure that may have fatal consequences (Abdel-Hameed, 1993). Examination of white blood cell concentrates can be useful for diagnosis of VL in AIDS (Izri *et al.*, 1996).

Diagnosis of canine VL uses the same parasitological procedures as for human VL, and can be easier (Lanotte, 1975). In addition samples from dogs obtained by biopsy or scarification of the margins of ulcers or of skin that is

apparently normal, can reveal amastigotes by giemsa-staining (Lestoquard & Donatien, 1936; Abranches *et al.*, 1991; Vexenat *et al.*, 1994).

1.7.3 Molecular biological diagnosis

Molecular biological techniques are another tool which are being used to corroborate the diagnosis of various diseases.

In VL, the sensitive DNA probe(Lmet2) which is specific for the *L.donovani*-complex (Howard *et al.*, 1991) has been shown to detect parasite DNA in various tissues of naturally infected dogs from Brazil (Vexenat *et al.*, 1994) and in popliteal lymph node aspirates in dogs in Pakistan (Rab & Evans, 1995). Furthermore, this probe has been shown to detect less than 100 organisms in experimentally infected or wild-caught sandflies, from Brazil and Ethiopia (Howard *et al.*, 1991; Howard *et al.*, 1990). Recently this technique was used to detect naturally infected sandflies from the endemic area of Teresina, Piaui, Brazil (Results and Vexenat *et al.*, 1994).

The technique, has also been adapted to a non-radioactive chemiluminescent detection system, (Wilson *et al.*, 1992)

The polymerase chain reaction (PCR) is another way to diagnose leishmaniasis, with high sensitivity. By amplification of *L.donovani* kDNA from a tiny biopsy obtained by spleen aspiration, it was possible to diagnose some patients (Smyth *et al.*, 1992) and kDNA of *Leishmania (V.) braziliensis* was recognised by a PCR test on blood from a patient with a clinical history of cutaneous leishmaniasis 30 years before (Guevara *et al.*, 1993). The LMet2 probe has been adapted to a colorimetric PCR assay (Qiao *et al.*, 1995). In this system the PCR product is detected by solution hybridization enzyme linked immunosorbent assay (PCR-SHELA) with high sensitivity and specificity for the *Leishmania donovani* complex. Although these results were obtained with tissue samples from experimental mice, similar methodology has given excellent results

for detection of *Onchocerca volvulus* DNA in human samples and without cross reactions.

1.7.4 Serology

A principal characteristic of a patient with progressive VL is the production of large quantities of immunoglobulin, mainly IgG, during the course of the disease. The non-specific immunoglobulin, can be detected by non-specific precipitation, when the aldehyde test is applied (Napier, 1922).

Various technologies have become available with improved sensitivities and specificities for the detection of specific antibodies:

a. complement fixation-test (CFT) which basically consists of the indirect detection of antibodies.

i. the serum is titred previously and a fixed amount of *Leishmania* antigen is added. If the antibody is present immune complexes will be formed.

ii. complement is added to the mixture. If the complexes are present, the complement will be consumed.

iii. finally, sensitized red cells are added and if these cells are not lysed, this indicates that the complement was consumed and consequently anti-*Leishmania* antibodies were present. Chung and Chang (1951) demonstrated a high correlation between CFT positive and parasitologically positive patients.

Nussenzweig *et al.* (1957) used this methodology widely in a serological survey of canine VL from Fortaleza, Ceara, Brazil.

b. indirect haemagglutination (IHA) is the demonstration of the presence of antibodies, captured by red cells previously sensitised with a specific antigen. Manson-Bahr (1967) using this methodology found an association between IHA

positive and active cases of human VL but the level of antibodies decreased after recovery of the patients.

c. immunofluorescent antibody test (IFAT) here the antigen is fixed onto a microscope slide. Consecutive addition of antibody and the IgG anti-animal species antibody, conjugated with fluorescein, will form a complex that is easily visible through an immunofluorescence microscope.

Initially this technique was used by Shaw & Voller (1964). The authors, using sera from human VL, found cross reactions antigenically with other trypanosomatids. Although several authors have demonstrated some cross reactions with malaria, Chagas disease and Hansen's disease (Latif *et al.*, 1979) and the standardization of this methodology has been difficult, it is actually widely used in different countries.

d. enzyme linked immunosorbent assay (ELISA); the use of plates, previously sensitised with whole or soluble antigen, permits a sequence of successive adhesions: antigen; antibody; IgG anti-animal species antibody, conjugated with an enzyme, and finally, after addition of the substrate, there is an easily visible chromogenic reaction. The amount of antibody is measured comparatively by optical density using a spectrophotometer.

The advantage of this technique (Hommel *et al.*, 1978) is that significant cross reactions with malarial or trypanosomal sera were not found. Furthermore, ELISA can be performed with tiny blood spot samples collected from the tip of the finger onto filter paper and it can be applied in seroepidemiological surveys. Due to the high correlation with patients who are parasitologically positive, this technique like the DAT (below) has been considered a way of avoiding the use of spleen aspiration.

e. dot enzyme linked immunosorbent assay (dot-ELISA): the dot-ELISA, described by Pappas *et al.* (1983) is a visually-read ELISA that uses small volumes of parasite antigen "dotted" onto nitrocellulose membrane. The substrate produces a dark insoluble precipitate, which contrasts with the white colour of the nitrocellulose membrane. The author only observed cross reaction with sera from Chagas disease patients. Protein A conjugate, has a great ability to bind to IgG and IgM (Lagone, 1978). Recently, Jaffe & Zalis (1988) exploited this characteristic in the dot-ELISA test with a protein A/peroxidase conjugate, obtaining good results. In the same year Dr. Patrick B. McGreevy, from the USAMRU, used the dot-ELISA test with protein A to evaluate the test for canine VL in Teresina, Piaui. (unpublished data).

In a recent study no cross-reactivity with sera from patients of malaria, tuberculosis, leprosy, amoebiasis and filariasis was observed, and besides the dot-ELISA was found to be more sensitive than ELISA, and inexpensive when applied in the field (Suman *et al.*, 1993). Dietze *et al.* (1995) also found dot-ELISA to be more sensitive and specific for canine VL than the conventional plate ELISA. Senaldi *et al.* (1996) suggested dot-ELISA may be suitable for production of a low-cost field kit, and they also adapted the system for antigen detection, although this was less effective.

f. direct agglutination test (DAT) is a simple and relatively inexpensive serological method (Harith *et al.*, 1986). As antigen promastigotes are used which are fixed and stained with comassie brilliant blue. In comparative studies performed by Gennene *et al.* (1992) the sensitivity was 80% but in 4 parasitologically positive patients the DAT was negative (Zijlstra *et al.*, 1992). A recent study (El Harith *et al.*, 1996) showed that DAT was valuable for specific diagnosis of PKDL.

The report a WHO Expert Committee (1990) has recommended the use of dot-ELISA and DAT under field conditions.

Serological recognition of a recombinant antigen, rK39, may correlate with the presence of active VL (Badaro *et al.*, 1996).

1.8 Treatment of the leishmaniasis

The first Brazilian report on treatment of leishmaniasis was by the great scientist Gaspar Vianna (1912) although British and French workers had demonstrated the effect of antimony in the treatment of African trypanosomiasis, (1906-1907). Tartar emetic (antimony potassium tartrate) was widely used in Asia, where mortality was reduced to 95%. Despite its toxicity this drug has also been used for the treatment of infantile VL (Mediterranean region). Due the high toxicity less hazardous derivatives soon appeared. Thus, the advent of the trivalent antimonial, Stibophen, was represented by "Fuadin, Reprodral", but in 1920 the improved pentavalent antimonials become available on the pharmacological market, as "Pentostam, Bayer 561 and Solustibosan". Pentostam, besides being, a drug with less toxicity, was demonstrated to be effective by intravenous administration. In Brazil, a similar drug was employed, Glucantime, (Meglumine Antimoniate).

In this same era, several other alternative treatments appeared: " Neostibosan (Ethylstibamine); Urea stibamine (Carbostibamine, Carbatine, Stiburea); Stilbamidine isethionate; Pentamidine isethionate (Lomidine, M & 800); Berberine chloride; Cycloguanil pamoate (Cycloguanil Embonate, Camolar); Quinacrine (Mepacrine); Amphotericin B (Fungizone); Monomycin; Rifampicin (Rifaldin) and aminosidine (Paromomycin), which is synonymous with the monomycin of topical application on lesions of *L. major* in BALB/c mice (El-On *et al.*, 1984, 1985) and has recently been used in human CL (El-on *et al.*, 1992).

At present, for treatment we have:

a. the first-line of the alternative drugs, the pentavalent antimonials (**Pentostam**) and meglumine antimoniate (**Glucantime**) (Bryceson, 1987; Navin *et al.*, 1992; Goodwin, 1995) but with awareness that:

i. it is a highly toxic drug (Veiga *et al.*, 1983; Rees *et al.*, 1980; Balzan & Fenech, 1992).

ii. in countries of the New World, where the drug mainly used is meglumine antimoniate (Glucantime) the reliable percentage of active drug contained in each batch is still unknown and what is more the quality varies depending on the manufacturer (Davidson & Croft, 1993).

iii. the product is easily altered by physical factors, such as temperature and light exposure, thus losing part of the activity (personal information from Dr.J.G. Dorea in hair from patients after antimony therapy; Dorea *et al.*, 1990; Dorea *et al.*, 1989).

iv. 2 hr after intramuscular administration of 10 mg Sb /kg , only 10 ug are found in each ml of blood and 24 hr later 80% of the antimony has been excreted through the urine (Croft, 1988; Rees *et al.*, 1980). Rapid drug excretion also occurs in dogs (Valladares *et al.*, 1996).

v. isolates of *L.donovani* have different sensitivities to Sb and resistance may be induced by inadequate use of the drug, (Grogl *et al.*, 1989, 1992; WHO, 1990; Gramiccia *et al.*, 1992). Thakur *et al.* (1988) found 10% of cases in India did not respond to Sb.

b. Allopurinol (Ribonucleoside) this drug must be used associated with an antimonial, because alone it is not effective (Kager *et al.*, 1981; Chunge *et al.*, 1985). Recently, Walton *et al.* (1983) used allopurinol orally in primates and

Saenz *et al.* (1989) showed clinical and parasitological cure, also administering the drug orally in patients with American cutaneous leishmaniasis.

c. Ketoconazole (anti-fungal, oral drug) Wali *et al.* (1990) reported that 4 of 5 human VL patients resistant to Sb were cured. This drug is producing promising results on the treatment of different clinical aspects of the leishmaniasis (el-Hassan *et al.*, 1992; Navin *et al.*, 1992; Norton *et al.*, 1992). Recently, dogs, naturally infected with *L. donovani*-complex, were treated in Texas, USA (Sellan *et al.*, 1993). Two promising oral drugs derivatives from the phospholipid (miltofosine and ilmofosine) were shown to be highly active as anti *L. donovani* agents in experimental studies performed on mice, and these may deserve further testing (Croft *et al.*, 1993).

d. Pentamidine (aromatic diamidine) this is an effective drug in VL but also highly toxic, causing development of diabetes (Bryceson, 1987). Thakur *et al.*, (1991), cured 312 patients that were resistant to Sb.

e. Amphotericin B (Fungizone) although this drug is highly toxic to the *Leishmania* parasite unfortunately in addition it is expensive and highly toxic in humans, producing hypomagnesaemia, hypotension, neurotoxicity and cardiotoxicity. Patients, must be hospitalized, the administration must be slow, by the intravenous route, with the possibility of forming dangerous thromboses. An alternative which reduces the risk of collateral effects, is the use of liposomal Amphotericin B (AmBisome) (Croft *et al.*, 1991; di Martino *et al.*, 1993). Lipid-associated amphotericin B has been shown to be effective in the treatment of VL. (Dietze *et al.*, 1993, 1995; Davidson *et al.*, 1994; Seaman *et al.*, 1995).

f. Aminosidine (Paromomycin) is an aminoglycoside antibiotic, which is anti-leishmanial *in vitro* (Neal, 1987). The collateral effects are mainly ototoxicity and nephrotoxicity. Various authors are using this drug, with satisfactory results (Chunge *et al.*, 1990; Scott *et al.*, 1992). The use of aminosidine to treat DCL caused by *L.aethiopica* was recently reported (Teklemariam *et al.*, 1994). Studies in Kenya, India and UK have shown that it is a safe and effective first line alternative to pentavalent antimony for treatment of newly diagnosed and unresponsive VL. CL in Honduras apparently fails to respond (Neva *et al.*, 1997). Further aspects of (aminosidine) therapy are reviewed in sections on experimental chemotherapy of canine VL (Results, Discussion).

1.9 Drug combinations

Although there are few studies on selection of drug combinations, several authors have demonstrated that the use of this option may reduce the long period of toxic drug administration. The combination of aminosidine/ stibogluconate for 20 days appeared to be an effective, economical and safe replacement for stibogluconate for 40 days (Chunge *et al.*, 1990; Seaman *et al.*, 1993; Thakur *et al.*, 1995).

Several authors have tested cytokines as a supplement to chemotherapy of VL, although the complexity of cytokine interactions is still not fully understood. Seven days treatment with IL-12 reduced liver parasite burden in mice.

1.10 Treatment of canine VL

There is no satisfactory treatment for canine VL. Repeated antimonial therapy is sometimes used in Europe but relapse is usual and carries the risk of encouraging disease transmission and the development of drug resistance (Gramiccia *et al.*, 1992). Antimonials have failed to be effective for canine VL in the New World (Marzochi *et al.*, 1985). Treatment of canine VL is discussed in detail in chapter 7.

1.11 Intervention and control

Intervention is action and in the leishmaniasis the expected result of an intervention is the control of the disease through interruption of the transmission cycle. The principal targets of intervention are the vectors, the parasite, the reservoir and the human host.

Sandflies are the only proven vectors of the leishmaniasis. Considering the lack of a really efficient drug without collateral effects and the difficulties of controlling the reservoir control of the vectors is considered to be an extremely important method of intervention.

Vioukov (1987) grouped the methods of control of sandflies as: **chemical methods** (principally insecticides), **biological methods** (parasites or predators of sandflies), **genetic methods** (eg. the possibility of eradicating the vector by sterilization of male sandflies) and **ecological methods** (attempting to alter or produce an adverse environment for sandflies).

1.11.1 Control of the vector

The most interesting point discussed by Vioukov is the decrease in the incidence of the leishmaniasis in almost all regions after the malaria control campaign. Although in a few countries the antimalarial operation did not produce a significant decrease in sandfly vectors (Greece and Iraq) in those cases the ecology of the sandflies was different to the ecology of the vectors of malaria (i.e., the sandflies were not exposed to the insecticides). In Iran, an increase in human CL occurred immediately after the cessation of antimalarial DDT spraying (Seyedi-Rashti & Nadim, 1975).

In Brazil, Nery-Guimaraes & Bustamante (1954) used peridomiciliary DDT spraying in a focus of CL in Rio de Janeiro and the incidence of human CL cases fell from 12.4% to 0.3%.

Deane *et al.*, 1955, evaluated the effect of DDT and they observed the immediately disappearance of sandflies indoors after spraying but 7 months later the population of sandflies returned to normal. This author in 1956 suggested that a high density of *Lu.longipalpis* is necessary to maintain the incidence of human cases.

Spraying of houses with DDT as part of a three-pronged attack, with the elimination of infected dogs and the treatment of diagnosed human VL, produced the almost total disappearance of the disease in the endemic areas of Ceara and Bahia, Brazil (Deane *et al.*, 1955, Alencar, 1961; Sherlock & Almeida, 1970). Spraying with DDT in a tropical forest (Brazil) eliminated *Lu.umbratilis* for 11 months (Ready *et al.*, 1985). Results on the use of deltamethrin, were obtained by Le Pont *et al.*, (1989) in Bolivia proving reduction in the vectors of VL, and recently Falcao *et al.*, 1991, showed that deltamethrin has a residual action during 12 months but only inside houses.

Lane (1991) emphasized that spraying methods have seldom been modified specifically for sandfly control and that antimalarial products were usually employed, and with the same strategies. According to the author, DDT was the insecticide with the best results in sandfly control although records of resistance already exist (El-Sayed *et al.*, 1989).

1.11.2 Control of the reservoir

A WHO report (1990), on the control of the leishmaniases described the use of strychnine in meat bait in Iraq to eliminate canids, producing a fall in the number of human cases of leishmaniasis but such a drastic measure is not always possible.

In northern Europe the treatment of symptomatic and asymptomatic seropositive dogs is routine but recently Gramiccia *et al.* (1992) reported the isolation of a strain that is resistant to antimonials, alerting us to the possibility of producing resistant parasites by improper and consecutive use of the drug.

Generally the dogs in the Mediterranean have great financial or sentimental value and the owners can offer treatment. The treatment is however, a palliative measure, as many dogs relapse, although the use of antimonials, once or twice a year, may reduce the infectivity to sandflies (WHO, 1990).

In some countries a measure which is proving efficient for serological screening of dogs is to associate the control of canine VL with campaigns against rabies.

In Brazil VL, is concentrated mainly in urban areas. Serological diagnosis for mass screening of dogs cover only 50% of the animals. Serology in use does not detect all the positive dogs and antimonials are not effective for treatment of infected animals (Marzochi *et al.*, 1985; Marzochi *et al.*, 1994).

Although large numbers of dogs are eliminated each year in endemic areas VL is increasing.

1.11.3 Control of human cases

The importance of treatment of human cases is unquestionable. The reason is that this disease is lethal in untreated children, although infections may be asymptomatic (Bryceson, 1987; Badaro *et al.*, 1986a,b,c). The possibility that human VL cases with amastigotes in normal skin could maintain a cycle man-sandfly-man has not been discarded (Sherlock & Miranda, 1992; Rab *et al.*, 1992).

There is no efficient drug without collateral effects but in Brazil the antimonials are still efficient for treatment of the human VL and few cases were registered as resistant.

The majority of fatal cases occur because the patients (mainly children from slums) arrive at medical centres almost dying.

Recently Grimaldi & Tesh (1993) emphasized that in theory, the control of leishmaniasis is possible by interruption of the transmission cycle and that different approaches have been used but with limited success. There is an urgent need for parallel interventions controlling the vector, eliminating the reservoir, and curing all the human cases or for a clear assessment of intervention priorities and how interventions should be delivered.

1.12 Objectives of the research project

1. To analyse epidemiological data on VL in the city of Teresina, Piauí.
2. To compare the following diagnostic procedures for canine VL:
 - a. serology (IFAT; ELISA; dot-ELISA; DAT) with serum samples and blood eluted from samples collected on filter paper.
 - b. parasitology (giemsa-staining of smears from skin biopsies, bone marrow and spleen aspirates).
 - c. new DNA based detection methods (DNA probe testing applicability in field work).
3. To determine, in a sample cohort whether asymptomatic infections are common in dogs, whether such carriers can act as a reservoir of infection and to study the ease of transmissibility to colony-bred *Lu.longipalis*.
4. To detect the presence of naturally infected sandflies, through individual dissection and application of DNA probing.

5. To establish a cohort of experimentally infected dogs with the following objectives:
 - a. to determine the number of infected sandflies required to transmit the infection from dog to dog by sandfly bite.
 - b. to determine the time required for experimentally infected dogs to become infective to sandflies.
 - c. to determine the time required for seroconversion.
6. To test aminosidine (paromomycin) as a method of treating naturally infected dogs.
7. To recommend improved strategies for disease control based on research findings from the project.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study area: Teresina, and epidemiological observations

Teresina is the capital of Piauí State in northeastern Brazil (5°09'S, 42°45'W). It is located strategically between the river Parnaíba and the river Poty (Figure 2.1). The climate is typically tropical and hot with temperatures oscillating between 22°C and 32°C (Figure 2.2A). The rainy season occurs between December and April with an accumulative rainfall of 157.37 mm and the dry season from May to November with 19.87 mm (Figure 2.2B). This area has scattered empty plots of land and gardens with many trees. The principal characteristic of this city is the contrasting mixture of classical houses of the rich, in many cases with pedigree dogs in kennels, and small or large slums mostly without even basic sanitation (Figure 2.3A, 2.3B).

The slum houses are usually constructed of mud on a wooden frame with a palm-thatched roof. It is very common to find chicken sheds and pig-sties nearby made of wooden stakes driven into the ground (Figure 2.4). Often one dog or more and/or cat may also be present.

The human population of the slums basically consists of immigrant unemployed who are very poor. Each family may have many children (Figure 2.5).

Epidemiological data were assembled initially from previous Ministry of Health (FNS) records and were then subsequently collected in collaboration with the FNS field workers. Details are given in the relevant section of the Results (chapter 3).

Figure 2.1

**Map of Piauí State, showing the location of the city of Teresina,
Piauí, Brazil**

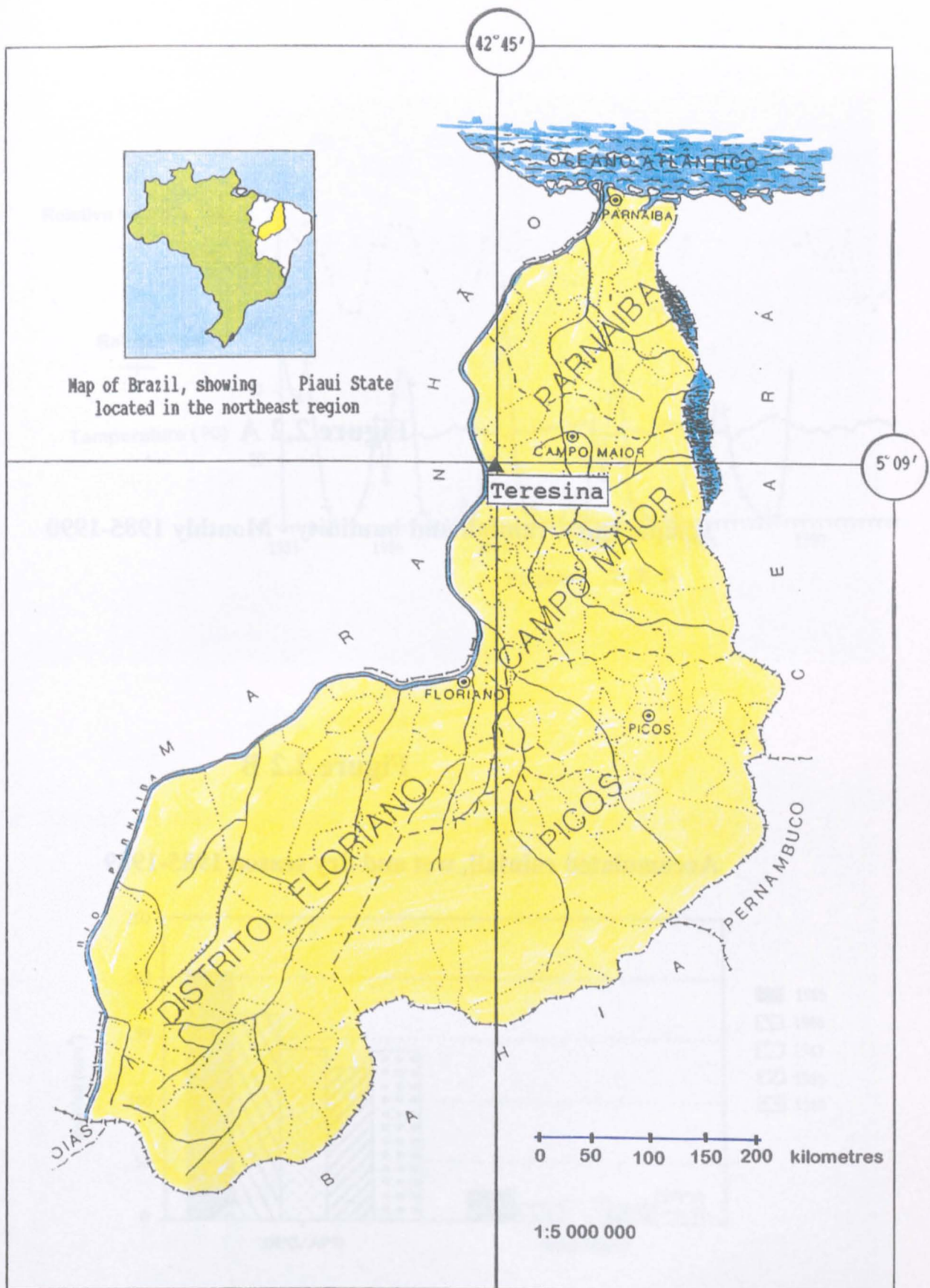


Figure 2.1

Figure 2.2 A

Temperature, rainfall and humidity - Monthly 1985-1990

Figure 2.2 B

Accumulated rainfall, wet and dry season 1985-1989

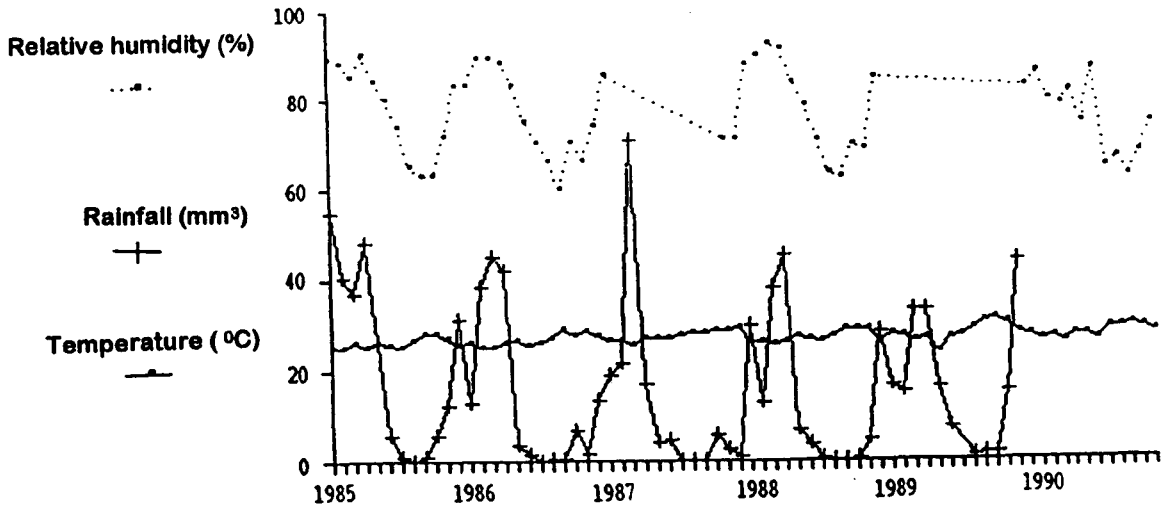


Figure 2.2 A

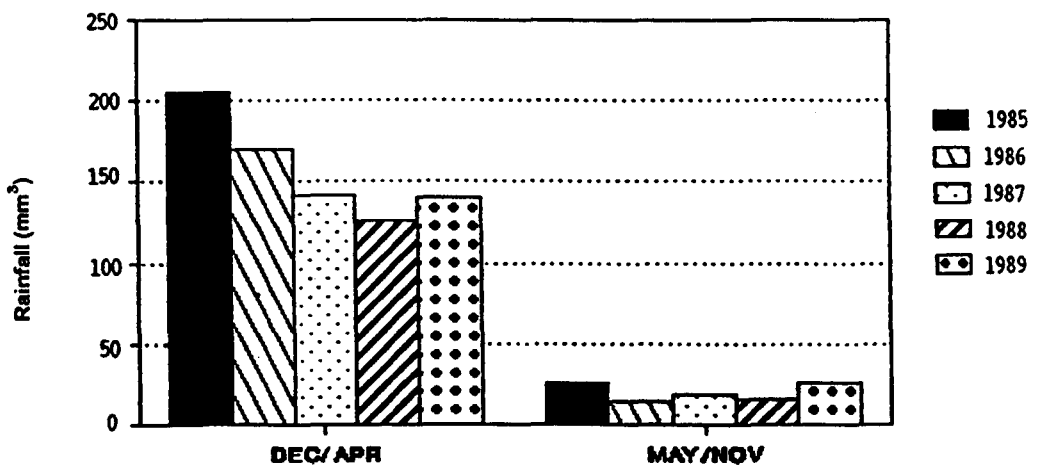


Figure 2.2 B

Figure 2.3A

Small slum located between two good quality buildings

Figure 2.3B

The lack of basic sanitation and drinking water is characteristic of the slums: residents queuing to obtain water



Figure 2.3A



Figure 2.3B

Figure 2.4

A typical house, with a chicken shed nearby made of wooden stakes driven into ground

Figure 2.5

A family representing three generations, the grandmother, the mother and six children



Figure 2.4



Figure 2.5

2.2 Sandflies

2.2.1 Capture and identification

Between September 1992 and November 1993, sandflies were collected by two sampling methods, pooters and CDC light-traps. The pooters for collecting sandflies were as described by Perfil'ev (1968) and Rioux *et al.*(1969) and were principally used for catching engorged females to investigate the presence of flagellate infections. The CDC light-traps (**Figure 2.6**) were used when flies were numerous as, for example, in pig-sties and chicken sheds (**Figure 2.7**).

The captures were always performed between 6 pm and 10 pm with pooters for catches on pigs, dogs, chickens, and man. Two or three CDC light-traps were placed inside chicken sheds, pig-sties and kennels, overnight, twice a week.

A proportion of the sandflies collected was placed in 70% ethanol and labelled. Specimens were identified using temporary mounts, (CMCP-10 low viscosity N.Y) and microscopical examination of mainly the genitalia and the armatures of the cibarium and pharynx.

In 1995 and 1996 further intensive captures were performed in rural and periurban areas around the city to extend information on the local sandfly fauna as part of a Wellcome Trust project on genetic diversity and behaviour of *Lutzomyia whitmani* (in collaboration with Diarmid Campbell-Lendrum).

Figure 2.6

CDC light-trap placed inside a chicken shed, operated overnight by a set of four 1,5v batteries

Figure 2.7

Typical chicken shed infested with *Lu. longipalpis* in Teresina



Figure 2.7



Figure 2.6

2.2.2 Laboratory colony

A laboratory colony of *Lu.longipalpis* was established from eggs produced by two batches of sandflies:

a) blood fed females collected in dog kennels.

b) flies collected by CDC light traps placed inside pig-sties and chicken sheds. Females were offered meals on golden hamsters anaesthetised with Ketalar (ketamine hydrochloride). After one day the engorged females were transferred by aspirator to plastic pots for oviposition. These plastic pots have humid gypsum in the bottom and lids with a hole covered with nylon. A small plug of absorbent cotton wool with a 50% solution of maize honey (commercially available food for children in Brazil) dissolved in distilled water was placed on the top of the nylon.

The first and the second larval stages were fed with a special food prepared with a mixture following:

a) dried pig faeces (9.5 parts in 10)

b) dried sandflies collected by CDC light traps (0.5 parts in 10)

c) distilled water (10 ml to 100 gms of a) + b))

After drying this mixture the food was ground and the powder used without sterilization. The other larval stages, third and fourth, were fed with dried *Daphnia* (marketed for aquarium fish).

When the adults emerged around 30 days after blood-feeding they were transferred to acrylic boxes where they were offered a blood meal, following the routine described above.

The average temperature in the insectary was 23°C during the day and 27°C at night.

The colony was used to perform xenodiagnoses and to determine the percentage of sandflies infected when fed on asymptomatic or symptomatic naturally infected dogs.

Initially, during the two first months the females of *Lu.longipalpis* were fed on human blood because there were no hamsters in the University. Hamsters obtained from a permanent colony located in the laboratory of parasitology of the University of Brasilia and maintained in conditions that prevented exposure to naturally infected sandflies, were used thereafter for the maintenance of the colony.

In preliminary observations of a sandfly colony maintained in the University of Brasilia, some relationship between the blood meal used to feed the female sandflies and the number of eggs oviposited was noted (unpublished data).

During maintenance of the new colony at Teresina 81 *Lu.longipalpis* females, 38 fed on human blood and 43 fed on hamsters, were separated individually and the eggs were counted after oviposition. A minimum of 29 and a maximum of 82 eggs were oviposited for each female when the blood meal was human and a minimum of 14 and a maximum of 58 eggs were oviposited by each female when the meal offered was golden hamster.

The colony produced an average of 1,500 adult flies per month, and 50% of them were females. This colony in all of its stages, was always free of fungi.

2.2.3 Detection of natural infections

Two methods were used for detection of natural infections in sandflies:

a. dissection of engorged females

i. after collection the sandflies were kept alive on a diet of raisins for between 4 and 7 days at a temperature of 24°C, until oviposition.

ii. before dissection, all the flies were briefly wetted in 2% neutral detergent in saline solution and washed (x3) in saline solution (**Appendix I**).

iii. the females were dissected individually in one drop of sterile saline solution under a stereoscopic dissecting microscope and then examined microscopically at x400 and x1000 magnifications.

iv. spermathecae and cibarium armatures of the dissected flies were also observed for species identification and the position of the promastigotes in the gut of flies was noted. The remaining portion of the gut after examination was transferred to Hybond N (Amersham) for parallel detection of infection, using the DNA probe.

v. some slides with promastigote infections were fixed with methanol and stained with giemsa solution (**Appendix I**) to examine the morphology of the parasites.

b. chemiluminescent DNA probe technology (Howard *et al.*,1991,1992 and McNerney *et al.*,1993b).

Flies captured with pooters from a single house where there were two human cases of VL and from several dog kennels were placed at -20°C for 2 min. Immediately after, when they were inactive but not dead, they were squashed onto Hybond N (Amersham) using a separate wooden toothpick pre-soaked in phosphate buffer saline (PBS, pH 7.2; **Appendix I**) for each specimen. The membranes were processed as described in section 2.3.3.

Further sandfly collections for DNA probing were subsequently made at several locations in the city, as described in the Results.

2.3 Diagnosis of canine visceral leishmaniasis

Eight groups of samples, or parts of them, were included in this study:

- a. 49 dogs from Teresina, Brazil, (**pilot study I**) were examined clinically, parasitologically, serologically and some dogs were also tested by detection of *Leishmania donovani*-complex DNA.
- b. 388 samples from blood spots collected on filter paper, from Teresina, Brazil.
- c. 129 sera from a Mediterranean endemic area, Corsica.
- d. 160 dogs from Teresina, Brazil, (**pilot study II**), were examined, clinically, parasitologically and serologically.
- e. 99 sera from The Institute of Health of the Federal District, Brasilia, Brazil.
- f. 27,436 blood samples collected as part of the dog rabies control programme in 1993 in collaboration with FNS, UFP and the Zoonosis Centre.
- g. a series of 25 dogs infected experimentally.
- h. 34,155 dogs examined in 1995 as part of a study to gather information on the distribution of dog breeds in the city and the prevalence of *L. chagasi* infection in different breeds.

In phase 1 a total of 110,000 dwellings were visited by 40 field assistants, over 60 working days, and the breed, age and sex of each household dog were noted.

In phase 2, after estimating the breed distribution by district a small sample of each breed was selected and serological diagnosis was performed.

2.3.1 Clinical diagnosis

The clinical examination of the dogs considered the following aspects:

- a. age
- b. sex
- c. breed
- d. external aspect, (normal, thin or fat)
- e. history of weight loss
- f. conjunctivitis/keratitis
- g. size of the claws
- h. depilation and exfoliative dermatitis
- i. external lesion (how many and where located)
- j. nasal haemorrhage

2.3.2 Parasitological diagnosis

The parasitological examination was made by microscopy of giemsa-stained skin impression smears and sternal bone marrow aspirates (**Figure 2.8**). The material for microscopical examination was obtained by scarification or biopsy of the margins of ulcers and occasionally by spleen-aspiration. These samples were collected from dogs previously anaesthetised with 0.5 ml ketamine chlorhydrate. Giemsa-staining of samples of different organs from the post-mortem examination of dogs was done to examine the parasitological distribution. After impression of the samples onto the slides, these were left for 2 min. at room temperature to dry and

Figure 2.8

Holding a dog to locate the site for sternal puncture



Figure 2.8

immediately fixed with methanol for 3 min. Then the slides were immersed in giemsa solution for 45 min and washed quickly in water. Observation was made with x1000 magnification using an oil immersion/objective.

2.3.3 Diagnosis by detection of DNA

a. selection of samples

Small drops of bone marrow, samples of skin, spleen and liver, without excessive blood contamination, were manipulated using a separate wooden tooth pick and placed onto Hybond N (Amersham) membranes.

The membranes were labelled and dried at room temperature for 1 hr, sealed in plastic bags and stored at room temperature until processing.

b. denaturation

The following solution/times were used:

- i. denaturing solution/10 min
- ii. neutralising solution/5 min
- iii. rinse in 2xSSC (**Appendix I**)

After drying at room temperature, the membranes were placed at 80°C for 2 hr and incubated at 37°C for 1 hr in proteinase K solution (**Appendix I**). All the membranes were stored dry at 4°C until hybridisation.

c. prehybridisation and hybridisation

Prehybridisation and hybridisation were processed inside commercial plastic bags and the volume of solution used per 50 cm² of membrane area was as given below.

i. the membranes were incubated in 5 ml of prehybridisation solution (**Appendix I**) for 30 min at 37°C.

ii. the excess of the previous solution was poured off and 10 ul of Lmet2 probe/ml (previously boiled for 5 min and transferred quickly to ice) were added immediately to fresh prehybridisation solution. The solution was mixed and incubated with the membranes overnight at 37°C.

d. detection

i. each membrane was rinsed (x2) with 50 ml of washing solution 1 (**Appendix I**) at room temperature.

ii. washed (x3) in 500 ml of wash solution 2 (**Appendix I**) for 15 min at 37°C.

iii. the membranes were rinsed in 50 ml of tris-buffer saline (TBS; **Appendix I**) covered with 20ml of blocking solution (TBSM; **Appendix I**) and incubated for 30 min at room temperature.

iv. the blocking solution was replaced and 10 ml of antibody solution (**Appendix I**) added, incubating at room temperature for 30 min.

v. the membranes were washed (x6) in 500 ml of wash solution 3 (**Appendix I**) for 30 min and 5 min in 50 ml of 2xSSC.

vi. after pouring off the excess solution, the membranes were placed, sample side up, on transparent plastic and sprayed with chemiluminescent substrate (**Appendix**

I). The membranes were covered with another transparent plastic sheet and removed before closing the bags.

vii. the plastic bags containing the membranes were placed in an autoradiography cassette together with an X-ray film for between 2.5 and 16 hr.

viii. After development of the film the presence of a dark signal, in the absence of background, indicated the presence of parasites of the *L. donovani*-complex.

e. PCR

Skin snip samples from the pilot study group of 49 dogs were tested by PCR using kinetoplast DNA minicircle primers in collaboration with Douglas Barker (Cambridge) but PCR was not adopted as a routine assay for other comparisons (see Discussion).

2.3.4 Serological diagnosis

Two different types of blood-samples were used for the serological investigations:

a. sera, obtained by centrifugation of venous blood at 4,000 rpm for 5 min. Before bleeding all dogs were secured by a knot placed under the mouth and behind the neck. Dogs that were difficult to handle were anaesthetised with ketamine chlorhydrate. Each sample of serum was mixed with 100% (v/v) of glycerol/sodium azide 0.04% (**Appendix I**) and stored at - 20°C.

b. antibody eluates from blood spots collected on filter paper (**Figure 2.9**). The blood was obtained by puncture at the point of the ear. The blood spots collected on filter paper were labelled and dried at room temperature for 6 hr, sealed in plastic bags and stored at - 20°C.

The serological methods used were the immunofluorescent antibody test (IFAT; (Walton *et al.*,1972), the enzyme linked immunosorbent Assay (ELISA), the dot enzyme-linked immunosorbent Assay (dot-ELISA; Pappas *et al.*,1985) and the direct agglutination test (DAT; Harith *et al.*,1986). Two newer serological tests, the FAST-ELISA and the latex agglutination (Nantulya, 1994) were also assessed (see Discussion).

Serology of a pilot group of 49 dogs was made to evaluate the following variables:

- i.** lyophilised reference antigen used in Brazil vs. antigen prepared with a strain isolated from a naturally infected dog in Teresina, Brazil.
- ii.** slides with antigen fixed with acetone vs. slides with antigen only dried at room temperature before use.
- iii.** the optimum dilution of the conjugate.
- iv.** to choose the best membrane for the dot-ELISA method.
- v.** to obtain preliminary information in the sensitivity and specificity of the serological methods.

Figure 2.9

Collecting blood spots from a dog onto filter paper



Figure 2.9

2.3.4.1 Immunofluorescent Antibody Test (IFAT)

Cultured promastigotes were washed (x3) with sterile PBS by resuspension and centrifugation at 3.400 rpm for 5 min at 4°C. The final pellet was resuspended in saline solution at 5×10^8 cells/ml and 5ul volumes of the cell suspension were immediately dispensed into each well of the microscope slides (Henley Essex) and the slides were allowed to dry in air. All the slides were wrapped in tissue paper, placed in plastic bags and stored at -20°C. Consecutive dilutions of serum samples, beginning with 1:50 were added into each well and the slides were incubated for 30 min at room temperature in a humid chamber. The solution used for diluting the serum samples was PBS/T/M (**Appendix I**). After incubation, the slides were washed (x3) for 10 min with PBST (**Appendix I**). Five ul of a 1:50 dilution of anti-dog Ig/FITC conjugate in PBST/M/1:10,000 Evan's Blue was added to each well and the slides were incubated for 30 min at 37°C in a humid chamber. After this incubation the slides were washed (x5) in PBS and allowed to air dry in the dark. Each slide was mounted in PBS/glycerol (**Appendix I**) and observed at x1000 magnification under a fluorescent microscope in a dark-room.

2.3.4.2 Enzyme Linked Immunosorbent Assay

Promastigotes cultured in liquid medium RPMI 1640 (**Appendix I**) with 10% of foetal calf serum (FCS) were harvested during the log-phase, washed (x3) in PBS and resuspended in sterile distilled water 1:40 (v/v). The parasites were frozen in liquid nitrogen and thawed in water at 37°C (x3) followed by ultrasonication (x5) of 15 seconds each in a Soniprep 150 MSE Scientific Instruments ultrasonic disintegrator (Fisons Ltd). This suspension was centrifuged at 30.000 rpm for 30 min at 4°C. The supernatant was used with a final protein concentration of 5 ug/ml in coating buffer solution (CB, pH 9,6; **Appendix I**). The concentration of protein was determined by the BCA Protein Assay Reagent (Pierce Chemical Company Cat.23225). The final concentration of the antigen

was determined through a checkerboard titration. Duplicated plates, with progressive dilutions of antigen, were exposed to progressive dilutions of standard negative and positive serum at (1:100 to 1:6400 dilution).

The plates were coated with 100 ul of the antigen in each well overnight and washed (x5) with PBS. Each well was blocked with 200 ul of CB/2% Marvel for 1 hr at 37°C, washed again (x5) with PBS and stored at -20°C until further use.

Two hundred ul of sera diluted 1:200 in PBS/M was added to each well of the ELISA plate and the plate was incubated for 1 hr at 37°C in a humid chamber and washed (x5) with PBS. Immediately each well was coated with 100 ul of peroxidase-conjugated, affinity purified, rabbit anti-dog IgG (H+L) conjugate, diluted at 1:1,000 in PBST/M, and incubated for 1 hr at 37°C.

The optimum dilution of the conjugate was found through a checkerboard titration. Duplicate plates, sensitised with a standardised antigen concentration and exposed to progressive dilutions of sera (negative controls and positive controls) were developed with 4 progressive dilutions of the conjugate, (1:500; 1:1000; 1:2000 and 1:4000) to select the most suitable.

The plates were washed again (x5) with PBS and 100 ul of substrate solution 1 (**Appendix I**) were added. The plates were left at room temperature for 5 min and immediately the chromogenic reaction was stopped with 50 ul of 2.5M H₂SO₄ (**Appendix I**). Each well was read spectrophotometrically at 490nm wave length.

2.3.4.3 Dot Enzyme-Linked Immunosorbent Assay

a. Preparation of antigen solution

The antigen used in this method was made with promastigotes washed (x3) in TBS and resuspended in TBS 10x at a dilution equivalent to 10^8 parasites/ml.

b. Preparation of membrane

Areas of 0.25 cm² of Immunodyne membrane, (Pall Immunodyne P/N BIAO30HC5 rating:3.0 micron lot.100941) were glued onto the teeth of 8 teeth combs. One ul of the antigen was dotted onto the centre of the membrane. The combs containing the membranes with the antigen were left at room temperature for 5 min.

c. Formation of immune complex

The combs were immersed in TBS/M blocking solution for 30 min. at room temperature and washed (x3) in distilled water. The combs were immersed in a microtitration plate containing the sera diluted 1:200 and incubated for 30 min at room temperature. The combs were washed (x5) in distilled water and placed into another plate with 100 ul/well protein A/peroxidase conjugate 1:1,000 in TBS/M and incubated for 15 min. Although some authors recommend a 1:2,000 dilution, we opted for the 1:1000 dilution because, when the conjugate was used at the lower dilution, some positive control sera gave low antibody titres (Gandhi, 1986; Jaffe & Zalis, 1988).

The combs were washed (x5) with distilled water.

d. Detection methods

The combs were transferred to another plate containing 100 ul of the substrate solution 2 (**Appendix I**), for 5 min at room temperature. Immediately the combs were transferred to 0.02M H₂SO₄ solution and then given a final wash in distilled water (x3), and dried between paper towels and the colour intensity of each spot evaluated visually (**Figure 2.10**).

2.3.4.4 Direct Agglutination Test

a. Preparation of antigen for DAT

Parasites of *L.donovani*, strain MCAN/BR/89/M12730 were used to prepare the DAT antigen. This antigen was prepared as described by Harith *et al.* (1988). Promastigotes were mass cultured in liquid medium RPMI:FCS and harvested by centrifugation at 5,000 rpm for 15 min at 4°C. They were washed (x5) by resuspension and centrifugation at 3,400 rpm for 10 min at 4°C in cold Locke's solution (**Appendix I**). The pellet was resuspended in trypsin solution (**Appendix I**) and incubated for 45 min at 37°C. The suspension was washed (x5) in Locke's solution and after the final wash the pellet was resuspended in cold Locke's solution to a concentration of 2×10^8 cells/ml. An equal volume of 2% (v/v) formaldehyde in cold Locke's solution was added to the pellet which was then left for 20 hr at room temperature. After centrifugation of the suspension at 5,000 rpm for 10 min, the pellet was washed (x2) in cold citrate saline solution (**Appendix I**) and resuspended to the same volume.

Commassie brilliant blue R-250 was added to obtain a final concentration of 0,1% (w/v), for 90 min and then the suspension was left stirring at moderate speed on a magnetic stirrer. Finally the parasites were harvested by centrifugation and the pellet washed (x5) in citrate saline solution. The pellet was then

Figure 2.10

The dot-ELISA test

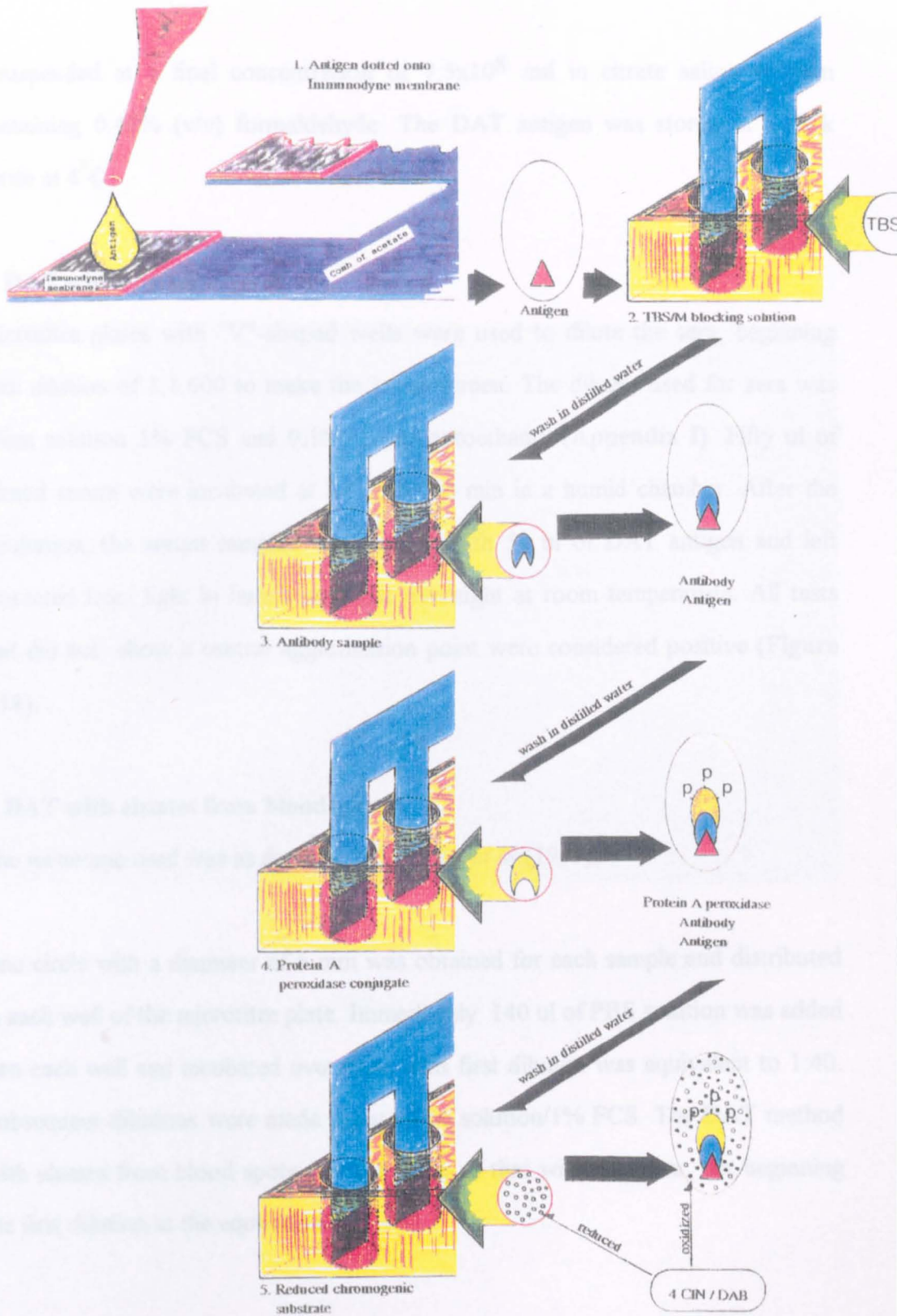


Figure 2.10

resuspended at a final concentration of 7.5×10^8 /ml in citrate saline solution containing 0.43% (v/v) formaldehyde. The DAT antigen was stored in a dark bottle at 4 °C.

b. DAT with dog sera

Microtitre plates with "V"-shaped wells were used to dilute the sera, beginning with dilution of 1:1,600 to make the initial screen. The diluent used for sera was saline solution 1% FCS and 0,1M 2-mercaptoethanol (**Appendix I**). Fifty ul of diluted serum were incubated at 37°C for 30 min in a humid chamber. After the incubation, the serum samples were mixed with 50 ul of DAT antigen and left protected from light in humid chamber overnight at room temperature. All tests that did not show a central agglutination point were considered positive (**Figure 2.11**).

c. DAT with eluates from blood spots.

The technique used was as described Coutinho *et al.*(1985).

One circle with a diameter of 6 mm was obtained for each sample and distributed in each well of the microtitre plate. Immediately 140 ul of PBS solution was added into each well and incubated overnight. This first dilution was equivalent to 1:40. Subsequent dilutions were made using saline solution/1% FCS. The DAT method with eluates from blood spots was the same as that with dog sera, but beginning the first dilution at the equivalent of 1:400.

Figure 2.11

DAT agglutination plate

2.4 Isolation and characterization of *Leishmania*

Parasitogones were isolated from naturally infected dogs using biphasic culture medium (Appendix B). The liquid phase was 1 ml of RPMI 1640 10%

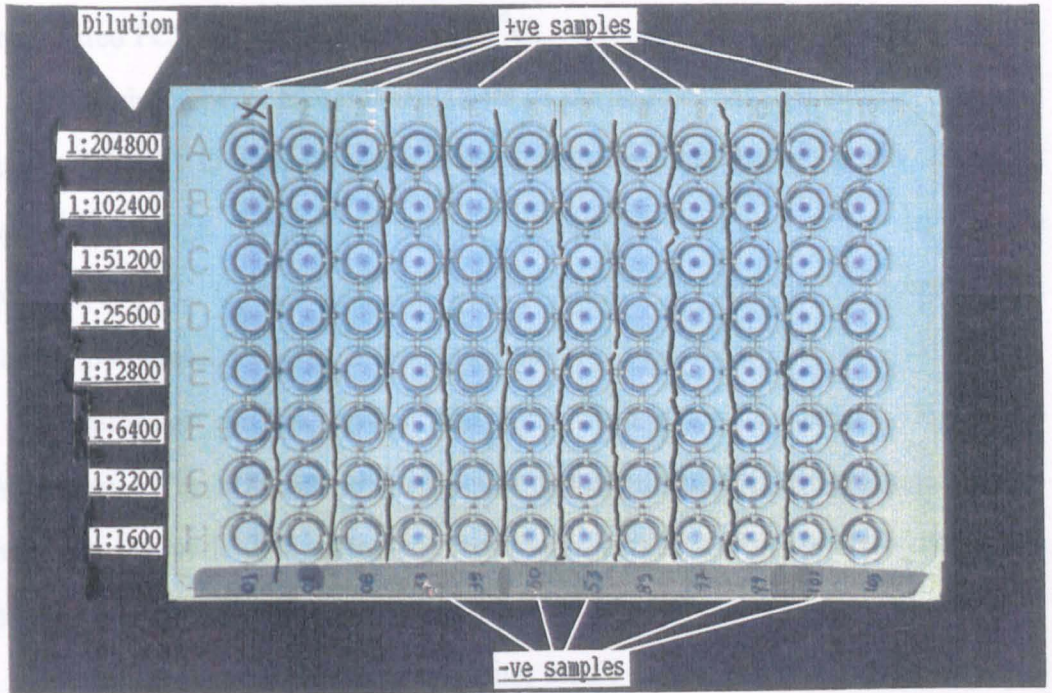


Figure 2.11

- isoenzyme electrophoresis
- development of the infection in sandflies
- behaviour of the organism in humans
- DNA probing

2.4 Isolation and characterization of *Leishmania*

Promastigotes were isolated from naturally infected dogs using biphasic culture medium (**Appendix I**). The liquid phase was 1 ml of RPMI 1640 10% inactivated FCS. Tubes were stored at 4°C.

Syringes of 20 ml containing 1 ml of sterile PBS with gentamicin, 50 ug/ml and 1 1/4"x 21 G gauge needles, were used to aspirate bone marrow, liver and spleen from dogs. Samples of material collected were cultured on biphasic culture medium at 24°C. Culture were examined weekly for the presence of promastigotes for up to 4 weeks.

Biopsies of skin, spleen and liver from naturally infected dogs were homogenised in PBS and the suspension was inoculated intraperitoneally into hamsters. Around 30 days after the inoculation parasites were isolated from spleen of the hamsters following the routine described above.

Isolates were cryopreserved using sterile cryoprotectant, Locke's/glycerol 16% (**Appendix I**) 1:5 (v/v).

Mass cultivation was performed using liquid medium RPMI 1640/10% FCS. Cultures were used for characterization of isolates by the following methods.

- a. isoenzyme electrophoresis
- b. development of the infection in sandflies.
- c. behaviour of the organism in hamsters
- d. DNA probing

The strains used to perform the characterizations by isoenzyme electrophoresis were isolated by Dr. Patrick McGreevy, during his experimental studies in Teresina, Piauí, in 1980. Those strains are cryopreserved in LSHTM/UK with the following codes:

MCAN/BR/89/ M12732	MCAN/BR/89/ M12737
MCAN/BR/89/ M12742	MCAN/BR/89/ M12727
MHOM/BR/89/ M12728	MCAN/BR/89/ M12729
MCAN/BR/89/ M12730	MCAN/BR/89/ M12731
MCAN/BR/89/ M12733	MCAN/BR/89/ M12734
MCAN/BR/89/ M12735	MCAN/BR/89/ M12736
MCAN/BR/89/ M12738	MCAN/BR/89/ M12739
MCAN/BR/89/ M12740	MCAN/BR/89/ M12741

2.4.1 Isoenzyme electrophoresis

After cultivation the promastigotes were harvested by centrifugation from culture medium RPMI 10% FCS. The parasites were washed in saline solution (x5) for 10 min at 4°C. After the last centrifugation, the supernatant was discarded. The pellet was mixed (1:1) with enzyme stabilizer (**Appendix I**) and frozen/thawed in liquid nitrogen (x3). This suspension was centrifuged at 30.000 rpm for 20 min.

Fifteen ul aliquots of the supernatant were dropped into liquid nitrogen and stored as frozen beads. The method used was a described by Evans (1989b). Five enzymes were used as follows:

Esterase (ES)	E.C.3.1.1.1
Nucleoside hydrolyase 1 (NH1)	E.C.3.2.2.2
Phosphogluconate dehydrogenase (6PGD)	E.C.1.1.1.44
Superoxide dismutase (SOD)	E.C.1.15.1.1
Mannosephosphate isomerase (MPI)	E.C.5.3.1.8

The solutions and staining conditions used for electrophoresis are in **Appendix II**.

2.4.2 Development of infection in sandflies

The distribution of some *Leishmania* isolates in xenodiagnosis sandflies, was observed at the time of dissection. Flies were washed for 1 min. in neutral detergent (one drop in 50ml saline solution) to remove hairs from the wings and in saline solution for 3 min. (x3). The gut was extracted and examined under the microscope at x450 magnification. Thus, the type of infection could be determined, whether hypopylarian, peripylarian or suprapylarian (**Figure 2.12**) as described by Lainson & Shaw (1979).

Figure 2.12

Classification of leishmanial parasites, according to Lainson and Shaw (1979)

Grouping of the Leishmanias according to their development in the sandfly vectors

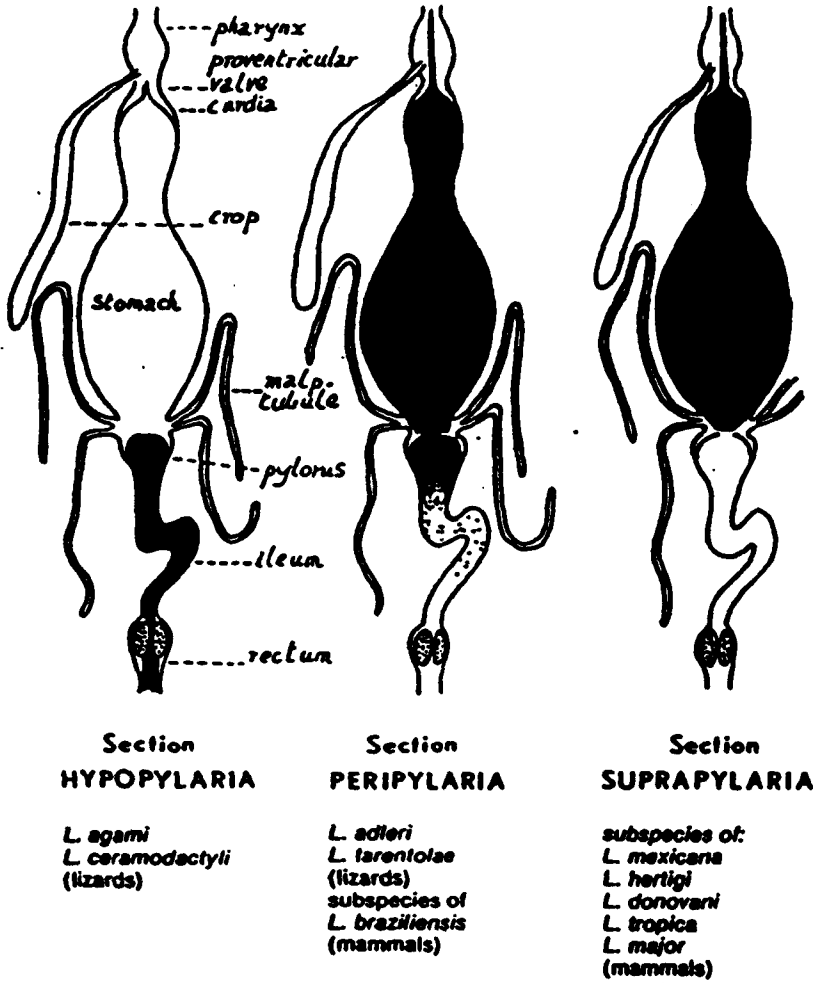


Figure 2.12

2.4.3 Behaviour of isolates in hamsters

Occasionally events beyond control, such as lack of electricity or lack of culture medium, meant that isolation of parasites could not be performed. Inoculation of tissue samples intraperitoneally into hamsters, was used as an alternative procedure when cultures were not available.

Samples of bone marrow, spleen, liver or biopsies from skin, were inoculated intraperitoneally into golden hamsters. After approximately 30 days, the animals were necropsied and examined carefully by giemsa-staining of different organs and/or isolation of the parasites into culture medium (section 2.4). Visceralization and the dissemination of infection were noted.

2.4.4 Characterization by DNA probing

When samples of bone marrow, biopsies of spleen, liver or skin from naturally infected dogs, were applied to Hybond N membranes, probing not only detected the parasite but also identified the species due to the specificity of the Lmet2 probe, which only recognises parasites of the *L.donovani*-complex when less than 10^4 target organisms are present, (Wilson *et al.*, 1992).

2.5 Experimental transmission

2.5.1 Pilot study

Two dogs were used to begin the studies of infections transmitted by experimentally infected sandflies.

a. Dog 1

A total of 20 *Lu.longipalpis* females were fed on a naturally infected dog, which had amastigotes disseminated throughout the skin. Seven days later, 7 flies had survived, only 4 of which were observed to feed when offered the internal part of the left ear of a dog of 70 days old, from a non-endemic area, Brasilia DF, Brazil. Immediately after the flies were fed, they were dissected to prove whether or not

they were infected. Serology was performed with sera collected before and during the experimental study, using the techniques described in **section 2.3.4** . After the twenty second day of infection, when a tiny erythema was observed at the site where the sandflies were fed, skin biopsies and xenodiagnoses were performed.

b. Dog 2

Flies collected in a kennel where a single naturally infected dog was present, were kept alive on a diet of 50% glucose solution (maize honey) at 24°C for 6 days. On the seventh day, thirteen female flies, were offered the belly of a young dog, 43 days old and 3 were observed to feed. This dog was the litter-mate of dog 1. All the specimens were dissected to prove whether or not they were infected.

The follow-up of the both dogs, was performed during the course of these experimental infections, by serology, skin biopsies, bone marrow puncture and xenodiagnosis.

This study was performed in an animal house, built specially for the purpose (**Figure 2.13**). All the windows were covered with mesh, to avoid entrance of sandflies.

2.5.2 Principal study

Twenty-three further young dogs that were clinically healthy and parasitologically negative by IFAT, were selected from a non-endemic area (Brasilia DF) for this experimental study.

Figure 2.13

Construction of the animal house



Figure 2.13

2.5.2.1 Prophylaxis and control of other infectious diseases

As prophylaxis and control of other infectious diseases, all the dogs were immunized with anti-rabies vaccine (Rai-Vaci) and (Vanguard 5/CV-L) against cinomosis, adenovirus type 2, coronavirus, influenza, parvovirus and canine leptopirosis. Flotril 2.5% (Enrofloxine antibiotic) was used occasionally for prophylaxis and control of respiratory infections. Lopatol (CIBA-GEIGY) was given as an anti-helminthic and TRIATOX to protect against ectoparasites, according to veterinary prescriptions.

All the dogs selected were transported by air to the city of Teresina (Piauí State) and housed in a specially designed, newly constructed kennel.

2.5.2.2 The kennel

This building was strategically designed considering principally that the city of Teresina is located in an endemic area of VL. Consequently to avoid natural infection of the study dogs, all the windows were covered with fine, sandfly-proof mesh and the internal and external walls were sprayed with residual deltamethrin every three months. ULV spraying with sumithion or malathion was also used inside and around the building twice a month. The kennel, located on the campus of the University of Piauí occupied an area of two hundred square meters (**Figure 2.14**).

Figure 2.14

The kennel house complete and showing the internal divisions and raised floor for hygienic maintenance of dogs

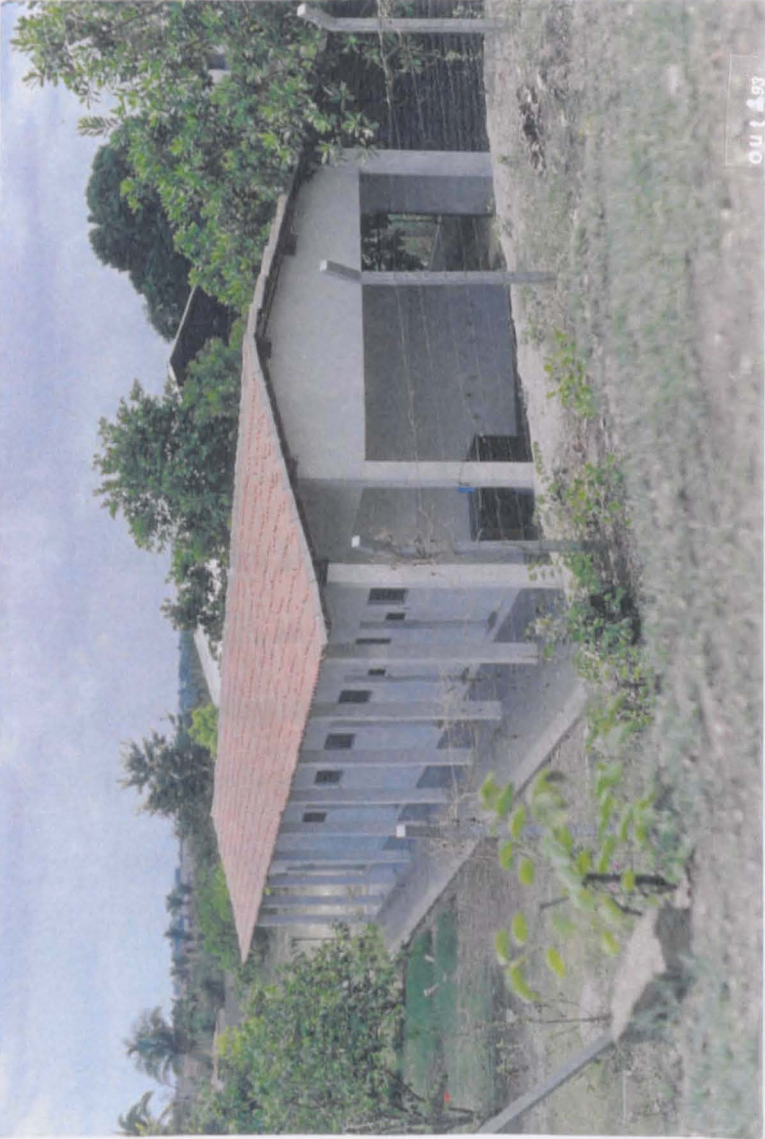


Figure 2.14

Five of a total of seven rooms had metal divisions with the objective of physically isolating 5 individual dogs or groups of three, dependent on the design of the experiment. Another improvement to the original structure was the construction of a second level at 80 cm from the floor, using a stainless steel grating (Figure 2.14). This assisted with cleanliness of the kennel, which was washed daily using neutral detergent and mild bleach solution.

2.5.2.3 Nutrition and maintenance

Dog ration (Purina plus) with milk and cooked chicken heads and feet were offered daily to each animal. Drinking water was always available.

2.5.2.4 Transmission

For the principal study colony-bred *Lu. longipalpis* were fed by xenodiagnosis on a naturally infected dog with amastigotes disseminated throughout the skin. Seven days later, after oviposition, groups of 7 to 15 surviving flies were fed on the internal part of the right ear of each experimental animal (see Results).

2.5.2.5 Follow-up

The follow-up post-infection was performed weekly during two months, at every fifteen days during 4 months and subsequently monthly.

For all the dogs a careful clinical examination was performed concentrating on the site where the sandflies had fed. Altered skin was suspected to indicate early presence of amastigotes. Presence of amastigotes in the skin was detected by giemsa staining smears obtained by skin scarification and by

xenodiagnosis using a colony-bred *Lu. longipalpis*, fed on the altered skin or where the infected flies had been fed. Both parasitological examination and xenodiagnosis on other part of the body of the experimentally infected dogs were used routinely during the follow-up to demonstrate dissemination of the disease. Giemsa staining of smears of bone marrow aspirates was also used as another follow-up parameter, to demonstrate systemic dissemination. Examination of spleen or liver aspirates was used exceptionally because of their hazard to the animal.

To demonstrate seroconversion, serological follow-up was performed using IFAT only, as described in section 2.3.4.1, based on the earlier comparison of serological methods that showed IFAT to be one of the most effective tests available.

2.6 Treatment trial with aminosidine

2.6.1 Selection of animal groups

A total of twenty-one dogs were treated. All animals had been brought to the Department of Parasitology for clinical, parasitological and serological examination because the owners suspected the presence of canine VL. Dogs were parasitologically proven to be infected with *L. chagasi* (Results) before entering the treatment trial and were allocated sequentially into the experimental groups. The owners of the dogs donated the animals to the trial in the knowledge that, in accordance with local public health recommendations, the only alternative was to have them destroyed.

The first group of dogs consisted of three adult Dobermann pinschers. The second group, of six adults, was comprised of two Dobermann pinschers, one German shepherd, one tawney boxer, one English cocker spaniel and one toy terrier. The third group of twelve adults comprised three Dobermann pinschers, one German shepherd, one 'fila Brasileiro', two toy terriers, one Irish setter, one short-haired dachshund, one bichon frise and two of unidentified breed.

2.6.2 Treatment

Treatment was with aminosidine sulphate (Gabbromicina, Farmitalia Carlo Erba) dissolved in sterile distilled water and administered by intra-muscular injection, in accordance with the manufacturer's instructions. Group one was given 20 mg/kg of body weight, daily, for fifteen days; group two, 80 mg/kg for twenty days, and group three, 40 mg/kg for thirty days.

The three groups were not treated simultaneously, results from group 1 were available before groups 2 and 3 were started. No specific control group of untreated animals was set up for this experiment, but during the course of this project several animals of similar clinical and parasitological status were maintained for experiments on the transmissibility of canine VL to sandflies (Vexenat *et al.*, 1993; Vexenat *et al.*, 1994). All such animals showed progressive symptomatic canine VL, none recovered, and towards the terminal stages of the disease they were humanely killed.

2.6.3 Clinical, parasitological and serological examination

All animals were examined clinically for signs of depilation and exfoliative dermatitis, external lesions, abnormal claws, and conjunctivitis/keratitis, and compared with the normal weight range for the breed (Table 1) (Pugnetti, 1988). Parasitological examination was by microscopy of Giemsa-stained impression smears of skin biopsies and sternal bone marrow aspirates. All animals included in the study had amastigotes in the skin and/or bone marrow. Bone marrow samples were obtained by elevation of the head of the dog in a sitting position and sternal puncture, which was rapid and well-tolerated. Serological examination was by the indirect immunofluorescent antibody test (IFAT) as described previously, based on comparisons of the IFAT, enzyme linked immunosorbent assay (ELISA) and the direct agglutination test (DAT) (Vexenat *et al.*, 1993). Clinical, parasitological and serological examination was performed prior to treatment, repeated five days after start of treatment and subsequently approximately every fifteen days, or less frequently for dogs with clinical recovery and long-term survival (Results). Impression smears of spleen and liver were examined post-mortem.

Possible adverse effects of treatment were followed by monitoring loss of appetite, weight loss, ocular changes, and lack of response to auditory or olfactory signals. True adverse effects were considered to be those occurring within one month of the end of treatment. Nevertheless we also noted and report here (Results) any event that occurred during follow up at any time since we could not predict long term effects of the drug.

2.7 Treatment trial with buparvaquone (Butalex)

Seven naturally infected dogs that had acquired infection in the urban endemic area of Teresina, Piauí state, Brazil were treated with Butalex® (Pitman-Moore). All animals were symptomatic and parasitologically positive. Treatment was by intramuscular injection into the upper rear leg, with 5 mg/kg of body weight, on days 0, 4, 8 and 12 (four doses, total period 12 days). The clinical and parasitological status of each animal was evaluated before, during and after treatment, as described above for the aminosidine trial (2.6.3).

RESULTS

CHAPTER 3: EPIDEMIOLOGY OF VISCERAL LEISHMANIASIS IN TERESINA

3.1 Prevalence and incidence of human and canine VL

A retrospective study (1981-1992), of epidemiological data on VL in Teresina, from the National Health Foundation, (NHF) of the Ministry of Health, gave a picture that was not encouraging with respect to the immediate control of this disease. The outbreak described by Costa *et al.* (1990) and the recent data described here, confirm the continuing importance of suburban endemic VL.

The human cases of VL that occurred in Teresina in each calendar month for the period 1981 to December 1994 are summarised in **Figure 3.1**. According to data available since 1985 in the records of the NHF, which recorded cases of human VL in the entire State of Piauí, the majority of registered human cases were concentrated in the city of Teresina. (**Figure 3.2**).

Incidence was calculated from demographic (census) data. The age and sex distributions of human cases of VL were analysed for comparison with the characteristics of other outbreaks. The analysis of incidence showed that the vast majority of human cases, 800 of 1045 (77%) were found in children under the age of 10 years. A comparison of the incidence by sex and age demonstrated that human VL predominated in males for those cases who were more than 10 years old, according to analysis of data on 1045 patients during the period 1987-1993. These data were statistically significant for ages 21 to 45 (R. Siddiqui, personal communication. (**Figure 3.3**))

Figure 3.1

**Reported cases of human visceral leishmaniasis in Teresina
Number of cases/month (1981-1993)**

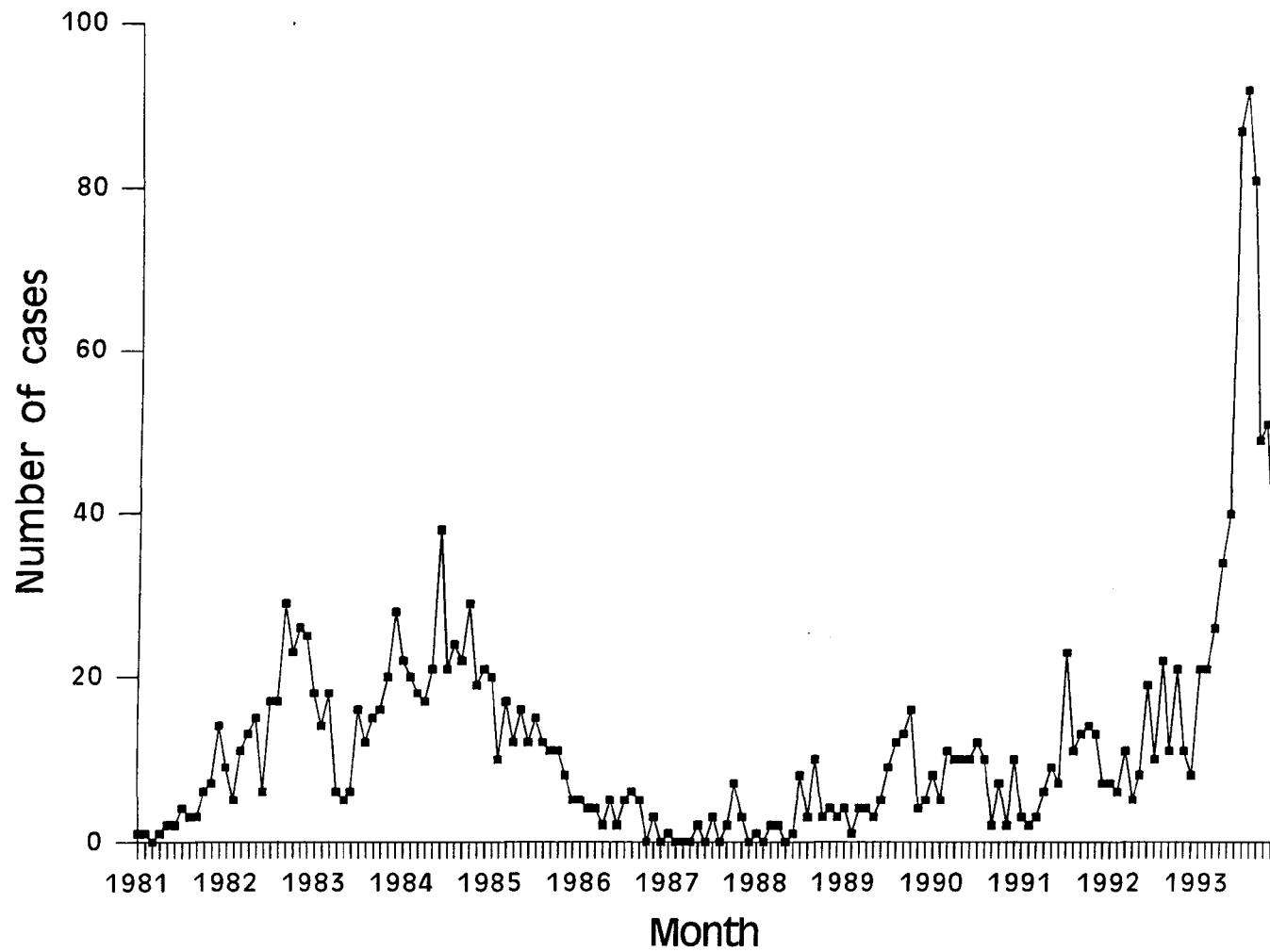


Figure 3.1

Figure 3.2

Cases of human visceral leishmaniasis in the state of Piauí and in the city of Teresina, 1985-1993

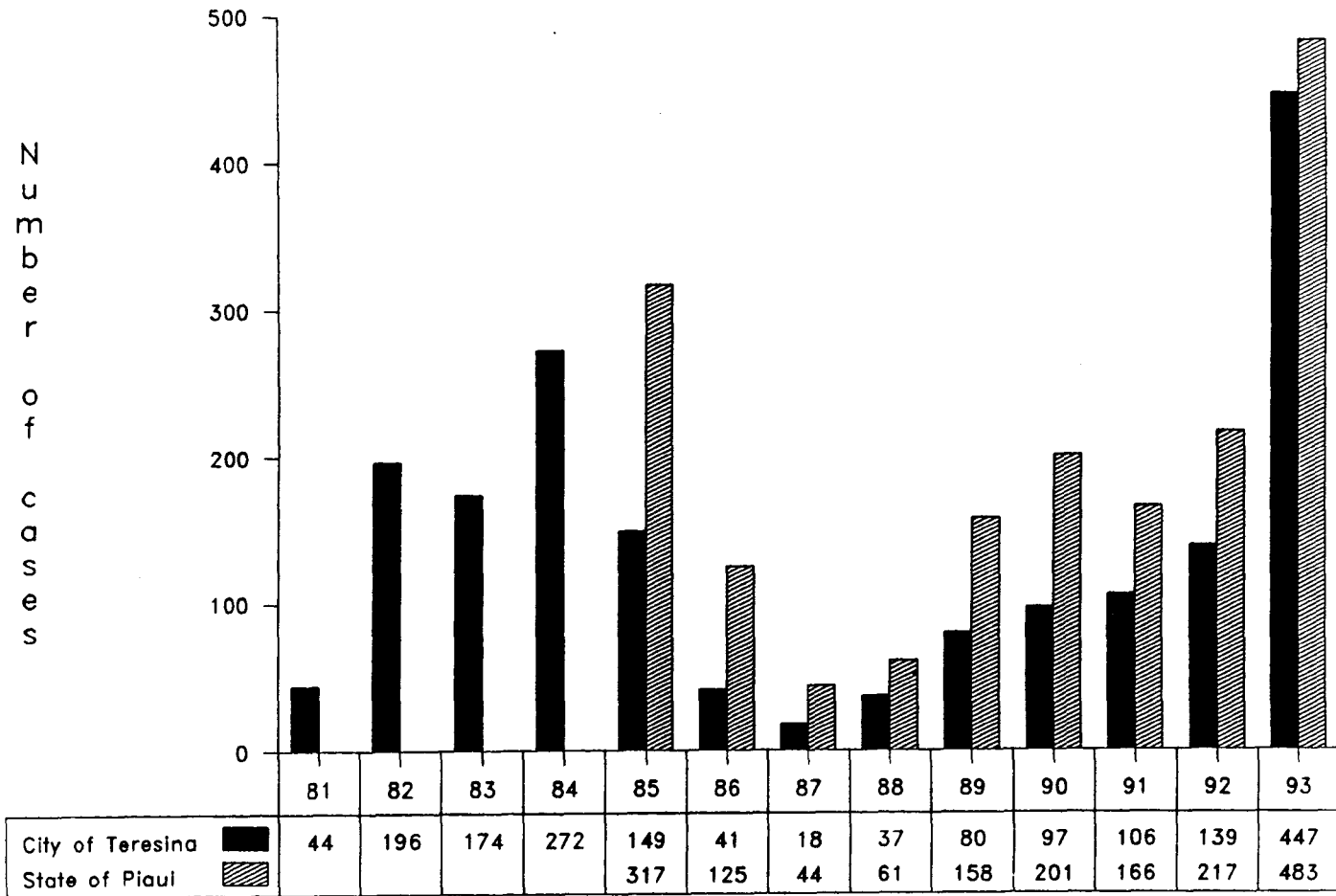


Figure 3.2

Figure 3.3

Incidence of human visceral leishmaniasis and age and sex distribution, Teresina 1987-1993

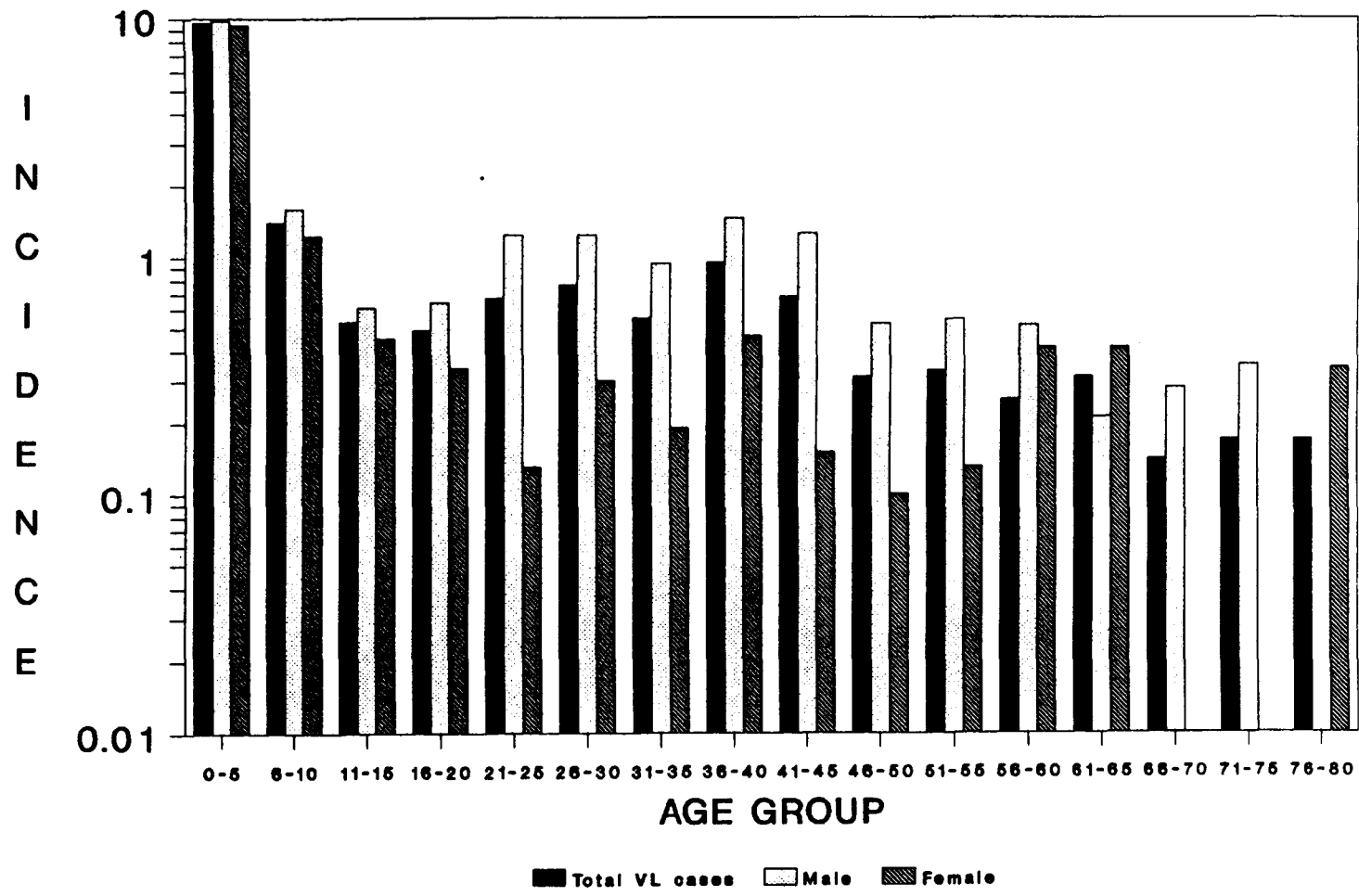


Figure 3.3

To compare the recent percentage of seropositive dogs with the percentage in previous years the data registered in the 1985-1991 records from the NHF were assembled together with the data for 1992 onwards from the NHF computer. According to all these records a total of 559,977 dogs were examined between 1985 and 1993. Of them 10,944 were diagnosed as seropositive. In addition a total of 27,936 of 36,527, (76%) of dogs from 1993, were examined using both IFAT and dot-ELISA as part of this project, during the rabies vaccination campaign. **(Table 3.1)**

To determine if there was a relationship between the epidemic of human VL and the percentage of seropositive dogs a comparative study was performed, using the data for human and canine VL of previous years found in NHF records and our more recent data. The parallel analysis of the number of human cases that occurred between 1982 and 1993, and the percentage of seropositive dogs showed an increasing trend in human VL, whilst the percentage of seropositive dogs has apparently been more or less stable for the last 6 years, although seropositivity rate apparently fluctuated somewhat between 1985 and 1987, perhaps in association with the intensive control campaign at that time **(Figure 3.4)**. In assembling these data we have assumed that the reliability of the serological tests did not change and that there was no major change in the way dogs were selected for examination.

To determine if there was some relationship between the presence of human VL and the records of seropositive dogs per district, the number of patients found in each district, was plotted against the number of seropositive dogs for January to September of 1993. **(Figure 3.5)**. No relationship was apparent between the presence of human cases and records of seropositive dogs. There were districts, in that period, where human cases were never notified but seropositive dogs were registered. **(Figure 3.6)**.

Table 3.1

Seropositivity rate (IFAT) in dogs 1985 - 1993

Year	dogs examined	dogs seropositive	percentage seropositive
1985	27,964	1.616	5.7
1986	48,084	644	1.3
1987	55,190	1.361	2.4
1988	61,912	1.134	1.8
1989	43,171	827	1.9
1990	120,459	1.903	1.5
1991	108,261	1.797	1.6
1992	58,409	885	1.5
1993*	40,861	1.796	4.4
Total	559,977	10.944	

(*) = includes 27,436 dogs examined by both IFAT and dot-ELISA

Figure 3.4

Seropositivity rates in dogs and number of human cases, Teresina, 1983-1993

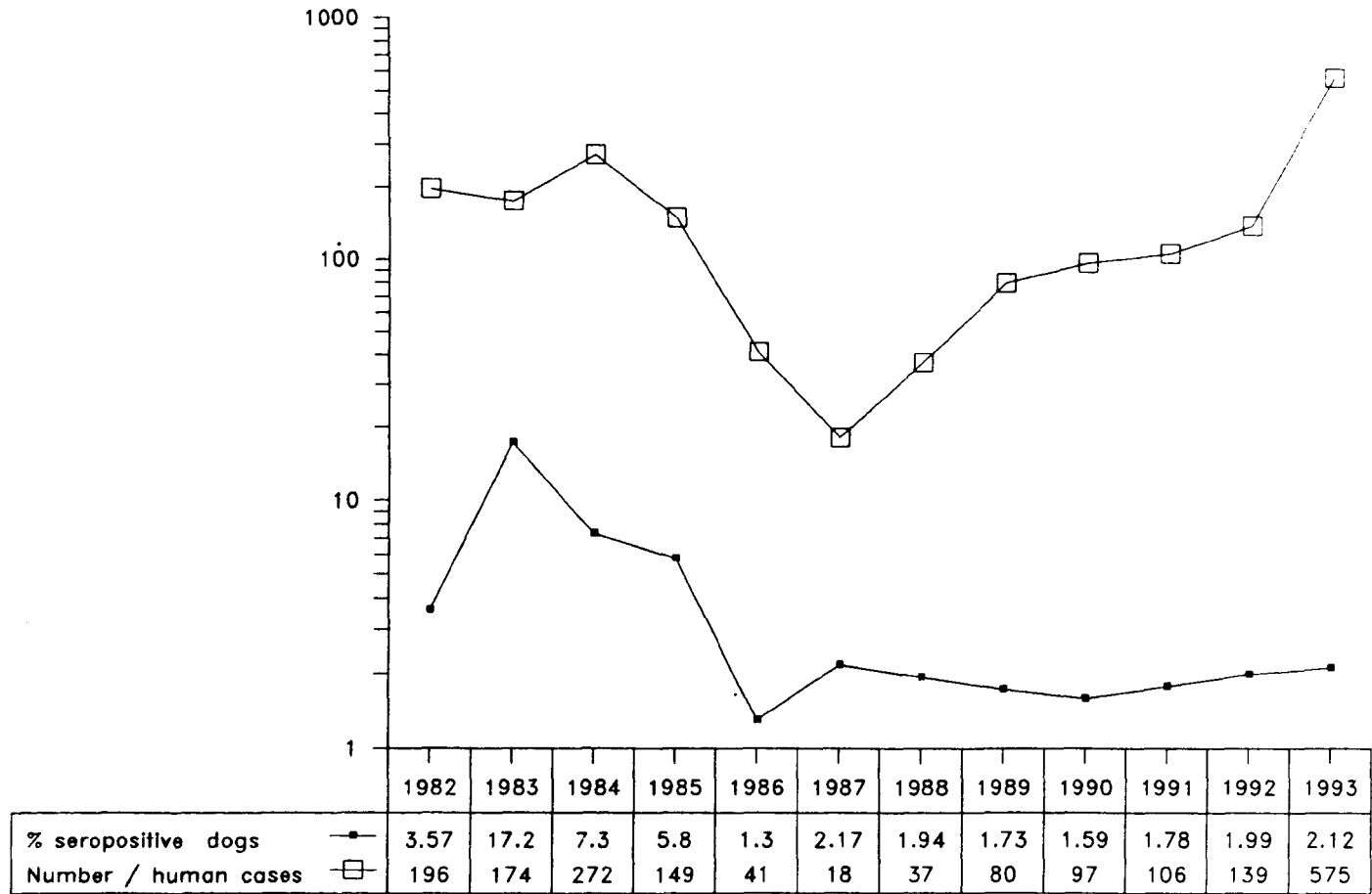


Figure 3.4

Figure 3.5

Distribution of human cases and seropositive dogs, Teresina, 1993

Districts

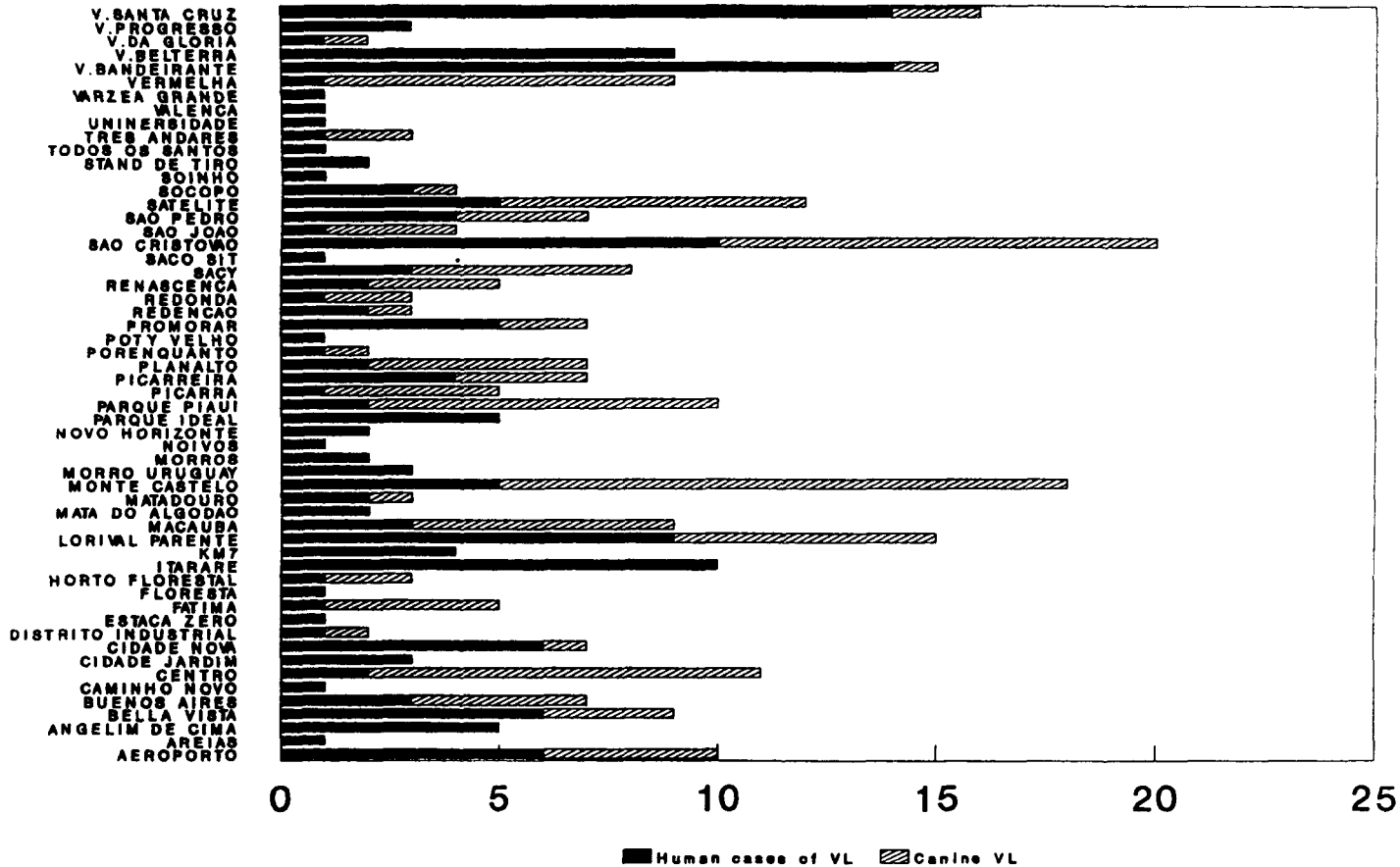


Figure 3.5

Figure 3.6

**Seropositive dogs in districts without records of human cases,
Teresina, 1993**

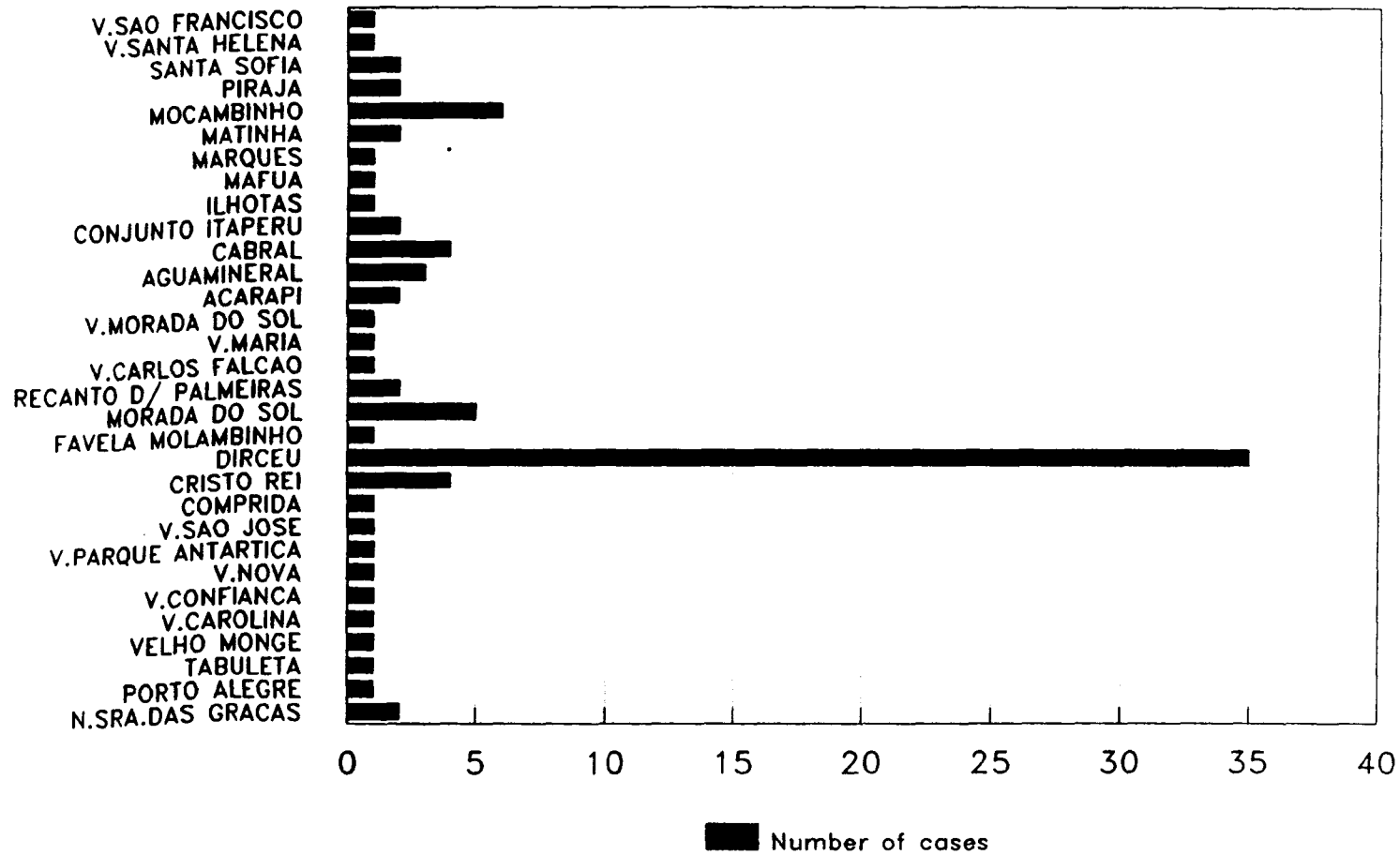


Figure 3.6

To perform an initial comparison between the structure of the canine population and that found for other endemic regions (Abranches *et al.*, 1991) a selected group of 209 dogs from Teresina, Piauí, which were studied as part of this project between 1992 and 1993, were grouped by breed. A total 51% of dogs were undetermined breeds (mongrels) and 49% were represented by 19 different breeds. The Dobermann pinscher, the German shepherd, crossbreeds of both and the Pekingese were the most abundant breeds among infected animals in this preliminary group (Table 3.2).

3.2 Characterization of *Leishmania* isolates

To characterize *Leishmania* parasites from Teresina by isoenzyme electrophoresis the isolates made by McGreevy were used, which were already available to us in the LSHTM cryobank. These parasites were isolated from one human case and from dogs with VL.

Electrophoretic enzyme profiles of 9 isolates (one human and eight from dogs) were distinct from the *L.(V.)braziliensis* strain, MHOM/BR/85/LTB300 and indistinguishable from the *L.chagasi* WHO reference strain, MHOM/BR/74/ PP75. No differences were seen between the *L. chagasi* isolates from Teresina in their enzyme profiles but this clearly deserves further study with a larger number of strains (Figures 3.7, 3.8 and 3.9).

The *Leishmania (V.) braziliensis* strain was used because it is common in some areas of CL and MCL in Brazil and dogs are found naturally infected with this species, although there are no records of visceralization produced by *L.(V.) braziliensis*, in dogs. *L.(L.) amazonensis* was not used as a control because this species has never been isolated from dogs.

Table 3.2

Breed of dogs represented in the diagnostic groups
Teresina. (1992-1993)

Breed	Observed number	+ve *	%
Doberman pinscher	29	26	25.4
German shepherd dog	11	9	8.8
Crossbreed d. and g.s.d	10	8	7.8
Great dane	7	6	5.8
Fila Brasileiro	6	2	1.9
Short-haired st.bernard	2	2	1.9
Rottweiler	2	2	1.9
Beagle	2	2	1.9
Irish setter	1	1	0.9
Afghan hound	1	1	0.9
Akita inu	1	1	0.9
Boxer	1	1	0.9
Pointer	1	0	0,0
Pekingese	14	12	11.7
Toy terrier(Pinscher)	5	3	2.9
Cocker spaniel	4	3	2.9
Puli	3	3	2.9
Basset	1	1	0.9
Fox terrier	1	0	0.0
Sub-total	102 (48.8%)	83	81.3
Undetermined	107 (51.1%)	84	78.5
TOTAL	209	167	79.9

* = dogs considered positive according to the reference standard

Figure 3.7

Electrophoretic patterns of MPI and SOD

Figure 3.7

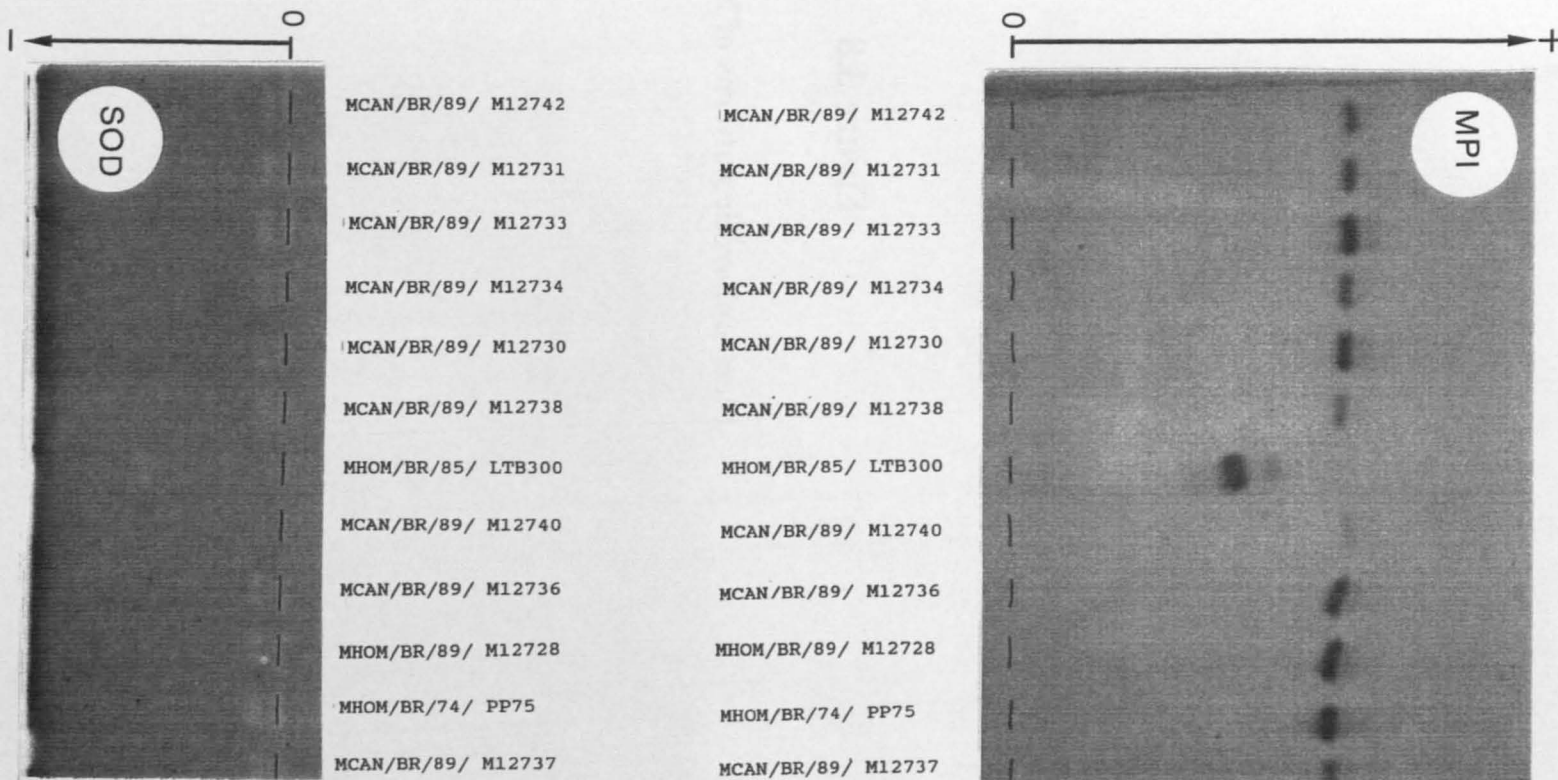


Figure 3.8

Electrophoretic patterns of NH1 and ES

Figure 3.8

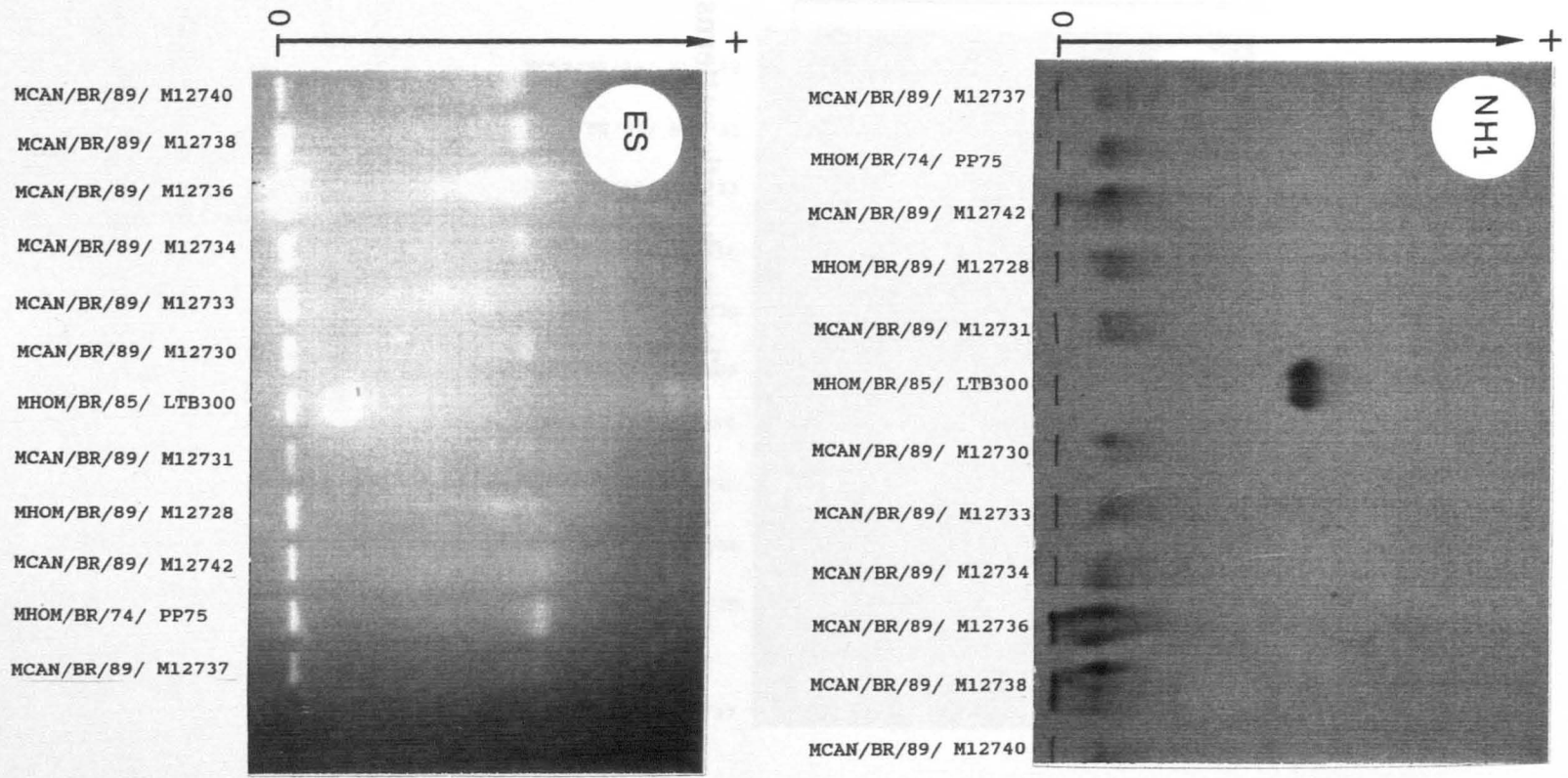


Figure 3.9

Electrophoretic patterns of 6PGD

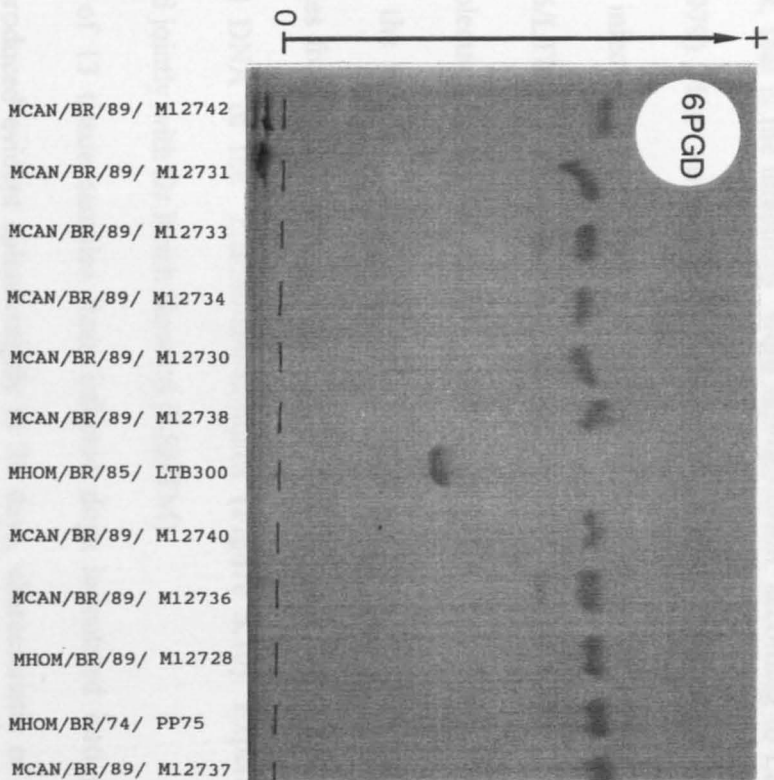


Figure 3.9

Another method used to characterize the parasites from naturally infected dogs was observation of the development of the promastigotes in the intestine of sandflies. Xenodiagnoses of 13 naturally infected dogs were performed and the flagellates found by dissection after oviposition were distributed in the stomach and the pharynx, that is the infections were *suprapylarian*, according to Lainson and Shaw (1979). For comparison the xenodiagnosis performed on an experimentally infected hamster with a strain of *Leishmania (V.) braziliensis* (MHOM/BR/85/LTB300) showed a *peripylarian* infection. (Table 3.3).

One molecular biological method, the Lmet2 DNA probe, was able not only to detect the parasites, but also to characterise them. When samples of infected sandflies from xenodiagnoses were applied to Hybond N membrane, the probe detected DNA of the *L.donovani*-complex (Figure 3.10). Experiments were performed jointly with Dr.Keith Howard (LSHTM).

Finally of 13 tissue samples from infected dogs inoculated into golden hamsters, all produced evident splenomegaly at 30 days, characteristic of the *L. donovani*-complex.

We conclude that all 9 isolates were *L. chagasi* by isoenzyme phenotype and behaviour in sandflies and hamsters.

3.3 Sandfly fauna

Studies of sandfly fauna and population dynamics had not been performed in this city. The only data were those published by Costa *et al.* (1990) and the records of the NHF (Table 3.4).

Table 3.3

Classification of flagellates according to distribution in the intestine of experimentally infected *Lu. longipalpis*, Teresina, Piaui.

Dog	Section
06a	suprapylaria
07a	suprapylaria
08a	suprapylaria
67	suprapylaria
68	suprapylaria
69	suprapylaria
74	suprapylaria
105	suprapylaria
112	suprapylaria
146	suprapylaria
147	suprapylaria
148	suprapylaria
170	suprapylaria
MHOM/BR/85/ LTB300	peripylaria

Figure 3.10

Positive *Lutzomyia longipalpis* (A1 - 6, B1 - 6, C1 - 6, and D1 - 6) and (E - 6) negative control, probed with Lmet2 chemiluminescent detection

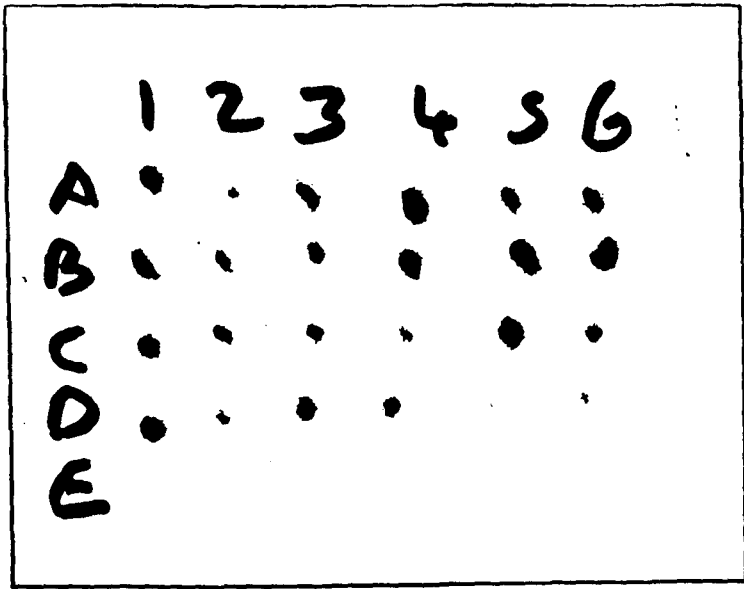


Figure 3.10

Table 3.4

Percentage of houses with *Lu. longipalpis*, January 1983-June 1986

Year	Study period (Jan-June; July-Dec)	Number of cases	Houses positive/ houses examined	%
1983	1	67	166/2.099	7,9
	2	97	37/416	8,9
1984	1	136	308/1.452	21,2
	2	136	34/920	3,7
1985	1	87	42/1.030	4,1
	2	62	30/1.029	2,7
1986	1	22	18/551	3,3

from Costa, C.H.N. *et al.* *Rev. Saude publ.*, S. Paulo, 24(5), 1990

A summary of the only entomological data found in the records of the NHF for 1987-1988 shows some captures of sandflies by district. Records of the presence of other sandfly species were also found but details on the methodology used to obtain those results are not known. (Table 3.5). Almost all the specimens collected were identified as *Lu.longipalpis* but *Lu.whitmani*, *Lu.migonei*, *Lu.intermedia* and *Lu.lenti*, were occasionally mentioned.

During this project large concentrations of sandflies were found in some parts of the city attacking children and adults at night and occasionally during the day. The bites were multiple and disseminated in some cases (Figure 3.11 A and B) In some houses sandflies were restricted to pig-sties and/or chicken sheds (Figure 3.12 and 3.13 and see below).

Captures of sandflies were performed between September-1992 and May-1993 to determine the distribution of *Lu.longipalpis* and to investigate the presence of naturally infected sandflies in the city of Teresina. During the project 36,395 sandflies were collected in 30 districts of the city. A total of 34,839 sandflies were identified as *Lu.longipalpis*, 1,516 *Lu.whitmani*, and 40 other sandflies species represented by 14 *Lu.shannoni*, 9 *Lu.lenti*, 8 *Lu.fischeri*, 3 *Lu.gomezi*, 2 *Lu.intermedia*, 1 *Lu.migonei*, 1 *Lu.pessoai*, 1 *Lu.bahiensis* and 1 *Lu.tupinambai*. 1,493 of 1516, (98%) of the *Lu.whitmani* specimens, were collected concomitantly with *Lu.longipalpis*, but only in two sites of capture, located in the districts of Cidade Satelite and Uninga (UFPI). In those sites, the common factor was the presence of a large number of goats. An interesting detail was that 305 of 382 (80%) of the females of *Lu.whitmani* were collected on the walls inside the houses and despite the presence of the goats, *Lu.whitmani* was never collected on those animals during these captures.

Table 3.5**Distribution of sandflies by district, Teresina, 1987-1988 and number of flies recorded**

District	1987	1988
Aeroporto	4	5
Angelim de cima		35
Catarina	2	
Cidade satelite	54	35
Horto florestal	2	
Ininga	5	
Jockey club	2	
Km 7	2	
Lorival parente	6	5
Memorare	71	76
Picarreira I	11	
Picarreira II		13
Pio XII		5
Planalto	7	2
Redencao	210	2
Sao cristovao	36	41
Sao joao		5
Sao pedro		1
Stand de tiro		3
Vicosa	18	
Vila bandeirantes		2
Vila operaria	2	3
Zona rural	114	

Figure 3.11A and B

Sandfly bites on a child and an adult



Figure 3.11

Figure 3.12

Sandflies captured during a single night, with CDC light-trap in a pig-sty

Figure 3.13

Sandflies on the body of a pig

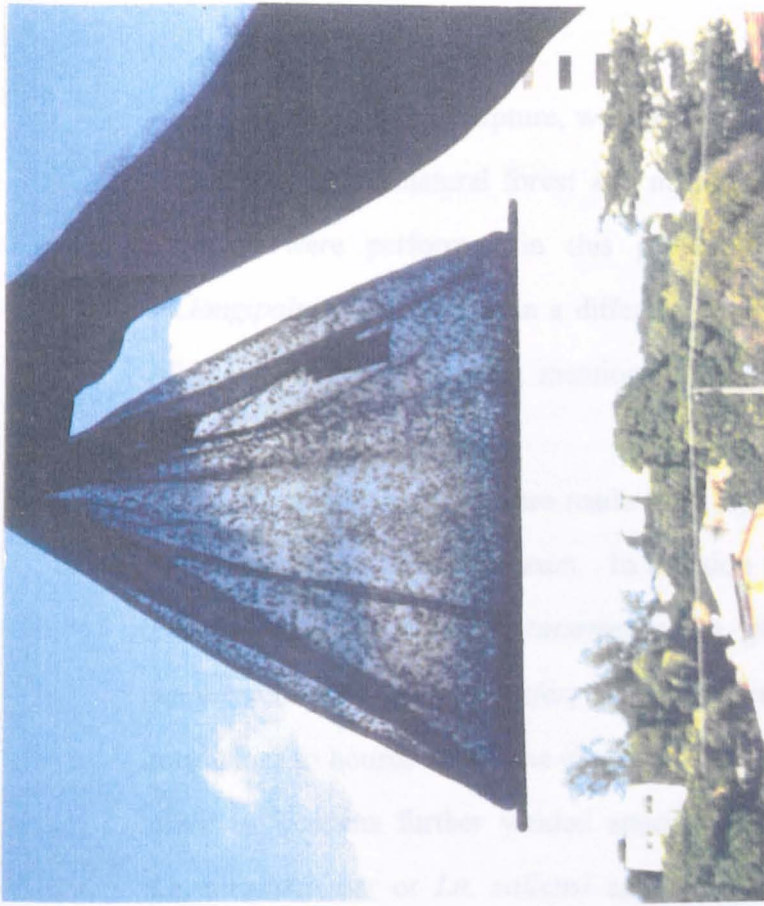
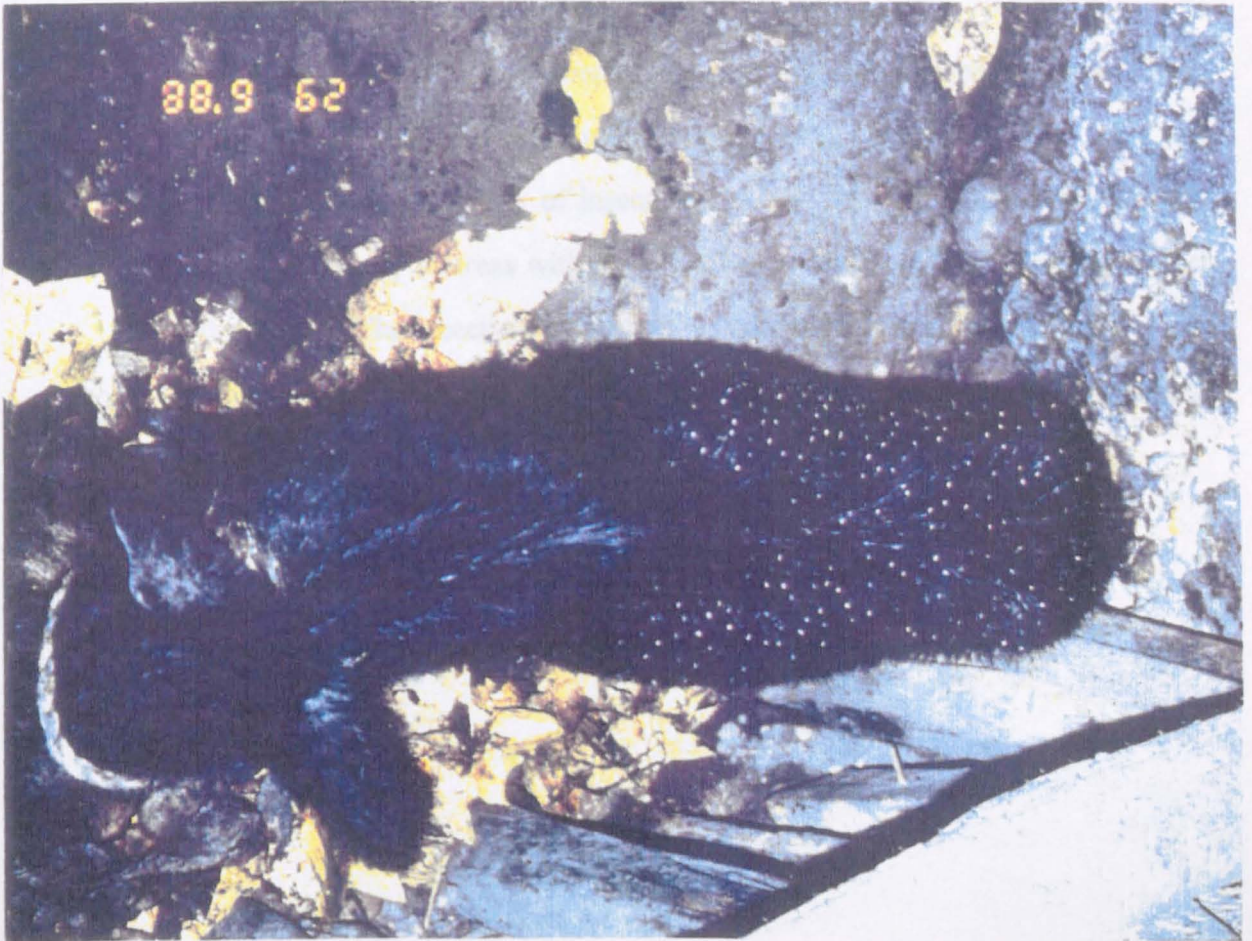


Figure 3.12

Figure 3.13



Another place of capture, was the Zoological Garden, which is in a riverine area, with residual natural forest and mature trees, on the outskirts of the city. Captures were performed in this place with the objective of knowing if *Lu.longipalpis*, was present in a different ecotope, with the other animal species. All the other sandfly species, mentioned above were found in this place. (Table 3.6).

Further collections were made from April to August 1995 in collaboration with Diarmid Campbell-Lendrum. In addition to *Lu. longipalpis*, *Lu. whitmani*, *Lu. shannoni*, *Lu. lenti*, *Lu. intermedia*, *Lu. gomezi* and *Lu. migonei*, examples were captured of *Lu. quinquefer*, *Lu. trinidadensis*, and *Lu. termitophila* in CDC traps close to houses within the city boundaries. CDC captures made in the forest close to Teresina further yielded specimens of *Lu. goiana*, *Lu. cortellezzi* or *Lu. corumbaensis* or *Lu. sallensi* and *Ps. wellcomei* or *Ps. complexa* (definite classification of these last two species was not possible as no males were captured.)

Despite the efforts of personnel of the NHF, spraying with insecticide was not constant and it did not cover the entire city, (Figure 3.14). This task depended on the availability of insecticide, which was not always sufficient and generally used only in areas with abundant human cases and/or reported presence of the vector. The insecticides used for a residual effect were deltamethrin and DDT, and sumithion and malathion for ultra- low volume (UBV) spraying. (Figure 3.15A and B).

Table 3.6

Distribution of sandflies by districts
Teresina, September 1992 - May 1993

Place	Method	Male	Female	
Total				
<i>Lu. longipalpis</i>				
Aeroporto	pooter	19	9 (6*)	28
Buenos aires	pooter	55	7 (6*)	62
Fatima	pooter	6	5*	11
Horto florestal	pooter	7	2*	9
Itarare I	pooter	3	1*	4
Jockey club	pooter/CDC	182	99 (16*)	281
Km 7	pooter/CDC	188	98 (8*)	286
Lourival parente	pooter	10	0	10
Mocambinho	pooter	3	1*	4
Monte castelo	pooter	15	7*	22
Morada do sol	pooter	121	551 (262*)	672
Morada nova	pooter	121	78 (51*)	199
Morro da Esperanca	pooter/CDC	15,892	960 (457*)	16,852
Picarreira	pooter	7	5*	12
Primavera	pooter	15	10 (9*)	25
Promorar	pooter	1	8*	9
Renacenca	pooter	12	0	12
Sao cristovao	pooter	67	33 (21*)	100
Socopo	pooter/CDC	331	122 (44*)	453
Sta Fe	pooter/CDC	7,300	1,541 (67*)	8,841
Taboleta	pooter	9	6*	15
Universidade (Kennel)	pooter	68	38 (12*)	106
Uruguay	pooter	43	28*	71
Vila Bandeirante	pooter/CDC	123	15*	138
Vila da paz	pooter	23	11*	34
Vila operaria	pooter/CDC	1,312	332 (54*)	1,644
Vila Sta. Helena	pooter	320	38*	358
Cidade satelite	pooter	32	13 (5*)	45
Ininga (UFPI)	pooter/CDC	4,201	320 (89*)	4,521
Zoological Gardens	pooter	15	0	15
<i>Lu. whitmani</i>				
Cidade satelite	pooter/CDC	55	77	132
Ininga (UFPI)	pooter/CDC	1,060	301	1,361
Zoological Gardens	pooter	19	4	23
other species				
Zoological Gardens	pooter	23	17	40
Total		31,658	4,737	36,395

* = All flies not identified were squashed onto Hybond N (Amersham) for detection of infection, using the DNA probe

Figure 3.14

Spraying a house



Figure 3.14



Figure 3.15 A

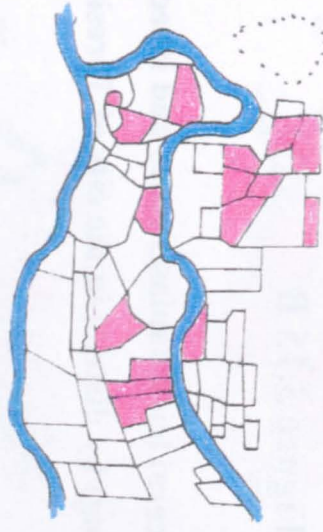
Distribution of human visceral leishmaniasis cases and insecticide (residual and UBV spraying) by district in the city of Teresina, 1987-1990.

1987

1988

1989

1990



INSECTICIDE

- Residual
- UBV
- Residual and UBV



NUMBER OF HUMAN

- 1
- 2 to 5
- 6 to 10
- 11 to 20
- 21 to 30
- 31 to 40
- More than 41

Figure 3.15 A

Figure 3.15 B

Distribution of human visceral leishmaniasis cases and insecticide (residual and UBV spraying) by district in the city of Teresina, 1991-1993.

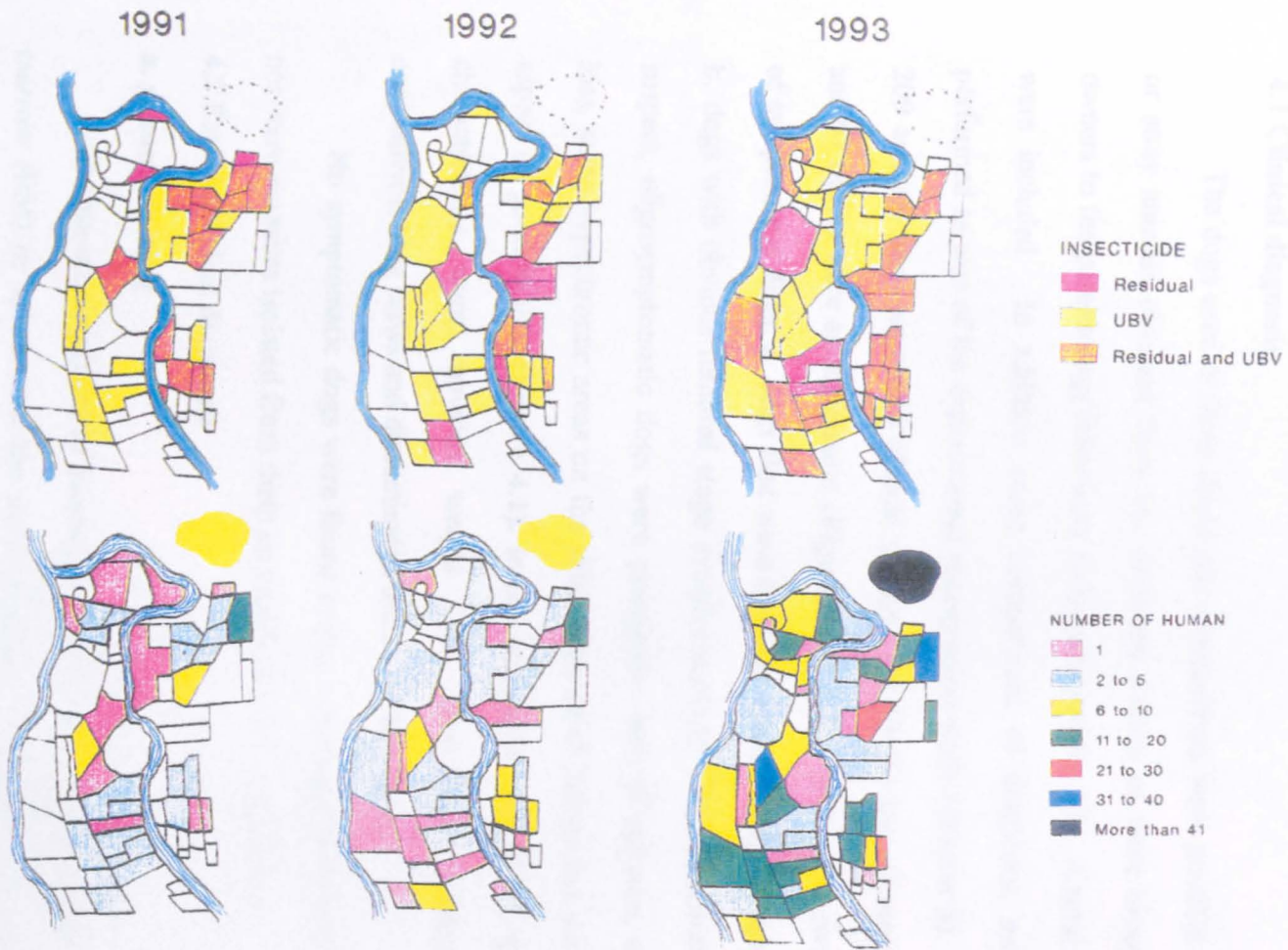


Figure 3.15 B

CHAPTER 4: COMPARATIVE DIAGNOSIS OF CANINE VISCERAL LEISHMANIASIS

4.1 Clinical diagnosis

The dogs used in these diagnostic comparisons were possible cases of VL or stray animals obtained from the Zoonosis Centre or were brought by their owners to the parasitology laboratory to be screened for VL. A total of 209 dogs were included. In addition some comparisons of diagnostic methods were performed as part of the experimental transmission study (chapter 6). Among the 209 animals there were two clinical groups: 112 (54%) were symptomatic dogs and 97 (46%) were asymptomatic (**Figures 4.1, 4.2**). There were two subgroups of symptomatic dogs: **a.** dogs that were clinically suspect (oligosymptomatic) and **b.** dogs with obvious terminal stage symptomatology. The characteristics of the suspect, oligosymptomatic dogs were principally: lack of appetite, slow weight loss, some hypochromic areas on the skin, some small lesions but with a general aspect of good health. (**Figure 4.1**). In the terminal stages of VL the principal characteristics were: obvious weight loss, generalized depilation, acute conjunctivitis, long claws and disseminated lesions, (**Figure 4.2**).

No symptomatic dogs were found in the control group from Brasilia, DF, nor were parasites isolated from them by parasitological examination.

4.2 Parasitological diagnosis

a. giemsa-staining

Giemsa-staining (Gm) of biopsies of skin lesions or of aspirates from bone marrow (BM) or spleen was the method used to determine the percentage of parasitologically positive dogs. It was not always possible to perform all these tests because sometimes the dog owners refused. In 81 of 112 (72%) symptomatic dogs and in 34 of 97 (35%) asymptomatic dogs, amastigotes were

Figure 4.1

A. asymptomatic Doberman pinscher, B. Cocker spaniel with tiny flaked area on snout



Figure 4.1

Figure 4.2

A. symptomatic dog, B. long claws of a symptomatic dog



Figure 4.2

demonstrated. Thus amastigotes were overall found more easily in the symptomatic group.

b. diagnosis by growth *in vitro*

Cultivation of samples from BM and/or liver aspirates in " Difco" blood agar medium was not the most efficient means of demonstrating the parasite but in some cases the dog was only parasitologically positive by isolation of parasites in culture.

c. xenodiagnosis

Xenodiagnosis was used to determine the percentage of sandflies which could become infected on a naturally infected dog and also to know if apparently normal skin from a naturally infected dog is infective to sandflies. The results are described as part of the experiments on transmissibility (chapter 5).

4.3 Diagnosis by detection of DNA

Preliminary evaluation (with M. K. Howard) of the chemiluminescent Lmet2 probe with post-mortem samples showed that it could detect *Leishmania donovani*-complex amastigotes widely disseminated in a parasitologically positive dog (**Figure 4.3**). Nevertheless a pilot study indicated that the Lmet2 DNA probe (radioactive or chemiluminescent technique) was not the most sensitive method of detecting parasites in tissue samples (BM, skin biopsies, and/or white blood cells). In comparing this technology with the known methods of parasitological diagnosis, only 5 dogs were diagnosed by the radioactive probe and 4 by the chemiluminescent method out of 20 parasitologically positive dogs. (**Table 4.1, Figure 4.4**).

Figure 4.3

***post mortem* examination of tissue samples from *Leishmania chagasi* infected dog by chemiluminescent DNA probe. A: skin: 1, scrotum; 2, right thigh; 3, left thigh; 4, right thorax; 5, left thorax; 6, left eye; 7, right eye; 8, left outer ear; 9, left inner ear; 10, right outer ear; 11, right inner ear; 12, nose; 13, mouth; 14, mouth; B: positive control; C: 1 spleen; 2, liver; 3, lung; 4, kidney; 5, lymph node; 6, heart; 7, testicle; 8, pancreas; 9, intestine.**

Table 4.1

Parasitological diagnosis compared with DNA probing for *Leishmania* infected dogs.

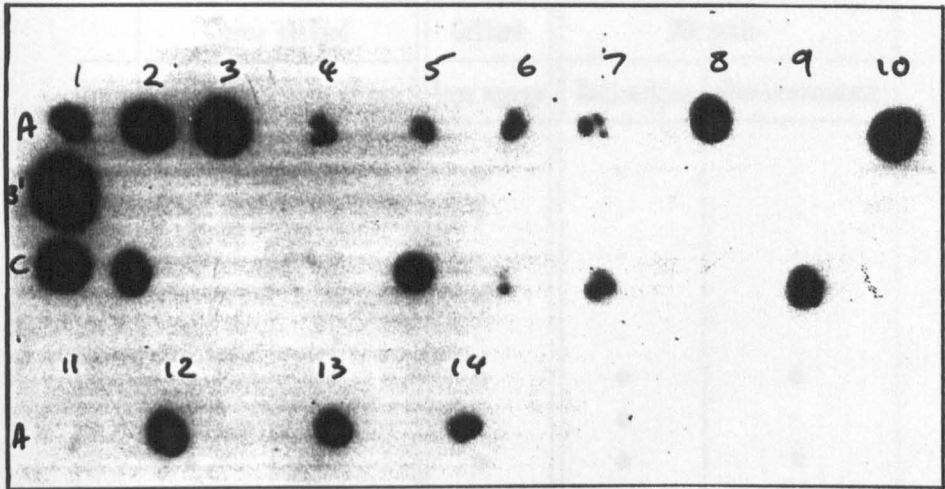


Figure 4.3

Table 4.1

Parasitological diagnosis compared with DNA probing for naturally infected dogs.

Dog	Giemsa stained		Culture	DNA probe	
	Bone marrow	Skin biopsy	Bone marrow	Radioactive	Chemiluminescent
81	●	●	●	-	-
12	●	●	●	-	-
13	●	●	-	-	-
15	●	●	●	-	-
16	●	-	-	-	-
18	●	●	●	●	●
21	-	●	-	●	-
23	●	●	●	●	●
33	-	●	●	●	●
34	●	-	●	-	-
35	●	-	-	-	-
37	-	●	●	●	●
39	-	●	●	-	-
48	-	●	●	-	-
41	●	●	●	-	-
42	-	●	-	-	-
44	●	●	●	-	-
45	●	●	-	-	-
46	●	●	●	-	-
49	-	●	●	-	-

● = +ve

- = -ve

Figure 4.4

Examples of *Leishmania donovani*-complex DNA detected in skin samples, by chemiluminescent (A, B and C) and (C1) radioactive Lmet2 probes.

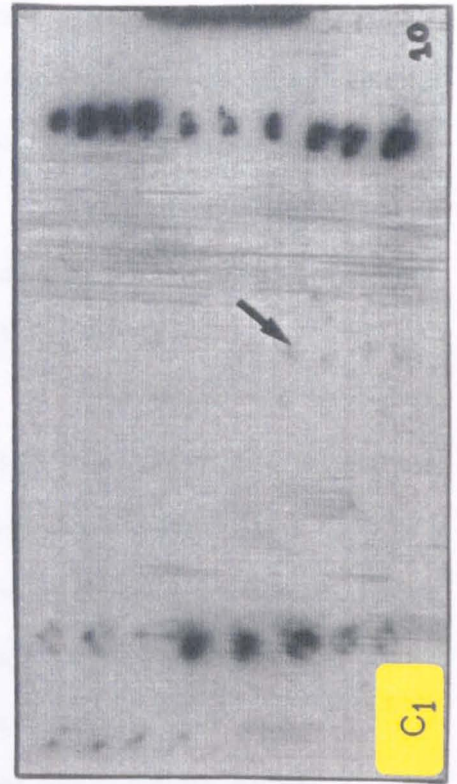
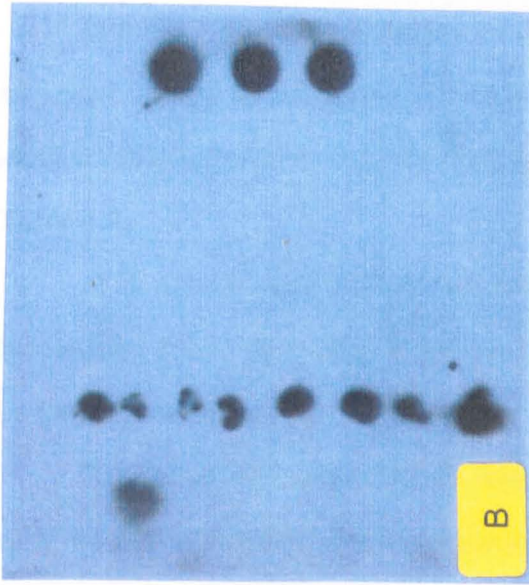


Figure 4.4

In a more detailed assessment of the Lmet2 probe (with samples obtained and tested through an EC sponsored visit by Dr M. Cabral) the probe was able to detect some positive bone marrow samples missed by parasitological examination (Table 4.2).

PCR assays using kDNA primers and agarose gel electrophoresis were discontinued due to difficulties in interpretation (Discussion).

4.4 Serological diagnosis

A total of 837 dog samples were used for the comparative serological study, excluding samples from the experimental infections (chapter 6).

- a. A Total of 111 samples, (99 sera from a non-endemic area, Brasilia DF, Brazil and 12 sera from London, UK), which were used as negative controls.
- b. 129 serum samples from a Mediterranean area, Corsica.
- c. 388 blood spot samples collected on filter paper, from Teresina, Brazil.
- d. 209 serum samples of a selected group of dogs from Teresina, Piaui. Blood spot samples were also collected from 49 dogs of this group to compare serology with serum or eluate samples.

4.4.1 Specificity and sensitivity

a. specificity

To determine the specificity of the serological methods 62 serum samples (50 sera from Brasilia DF, Brazil and 12 sera from London UK) were initially used.

Non-specific antibodies were detected in 2 of 50 sera from Brasilia DF, Brazil, by IFAT and dot-ELISA and ELISA; and in 4 of the 12 sera from London UK by ELISA only, whereas all of the control sera were negative by DAT.

Table 4.2

The diagnosis of canine VL: results obtained with 18 dogs with, and 37 dogs without clinical signs of the disease from Teresina, Brazil

	Number of dogs	Lmet 2 DNA probe	Giemsa and/or culture	IFAT
Dogs with clinical signs	12	+	+	+
	5	+	-	+
	1	-	+	+
Positive		17/18	13/18	18/18
Dogs without clinical signs	7	+	+	+
	13	+	-	+
	4	+	-	-
	1	-	+	+
	7	-	-	+
	5	-	-	-
Positive		24/37	8/37	28/37
Control dogs (Glasgow)		-	-	-
Negative		16/16	16/16	25/25

The specificity was determined by the following formula:

$$\frac{\text{Number of negative dogs that are also test -ve (N-)}}{\text{Total number of negative dogs}} \times 100$$

Thus, specificities of the serological methods based on these controls were:

ELISA 90.3%; IFAT and dot-ELISA, 96.7% and DAT 100%, (**Figure 4.5**). In an additional group of negative control dogs from Brasilia DF, Brazil, which was analysed only by IFAT and dot-ELISA, non-specific antibodies were detected in 2 of 49 serum samples by both tests and the specificity was therefore 95.9%. On the basis of the specificities of >95% and the low sensitivity of DAT (below) IFAT and dot-ELISA were selected as the most appropriate serological assays.

Surprisingly a high level of non-specific antibodies was detected by ELISA on serum samples from female dogs in London UK (**Figure 4.6**).

b. sensitivity

To determine the sensitivity of the different tests used, a reference standard for dogs considered to be positive was selected as those that were **parasitologically positive or had a positive DAT or had both IFAT and dot-ELISA positive.**

Figure 4.5

Comparative specificity of IFAT, dot-ELISA, ELISA and DAT

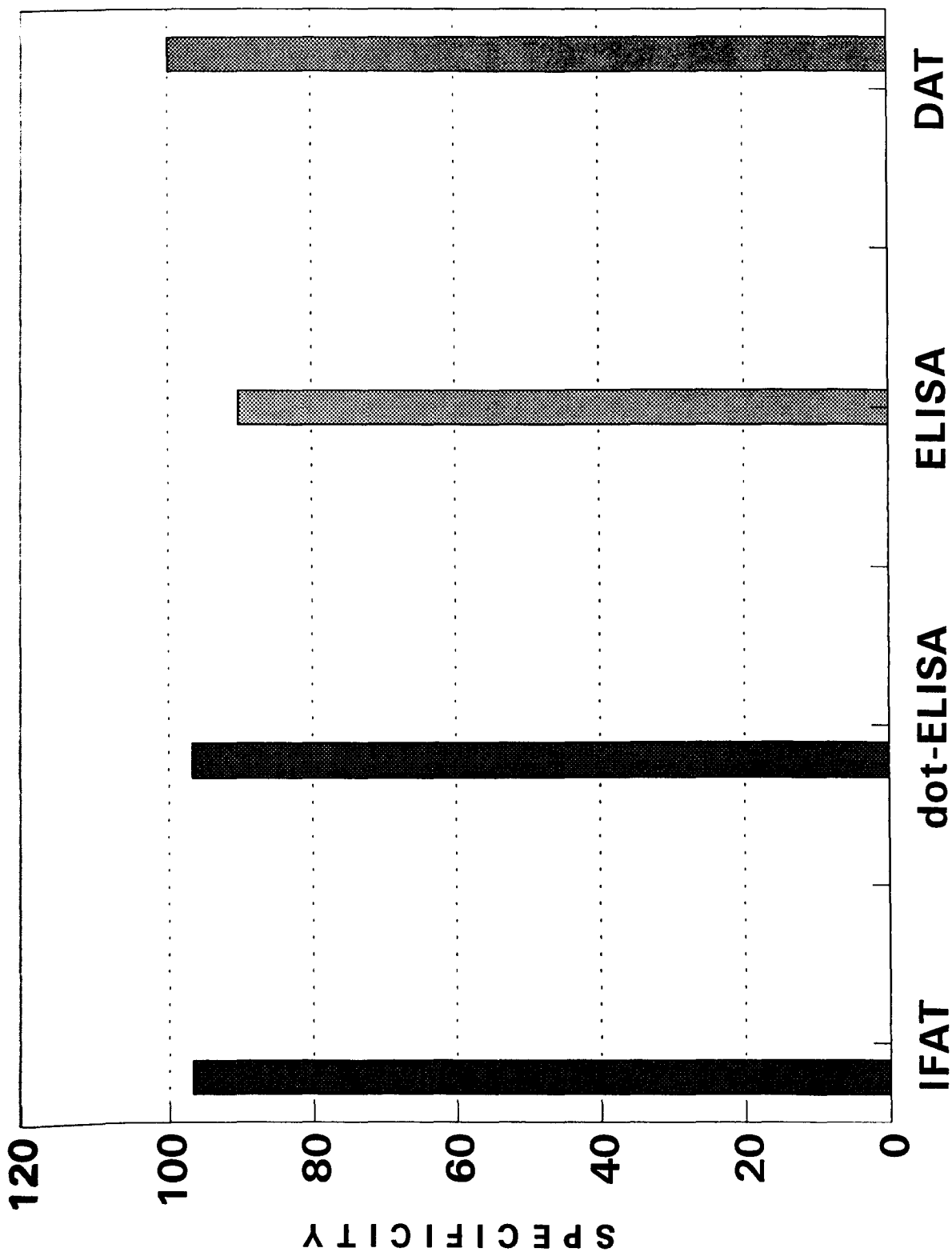
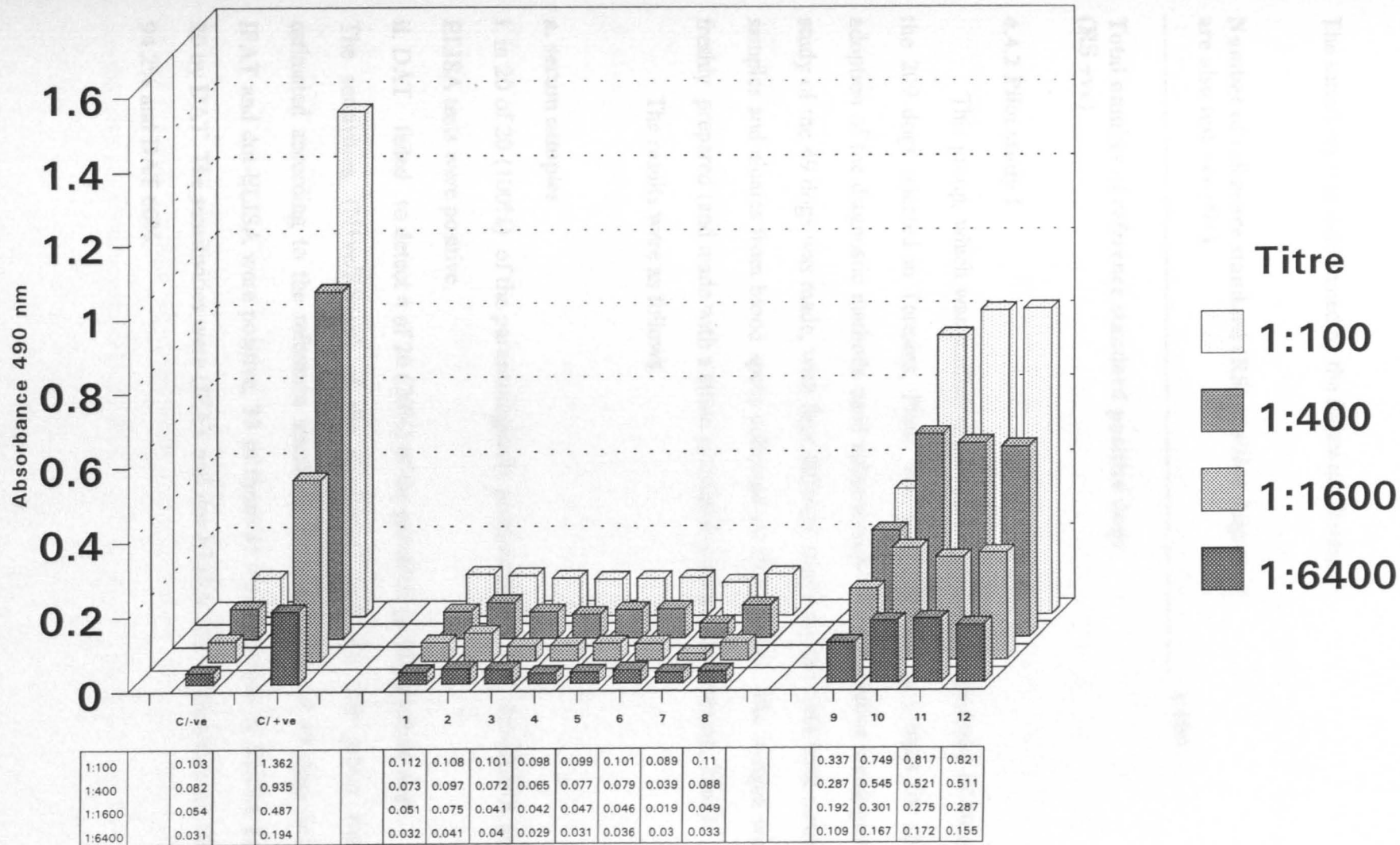


Figure 4.5

Figure 4.6

**Non-specific antibodies detected by ELISA in dog sera from
London**



1 to 8 male dogs / 9 to 12 female dogs

Figure 4.6

The sensitivity was determined by the following formula:

$$\frac{\text{Number of reference standard (RS) positive dogs that are also test +ve (N+)}}{\text{Total number of reference standard positive dogs (RS +ve)}} \times 100$$

4.4.2 Pilot study I

This group, which was studied when the project began, consisted of 49 of the 209 dogs selected in Teresina, Piauí. The results served as a basis for the adoption of the diagnostic methods used subsequently. A comparative serological study of the 49 dogs was made, with four different methods, and with both serum samples and eluates from blood spots collected on filter paper. The antigen was freshly prepared (and made with a strain isolated from a dog in Teresina, Piauí).

The results were as follows:

a. serum samples

i. in 20 of 20 (100%) of the parasitologically positive dogs IFAT, dot-ELISA and ELISA tests were positive.

ii. DAT failed to detect 4 of 20 (20%) of the parasitologically positive dogs.

The sensitivities, (N+ve/RS+ve) of the serological tests for this group were estimated according to the reference standard adopted: in 35 of 49 dogs both IFAT and dot-ELISA were positive, 33 of those 35 were positive by ELISA and 20 by DAT. The sensitivities were (IFAT and dot-ELISA 100%) therefore ELISA 94.2% and DAT 60%.

b. eluates from blood spots

i. in 20 of 20 (100%) of the parasitologically positive dogs IFAT and dot-ELISA tests were positive.

ii. in 19 of 20 (95%) of parasitologically positive dogs the ELISA was positive.

iii. DAT failed to detect 4 of 20 (20%) of parasitologically positive dogs.

For the eluates from the blood spots the sensitivities of the serological tests in comparison with the serum samples and according to the formula $(N+ve/RS+ve)$ were therefore: IFAT and dot-ELISA 85.7%, ELISA 82.8% and DAT 60%.

c. parasitology

The same formula gave a sensitivity of 57.1% for the parasitological methods.

The results of these comparisons are summarized in **Figure 4.7 A and B**.

d. source of antigen

When these 49 eluate samples were analysed by IFAT using the freshly prepared local strain antigen and another antigen manufactured in Brazil, which is used routinely, the results were as in **Table 4.3**.

4.4.3 Serology with samples from blood spots collected on filter paper in Teresina, Piaui.

Samples collected from dogs, without parasitological examination, were analysed comparatively using IFAT, dot-ELISA, ELISA and DAT. A total of 44 of 388 (11.3%) samples were positive by IFAT and dot-ELISA, 32 of these 44 seropositive dogs were positive by ELISA and 13 of the 44 samples were positive by DAT.

Figure 4.7 A

Comparative sensitivity between 4 serological tests and parasitological diagnosis using serum samples and eluates from blood spots. Parasitologically +ve dogs as reference standards (RS)

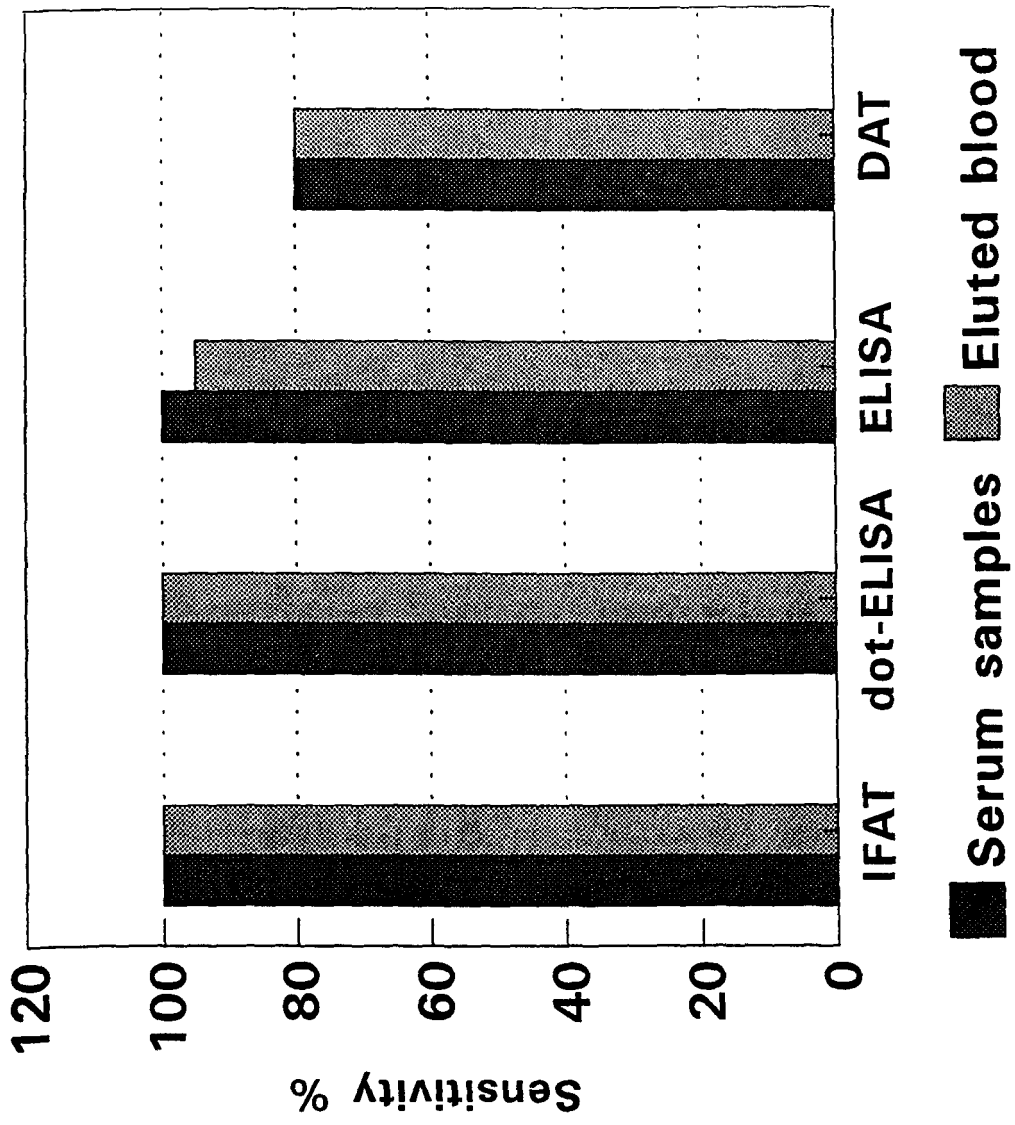


Figure 4.7 A

Figure 4.7B

Comparative sensitivity between 4 serological tests and parasitological diagnosis using serum samples and eluates from blood spots. Parasitologically +ve and/or serologically +ve dogs as reference standard (RS)

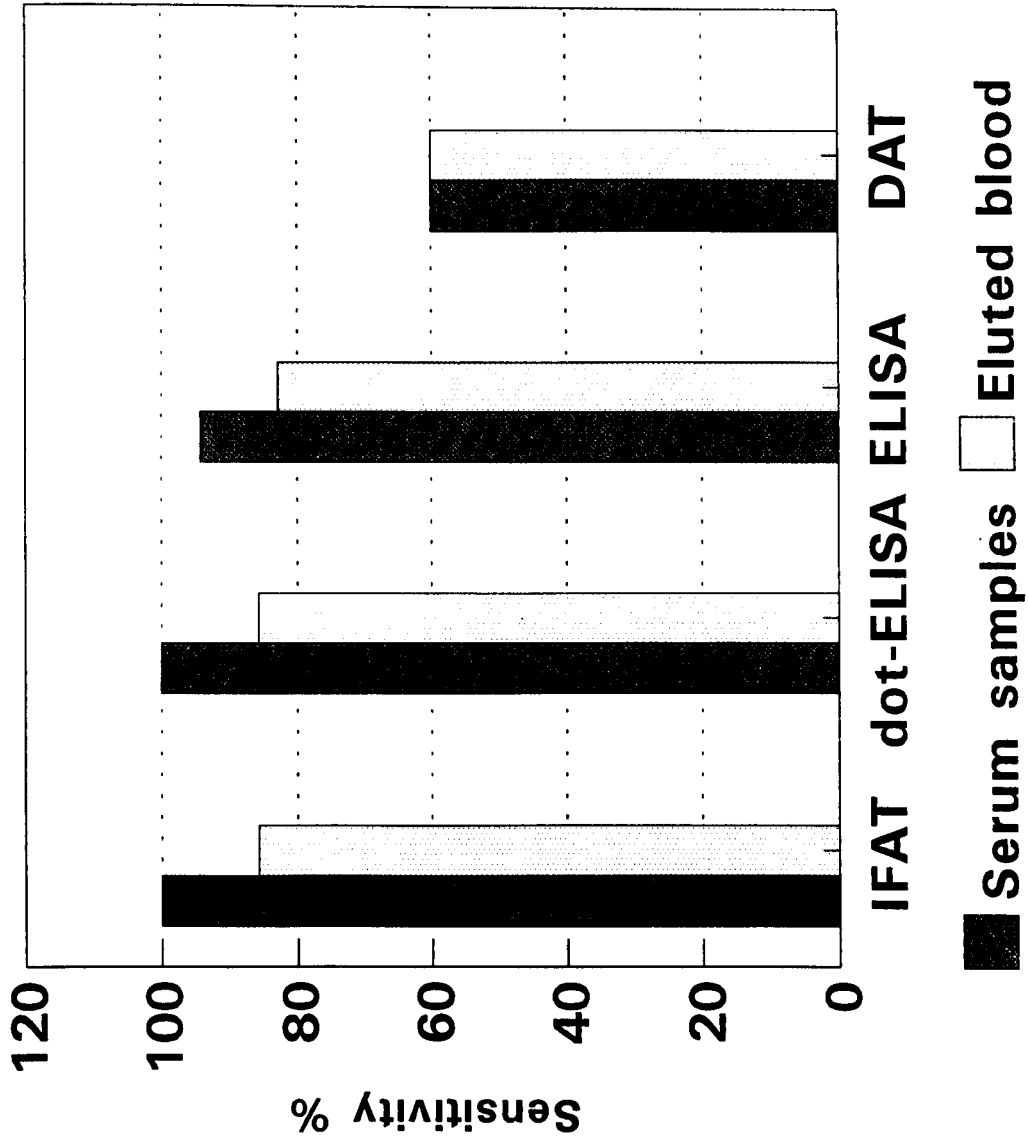


Figure 4.7 B

Table 4.3

Comparison of local and commercial antigens (in IFAT)

	Fresh antigen from local strain	Commercial antigen used routinely in Brazil
IFAT +ve	30	25
IFAT -ve	19	24
<hr/>		
% +ve	61.2	51.0

The respective sensitivities were (assuming IFAT and dot-ELISA to be 100%) were ELISA 72.7% and DAT 29.5%.

4.4.4 Serum samples from an endemic area in Corsica.

In a comparative analysis performed with 129 serum samples from an endemic area in Corsica, the following results were found:

Of 20 samples from dogs parasitologically positive by detection of *L.donovani*-complex DNA (radioactive methodology, in collaboration with Dr I. A. Frame) and/or by giemsa staining and microscopy, 19/20, (95%) were positive by dot-ELISA, 18 (90%) by IFAT and ELISA and the DAT failed to detect antibodies in 5 of 20 (25%) of positive dogs.

A total of 29 samples were considered (RS+ve) positive (27 samples with IFAT and dot-ELISA positive, 1 sample positive only by detection of *L.donovani*-complex DNA and another only DAT positive). The sensitivities of the respective tests in this group of dogs was as follows:

IFAT and dot-ELISA 93.1%, ELISA 86.2% and DAT 62%. The sensitivity of the parasitological method used was 58.6% for microscopical examination of bone marrow aspirate (BM) and 48.2% for the Lmet2 probe. (Figure 4.8).

4.4.5 Study II

This study was the continuation of Pilot study I. A total of 160 dogs from Teresina were analysed by only two serological tests (IFAT and dot-ELISA) and results were compared with clinical and parasitological examinations of all the

Figure 4.8

Comparative sensitivity between 4 serological tests, parasitological diagnosis and DNA probing, with serum samples from an endemic area of Corsica. Reference standard (RS) as defined in the text.

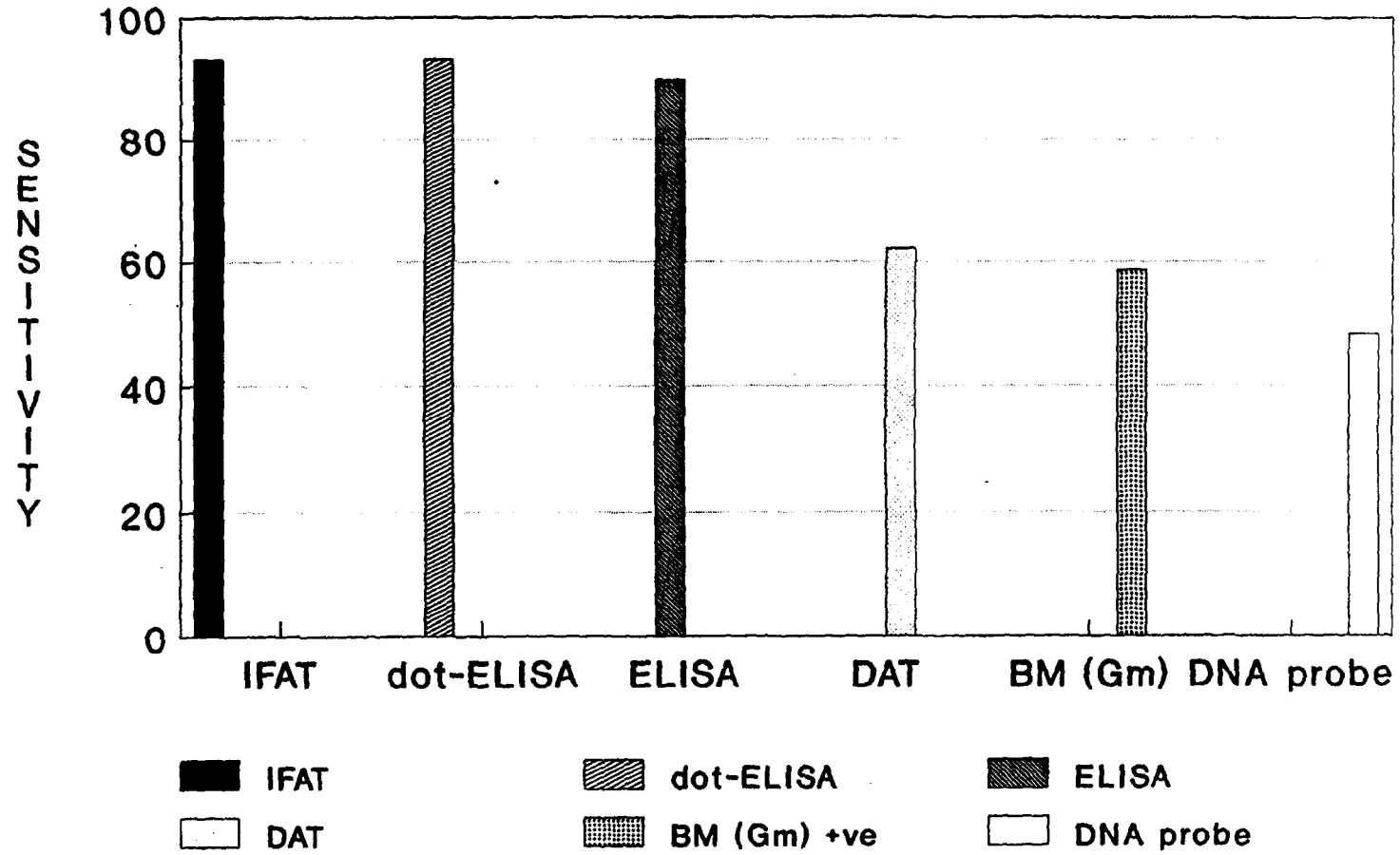


Figure 4.8

dogs. The ELISA test was not used at this second stage, because the specificity was less than IFAT and dot-ELISA and nor was the DAT, because of its lower sensitivity and as it was not possible to manufacture sufficient DAT antigen in the Parasitology laboratory during the other aspects of this study. The titres of seropositive dogs varied between 1:100 and 1:6400 by IFAT, and between 1:200 and 1:12400 by dot-ELISA; in 130 of 160 serum samples a comparative analysis of titres between IFAT and dot-ELISA was performed (**Figure 4.9**).

In this group 129 of 160 (80.6%) of the dogs were serologically positive but, parasites were demonstrated in 3 of 31 (9.6) of the seronegative dogs. (**Figure 4.10, IFAT**). The summary of the analysis of antibody titres, parasitology and clinical assessment (asymptomatic, symptomatic) is shown in **Table 4.4**.

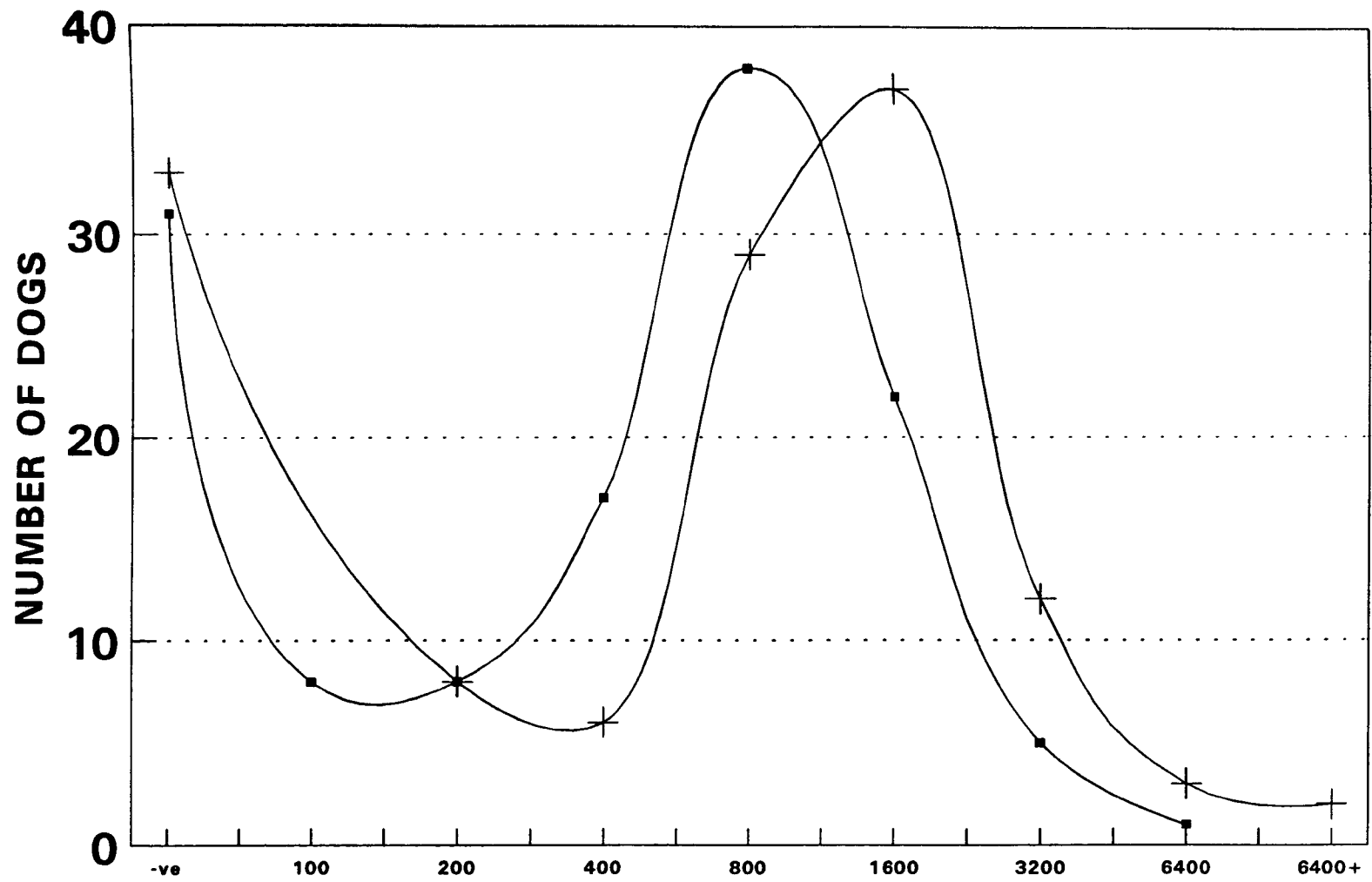
The comparison between the sensitivity of the parasitological and serological methods was as follows: serology, 129 of 132 RS+ve dogs (97.7%) were serologically positive; combined parasitological methods, 95 of 132 RS+ve (71.9%) for this group of dogs.

4.4.6 Comparative diagnostic analysis for all dogs.

To perform these comparative analyses of sensitivity, we split the reference standard (RS) as before into **RS parasitologically positive dogs** and **RS parasitologically positive dogs and/or dogs that had positive DAT or had both IFAT and dot-ELISA positive**. In this group no dog was positive only by the DAT.

Figure 4.9

Comparative titers of IFAT and dot-ELISA with serum samples of dogs from Teresina



IFAT	31		8		8		17		38		22		5		1		
dot-ELISA	33				8		6		29		37		12		3		2

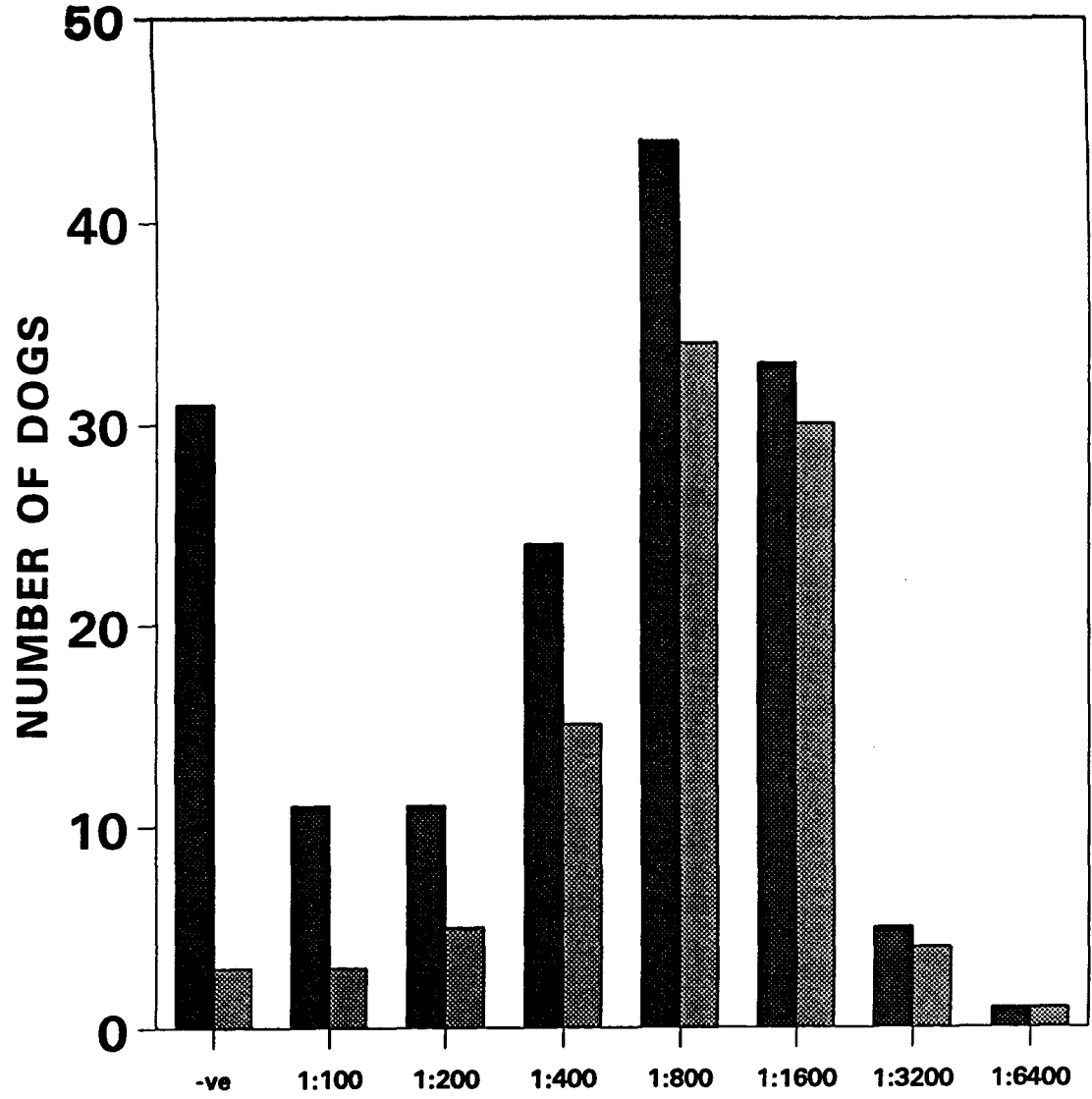
SEROLOGICAL TITRE

■ IFAT + dot-ELISA

Figure 4.9

Figure 4.10

Antibody distribution and parasitologically positive dogs, study II



Number of dogs	31	11	11	24	44	33	5	1
Parasit/ + ve dogs	3	3	5	15	34	30	4	1
	1							

Serological titre by IFAT

■ Number of dogs
 ▨ Parasit/ + ve dogs

Figure 4.10

Table 4.4**Comparison of antibody titre and parasitological status in asymptomatic and symptomatic dogs**

ASYMPTOMATIC DOGS				SYMPTOMATIC DOGS			
IFAT titre	Number of dogs	Parasitologically positive dogs	Parasitologically positive dogs (%)	IFAT titre	Number of dogs	Parasitologically positive dogs	Parasitologically positive dogs (%)
-ve	31	3	9.6	1:100	4	2	50
1:100	7	1	14.2	1:200	6	4	66.6
1:200	5	1	20	1:400	17	11	64.7
1:400	7	4	57.1	1:800	23	22	95.6
1:800	21	12	57.1	1:1600	23	21	91.3
1:1600	10	9	90	1:3200	5	4	80
1:6400	1	1	100				

This comparative analysis of diagnostic methods showed that serology is not 100% sensitive but it is the best, followed by the parasitology, and then symptomatology. The results with the LMet2 DNA probe, but with a smaller group of animals, are described above (section 4.3) (**Figure 4.11**).

Comparison of the serological methods showed that the IFAT and the dot-ELISA were the most sensitive tests for detection of parasitologically positive dogs, followed by the ELISA and the DAT (**Figure 4.12**).

Comparison of the parasitological methods showed that for each of the techniques used the sensitivity was never more than 60%, in comparison with the reference standard (RS) as defined above. Although demonstration of the parasite by culture of samples from spleen aspiration and BM puncture was more sensitive than the examination of the same samples by giemsa staining and microscopy, culture is not practical for routine used and from wild animals, gives increased contamination (Vexenat *et al.*, 1986) (**Figure 4.13**).

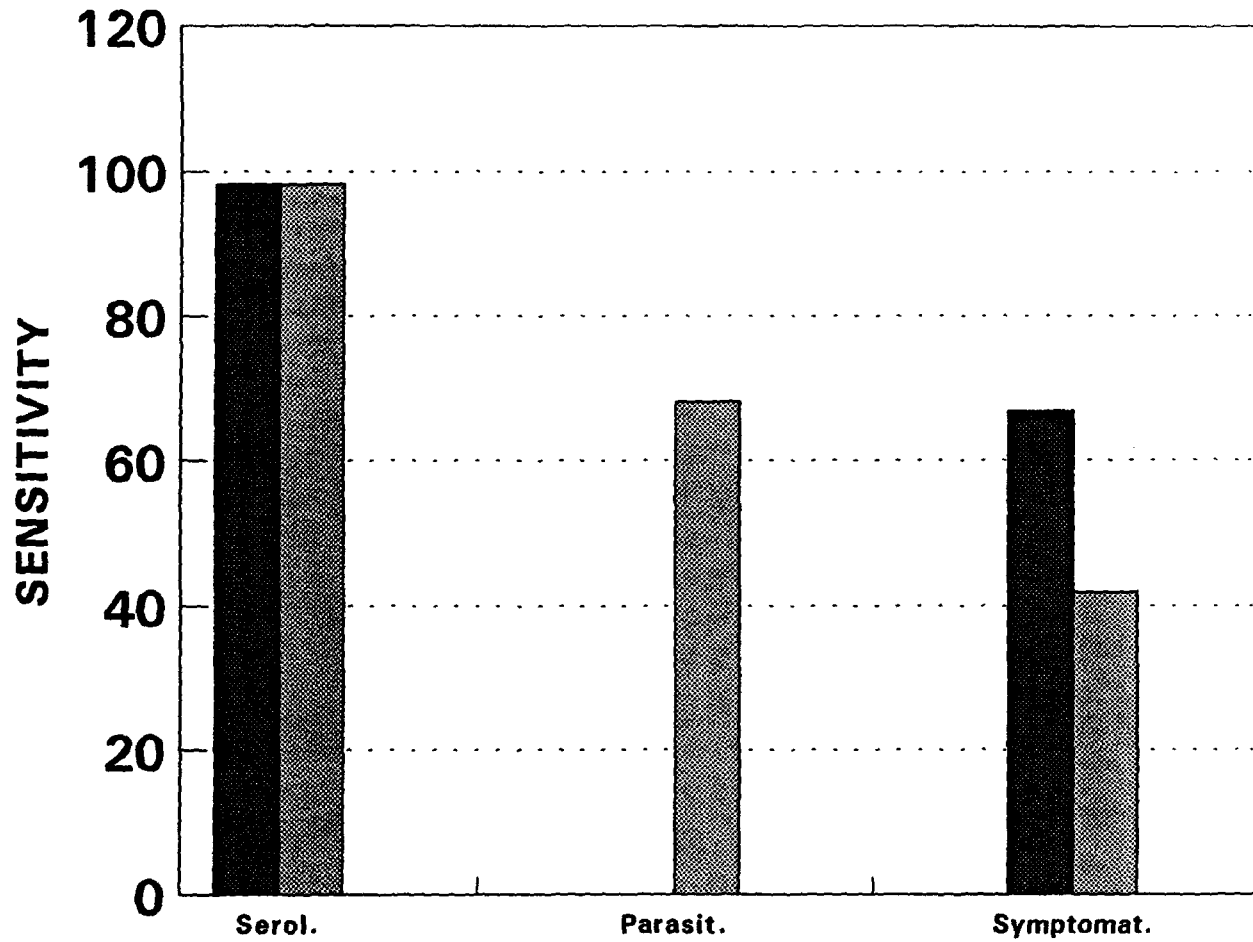
Two particularly important results from these comparisons between the demonstration of the parasite and the detection of the presence of antibodies, were that:

- a. 6.6% of the serologically negative dogs were parasitologically positive.
- b. the chance of demonstrating the parasites increased with increasing antibody levels. (**Figure 4.14, IFAT**).

The significance of these observations will be discussed below.

Figure 4.11

Comparative sensitivity of diagnostic methods



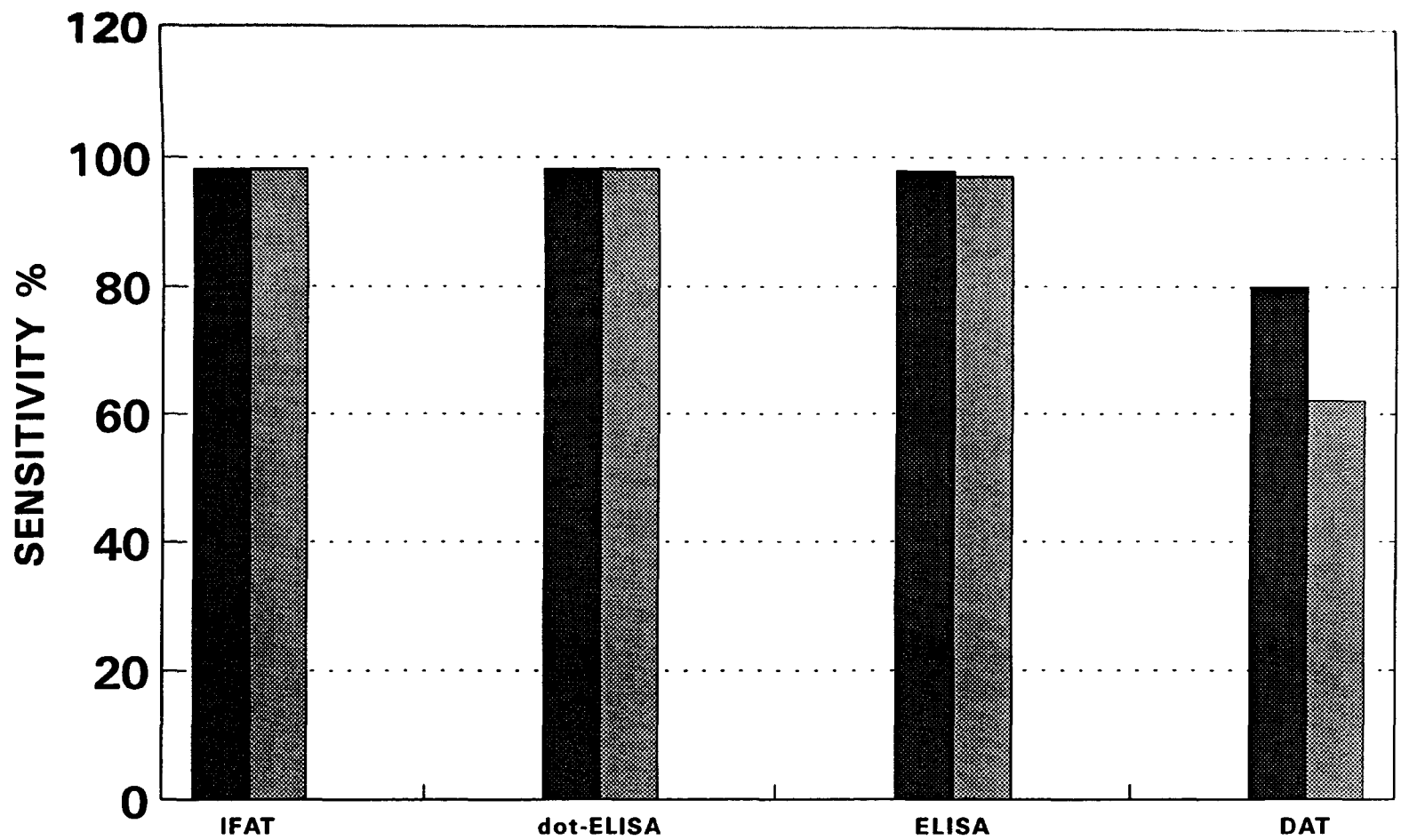
Paras/ + ve	98.2		68.2		66.9
Paras/serol/ + ve	98.2		68.2		41.9

Reference standard: Paras/ + ve Paras/serol/ + ve

Figure 4.11

Figure 4.12

Comparative sensitivity of serological methods



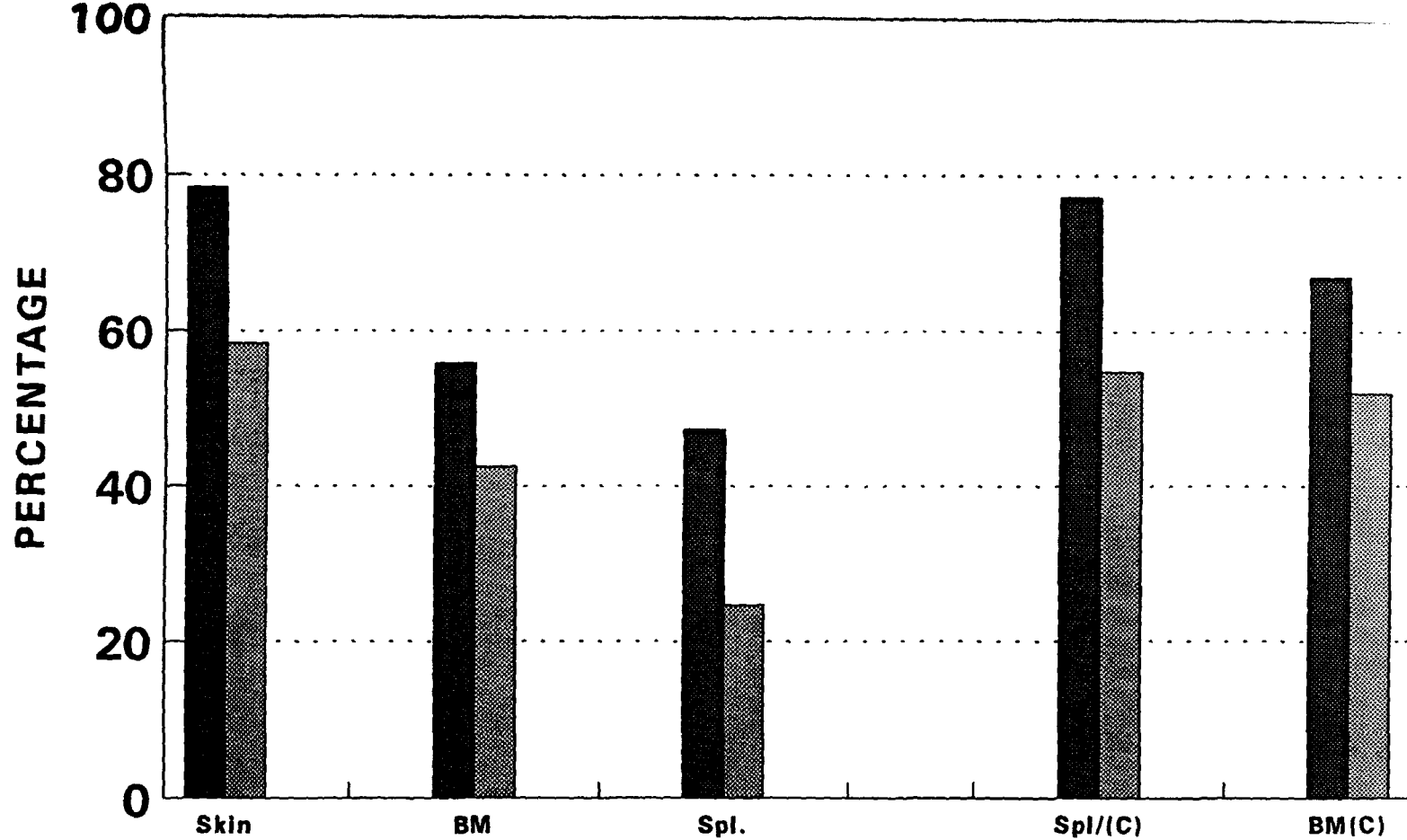
Paras/ + ve	98.2		98.2		97.9		80
Paras/serol/ + ve	98.2		98.2		97.1		62.1

Reference standard: Paras/ + ve Paras/serol/ + ve

Figure 4.12

Figure 4.13

Comparative sensitivity of parasitological methods



Paras/ + ve	78.4		55.9		47.3			77.2		67
Paras/serol/ + ve	58.4		42.5		24.6			54.8		52

Reference standard: Paras/ + ve Paras/serol/ + ve

Figure 4.13

Figure 4.14

Comparison of antibody level by IFAT and results of parasitological examination.

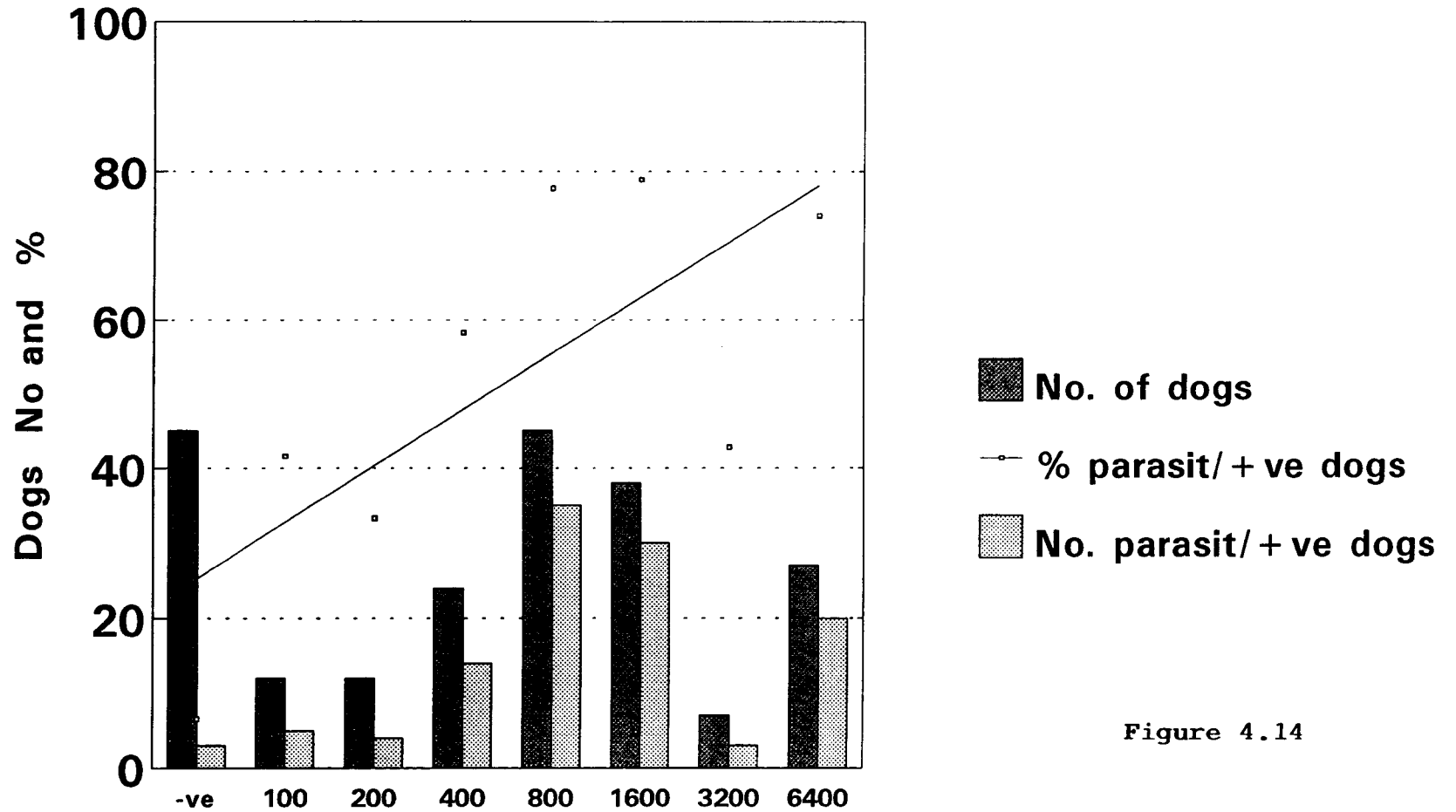


Figure 4.14

No. of dogs	45	12	12	24	45	38	7	27
% parasit/+ve dogs	6.6	41.6	33.3	58.3	77.7	78.9	42.8	74
No. parasit/+ve dogs	3	5	4	14	35	30	3	20

CHAPTER 5: TRANSMISSIBILITY OF CANINE VISCERAL LEISHMANIASIS

5.1 Detection of natural infections in sandflies

In a preliminary attempt to detect naturally infected sandflies and to investigate whether they were found in association with naturally infected dogs, collections of females were made in three places:

(a) a communal kennel with approximately 20 dogs of unknown origin, of unidentified breeds, and with an unknown percentage of infected dogs. Here engorged female sandflies were collected by pooter.

(b) a slum house with one pig-sty, one chicken shed and one parasitologically positive dog. Engorged female sandflies were separated from collections made by CDC light-trap. A large proportion of the sandflies examined may have fed on pigs or chickens.

(c) a luxury house with a kennel and one infected dog that had disseminated amastigotes in the skin. Here engorged female sandflies were collected on the wall inside the kennel.

When dissected between 5 and 7 days after collection positivity rates by microscopical examination in the sandflies were between 0.6 and 67.3% (**Table 5.1**).

The identification of naturally infected sandflies by dissection is an arduous and time-consuming task. To test the chemiluminescent DNA probe technology in the field 200 female sandflies were collected inside and outside a house where

Table 5.1

Natural infections in sandflies detected by dissection 5 - 7 days after capture

Place and source	Flies +ve	Flies -ve	% +ve
A communal kennel with approximately 20 dogs of unknown origin, unidentified breeds and with an unknown percentage of infected dogs (flies collected by pooter).	02	16	11.1
A slum house with one pig-sty, one chicken shed and one parasitologically positive dog (flies collected by CDC light-trap).	05	790	0.6
A luxury house with one infected dog in a kennel (engorged flies collected on the internal wall).	33	16	67.3
Totals	40	822	

there was an untreated human case of VL waiting for hospitalisation and also a serologically positive dog. The sandflies were processed as described in the Materials and Methods. In two of 200 flies examined DNA of the *Leishmania donovani*-complex was detected by the Lmet 2 probe.

To determine the percentage of kennels with naturally infected sandflies using the DNA probe method (through which it is possible to examine a large number of flies) another set of 1,234 female sandflies were collected and squashed onto Hybond N (Amersham). The flies were collected in kennels located in different houses and different districts of the city of Teresina. When a large quantity of sandflies was collected at a site only the females which had fed or apparently had eggs in the abdomen were used. In this case the radioactive Lmet2 DNA probe was used as radioactive facilities were available; occasionally the chemiluminescent method had given background on the filters and we wished to guarantee an absolutely clear result at the first attempt.

5.2 Xenodiagnosis studies of naturally infected dogs

Early in the Teresina project colony-bred *Lu longipalpis* were fed on altered skin of 13 naturally infected (parasitologically +ve) dogs. Dissection after 5 - 7 days showed that between 11% (1 out of 9) and 78% of flies became infected and with 10 of the 13 host animals more than 50% of flies contained promastigotes when they were dissected (**Table 5.2**). This compares with a rate of 100% obtained by Molina on xenodiagnosis of patients with VL, but all these subjects were suffering from AIDS (Molina *et al.*, 1994b).

On one dog (8a, **Table 5.2**) a second batch of flies was fed on apparently normal skin. Although the infection rate was reduced 6% (4 out of 65) flies became infected, as compared to 35% (57 out of 163) fed on altered skin ("skin lesions") of the same animal. This result indicated that sandflies very easily acquired infection from parasitologically positive dogs if they fed on skin lesions

Table 5.2**Xenodiagnoses on 13 naturally infected dogs**

Dog no.	Flies +ve	% +ve
	Flies fed	
6a	32/45	71%
7a	76/121	63%
8a	57/163	35%
8a*	4/65	6%
67	41/53	77%
68	34/63	65%
69	7/9	78%
74	69/124	56%
105	9/16	56%
112	1/9	11%
146	92/132	70%
147	61/83	73%
148	42/69	61%
170	12/46	26%

*** This batch only fed on apparently normal skin, all other batches fed on altered skin.**

and suggested that host clinical and parasitological status should be compared in more detail with host infectivity to sandflies. The comparison of serological positivity and parasitological positivity had already indicated an association between serological titre and parasitological positivity (chapter 4, **Figure 4.14**) and, by implication, between serological titre and infectivity.

A group of 40 IFAT positive dogs was examined clinically (classified as symptomatic or asymptomatic) and parasitologically (sternal puncture aspirates and skin) and then used for comparative xenodiagnoses, with one batch of flies fed on skin lesions (altered skin) and a second batch fed on apparently normal skin. Artificial xenodiagnosis was performed in parallel by membrane feeding and heparinised blood samples were also cultured (Materials and Methods). It was intended that the membrane feeding and the culture of heparinised blood samples would indicate, by comparison with direct xenodiagnoses, whether sandflies had acquired infection from the skin of the dogs or, possibly, from amastigotes circulating in blood/infected blood cells (**Table 5.3**).

Comparisons derived from the data shown in **Table 5.3** are summarised in **Table 5.4**.

Certain simple observation may be made from the comparisons summarised in **Table 5.4**, as follows:

1. Both microscopy of giemsa stained impression smears and culture of skin biopsies were more sensitive with altered skin than normal skin for detecting *L. chagasi* infection in symptomatic dogs.
2. In this particular study with symptomatic dogs culture of skin biopsies did not detect any infections that were missed by microscopy of giemsa stained impression smears.

Table 5.3

Results of parasitological examination, direct and indirect xenodiagnoses on skin lesions or apparently normal skin of symptomatic or asymptomatic dogs

Table 5.3

Dog	Giemsa			Culture				Direct xeno (+/exam)				Membrane fed
	NL	NS	BM	NL	NS	BM	BC	NL	%	NS	%	BLOOD
01	+	+	+	+	+	+	-	45/55	81	12/68	17	0/35
02	SL	-	-	ND	-	-	-	ND		0/32	0	0/30
03	SL	-	+	ND	+	+	-	ND		0/47	0	0/23
04	+	+	+	+	+	+	-	122/145	84	76/99	76	0/45
05	SL	-	-	-	-	-	-	ND		0/43	0	0/32
06	+	+	-	+	+	+	+	387/422	91	202/632	31	0/122
07	SL	-	+	ND	-	-	-	ND		0/21	0	0/32
08	SL	-	+	ND	-	-	-	ND		0/22	0	0/30
09	SL	+	-	ND	-	+	-	ND		4/29	13	0/34
10	SL	+	+	ND	+	+	-	ND		6/41	14	0/20
11	SL	-	-	ND	-	+	-	ND		0/33	0	0/24
12	SL	-	-	ND	-	+	-	ND		0/22	0	0/20
13	SL	-	-	ND	-	+	-	ND		0/23	0	0/32
14	SL	+	+	ND	+	+	-	ND		22/24	91	0/23
15	SL	-	-	ND	-	+	-	ND		2/34	5	0/21
16	+	-	-	+	-	-	-	15/18	83	0/21	0	0/34
17	+	-	-	+	-	-	-	12/19	63	0/35	0	0/23
18	-	-	-	-	-	-	-	0/30	0	0/24	0	0/32
19	+	-	-	+	-	-	-	0/21	0	0/34	0	0/37
20	SL	+	-	ND	+	+	+	ND		32/36	88	0/42
21	+	-	-	-	-	-	-	4/25	16	0/35	0	0/32
22	+	-	-	-	-	+	-	5/34	14	0/26	0	0/28
23	SL	-	-	ND	-	+	+	ND		0/23	0	0/26
24	SL	-	-	ND	-	+	-	ND		0/25	0	0/20
25	SL	+	-	+	-	+	-	ND		21/28	75	0/32
26	+	-	-	-	-	+	-	6/18	33	0/22	0	0/25
27	+	-	-	-	-	+	-	2/26	7	0/27	0	0/22
28	-	-	+	-	-	-	-	0/33	0	0/23	0	0/30
29	+	-	+	+	-	-	-	3/30	10	0/21	0	0/19
30	+	-	+	+	-	-	-	8/34	23	0/31	0	0/34
31	+	-	+	+	-	+	-	12/18	66	0/24	0	0/26
32	SL	-	+	ND	-	+	-	ND		0/32	0	0/21
33	SL	-	-	ND	-	+	-	ND		0/23	0	0/25
34	+	+	+	+	+	+	-	10/29	34	2/31	6	0/24
35	+	+	-	+	+	+	+	15/19	78	4/28	14	0/20
36	+	+	-	+	+	+	-	18/25	72	5/42	11	0/21
37	+	+	-	+	+	+	-	27/38	71	3/29	10	0/32
38	+	+	-	+	+	+	+	38/43	88	13/67	19	0/41
39	+	-	-	+	-	-	-	5/32	15	0/48	0	0/38
40	+	-	-	+	-	-	-	3/22	13	0/32	0	0/26

SL = skin lesion (altered skin); NS = normal skin; NL = no skin lesion (altered skin) present; BM = examination of bone marrow aspirate (by giemsa staining and microscopy); BC = blood culture; ND = not done. Dogs recorded as NL in column 2 (SL) correspond with animals designated asymptomatic.

Table 5.4**Comparison of clinical and parasitological status in 40 seropositive dogs**

Examination	Clinical status	
	Symptomatic (22) (+ve/examined)	Asymptomatic (18) (+ve/examined)
Microscopy: altered skin	20/22	-
Microscopy: normal skin	8/22	5/18
Culture: altered skin	16/22¹	-
Culture: normal skin	8/22¹	4/18²
Culture: blood	3/22	2/18
Microscopy: bone marrow	7/22	6/18
Culture: bone marrow	12/22³	14/18⁴
Xenodiagnosis: altered skin	19/22	-
Xenodiagnosis: normal skin	8/22	6/18

1 No additional infections detected as compared to microscopy

2 One additional infection detected but two missed in comparison with microscopy

3 Eight additional infections detected but three missed in comparison with microscopy

4 Ten additional infections detected but two missed in comparison with microscopy

3. In contrast culture of bone marrow detected 8 additional infections, as compared to microscopy of bone marrow, with the 22 symptomatic dogs.
4. Only 5 of the 40 dogs, 3 symptomatic and 2 asymptomatic, were positive by blood culture.
5. Overall similar proportions of asymptomatic dogs were found to be infected by the parasitological methods and xenodiagnosis, except that altered skin could not be examined in these animals.

A simple attempt was made to see if parasitologically positive skin and bone marrow were associated in this group of 40 seropositive dogs, as shown in **Table 5.5**. Consistent with observations on the cohort of experimentally infected dogs (Chapter 6) 13 of 31 dogs that were bone marrow positive were skin negative and 6 of 24 dogs that were skin positive were bone marrow negative.

Similarly, the parasitological status of skin was compared with the result of xenodiagnosis (**Table 5.6**). Here there was a close association between skin positivity and xenodiagnosis positivity in that 23 of 25 skin positive dogs were xenodiagnosis positive; only 2 of these were xenodiagnosis negative and only 1 skin negative dog was xenodiagnosis positive.

By way of further comparison 12 bone marrow positive dogs were xenodiagnosis negative and 12 of 31 bone marrow positive dogs were xenodiagnosis positive and 5 of 24 xenodiagnosis positive dogs were bone marrow negative (**Table 5.7**). These comparisons suggest that parasitologically skin is a clear indicator of ability to transmit infection to sandflies.

Table 5.5

Comparison of associations between parasitological status of skin and bone marrow (BM) in naturally infected dogs used for xenodiagnoses

	BM +ve	BM -ve	
Skin +ve	18	6	24
Skin -ve	13	3	16
	<hr/> 31	<hr/> 9	<hr/> 40
			<hr/> $\chi^2 = 0.2$

Fisher's exact test = 0.4

Table 5.6

Comparison of association between result of xenodiagnoses and parasitological status of skin

	Skin +ve	Skin -ve	
Xeno +ve	23	1	24
Xeno -ve	2	14	16
	25	15	40
			$\chi^2 = 28$

Fisher's exact test = 30.3

Table 5.7

Comparison of association between result of xenodiagnoses and parasitological status of bone marrow

	BM +ve	BM -ve	
Xeno +ve	19	5	24
Xeno -ve	12	4	16
	31	9	40
			$\chi^2 = 0.1$

Fisher's exact test = 0.3

Xenodiagnosis was performed on 3 of the 5 dogs that were parasitologically positive by blood culture. In these three cases 91% (387 of 422), 78% (15 of 19) and 88% (38 of 43) sandflies became infected. All three animals had, however, parasitologically positive skin (**Table 5.3**). It cannot be concluded, therefore, that the blood infections contributed to the high numbers of sandflies that acquired infection when fed on these animals, although it may have done. Surprisingly, none of the total of 251 sandflies acquired infection when fed artificially by membrane feeding on blood derived from these five animals that were parasitologically positive by blood culture. This may suggest that sandflies could not readily acquire infection from the small quantity of blood that they took up, in comparison with the relatively large volume placed into culture tubes, and supports the conclusion that sandflies normally acquire infection from amastigotes in the skin of infected dogs. Alternatively, the process of membrane feeding may have destroyed the infectivity of blood-borne amastigotes. This is unlikely, however, as amastigotes derived from hamsters by trituration and filtration through glass wool and mixed with defibrinated, heat-inactivated (56°C, 30 m) dog blood were able to convey infection to all sandflies that fed on this control suspension by membrane feeding (amastigotes numerous, not counted; 20 flies dissected).

Table 5.8 summarises the data on infectivity to sandflies (transmissibility), clinical status of the dog (symptomatic or asymptomatic) and the condition of the skin at the site at which sandflies fed. It can be seen that for symptomatic dogs altered skin or skin with lesions was more infective to sandflies than normal skin, in that 86% of xenodiagnoses on altered skin were positive and 36% on normal skin were positive. Although a high percentage of sandflies fed on altered skin acquired infection (64.9%) a considerable proportion (22.7%) also acquired infection from normal skin. For asymptomatic animals, in which altered skin was not available as a site for xenodiagnosis, 16.2% of sandflies still acquired infection

Table 5.8

Comparison of infectivity, clinical status and skin condition at site of xenodiagnosis

Clinical status of dog and site of xenodiagnosis	Xenodiagnosis result	
	No of dogs + No of dogs examined	No of flies + No of flies examined
<hr/>		
Symptomatic		
skin lesions	19/22 (86%)	737/1136 (64.9%)
normal skin	8/22 (36%)	317/1399 (22.7%)
Asymptomatic		
normal skin	6/18 (33%)	87/538 (16.2%)

from 33% (6 of 18 of the asymptomatic dogs). Overall and consistent with comparative data on naturally infected animals, these data clearly indicate that asymptomatic dogs cannot be excluded as a significant source of infection to sandflies considering that a single infected sandfly may transmit infection to a naive host (Chapter 6). The facility with which flies acquire infection from dogs implies that they are an overwhelmingly important source of infection and potential reservoir host, if the same sandfly populations are vectors of canine and human VL.

Figure 5.1 shows the percentages of sandflies infected, for individual dogs, with the three groups of animals shown in **Table 5.7**.

Figure 5.1

Percentages of sandflies that were infected following xenodiagnoses of skin lesions (altered skin) or normal skin for symptomatic or asymptomatic dogs that were serologically positive (by IFAT)

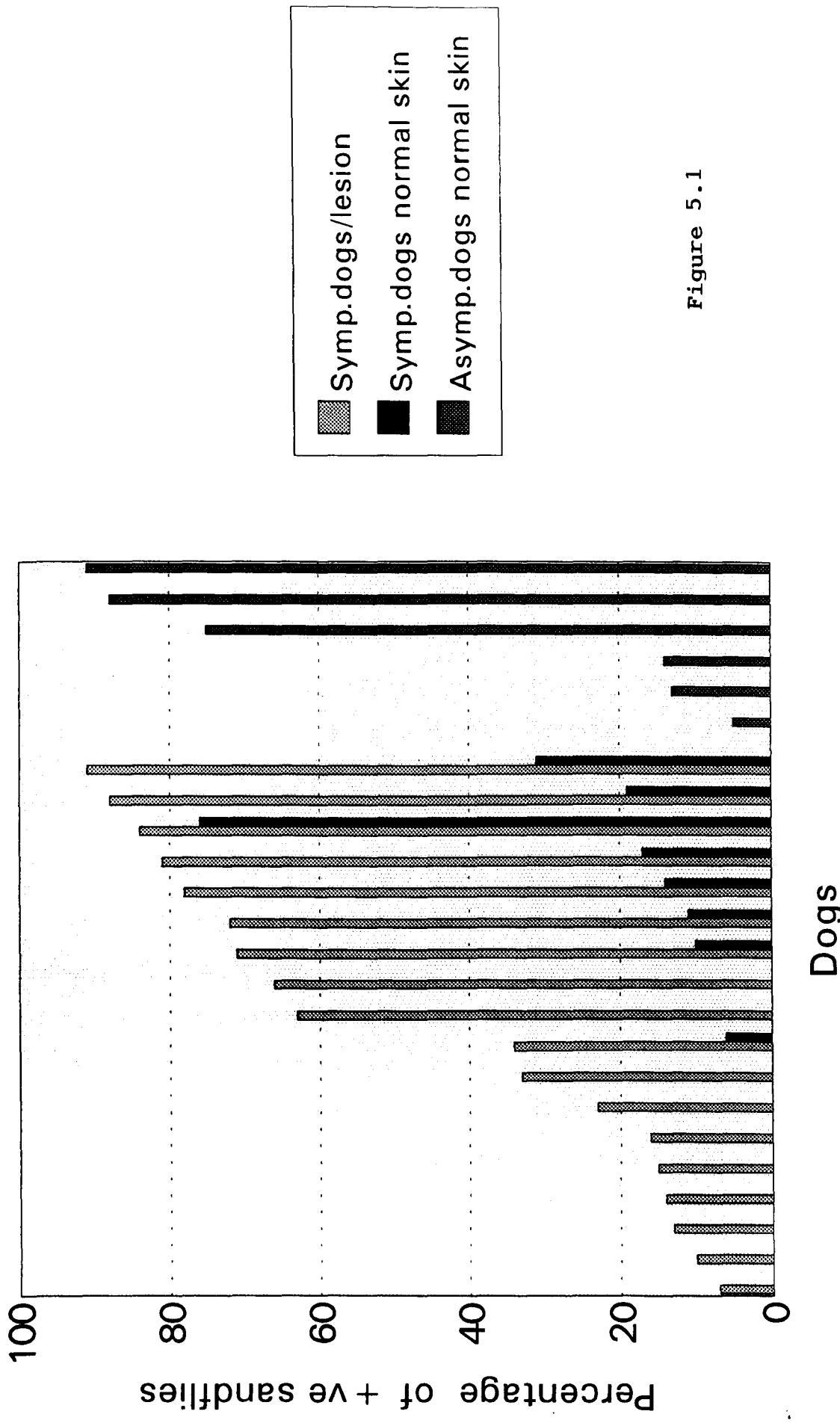


Figure 5.1

CHAPTER 6: EXPERIMENTAL TRANSMISSION

The results of the experimental infection of a cohort of dogs are summarised in **Table 6.1**.

6.1 The infective bite

In one (dog D1) of two pilot animals used in this study cohort of 25 dogs a single engorged fly of seven (which were collected in a luxury house with one infected dog in a kennel) was found to be parasitologically positive by microscopical examination and it was concluded that this fly was responsible for transmission. Within 22 days after the infective feed, a tiny erythema was noted at the site of the bite (left inner ear of a young dog of 70 days old) and immediately skin biopsies and xenodiagnosis were performed on this site. To perform the xenodiagnosis exactly on the suspect site, the ear was covered and only the lesion exposed to sandflies (**Figure 6.1A**). Large numbers of amastigotes were found by microscopy of giemsa stained impression smears of biopsies; 7 of 14 sandflies dissected 5 days after feeding were positive.

Follow-up xenodiagnoses were done on three separate occasions, weekly, biweekly or monthly (see results below) according to the time post-infection using local colony-bred sandflies. To detect any early skin dissemination comparative xenodiagnoses were performed at other sites: 43 of 73 flies that fed on the lesion were positive by microscopical examination and all 187 flies fed on the right ear and abdomen were negative. **Figure 6.1B** illustrates xenodiagnosis on the abdomen. **Figure 6.2** shows the lesion in dog D, at 22, 38 and 65 days post infection. Regrettably this animal died, at 145 days post infection, during parasitological examination by spleen puncture, without evidence of dissemination of the infection from the infected lesion, and with IFAT and dot

Table 6.1

Summary of parasitological and serological examinations on a cohort of 25 dogs experimentally infected by sandfly bite. Dogs are grouped according to course of infection, not chronologically (see text)

Sex	Dogs	Weeks post-infection									
		3-4	5-10	11-16	17-20	21-24	25-28	29-32	33-36	37-40	50
♀	06	▲□○	▲■○		▲□		△□○			△□○	
♀	07	△■○	▲■		▲■○		▲■○	△□○		△□○	
♂	15	▲■○	▲■		▲□	▲□		▲□○		△□○	
♂	M2	△□○	▲■			▲■		▲■○			
♂	17	△□○	▲□		▲■○						
♀	11	△■○	▲■			▲■		▲□○		△□○	
♀	13	▲■○	▲■			▲□		△□○		△□○	
♀	05	▲■○	▲■			▲■	△□○			△□○	
♀	V2	▲■○	▲□			▲■		▲■○	□		
♀	M1	△□○	△□		▲■	▲■○					
♂	D1	▲■○		▲■		▲■○					
♀	M3	▲□○	▲□	▲■○	○						
♀	D2	△□○		▲■○		▲■			▲■●	○	
♂	02	▲□○	▲■	▲■○			△□●			△□●	
♂	09	△□○	△□		▲■○	▲■		▲□●		△□●	
♂	P1	△□○	△□○	●				△□●			
♀	P2	△□○	▲■○	▲■●		▲■●		▲□			
♀	P3	△□○	△□			△□○	△□●	△□			
♀	P4	△□○	△□			△□○	▲■●	▲□●			
♀	V1	▲□○	▲□		▲■	▲□		△□●			
♀	M4	▲■○		▲■○							
♀	M5	△□○		△□●							
♂	M6	△□○		△□	▲■●						
♂	M7	△□○		▲■○						○	
♂	M8	△□○		▲■○						○	

▲ = Skin +ve
 ■ = Xenodiagnosis +ve
 ● = Bone marrow +ve
 △ = Skin -ve
 □ = Xenodiagnosis -ve
 ○ = Bone marrow -ve
 ■ (shaded) = Seroconversion
 □ (shaded) = Seroreversion
) = Death

Summary of parasitological and serological examinations on a cohort of dogs experimentally infected by sandfly bites. These are grouped according to the course of infection, not chronologically (see text).

Table 1 (continued)

Dog	1-4	5-10	11-14	15-21	22-28	29-35	36-42	43-49	50-56
Q 86	ABO	ABO							
Q 87	ABO	ABO							
Q 15	ABO	AB							
Q 82	ABO	AB							

Figure 6.1 A

Xenodiagnosis with sandfly access restricted to a skin lesion (Dog D1)

D1)

Dog	1-4	5-10	11-14	15-21	22-28	29-35	36-42	43-49	50-56
Q 11	ABO	AB							
Q 13	ABO	AB							
Q 88	ABO	AB							
Q 89	ABO	AB							
Q 90	ABO	AB							
Q 91	ABO	AB							
Q 92	ABO	AB							
Q 93	ABO	AB							
Q 94	ABO	AB							
Q 95	ABO	AB							
Q 96	ABO	AB							
Q 97	ABO	AB							
Q 98	ABO	AB							
Q 99	ABO	AB							
Q 100	ABO	AB							

Figure 6.1 B

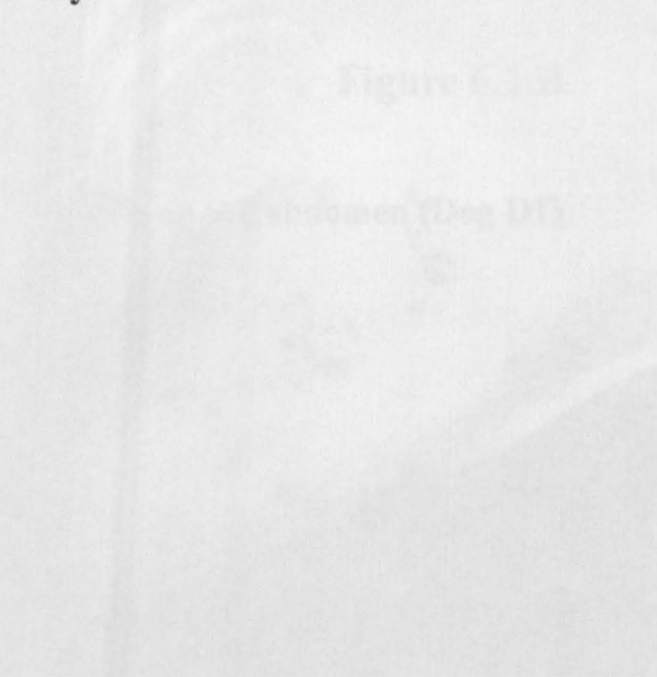
Xenodiagnosis on the abdomen (Dog D1)

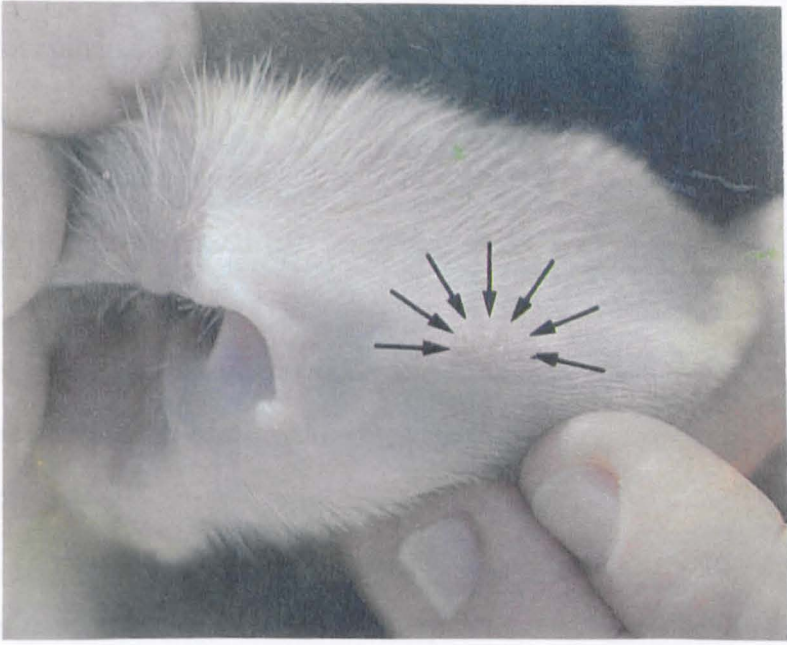
Dog	1-4	5-10	11-14	15-21	22-28	29-35	36-42	43-49	50-56
Q 101	ABO	ABO							
Q 102	ABO	ABO							
Q 103	ABO	ABO							
Q 104	ABO	ABO							
Q 105	ABO	ABO							
Q 106	ABO	ABO							
Q 107	ABO	ABO							
Q 108	ABO	ABO							
Q 109	ABO	ABO							
Q 110	ABO	ABO							
Q 111	ABO	ABO							
Q 112	ABO	ABO							
Q 113	ABO	ABO							
Q 114	ABO	ABO							
Q 115	ABO	ABO							
Q 116	ABO	ABO							
Q 117	ABO	ABO							
Q 118	ABO	ABO							
Q 119	ABO	ABO							
Q 120	ABO	ABO							



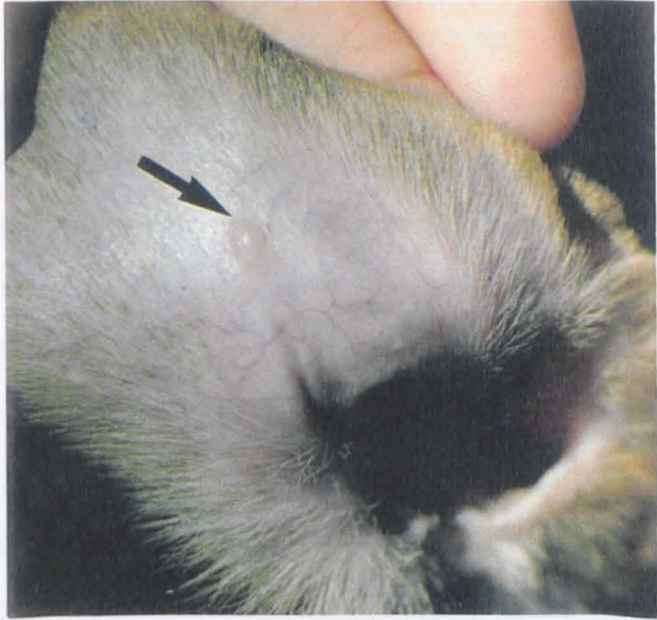
Figure 6.2 A, B, C

Lesions at the site of an infective sandfly bite at 22 days, 38 days and 65 days





A



B



C

Figure 6.2

ELISA titres of 1:100 (which became positive at day 129). Observations on the second pilot animal dog D2 are incorporated into the description of Results for the overall study cohort.

6.2 The incubation period

The incubation period (IP) is defined as the time from initial infection to the point at which signs of disease appear (or are diagnosed). This was determined by clinical examination for abnormalities at the site of the infective bite (or elsewhere) with confirmation that amastigotes were present in the skin or in the bone marrow.

In 12 of 25 (48%) of the infected dogs the IP, for the experimental procedures used here, was shown to be between three and four weeks after the infective bite. In 3, 5, 3 and 2 of 25 of the other infective dogs the IP was 5-10, 11-16, 17-20 and 25-28 weeks respectively (**Table 6.1**). The overall rate of infection in this study was thus 100%, although the persistence and course of infections varied. In this case the incubation period and prepatent periods were approximately the same as appearance of skin alterations usually coincided with the detection of amastigotes.

6.3 The latent period

The Latent Period (LP) is defined as the time from initial infection to the point at which the individual becomes infectious to others (here taken to be infective to sandflies); in this study it was recorded for 22 of 25 (88%) of the infected dogs. Early infection of *Lu. longipalpis* was obtained through xenodiagnosis performed on the transmission site. All the flies were examined after 6 days to determine the LP and the percentage of positive flies. The LP was demonstrated to be 3-4, 5-10, 11-16, 17-20, and 25-28 weeks in 8 (32%), 4, 4, 5 and 1 dog respectively (**Table 6.1**). It should be noted, however, that not all dogs that previously had negative xenodiagnoses were rexenodiagnosed at each time

point. LP was not determined by xenodiagnosis for 3 (12%) of the cohort: P1, P3, M5 (died), at the end point shown in **Table 6.1**.

6.4 The infectious period

The infectious period (INFP) defined as the period of time during which an individual is infectious to others (here considered to be infectivity to sandflies), was also demonstrated using xenodiagnosis. The INFP for experimental canine VL, was observed in 22 of the 25 infected dogs (although some dogs are still alive and infectious). The majority of dogs were infectious from weeks 5-10 to weeks 17-20. Comparisons between the rates of infection for sandflies showed, although it was possible to obtain early infection, that the highest rate was 5-10 weeks post-infection. Note, however, that few of the dogs had developed disseminated cutaneous infections (6.5, below).

6.5 Metastasis/skin dissemination

Metastasis and/or skin dissemination were demonstrated by periodic parasitological examination of bone marrow and altered or normal skin in various parts of the body of each infected dog (Materials and Methods).

This clinical development of canine VL was observed in only two of the 25 infected dogs, although clinical observation is continuing. The dog D2 was parasitologically positive in normal skin at day 65 post-infection and never recovered (**Figure 6.3**), and in dog 05, but in this case disseminated amastigotes and skin lesions on the ear were a temporary feature during pregnancy prior to the birth of four puppies (**Figure 6.4**).

Figure 6.3

Dog 2. A, 43 days old, B, 5 months after infection and C, 8 months after infection



A



B



C

Figure 6.3

Figure 6.4

Dog 05: Skin lesions at days 66, 89, 130 (4 pups born) 150 and 240

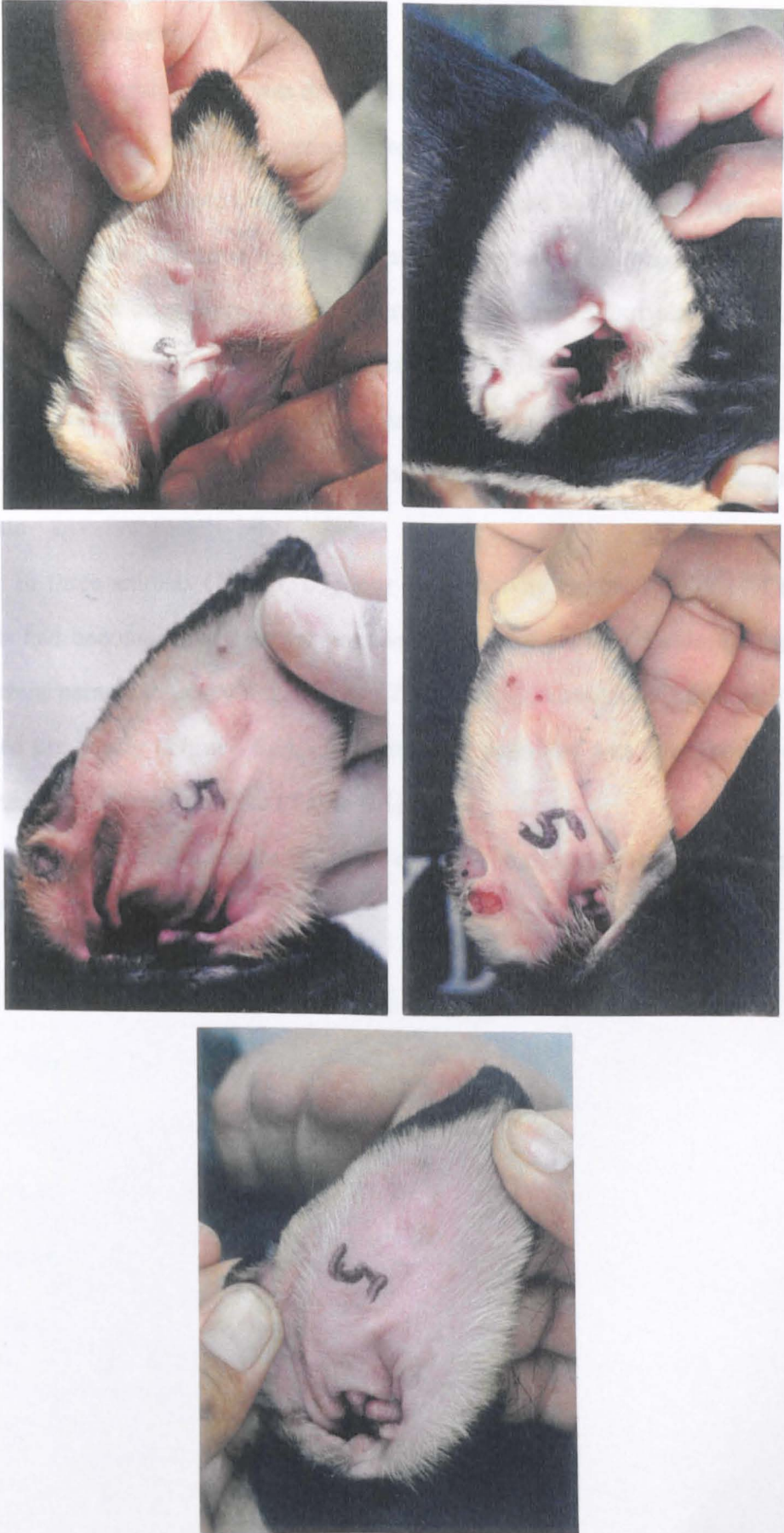


Figure 6.4

6.6 Seroconversion

Seroconversion (SC) was detected in 20 (80%) of the infected dogs.

The indirect immunofluorescent test (IFAT) was used to determine the time of SC and to estimate the antibody titre, as this had been shown previously to be one of the most effective tests. The minimal period to SC was eleven weeks. Serologically -ve dogs were not only parasitologically +ve, but also infective to sandflies. The comparison of the presence of antibodies and the infective rate for sandflies, demonstrated that 60% of the infected dogs were infective to sandflies (fed on skin lesions) during the seronegative phase with 32% of sandflies acquiring infection.

In three animals (11, 13, 05) low seropositivity reverted. None of these animals had become bone marrow positive at the end point in **Table 6.1** and all three were parasitologically negative at 37-40 weeks (although infection had been detected previously (**Figure 6.5**). Bone marrow positivity was only seen in dogs that were serologically positive (**Table 6.1**).

Examples of skin lesions for other dogs in the cohort are shown in **Figures 6.6A-G**.

Figure 6.5

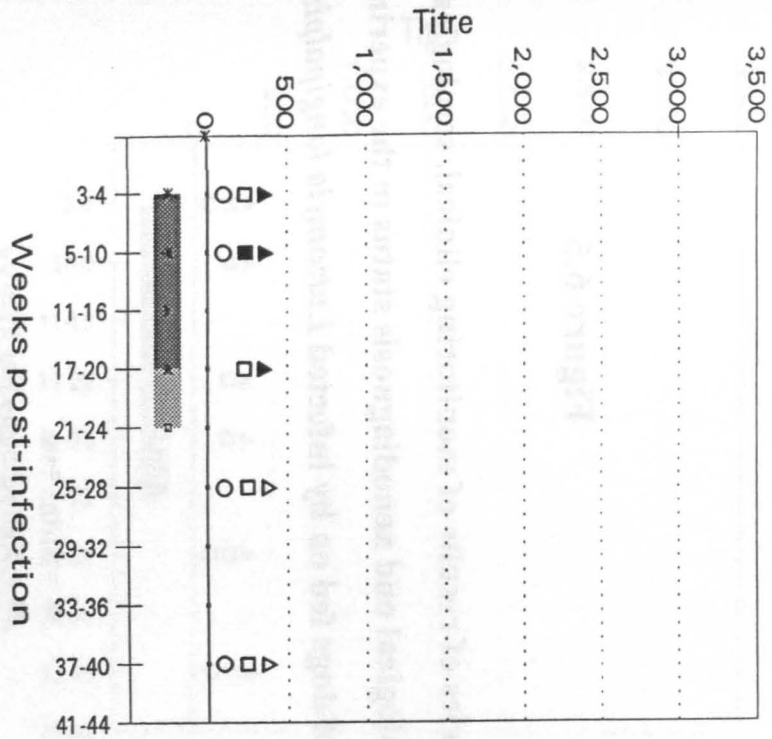
Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve

- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▣ = Nodule
- = Lesion
- ▤ = Scar

Dog 06



Dog 07

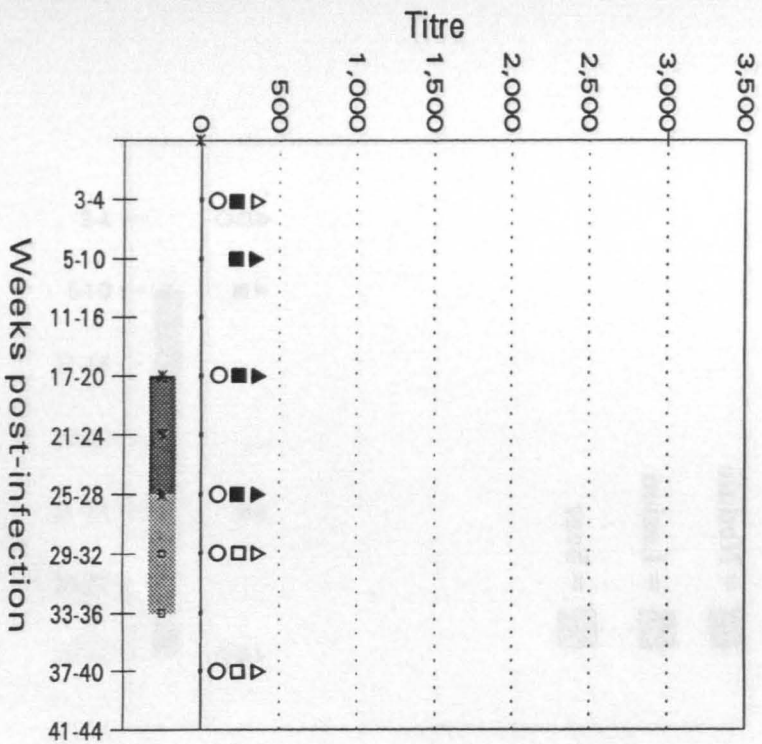


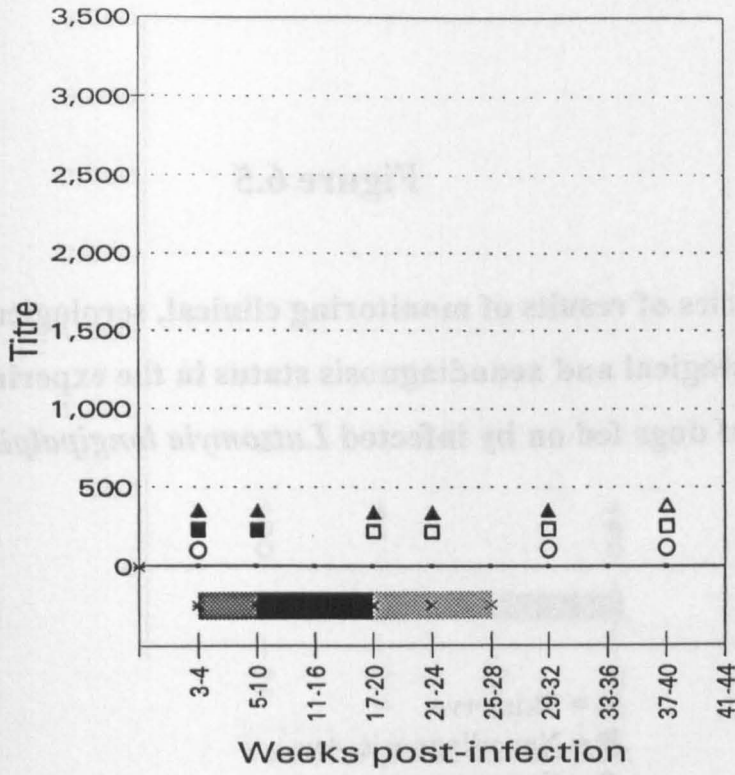
Figure 6.5

Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve
- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▨ = Nodule
- = Lesion
- ▨ = Scar

Dog 15



Dog M2

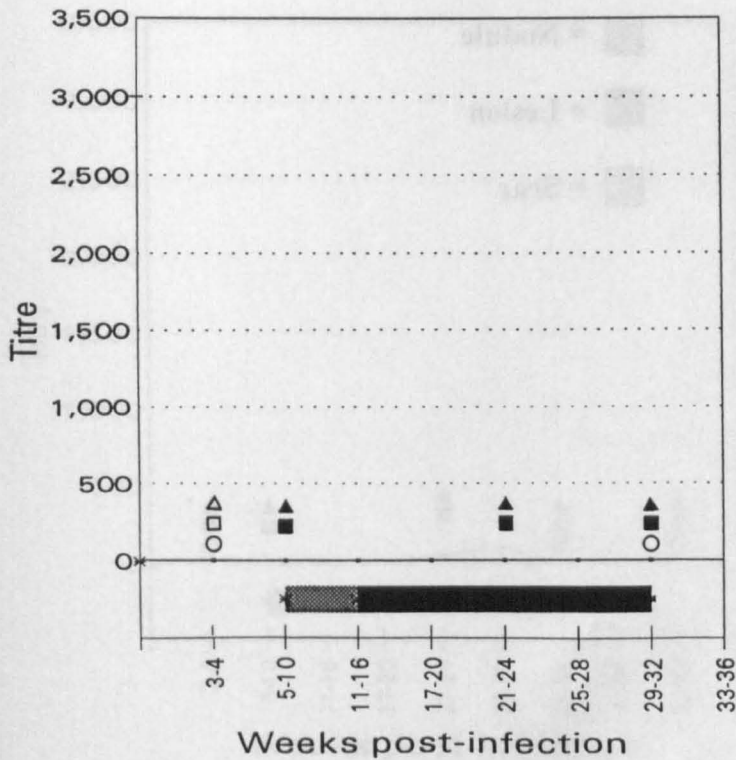


Figure 6.5

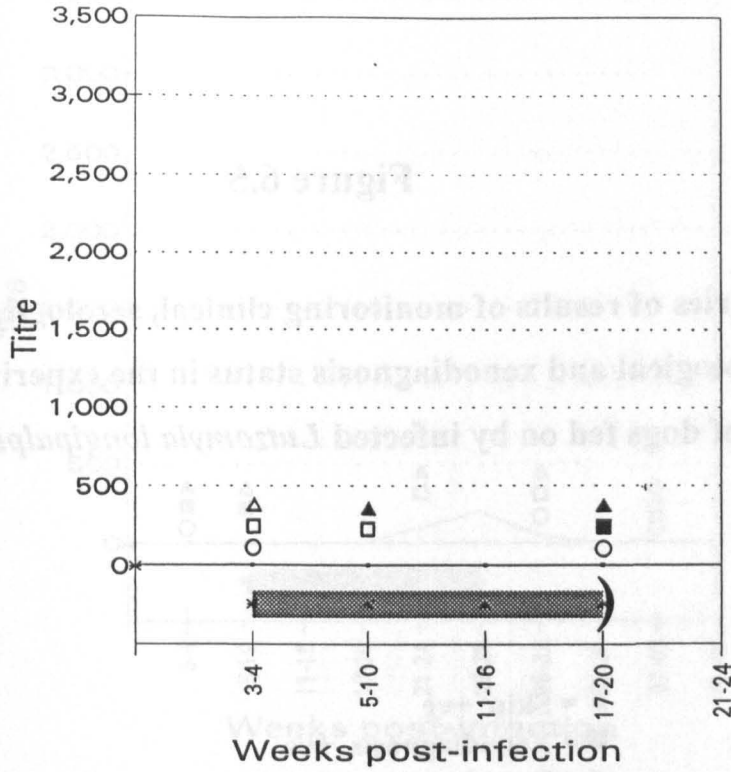
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- = Bone marrow +ve

- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▨ = Nodule
- = Lesion
- ▨ = Scar

Dog 17



Dog 11

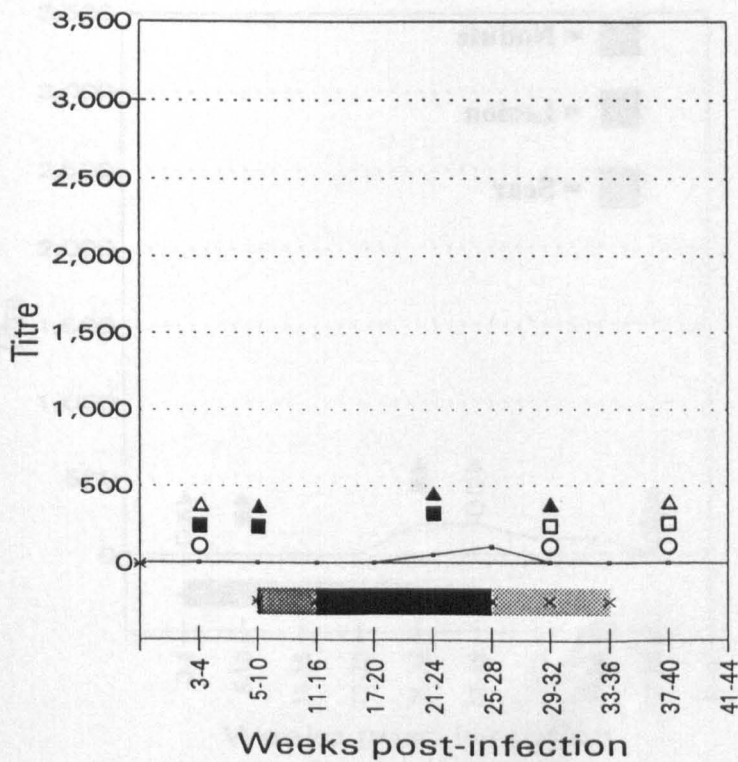


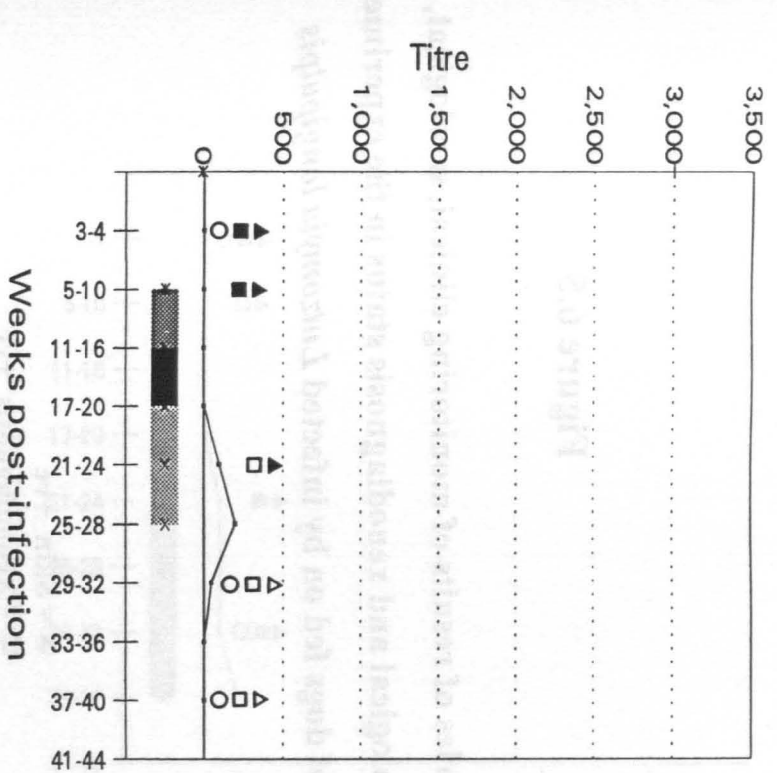
Figure 6.5

Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve
- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▨ = Nodule
- = Lesion
- ▨ = Scar

Dog 13



Dog 05

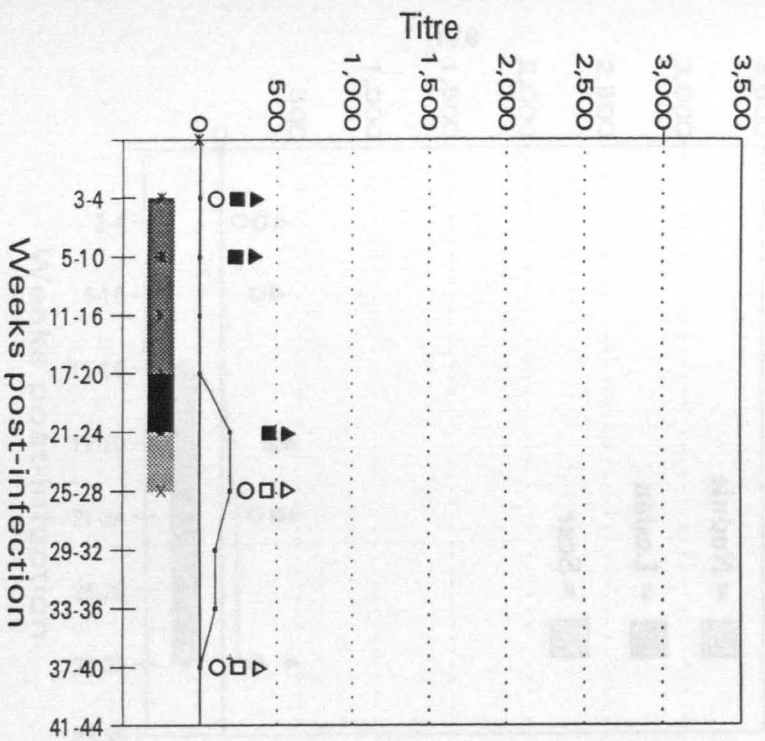


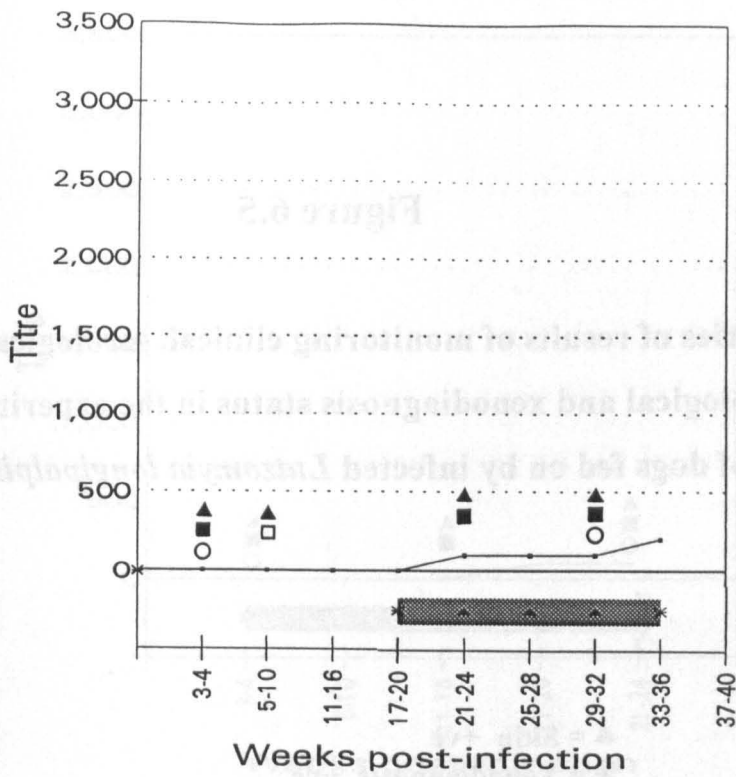
Figure 6.5

Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve
- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▨ = Nodule
- = Lesion
- ▨ = Scar

Dog V2



Dog M1

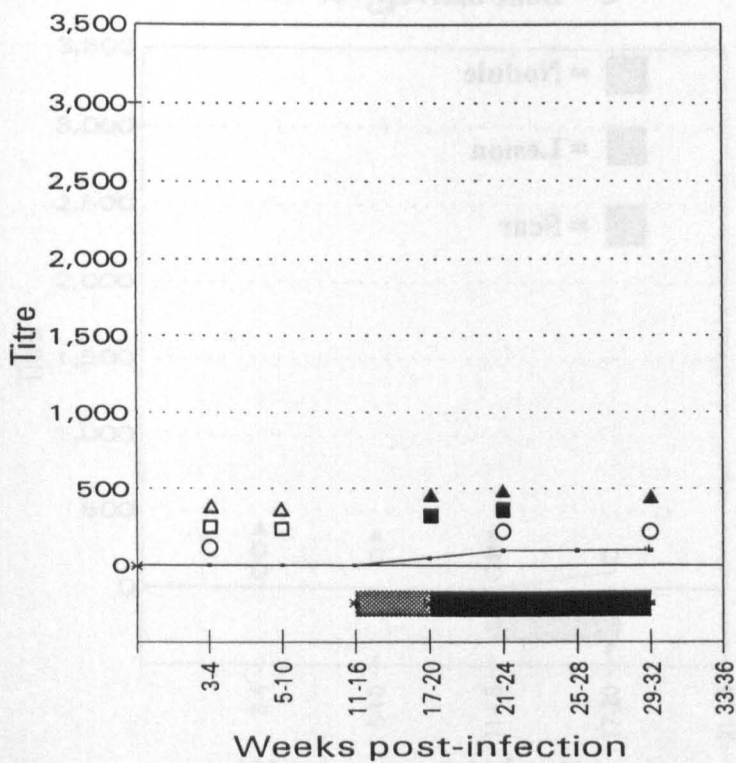


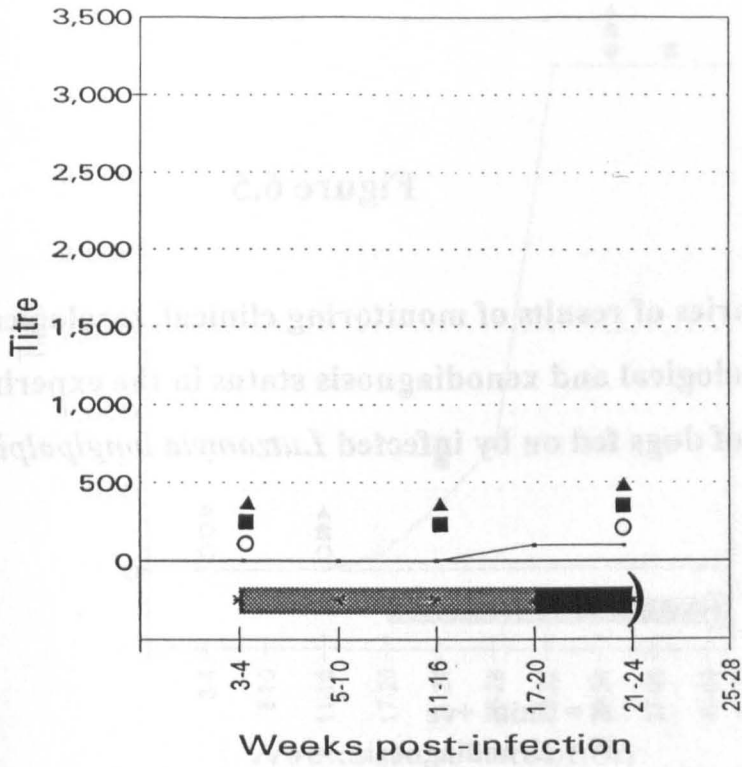
Figure 6.5

Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve
- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▨ = Nodule
- = Lesion
- ▩ = Scar

Dog D1



Dog M3

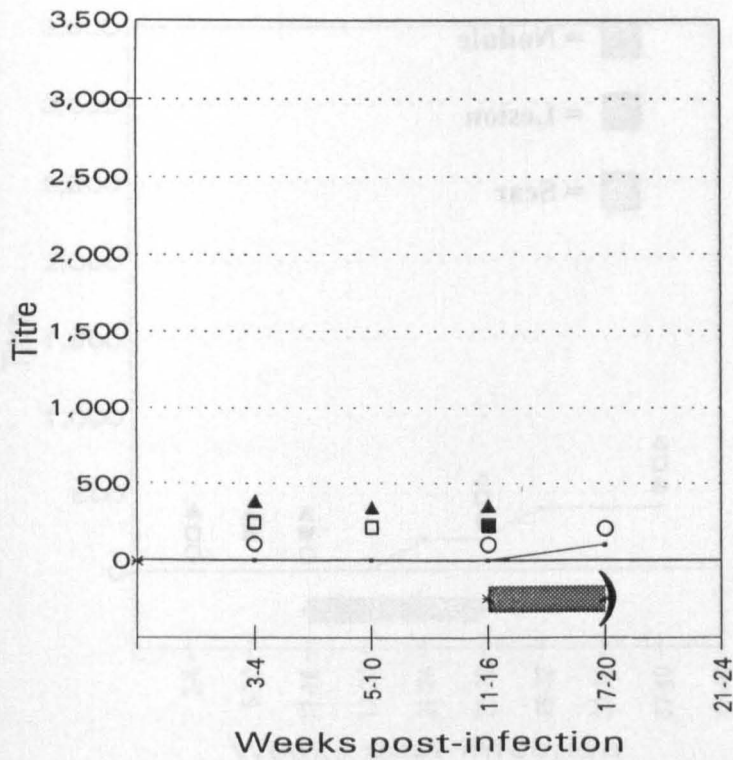


Figure 6.5

Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve
- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▨ = Nodule
- = Lesion
- ▨ = Scar

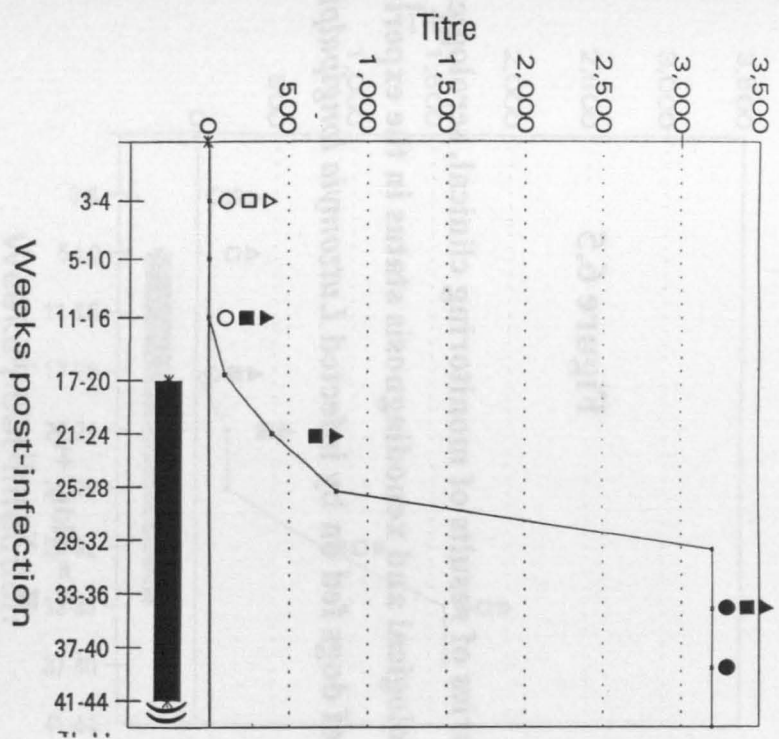
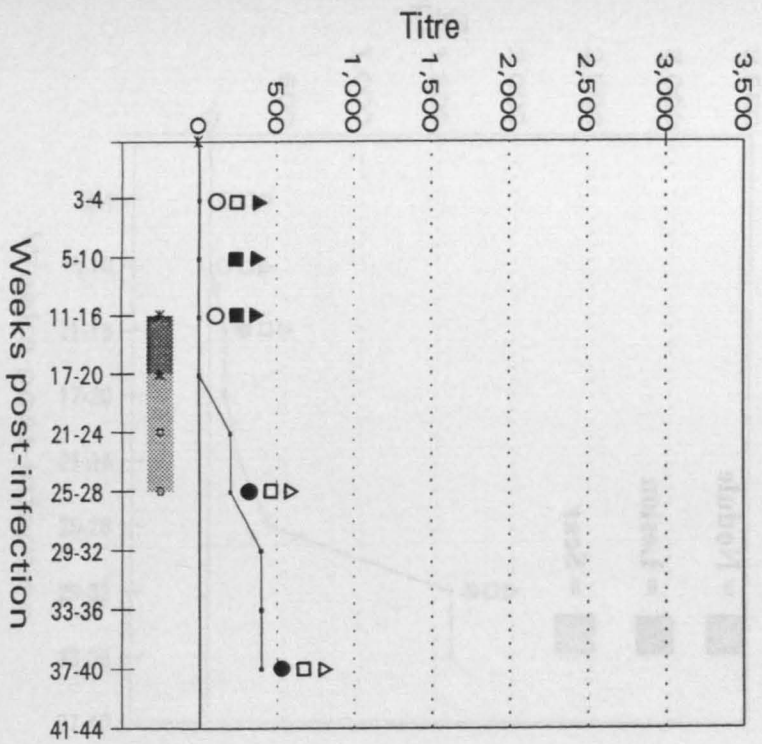


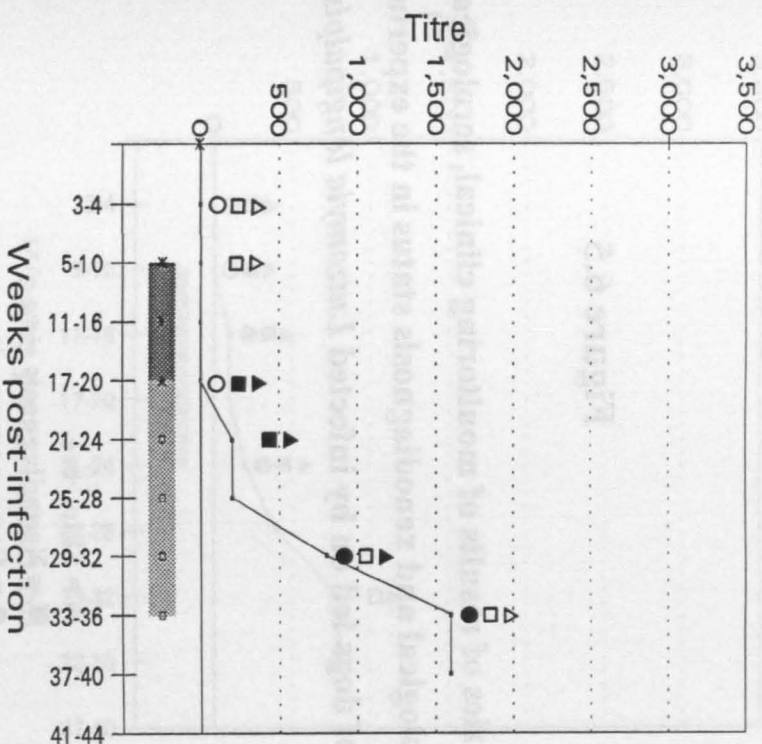
Figure 6.5

Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

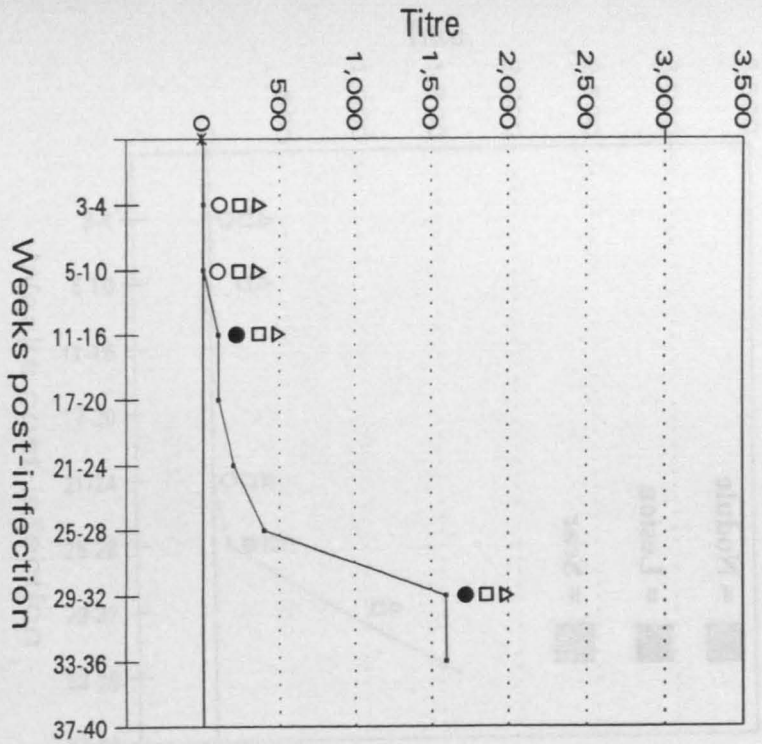
- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve

- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▨ = Nodule
- = Lesion
- ▨ = Scar



Dog 09



Dog P1

Figure 6.5

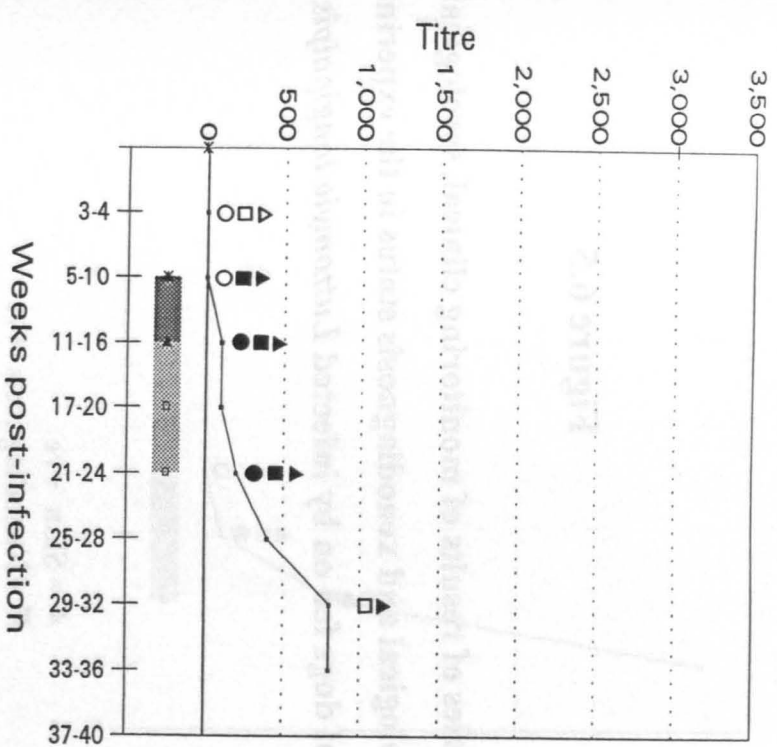
Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve

- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▨ = Nodule
- = Lesion
- ▨ = Scar

Dog P2



Dog P3

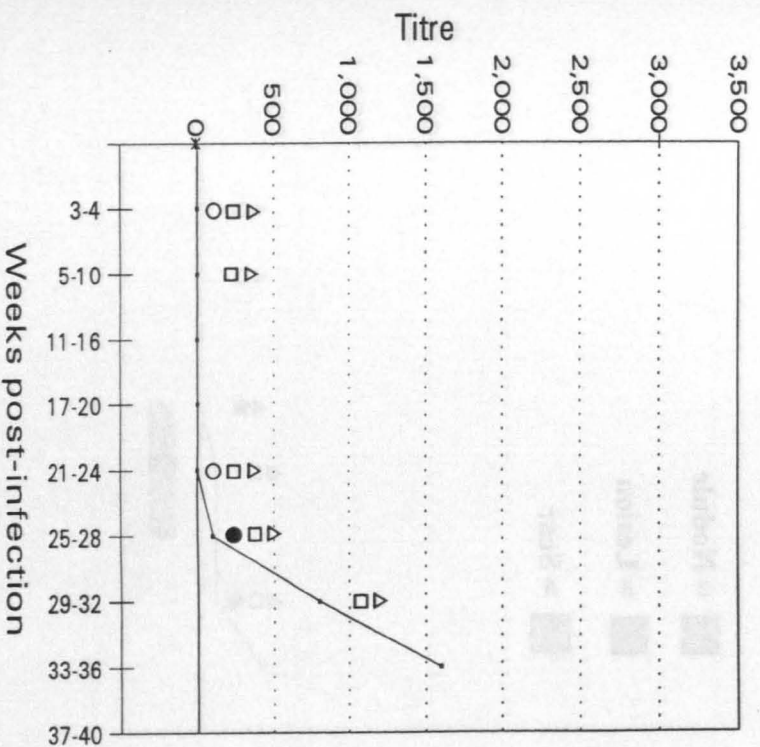


Figure 6.5

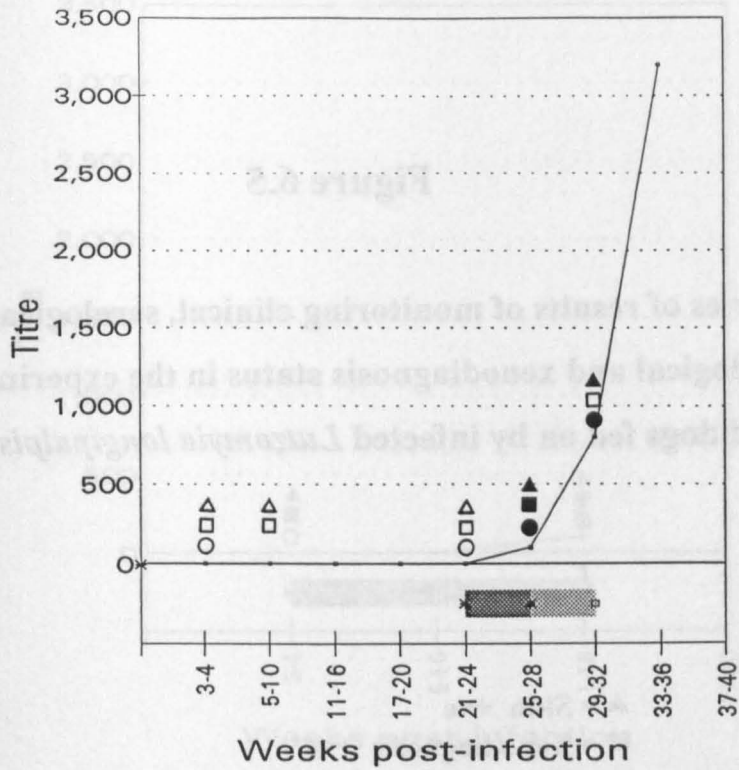
Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve

- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▨ = Nodule
- = Lesion
- ▩ = Scar

Dog P4



Dog V1

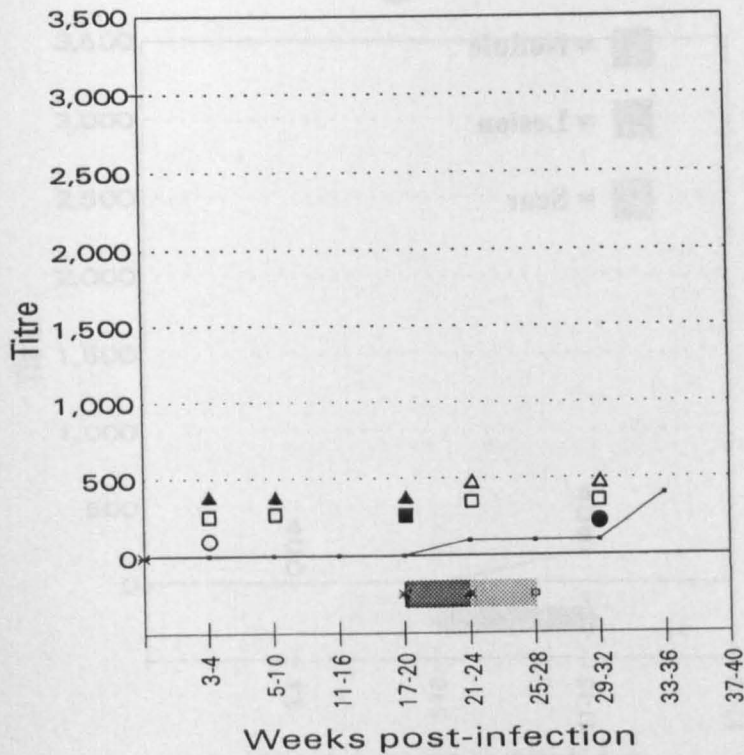


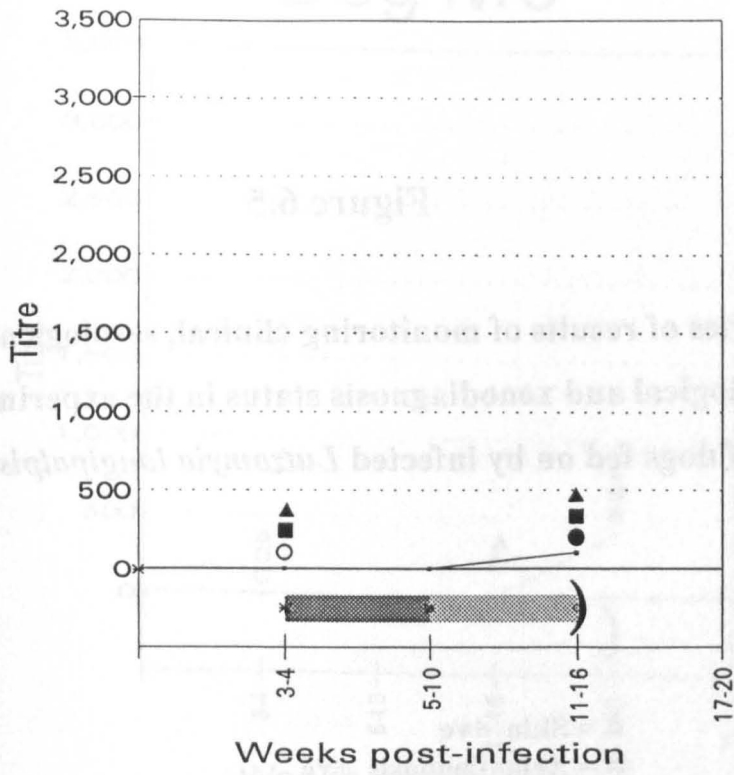
Figure 6.5

Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve
- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▨ = Nodule
- = Lesion
- ▩ = Scar

Dog M4



Dog M5

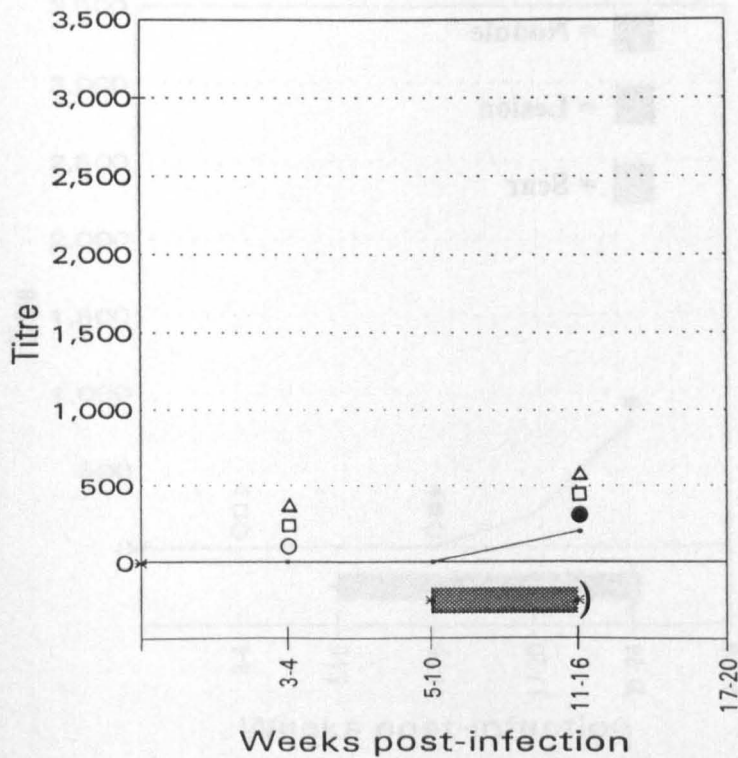


Figure 6.5

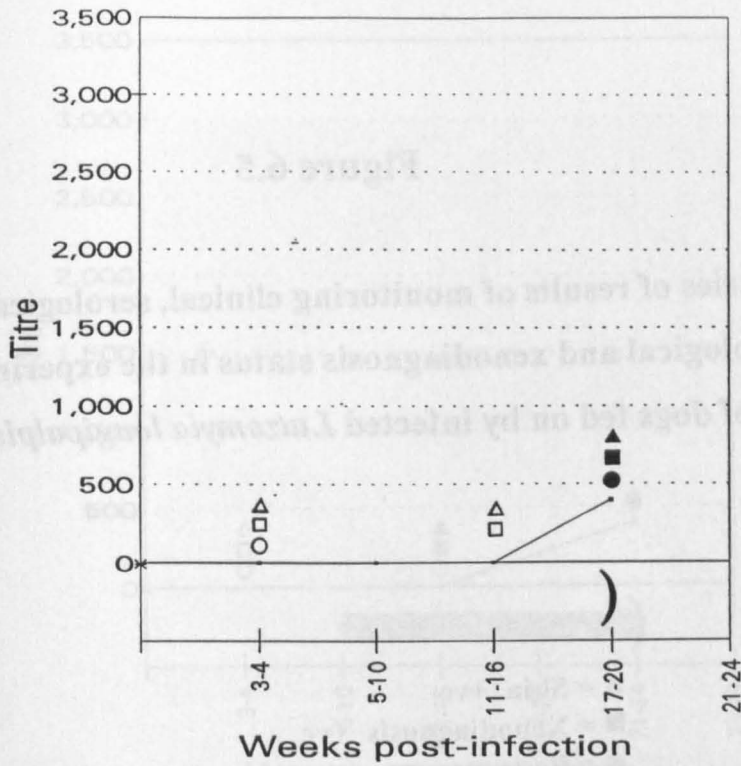
Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve

- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▣ = Nodule
- = Lesion
- ▤ = Scar

Dog M6



Dog M7

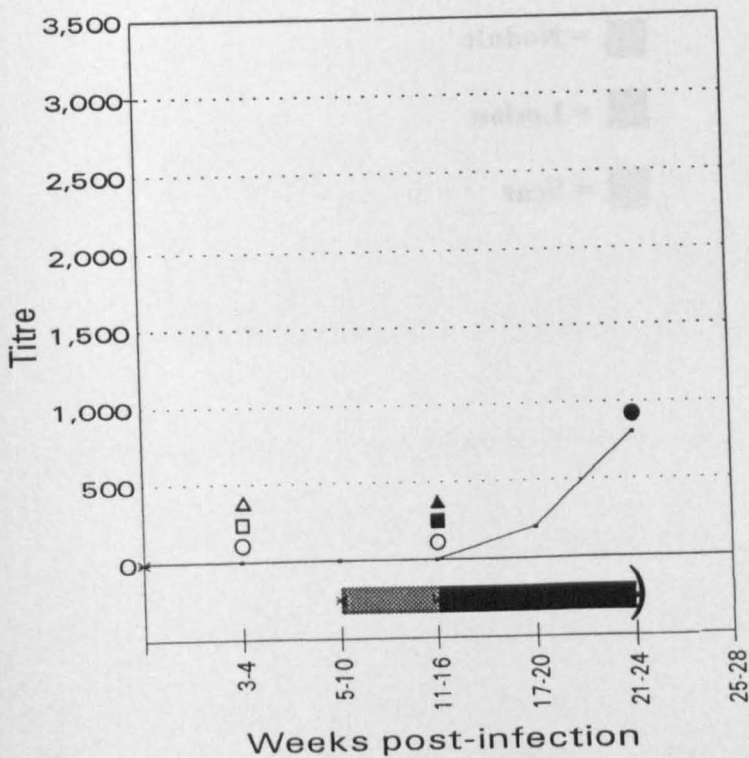


Figure 6.5

Summaries of results of monitoring clinical, serological, parasitological and xenodiagnosis status in the experimental cohort of dogs fed on by infected *Lutzomyia longipalpis*

- ▲ = Skin +ve
- = Xenodiagnosis +ve
- = Bone marrow +ve

- △ = Skin -ve
- = Xenodiagnosis -ve
- = Bone marrow -ve

- ▨ = Nodule
- = Lesion
- ▨ = Scar

Dog M8

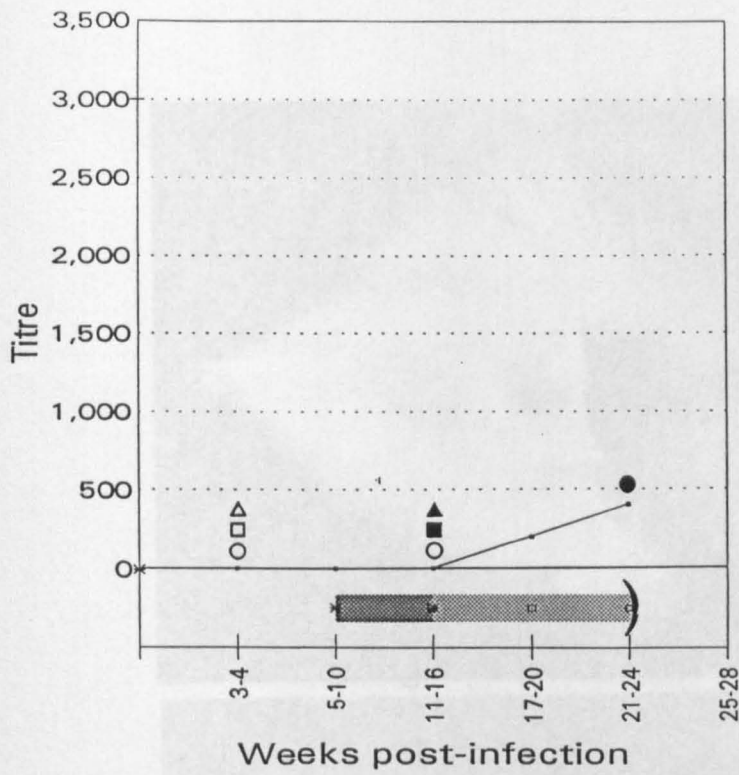


Figure 4.10 A

Figure 6.6A

Dog 06: days 89 (upper) and 240 (lower) after infective feed



Figure 6.6 A

Figure 6.6B

**Dog 07: days 66, 66 (possible, but unconfirmed secondary lesion),
120, 150, 170 and 240 after infective feed**



Figure 6.6C

Dog 15: days 30, 66, 89, 120 and 240 after infective feed



Figure 6.6 C

Figure 6.6D

Dog 11: days 66, 89, 194 and 240 after infective feed



Figure 6.6 D

Figure 6.6 E

Dog 13: days 66, 75, 89, 120, 162 and 240 after infective feed



Figure 6.6 E

Figure 6.6 F

Dog 02: days 66, 120, 193 and 240 after infective feed

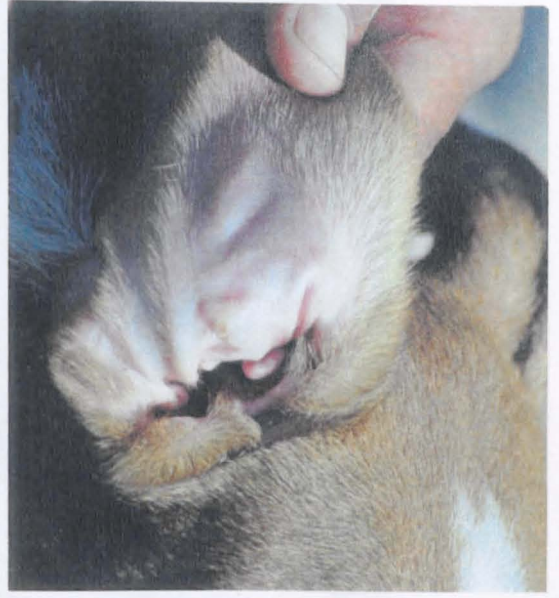


Figure 6.6 F

Figure 6.6 G

Dog 09: days 66, 89, 155, 190 and 240 after infective feed



Figure 6.6 G

CHAPTER 7: DRUG TREATMENT OF CANINE VISCERAL LEISHMANIASIS

7.1 Treatment of canine VL with aminosidine (paromomycin)

The dog is considered to be an important reservoir of infection for human VL, except in endemic areas of the Old World where the disease agent is *L. donovani*, and not *L. infantum/chagasi*. Although residual or ultra low volume insecticide spraying may be useful for attacking the sandfly vectors there are two principal difficulties obstructing the direct control of canine VL.

Firstly, diagnosis of canine VL is unreliable, due to the lack of a proven, highly sensitive, specific, simple assay for antibodies that can be performed on site during household visits. The alternative approach of parasitological diagnosis requires intensive effort, and diagnostic DNA probes for field use have yet to be perfected.

Secondly, there is no effective drug for the treatment of canine VL. Pentavalent antimonials, which are of great value for treatment of the human disease (Marsden, 1985; Olliaro and Bryceon, 1993) are used in some countries of the Mediterranean region to treat symptomatic canine VL or asymptomatic dogs that are serologically positive. Clinical signs of canine VL may disappear, although infections usually relapse soon after antimonial treatment is withdrawn. Treatment is often repeated every few months but such repeated use of non-curative doses of antimonials is hazardous: drug-resistant organisms are likely to arise, which may be transferred to humans, and apparently healthy infected dogs may act as a prolonged source of infection to sandflies (Gradoni *et al.*, 1987; Gramiccia *et al.*, 1992). Attempts to use antimonials for the treatment of canine VL in the New World have been unsuccessful (Marzochi *et al.*, 1985).

During the last decade there has been renewed interest in the anti-leishmanial activity of the aminoglycoside antibiotic aminosidine (paromomycin) (Schillings and Schaffner, 1961). The drug is active *in vitro* and *in vivo* against various *Leishmania* species (Neal, 1968; Neal *et al.*, 1995). Clinical studies have confirmed that it is a very useful alternative to antimonials for the treatment of human VL, although like antimonials it requires parenteral administration and is not absorbed through the alimentary tract (Chunge *et al.*, 1990; Scott *et al.*, 1992; Hassan *et al.*, 1995). Single-agent aminosidine treatment was found to have limited efficacy in cutaneous leishmaniasis in South America (Soto *et al.*, 1994). Aminosidine has been used with great success in three clinical trials of combination therapy with antimonials for human VL (Chunge *et al.*, 1990; Thakur *et al.*, 1992, 1995; Seaman *et al.*, 1993).

The aims of these experiments (Materials and Methods) was to follow clinical recovery, relapse, clinical and parasitological cure in symptomatic canine VL treated with aminosidine.

7.1.1 Treatment with 20 mg/kg/day for 15 days

The most striking observation from this preliminary group of three dogs was the early and dramatic clinical improvement, with the disappearance of conjunctivitis, increase in appetite, weight gain and general improvement in skin and coat condition. All three animals were considered to be underweight prior to commencement of treatment, although there was no evidence that they had been denied food and apparent loss of appetite/weight loss was one reason why the owners had asked for the animals to be examined. The only detectable side-effect was a temporary loss of hair over five days during the second week of treatment prior to replacement by a healthy coat. At the end of treatment the three animals were parasitologically negative by examination of both bone marrow and skin. Between 50 days (dogs 1 and 2) and 100 days (dog 3) after initiation of treatment,

however, amastigotes were again found, first in the skin and then in the bone marrow (**Figure 7.1**). About the same time, or slightly later, symptoms of VL returned becoming severe with weight loss, disseminated dermatitis, hair loss, and conjunctivitis. Deterioration was progressive and all three animals were killed at day 157, as there was no prospect of recovery, to prevent suffering. The dramatic weight gain, to levels typical for healthy examples of this breed, that accompanied clinical improvement, and subsequent decline in bodyweight associated with relapse are shown in **Figure 7.2**.

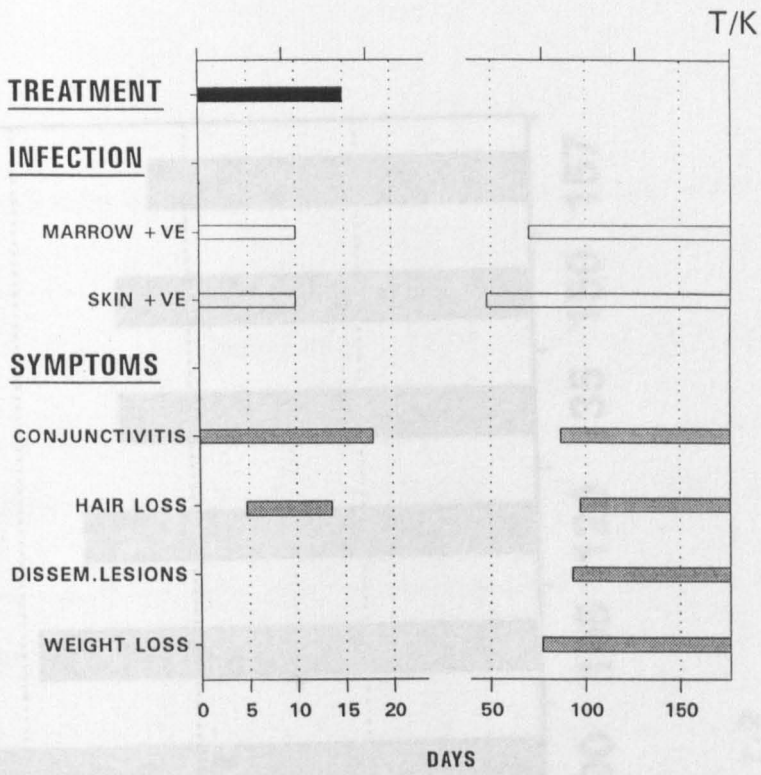
7.1.2 Treatment with 80 mg/kg/day for 20 days

In view of the relapse of all three dogs in group 1 it was decided to use a higher dose (80 mg/kg) and a slightly prolonged schedule for treatment (20 days) in a second group of animals. A third group (below) was also set up with an intermediate dose level (40 mg/kg) and a yet more prolonged schedule (30 days). Dogs in the 80 mg/kg group either had severe VL (symptomatic) or less severe but obvious signs of VL (oligosymptomatic; **Table 7.1**) and all had amastigotes in both skin and bone marrow. Response to treatment was not uniform. Two dogs died five days or one day before treatment ended, and a third dog died four days after treatment had ended. All three of these animals showed adverse effects including appetite loss, weight loss, acute dehydration (sunken eyes), loss of scent perception and deafness; two also had keratitis. Weight loss as a possible adverse effect of treatment is shown in **Figure 7.3**. There were no detectable amastigotes in liver or spleen post-mortem.

Figure 7.1

Aminosidine 20 mg/kg/day for 15 days: relapse after a period of improvement (dogs 2, 3), T/K = terminal phase/killed

DOG 2



DOG 3

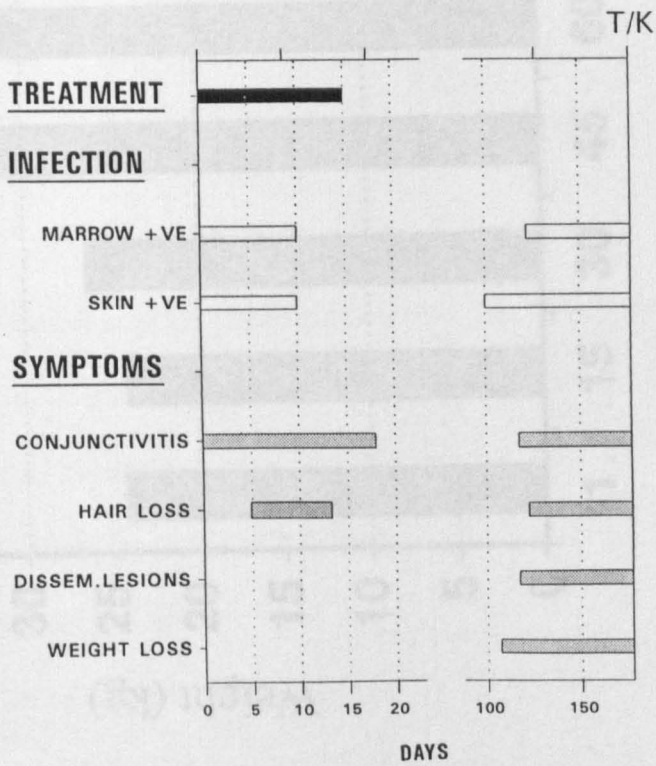


Figure 7.1

Figure 7.2

Mean weight fluctuations in three Dobermanns following treatment with aminosidine (20 mg/kg/day/15 days) and in conjunction with subsequent relapse (see Figure 7.1)

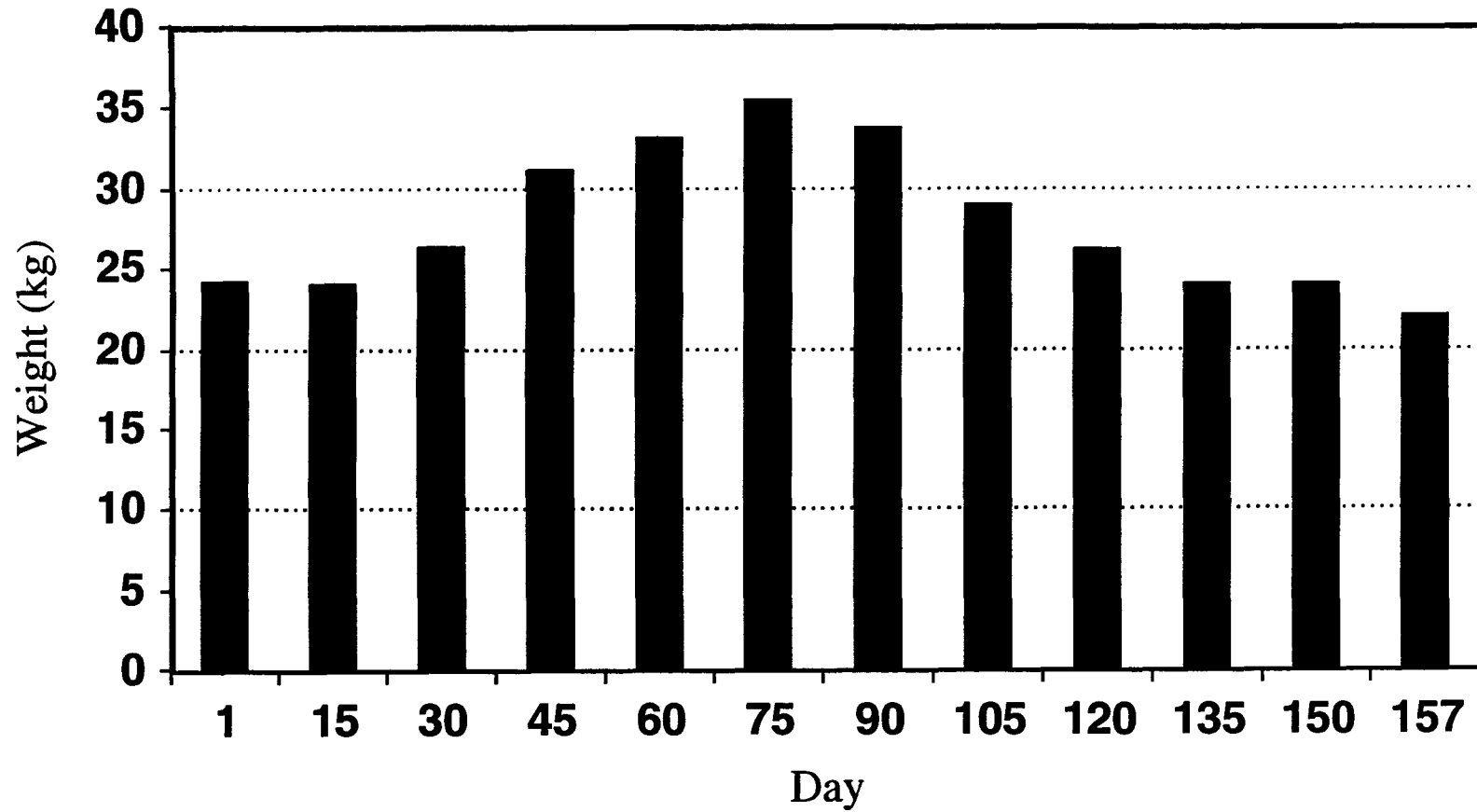


Figure 7.2

Table 7.1

Summary of results of aminosidine treatment of canine visceral leishmaniasis

Dose	No. of dogs	CCBT	AE	D	R/D	CPC (%)
20 mg/kg/day 15 days	3	Symp	0/3	0/3	3/3	0/3
80 mg/kg/day 20 days	6	Oligo 4	1/4	1/4	2/4	1/4
		Symp 2	2/2	2/2	-	-
40 mg/kg/day 30 days	12	Oligo 7	3/7	1/7	3/7	3/7
		Symp 5	1/5	1/5	4/5	0/5

CCBT = clinical condition before treatment

AE = adverse effects occurring within one month of the end of treatment (later clinical events were also recorded)

D = died within one month of treatment

R/D = relapsed and died

CPC = clinical and parasitological cure

Figure 7.3

Decrease in the body weight of a dog during treatment with aminosidine at 80 mg/kg/day for 20 days

Decrease of the corporal weight of a dog, during treatment with Aminosidine 80 mg/kg/day/20 days

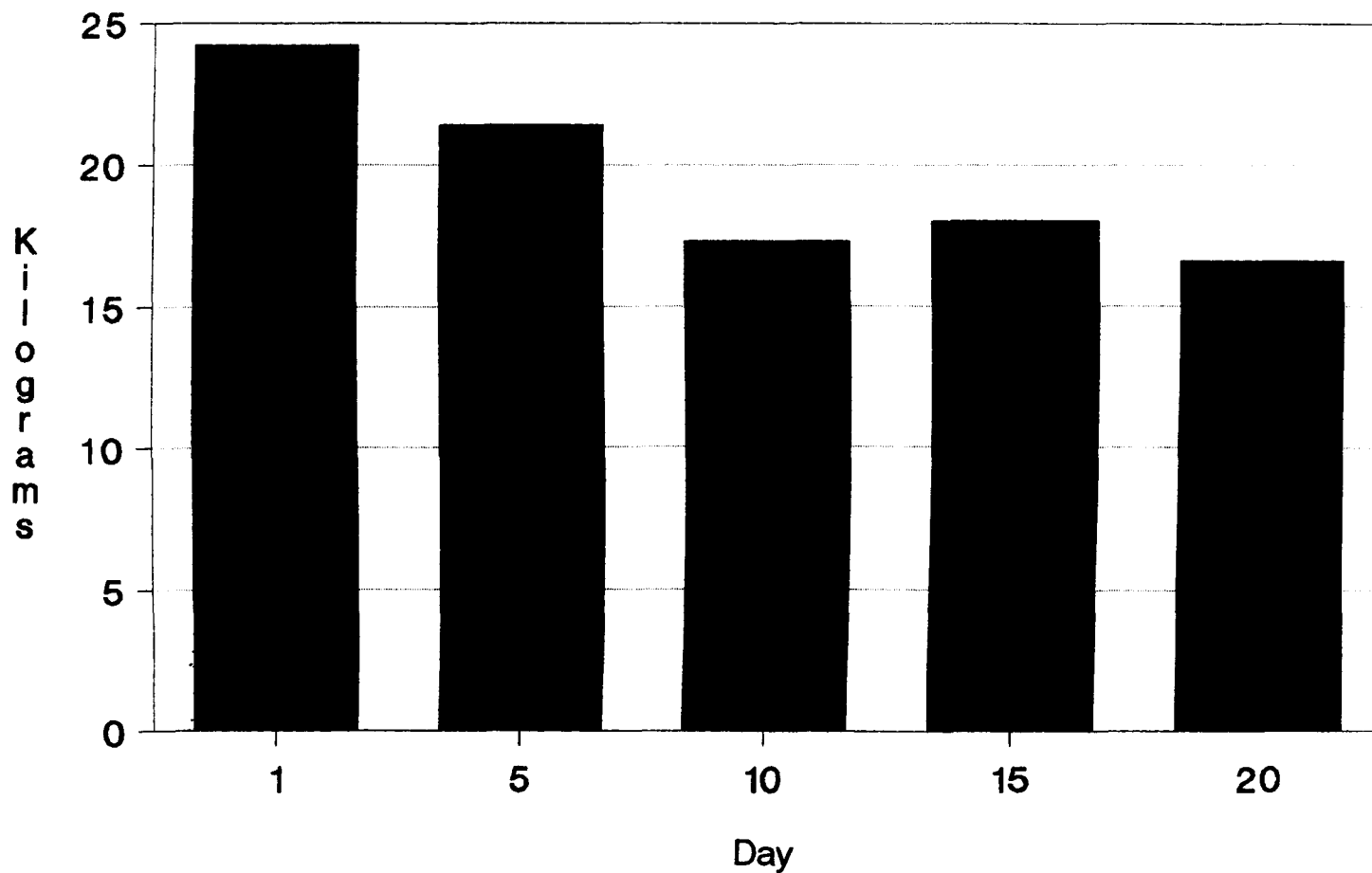


Figure 7.3

A fourth dog survived treatment with dramatic clinical improvement including restored appetite, weight gain and disappearance of oedema, was apparently entirely healthy apart from incipient blindness that began around day 60, but died suddenly of unknown causes. A single atypical amastigote was found post-mortem in the liver. A fifth dog also recovered dramatically, relapsed and became parasitologically positive seven months after treatment began; deafness and blindness were noted during the terminal phase of the disease.

One dog in this group survived and after four years is in excellent general health, parasitologically negative, but with defective vision that arose two years after treatment (**Figure 7.4**).

7.1.3 Treatment with 40 mg/kg/day for 30 days

One dog lost weight and died before treatment was complete and a second died shortly after treatment; amastigotes were found in both animals post-mortem. Two dogs did not clear their skin infection during treatment, were killed thirty days after treatment and had amastigotes in both liver and bone marrow post-mortem. Five dogs appeared to have cleared their infections, with weight gain and dramatic clinical improvement, but relapsed between one and four months later with progressive canine VL and parasites in skin and bone marrow.

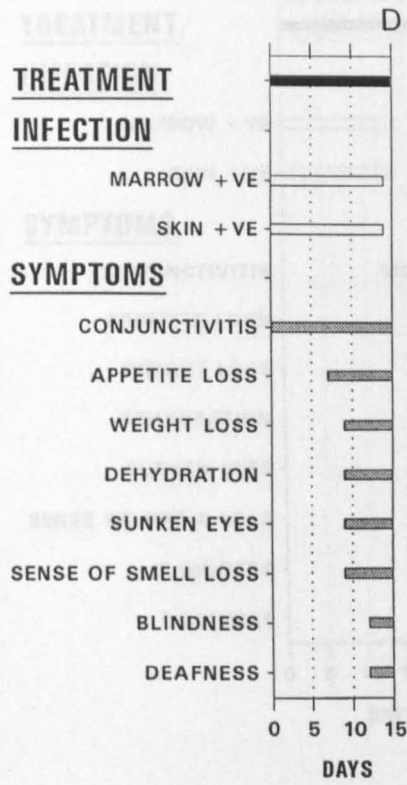
Three of the twelve dogs have survived for more than four years with no detectable *L. chagasi* infection (**Figure 7.5**).

Possible side-effects in this group were weight loss in one dog that died during treatment, signs of deafness in four dogs (beginning at between days 25 and 60), and keratitis leading to partial or total blindness in six dogs (beginning at between days 28 and 500). Two of the three dogs that have survived for more than four years and are apparently totally cured of infection have no detectable side-effects.

Figure 7.4

Summary of treatment with aminosidine at 80 mg/kg/day for 20 days: dogs 1-6, D = died, T/D = terminal phase/died, S.A. = still alive. Late occurring blindness and/or deafness were not necessarily attributable to preceding aminosidine therapy (see text)

DOG 1



DOG 2

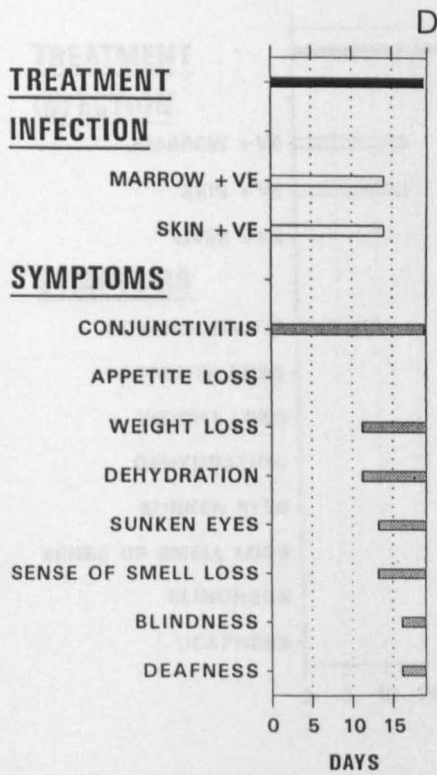
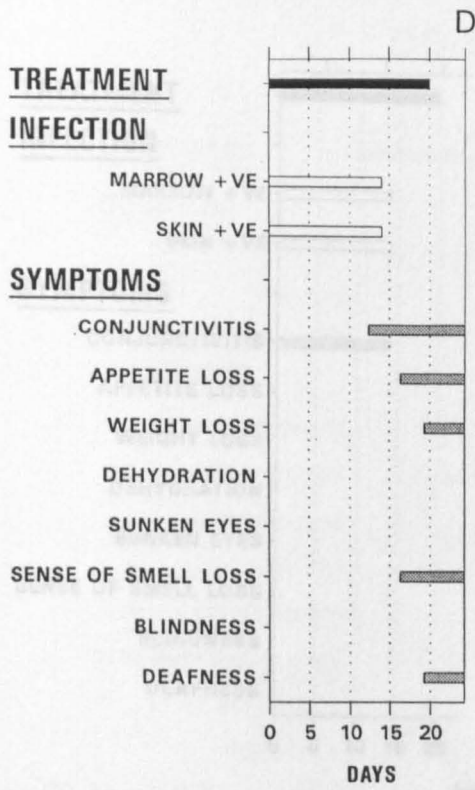


Figure 7.4

DOG 3



DOG 4

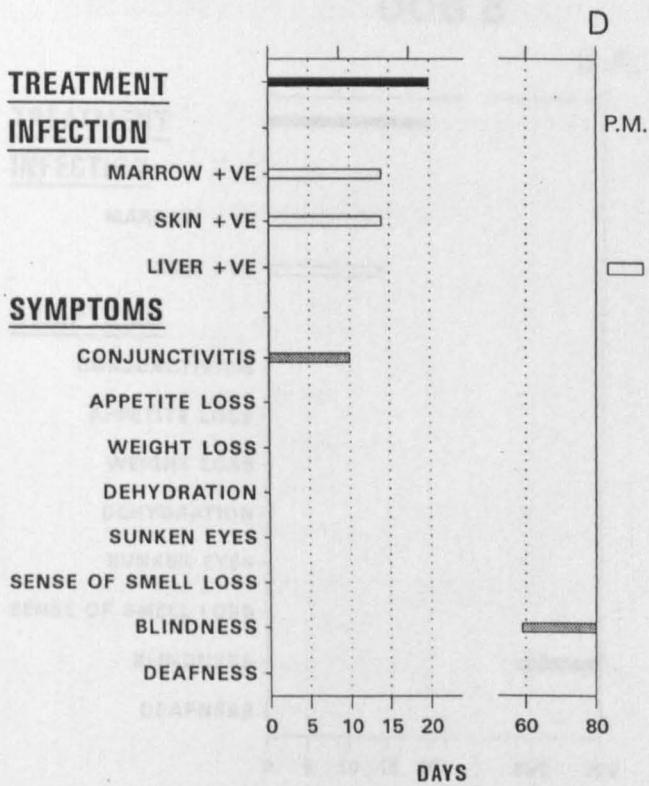
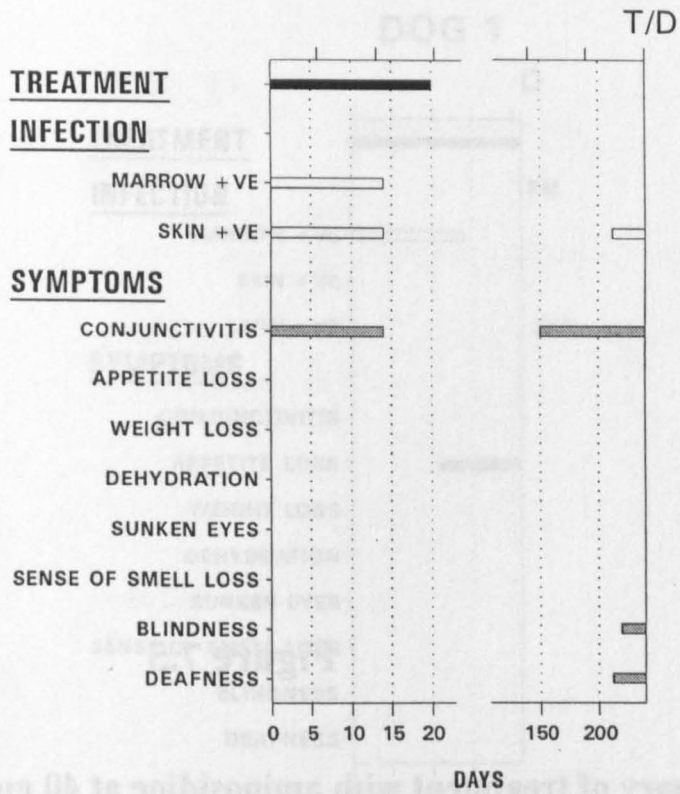


Figure 7.4 (continued)

DOG 5



DOG 6

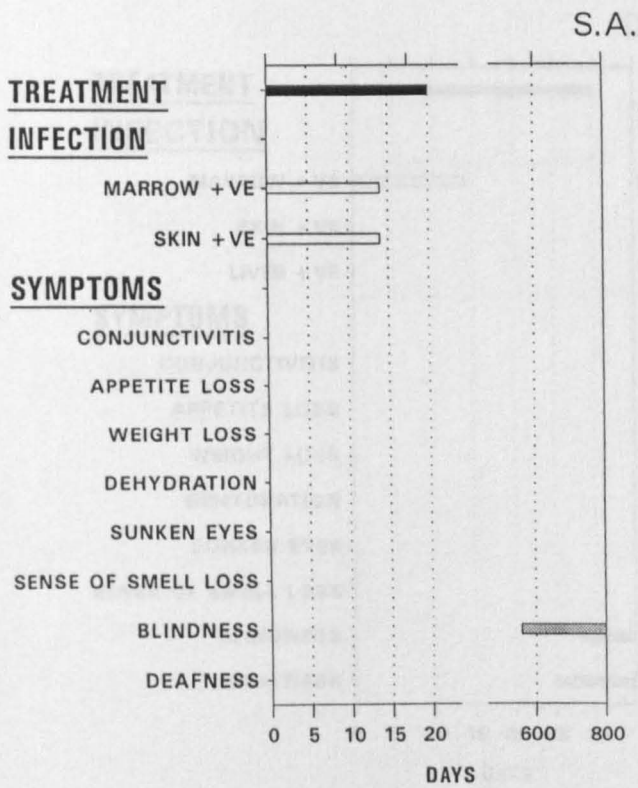
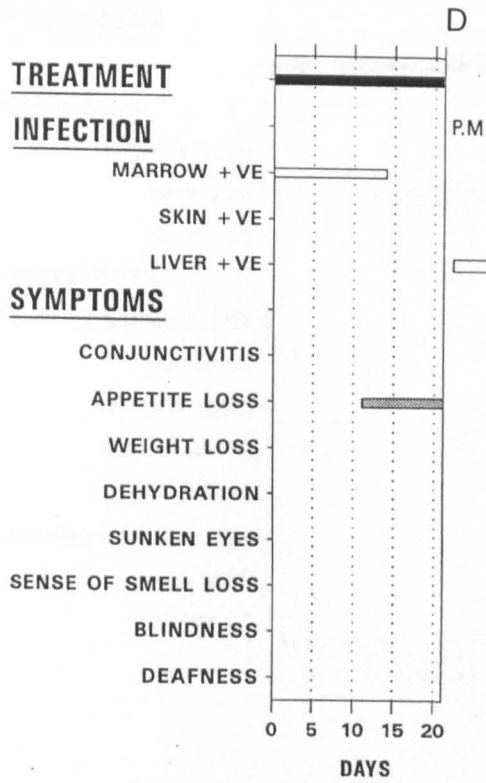


Figure 7.4 (continued)

Figure 7.5

Summary of treatment with aminosidine at 40 mg/kg/day for 30 days: dogs 1-12, D = died, S.A. = still alive (Note dogs are not shown in chronological order of recruitment)

DOG 1



DOG 2

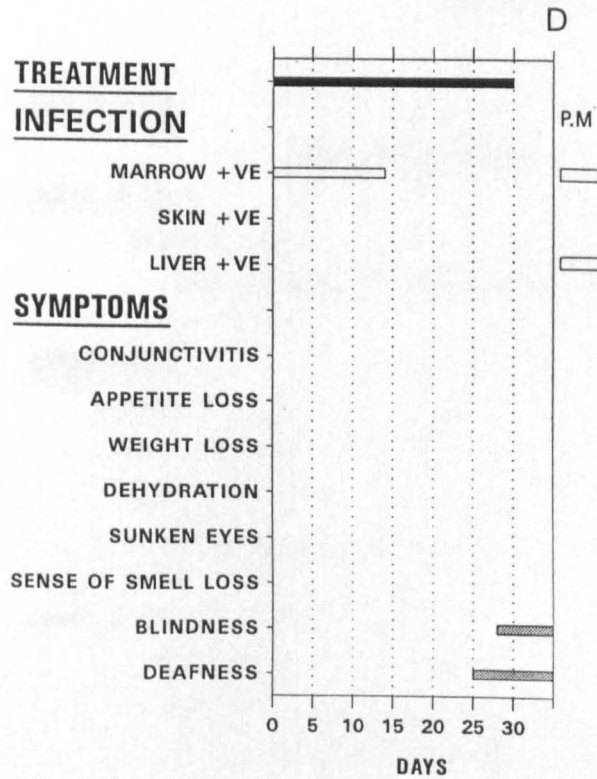
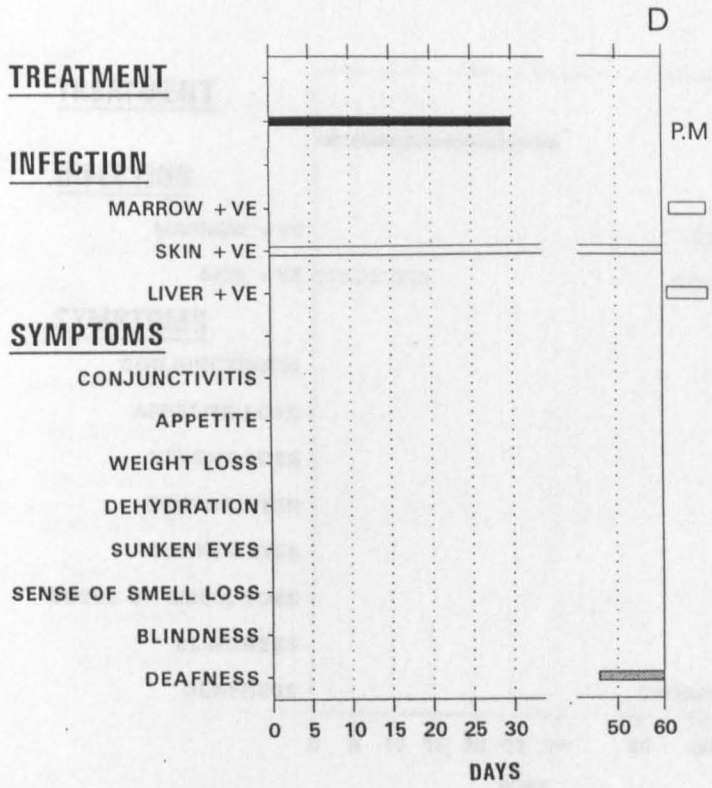


Figure 7.5

DOG 3



DOG 4

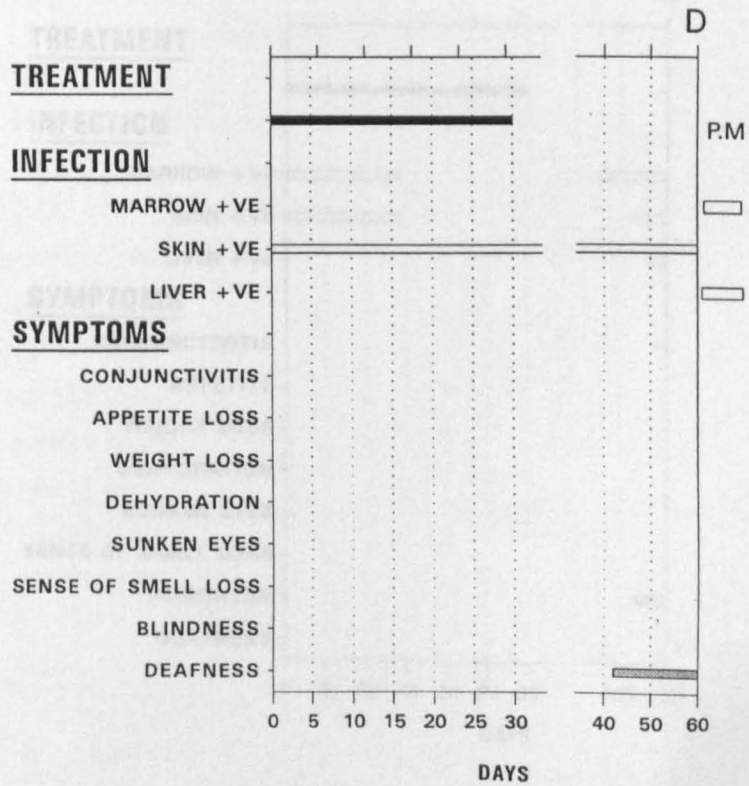
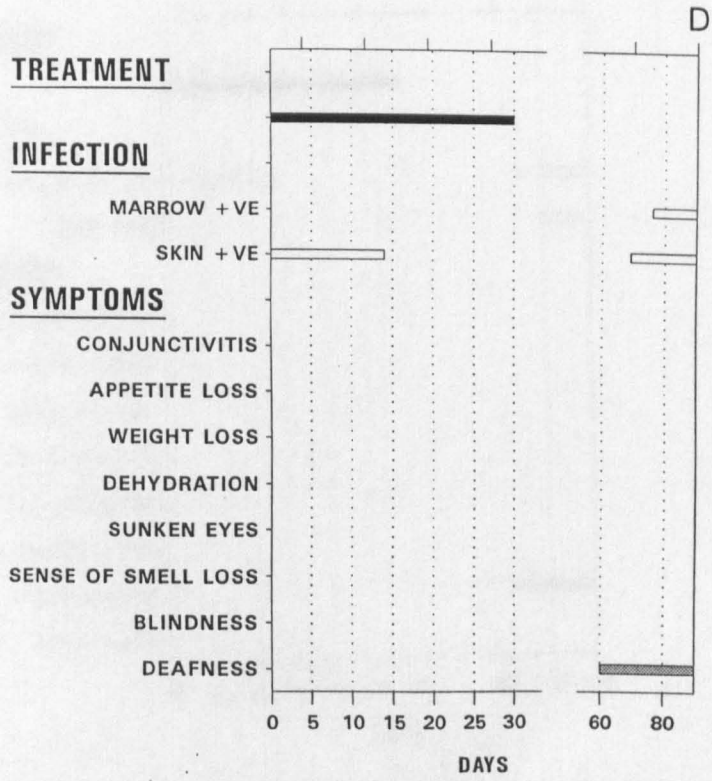


Figure 7.5 (continued)

DOG 5



DOG 6

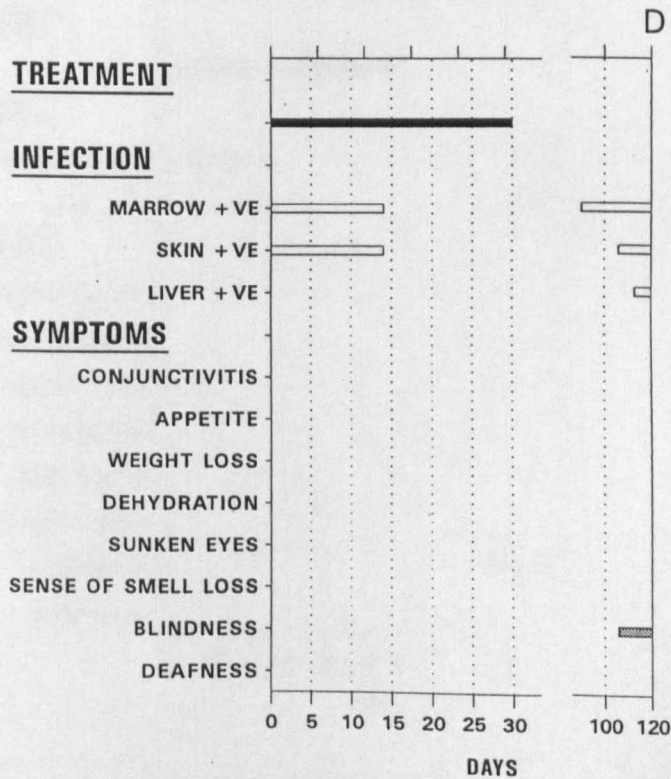
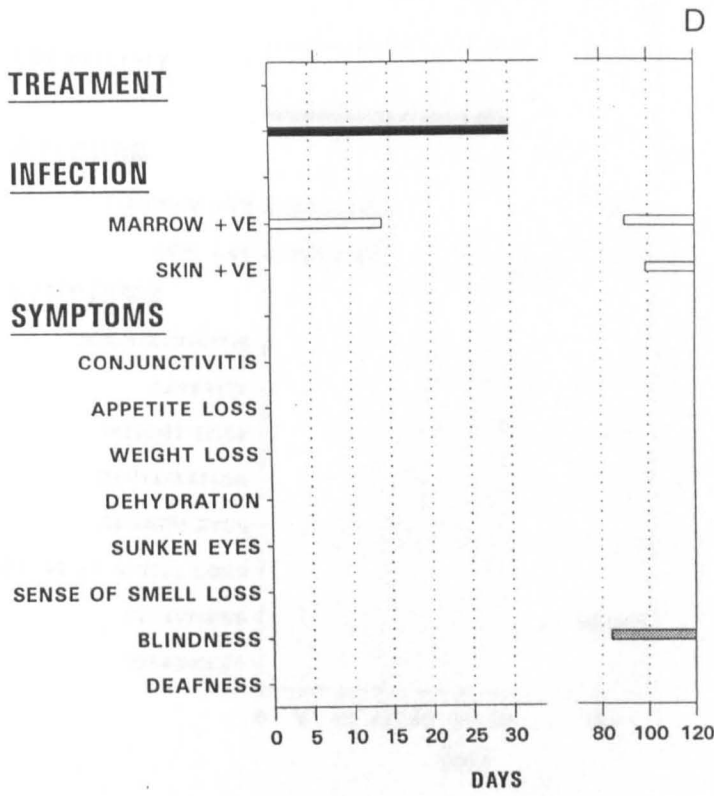


Figure 7.5 (continued)

DOG 7



DOG 8

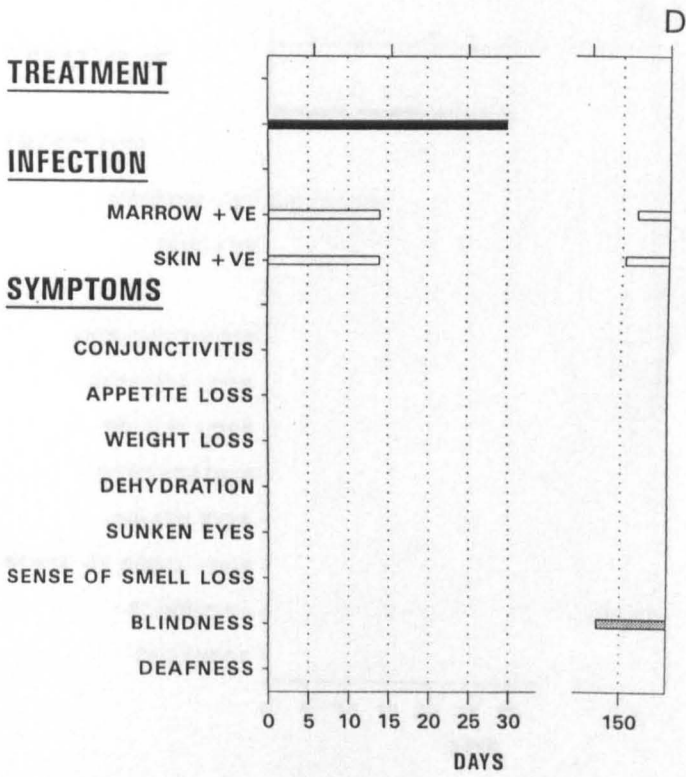
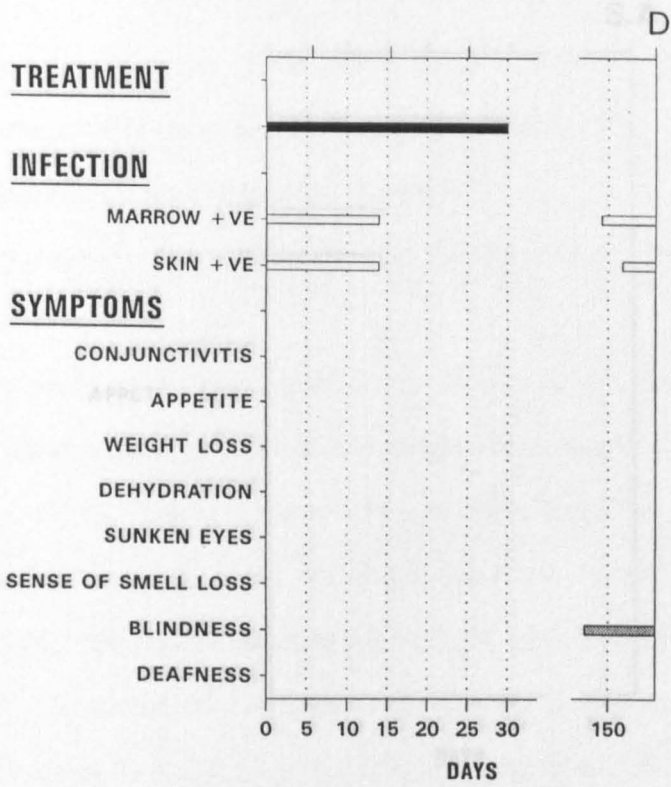


Figure 7.5 (continued)

DOG 9



DOG 10

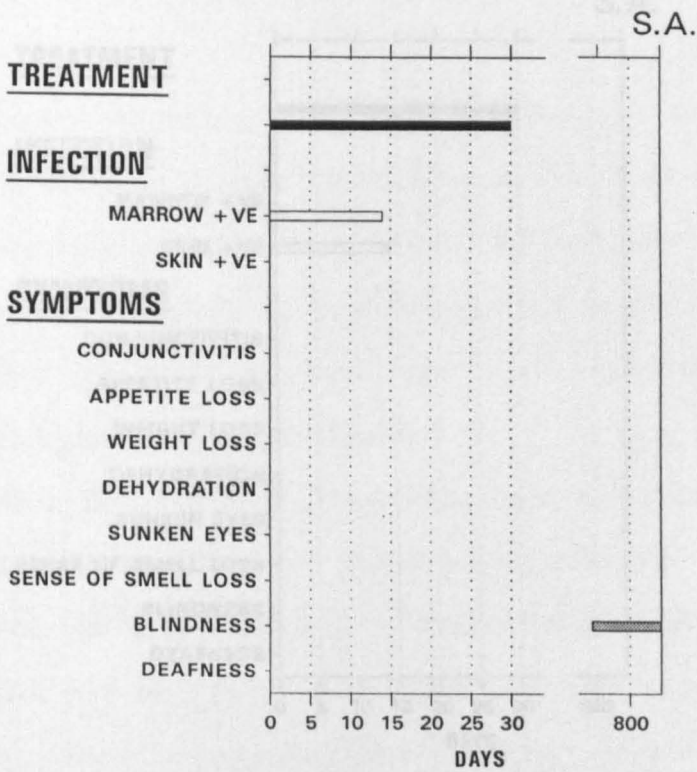


Figure 7.5 (continued)

DOG 11

S.A.

TREATMENT

INFECTION

MARROW + VE

SKIN + VE

SYMPTOMS

CONJUNCTIVITIS

APPETITE LOSS

WEIGHT LOSS

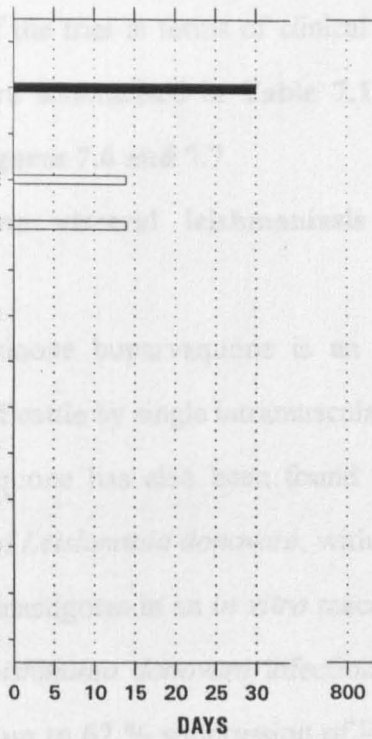
DEHYDRATION

SUNKEN EYES

SENSE OF SMELL LOSS

BLINDNESS

DEAFNESS



DOG 12

S.A.

TREATMENT

INFECTION

MARROW + VE

SKIN + VE

SYMPTOMS

CONJUNCTIVITIS

APPETITE LOSS

WEIGHT LOSS

DEHYDRATION

SUNKEN EYES

SENSE OF SMELL LOSS

BLINDNESS

DEAFNESS

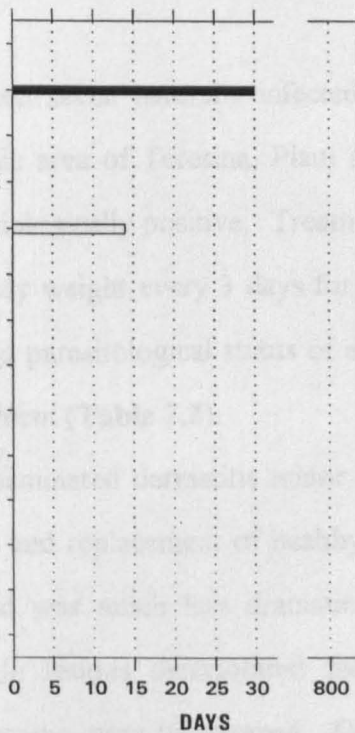


Figure 7.5 (continued)

The overall Results of the trial in terms of clinical status before treatment and outcome of treatment are summarised in **Table 7.1**. Examples of clinical improvement are shown in **Figures 7.6 and 7.7**.

7.2 Treatment of canine visceral leishmaniasis with buparvaquone (Butalex)

The hydroxynapthoquinone buparvaquone is an effective treatment for *Theileria* (East-coast fever) of cattle by single intramuscular injection of 2.5 mg/kg (McHardy, 1985). Buparvaquone has also been found to be a promising lead compound for the treatment of *Leishmania donovani*, with ED₅₀ values of between 0.12 and 0.005 µM against amastigotes in an *in vitro* macrophage model (Croft *et al.*, 1992). Treatment of *Leishmania donovani* infection in the BALB/c mouse model was disappointing but up to 62 % suppression of liver amastigote numbers was achieved by subcutaneous administration with 100 mg/kg/day for 5 days. These results suggested that buparvaquone should be tested in a different animal model.

Accordingly we treated seven naturally infected dogs that had acquired infection in the urban endemic area of Teresina, Piaui state, Brazil. All animals were symptomatic and parasitologically positive. Treatment was by intramuscular injection with 5 mg/kg of body weight every 3 days for a total period of 12 days (four doses). The clinical and parasitological status of each animal was evaluated before, during and after treatment (**Table 7.2**).

Where there was disseminated dermatitis minor clinical improvement was seen with partial loss of hair and replacement of healthy hair. This improvement occurred in two animals and was much less dramatic than that obtained with aminosidine (above). Single lesions deteriorated following this schedule of buparvaquone and lesions became more widespread. Dogs died with progressive canine VL or were killed to prevent suffering.

Figure 7.6

Clinical improvement following aminosidine treatment: top, before treatment; centre, 6 months after treatment; lower, possible side effect 6 months after treatment (80 mg/kg, dog 6)



Figure 7.6

Figure 7.7

Clinical improvement following aminosidine treatment: top, before treatment; lower, one month after treatment (80 mg/kg, dog 4)

Table 7.3

Treatment of severe skin with various antibiotics and antifungal agents



Figure 7.7

Table 7.2

Treatment of seven dogs with naturally acquired symptomatic canine visceral leishmaniasis by buparvaquone (5 mg/kg, intramuscularly, four doses over 12 days)

<u>BREED</u> (age, sex, weight)	<u>CLINICAL CONDITION</u> ¹ (parasitological status) ²	
	<u>Before treatment</u>	<u>After treatment</u>
1. Mongrel (2 yr, female, 18 kg)	Ear lesion (skin, b.m., liver +ve)	Lesion extended (skin, b.m., liver +ve)
2. Basset (3 yr, female, 13 kg)	Nose lesion (skin +ve)	Lesion extended (skin +ve)
3. Mongrel (1 yr, female, 7 kg)	Ear lesion (skin, b.m. +ve)	Disseminated lesions (skin, b.m. +ve)
4. Mongrel (1 yr, male, 10 kg)	Lip lesion (skin, b.m. +ve)	Hair replacement, lesion extended, second lesion (skin, b.m. +ve)
5. Pekinese (2 yr, female, 4 kg)	Lip lesion (skin +ve)	Lesion extended (skin +ve)
6. Mongrel lesion (3 yr, female, 5 kg)	Skin lesion (skin, b.m. +ve)	Hair replacement, extended and multiple (skin +ve)
7. Mongrel (3 yr, male, 6 kg)	Ear lesions (skin, b.m. +ve)	Lesions extended (skin +ve)

¹ All animals died or killed between days 41 and 85.

² b.m. = bone marrow, by microscopy of Giemsa-stained impression smears.

CHAPTER 8: PRELIMINARY RESULTS ON THE APPLICABILITY OF PYRETHROID INSECTICIDES TO VECTOR CONTROL (1993-1994)

8.1 Study design

After consultation with the technical personnel of Zeneca Public Health, a protocol was elaborated, with the objective of preliminary testing of the efficiency of a pyrethroid insecticide. A comprehensive study was not possible with the scope and duration of the present project.

8.1.1 Focal ultra low volume (ULV) spraying of pigsties with lambda-cyhalothrin (ICON) (by "Thermal Fog" with diesel diluent)

The necessary requirement for the accomplishment of the protocol was that there had to be test sites with large numbers of sandflies, such as infested domestic animal shelters. Sandflies were collected at the chosen sites during 5 months, June-October 1993. The sites were 3 pig-sites with similar characteristics, according to the protocol. A total of 90,493 sandfly specimens were collected, 71,899 (79.4%) males and 18,494 (20.4%) females (**Table 8.1**). An interesting aspect, which deserves to be emphasized, was that in one of the sites of capture the number of collected sandflies reduced considerably with the removal of the pig from the pig-sty. (The pig was sold to pay the electricity bill.)

The impact of focal spraying of ultra low volume (ULV) lambda-cyhalothrin (ICON) on the three pigsties previously monitored, as summarised in **Table 8.1**, is shown in **Figure 8.1**. Site A, B and C correspond, respectively, with Morro da Esperanca, Universidade and Vila Operaria (**Table 8.1**). Spraying was done in January 1994 after a new pig had been given to residents at site A. An immediate, but not total, reduction in fly numbers (flies) CDC trap (night) is indicated in **Figure 8.1**, 1 day after focal ULV spraying. After 7 or 15 days,

Table 8.1

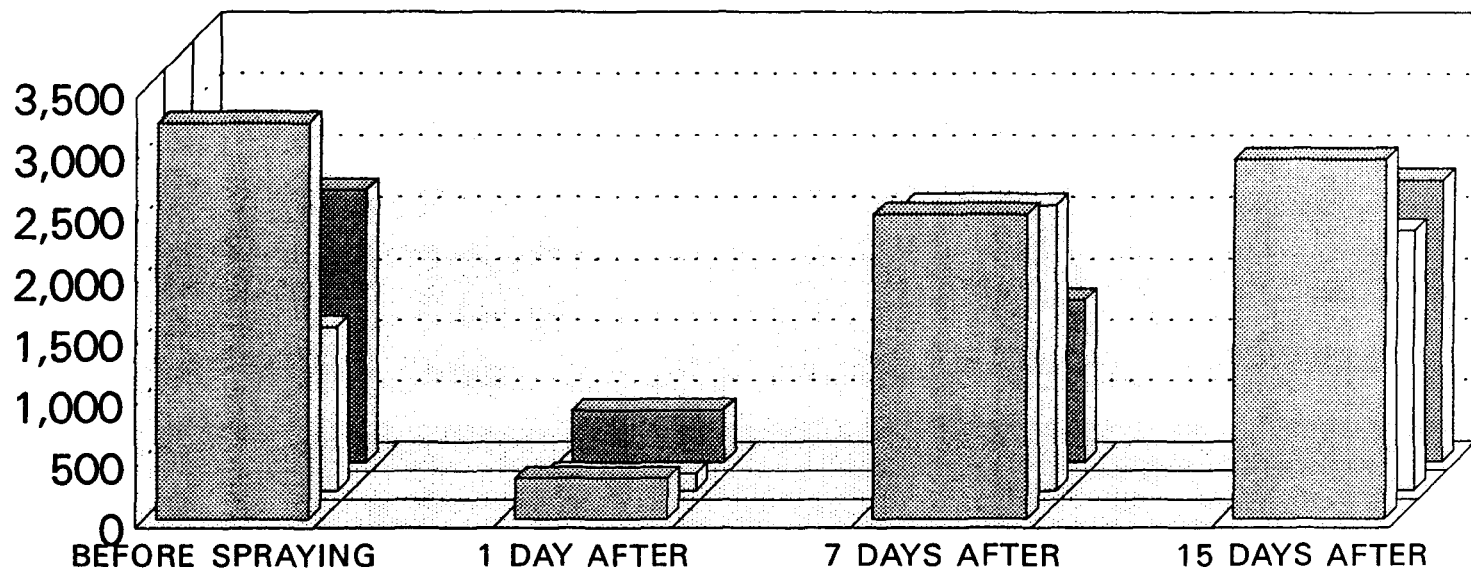
Results of collection performed in 3 pig-sties
Teresina, Piaui, June - October / 1993.

Month	Total of captures performed	Place	Method	Male	Female	Total
june	3	*	CDC(3)	8,943	1,590	10,533
	3	**	CDC(2)	3,528	480	4,008
	3	***	CDC(1)	5,380	2,390	7,770
july	3	*	CDC(3)	10,114	3,101	13,215
	3	**	CDC(2)	3,230	679	3,909
	3	***	CDC(1)	3,201	1,408	4,609
august	3	*	CDC(3)	7,413	1,603	9,016
	3	**	CDC(2)	2,521	483	3,004
	3	***	CDC(1)	3,001	989	3,990
september	3	*	pooter .	167	45	212
	3	**	CDC(5)	8,142	1,810	9,952
	3	***	CDC(1)	3,818	630	4,448
october	3	*	pooter .	98	32	130
	3	**	CDC(5)	9,328	2,313	11,641
	3	***	CDC(1)	3,115	941	4,056
Total				71,899	18,494	90,493

- . = Without pig in the pigsty
- * = Pig-sties /Morro da Esperanca (centre of the city)
- ** = Pig-sties /Universidade (University)
- *** = Pig-sties /Vila Operaria (peripheral area of the city)

Figure 8.1

Impact of focal ULV spraying with lambda-cyhalothrin (ICON) on numbers of *Lutzomyia longipalpis* in three pigsties



SITE C	2,220		421		1,328		2,301
SITE B	1,340		140		2,330		2,120
SITE A	3,224		335		2,490		2,932

SANDFLIES COLLECTED WITH CDC OVERNIGHT

SITE A SITE B SITE C

Figure 8.1

however, fly numbers were restored presumably by immigration or emergence, the implication being that only total (and repeated) coverage by ULV spraying would be a realistic vector control strategy in the study area.

8.1.2 Residual activity of ICON against *Lutzomyia longipalpis* in sprayed pigsties and chicken houses (1995)

In accordance with the manufacturer's instructions the internal and external walls of two pigsties and two chicken houses were sprayed with ICON using 10% wettable powder at 30 mg of active ingredient/m². Both the pigsties and chicken houses were constructed of wood and large numbers of *Lu. longipalpis* had been captured there, independently of the season of the year. All the internal and external surfaces were sprayed. One additional pigsty and one chicken house with similar characteristics were selected as unsprayed controls.

Retention of residual activity was tested by 'cone-tests' (WHO, 1978) using the stable colony of *Lu. longipalpis* that had been established at the Parasitology Laboratory of the University of Teresina. Sixty sandflies were used in each cone and they were exposed to the wall surface for 5 minutes, after which surviving sandflies were taken back to the laboratory and the numbers of survivors at 24 hrs was recorded. The results are summarised in **Figures 8.2 and 8.3**, as means for three cones of 60 sandflies at each exposure.

After 4 months a high level of residual activity was retained, especially on the internal walls of the chicken houses, which were protected from the weather and light. Activity was lost more rapidly from the external walls of the chicken houses and from both the external and internal walls of the pigsties, possibly because internal walls were periodically cleaned by the owners and the lower walls were abraded by contact with the pigs.

Figure 8.2

Monitoring residual activity of ICON (10 % csp, 30 mg ai/m²) on the internal and external walls of two pigsties. Mean mortality for 3 cones of 60 sandflies, 24 hrs after exposure

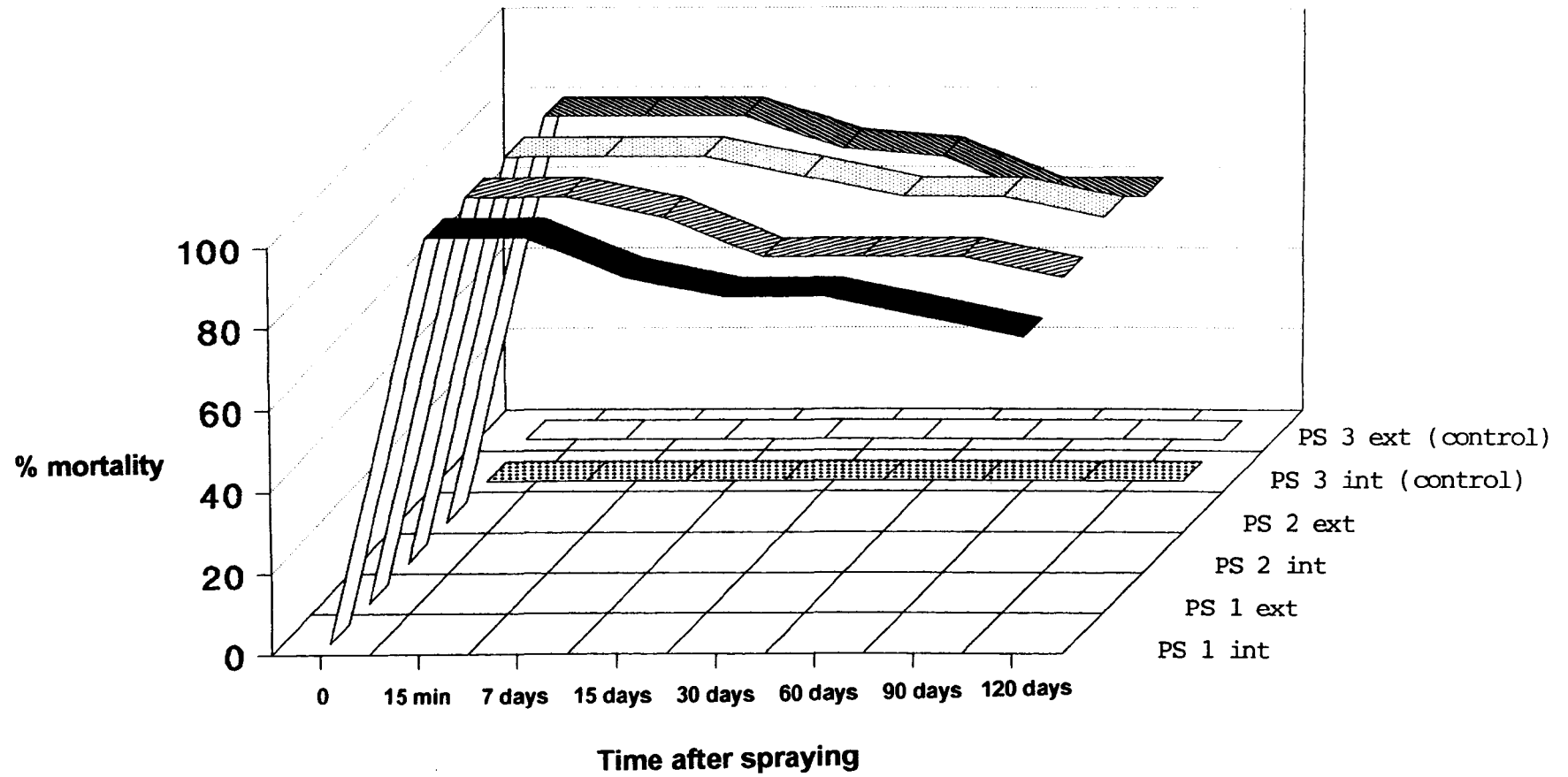


Figure 8.2

Figure 8.3

Monitoring residual activity of ICON (10% wp, 30 mg ai/m²) on the internal and external walls of two chicken houses. Mean mortality for 3 cones of 60 sandflies 24 hrs after each exposure

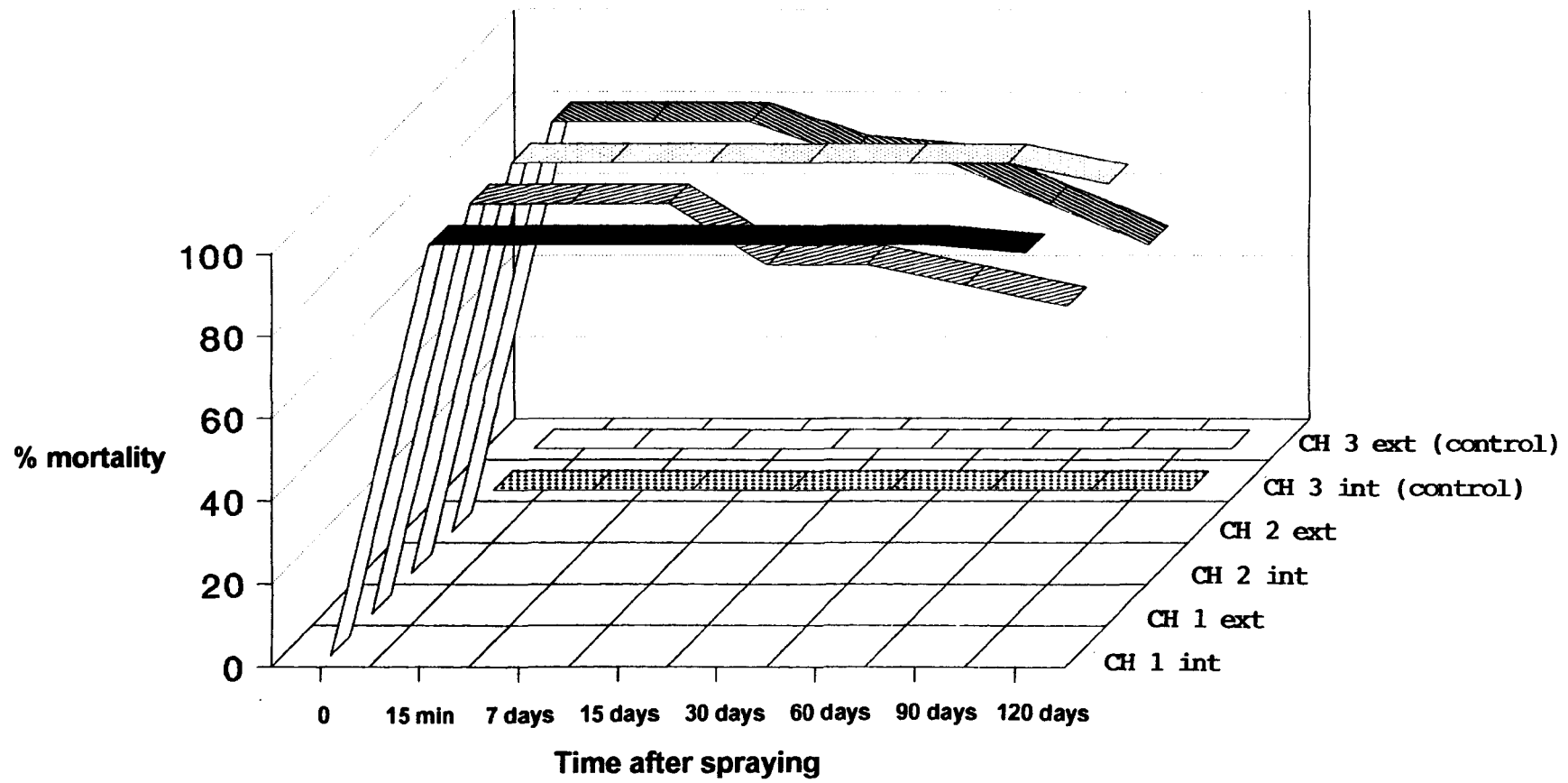


Figure 8.3

8.1.3 Laboratory comparisons of efficacy of pyrethroid insecticides and DDT

Comparisons of the efficacy of three pyrethroids ICON (0.1 %), deltamethrin (DM, 0.25 %) and permethrin (PE, 0.25 %) and DDT (4 %) were performed at the London School of Hygiene and Tropical Medicine using test papers in standard WHO exposure tests.

Two initial tests compared mortality ('knock-down') produced by ICON and DDT after 5 mins, 15 mins, 30 mins or 60 mins exposure and observation at 1 hr, 2 hrs, 12 hrs, 24 hrs, 48 hrs, 72 hrs and 96 hrs. ICON appeared to be more effective than DDT (**Figure 8.4**).

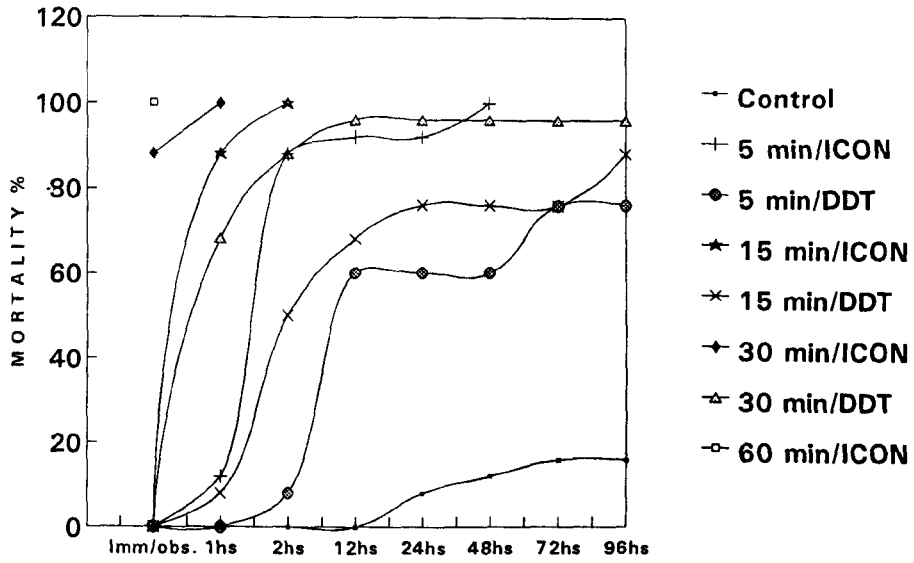
Mortality ('knock down') due to exposure to each of the three pyrethroids ICON (0.1 %), DM (0.025 %) and PE (0.025 %) for 5 min, 15 min and 30 min was compared, 24 hrs after exposure, with approximately 50 sandflies for each insecticide, per exposure group (**Figure 8.5**). On the basis of this test there seemed little difference between the three pyrethroid formulations.

When more sandflies became available more extensive comparisons were performed including ICON, DM, PE and DDT in the same tests and recording mortality ('knock down') after 1 hr and 24 hrs, with exposure times of 5 min, 15 min and 30 min (**Figure 8.6**). Results of observations at 24 hrs, which are a better indicator of mortality rather than temporary 'knock-down' indicated that ICON was the most effective insecticide at short exposure times (**Figure 8.6B**).

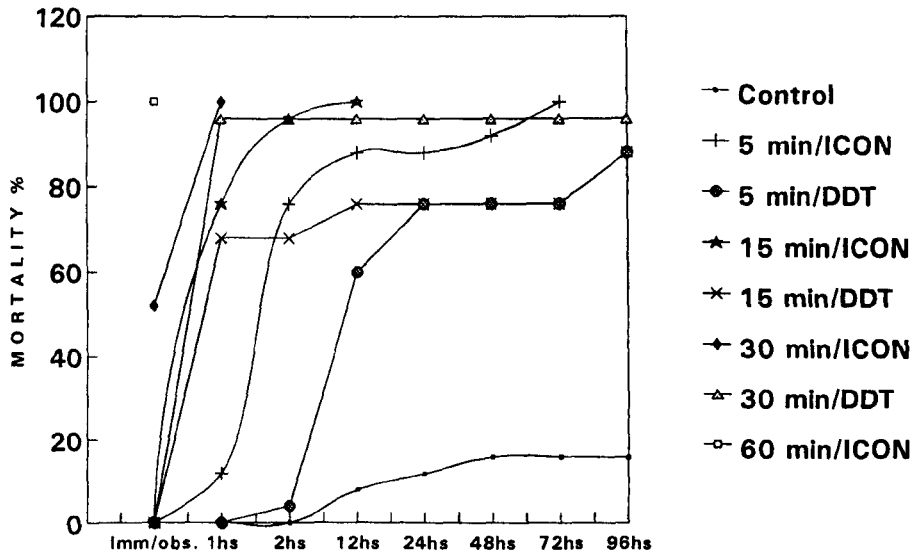
Finally the efficacy of ICON with engorged and non-engorged *Lu. longipalpis* was compared and no difference in susceptibility detected (**Figure 8.7**).

Figure 8.4

Comparative mortality ('knock-down') following exposure of *Lu. longipalpis* to ICON (0.1 %) and DDT (4 %) in standard WHO tests; 200 sandflies per insecticide and approximately 30 per each of the seven exposure groups: two test results, A and B, shown



A



B

Figure 8.4

Figure 8.5

**Comparative mortality ('knock-down') following exposure of *Lu. longipalpis* to ICON (0.1 %), DM (0.025 %) and PE (0.25 %)
(see text)**

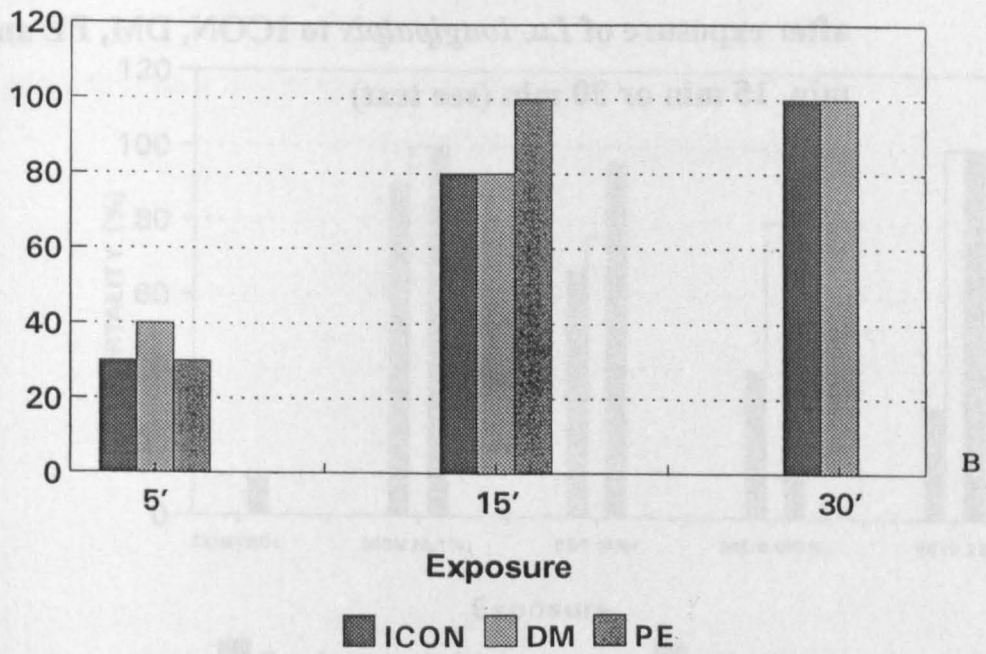
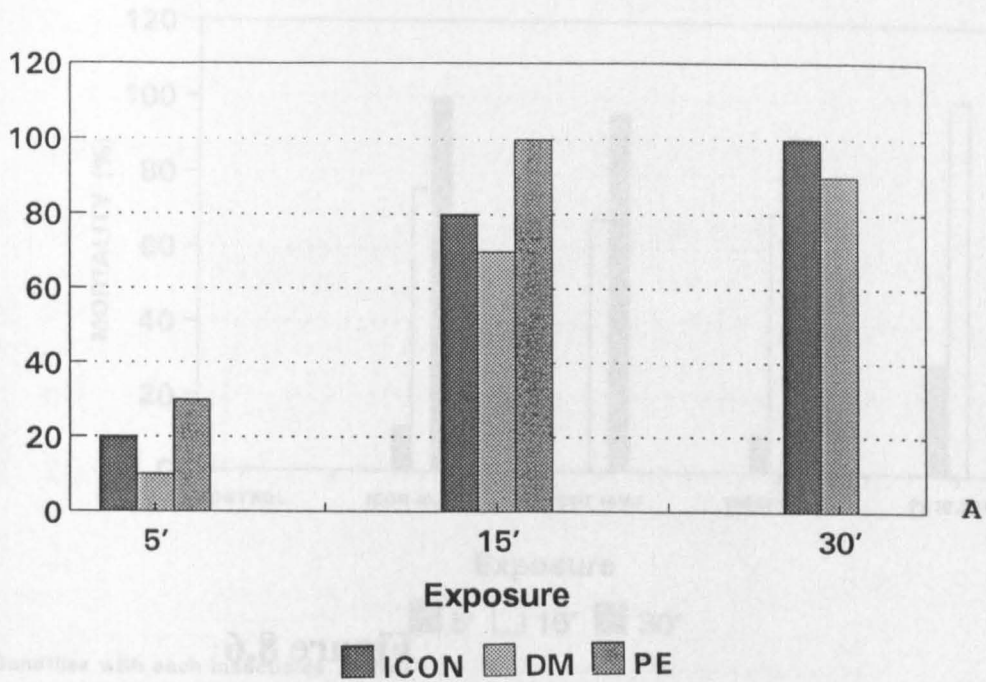
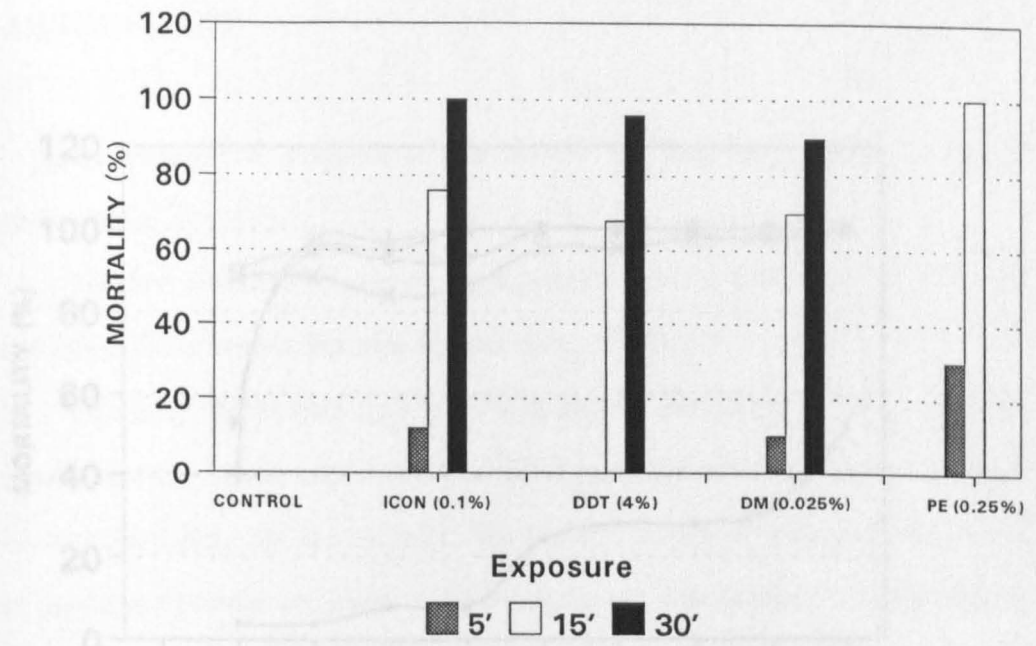


Figure 8.5

Figure 8.6

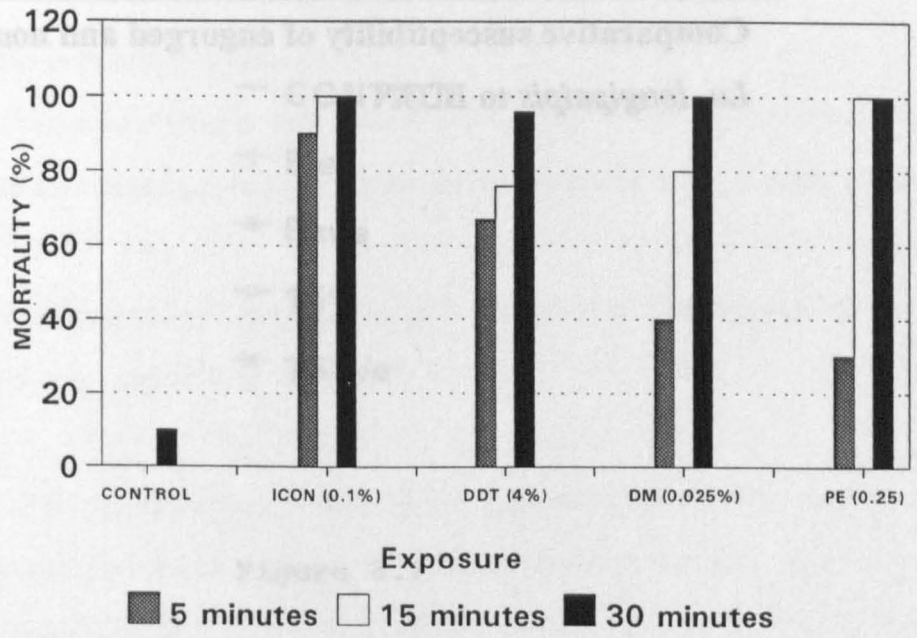
Comparative mortality ('knock-down') at 1 hr (A) and 24 hr (B) after exposure of *Lu. longipalpis* to ICON, DM, PE and DDT for 5 min, 15 min or 30 min (see text)



Sandflies with each insecticide = 200

A

INSECTICIDE	5'	15'	30'	5'	15'	30'	5'	15'	30'
CONTROL	0	0	0	0	0	0	0	0	0
ICON	12	75	100	12	75	100	12	75	100
DDT	0	68	95	0	68	95	0	68	95
DM	10	70	90	10	70	90	10	70	90
PE	28	98	0	28	98	0	28	98	0



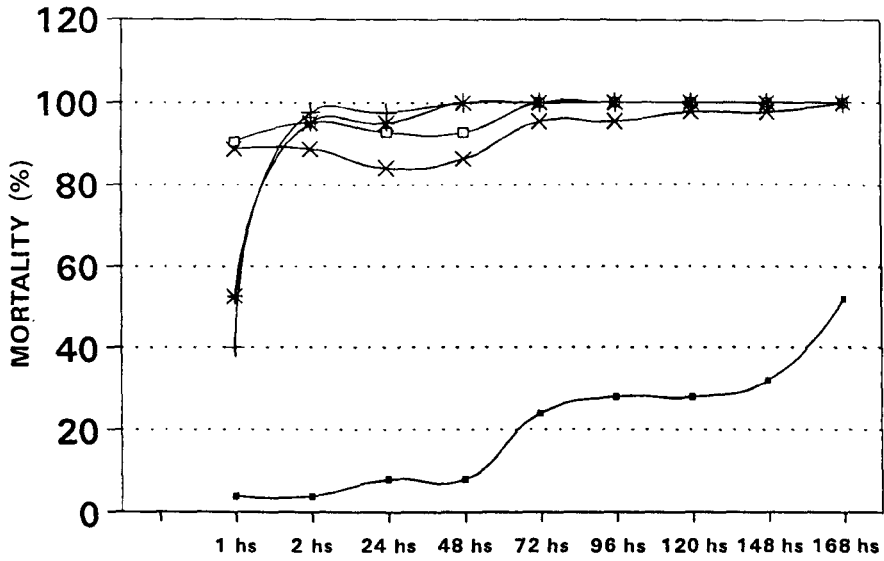
Sandflies with each insecticide = 200

B

Figure 8.6

Figure 8.7

**Comparative susceptibility of engorged and non-engorged
Lu. longipalpis to ICON**



CONTROL		4	4	8	8	24	28	28	32	52
5'e		40	97.5	97.5	100	100	100	100	100	100
5'n/e		52.5	95	95	100	100	100	100	100	100
15'e		90.4	95.2	92.8	92.8	100	100	100	100	100
15'n/e		88.6	88.6	84	86.3	95.4	95.4	97.7	97.7	100

— CONTROL

+ 5'e

* 5'n/e

□ 15'e

× 15'n/e

Figure 8.7

9: DISCUSSION

9.1 Epidemiology of visceral leishmaniasis in Teresina: human visceral leishmaniasis

The first outbreak of human VL registered in Piauí State was in 1984, and the principal focus was in Teresina (Costa *et al.*, 1990).

Curiously, Sherlock's review (1987) on the periodicity of VL in Brazil, predicted another outbreak in 1993 or 1994, emphasising that this cycle occurs every ten years, although the causes of the periodic epidemic cycle are not known. The prediction proved accurate; a second outbreak was registered in the city of Teresina in 1993.

The reason for the first outbreak in Teresina, according to Costa *et al.* (1990) was mainly due to:

- a. the increase in human population density;
- b. the lack of serious problems with malaria and Chagas disease, which meant that the city was not widely sprayed and consequently there was no indirect control of VL;
- c. the possible presence of populations of *Lu. longipalpis* that were particularly anthropophilic as suggested by Ward *et al.*, (1983).

In addition the immune status of the population, that is the number of individuals not previously exposed to infection (herd immunity), may be an important factor.

The strategic geographical location of Teresina, as an obligatory route for land communication between neighbouring states, the long season of drought in

the states of Piauí and Ceará, and the existence of a specialised hospital has led to increasing immigration of the sick and the unemployed.

A comparison between the time at which the increase in human VL occurs in each year (1985-1993) with the rainfall and humidity shows an association. This association has been shown to be statistically significant (Qiao, personal communication). This might be explained by the increase in the density of sandflies during the rainy season.

The spraying in 1993 and in previous years was palliative and not preventative, suggesting that the fall in the number of cases towards the end of each year was dependent on the seasonal transmission and not on spraying against the sandfly vector (Deane & Deane, 1955).

The analysis of the incidence of human VL, showed a predominance in children who were less than 5 years old. This might be because at this age there is more exposure to the vector (Costa *et al.*, 1990) but could also be explained by lack of immunocompetence, perhaps induced by malnutrition (Actor, 1960; Badaro *et al.*, 1986a and b; Cerf *et al.*, 1987; Evans *et al.*, 1992).

Another interesting observation was the difference in incidence between the ages of 11 and 55 years, for males and females. Two reasons might be considered for the higher incidence of VL in males, greater exposure to sandflies or hormonally associated reduction in susceptibility of females during their reproductive life (Alexander & Stimon, 1988a,b).

9.2 Canine visceral leishmaniasis

Among the 209 dogs (107 mongrels and 102 of known breeds - not selected to represent the distribution of breeds in the city) that were studied with comparative diagnostic methods in Teresina, 84 of 107 (78%) of the mongrel dogs were RS+ve and the Doberman Pinscher, German Shepherd, their crossbreed, which is favoured as watchdogs, and the Pekingese "toy dog" were the breeds

most affected by VL. Similar results were reported by Abranches *et al.* (1991) for known breeds, but only 7% of mongrel dogs were reported as positive.

Although the number of human VL cases increased in 1993, the percentage of seropositive dogs did not. In the districts of the city no association was apparent between the number of seropositive dogs and the presence of human VL, since some districts with human VL had no records of canine infection and other with seropositive dogs had no record of human VL.

It is possible that:

- a. some people acquired infection in other or neighbouring districts.
- b. some infected dogs were not detected by the serology (see below), either because the methods were not sensitive enough or because samples were not obtained from all dogs in each district or because samples were taken from dogs at a time of year that was not relevant to the human cases recorded. The dog sera were taken in March of 1993; the human cases used in the comparison were also from 1993, ideally this comparison should be repeated with human cases that subsequently arose, in 1994.
- c. in districts with human VL and with infected dogs, there was a high density of sandflies and a high population of susceptible children less than 5 years old.
- d. in districts with infected dogs but no human VL there were fewer sandflies, less exposure to sandfly bites or fewer susceptible people.

It has been reported that naturally infected dogs are not associated with the (risk of) acquisition of human VL (Costa *et al.*, 1989; Evans *et al.*, 1992) but for the reasons indicated above (**a**, **b**) it is difficult to test this association.

9.3 Other domestic reservoirs

Although *L. chagasi* has never been reported in goats, these and pigs and equine hosts must be investigated. They are certainly used as sandfly congregation (lekking) sites. Brazil (1982) found a typical amastigote in the skin from one pig, but the species was not identified; equines are known to be susceptible to *L. braziliensis* (Vexenat *et al.*, 1986; Aguilar *et al.*, 1989).

Cats have not been shown to be an important reservoir. Sherlock and Miranda (1992) found a typical amastigote by giemsa staining but *L. chagasi* has never been identified. Deane examined 142 cats and all were negative (Ward, 1977).

Negative results from the examination of *Rattus rattus*, in an endemic of VL from Bahia (Sherlock, 1988) is not a reason to ignore this rodent or other peridomestic animals as a reservoir, since the rat has been associated in South-West Saudi Arabia with confirmed cases of VL (Ibrahim *et al.*, 1992; Bettini *et al.*, 1980; Pozio *et al.*, 1981). It is assumed that foxes in Piauí State carry VL but we have not studied this and their epidemiological importance to endemic VL in Teresina is not known

9.4 Characterization of *Leishmania* isolates

The parasites isolated from dogs and from one human VL case in Teresina were identified as *L. chagasi*, this was confirmed by enzyme electrophoresis and by the chemiluminescent DNA probe.

More human strains must be characterised; dog strains, isolated from bone marrow and/or liver and/or spleen must be compared with those isolated from skin to determine if dermatropism is characteristic of the parasite strain, or the condition (breed, age, nutritional status, etc.) of the dog. Ponce *et al.* (1991) have reported *L. chagasi* as an agent of uncomplicated human cutaneous leishmaniasis in Honduras.

9.5 Sandflies: fauna and distribution

Preliminary observations demonstrated that *Lu.longipalpis* is widely distributed all over the city.

Lu.whitmani was the second most abundant species but only found in a few isolated districts. Another 9 species (section 3.2) were found only in the Zoological garden on the outskirts of the city. Further collections (data not shown) were made in conjunction with Dr Diarmid Campbell-Lendrum.

Large numbers of sandflies were associated with the presence of pigs and/or chickens. Removal of the domestic pig from one sty caused an immediate and dramatic decline in sandfly numbers (**Results**). Morrison *et al.*, (1994, 1995) demonstrated that cows and pigs were the preferred host of *Lu.longipalpis* and suggested that this species is an opportunistic anthropophilic sandfly.

In one interesting site (Santa Fe district) we collected 8,841 sandflies in 5 days from an isolated house located on the corner of a block, without pigs or chickens. Surprisingly, sandflies were never found in neighbouring houses nor in various types of natural vegetation within a 100 metre range of the house. Two healthy adults and 2 healthy children, who were badly bitten by sandflies, were living in this house. There was also one serologically positive dog, which was repeatedly negative parasitologically on skin, bone marrow and spleen samples. Flagellates were not found in 67 engorged sandflies collected from where the serologically positive dog slept.

This house was an example of a high population of sandflies, with a seropositive and possibly infected dog but no easily transmissible source of infection. Serological follow-up of the family group was negative during 8 months of observation.

9.6 Natural infection in sandflies

Naturally infected sandflies were most often associated with the presence of dogs with amastigotes disseminated in the skin.

The (chemiluminiscent) DNA probe was found to be a useful new tool for the detection of infected sandflies. Using the probe it is possible to examine large numbers of flies and characterise automatically the species of the parasite.

Specific probes for other species of *Leishmania* are needed, especially in areas where the sandflies are found infected with morphologically similar flagellates (Hoch *et al.*, 1986).

67% of engorged sandflies collected in a kennel where there was a parasitologically positive dog with amastigotes disseminated in the skin were infected. This could be epidemiologically important as such a dog could act as an epicentre of transmission. If high infectivity to sandflies coincides with the beginning of the annual transmission season, neighbouring dogs could be infected and each become another epicentre, possibly for the following transmission season. This chain of infection sooner or later will affect children of surrounding slums, which are common throughout the city.

Of a collection of 1234 *Lu. longipalpis*, from 24 houses with dog kennels, dispersed throughout the city of Teresina, 3 flies were clearly probe positive - a rate of 0.24%.

9.7 Comparative diagnosis

9.7.1 Clinical diagnosis

Symptomatology in the group of studied dogs was not diagnostic for the presence or absence of the infection, as only 70 of 167 (42%) of the RS+ve dogs were symptomatic. Abranches *et al.*, (1991) found 46% of suspected dogs clinically symptomatic but 54% of seropositive dogs asymptomatic, suggesting that canine VL has a prolonged asymptomatic period. Long prepatent states can result

in the maintenance of the parasites for several years, sometimes with spontaneous recovery of the infected dog (Lanotte *et al.*, 1970).

9.7.2 Parasitological diagnosis

A total of 115 of 167 (68%) of the RS+ve dogs were parasitologically positive, when results of all parasitological methods were combined.

L. chagasi has the capacity to invade almost all internal organs of an infected dog (Vexenat *et al.*, 1994) but this is not always seen in the infected dogs from Teresina. In our observations 64% of the parasitologically positive dogs had amastigotes demonstrated in the skin, which, as explained below, is the reason for high infectivity to sandflies.

Evans *et al.* (1990) reported the detection of parasites in 40% of seropositive dogs but amastigotes were not searched for in the skin.

Abranches *et al.* (1991) reported that the search for amastigotes in popliteal lymph nodes was most efficient, in 49 of 65 (75%) of dogs examined, and amastigotes were present in 20% of the seropositive dogs by examination of healthy skin.

Our preliminary observations show that the presence of amastigotes is most common in the skin and positive skin was most frequent in dogs of known breed (but not in a representative sample).

It is possible that in luxurious houses where dogs are used as guard dogs, generally better fed and susceptible breeds, that life-expectancies of animals with a chronic abundant skin infection are much greater than in the slums.

9.7.3 Diagnosis by detection of DNA

Although the radioactive and recently the chemiluminescent DNA probe have been described as tools for identifying parasites of the *L. donovani*-complex, and they are extremely useful for screening large numbers of sandflies (as discussed above), the sensitivity for diagnosis of canine VL with skin biopsy

samples was overall not greater than the traditional serological and parasitological methods. With bone marrow aspirates some infection missed by microscopy could be detected.

Blood in tissue samples inhibits sensitivity of the probe (McNerney *et al.*, 1993a,b) and treatment of membranes with protease before hybridization restores a positive signal. Further studies are required with this modified method.

Several authors have described the PCR technique as an alternative for diagnosis of VL, the results obtained are still questioned, mainly due to the high rate of contamination (Kwok & Higuchi 1989, and Wilson 1993). Our pilot study with a kinetoplast DNA probe was not encouraging, partly because of the agarose detection method.

Recently Qiao *et al.* (1994) described a rapid, convenient and simple method for detection of PCR product the PCR-SHELA (PCR-solution hybridization-enzyme linked immunoassay). This technique gives a high sensitivity and specificity for the *L. donovani*-complex. These results were obtained experimentally on mouse samples. The endemic area of Teresina, could be an excellent situation to perform a better evaluation. Similar methodology showed excellent results for detection of *Ochocerca vulvulus* DNA in human samples without cross-reactions (Nutman *et al.*, 1994).

9.7.4 Serology

9.7.4.1 Specificity

DAT showed 100% specificity when serum samples from two non-endemic areas of VL were used, Brasilia, DF, Brazil and London, UK. Serum samples from dogs with other diseases were not compared, except that two serum samples from symptomatic dogs with *Dirofilaria* sp. demonstrated by giemsa from ear skin biopsies, were serologically negative. More studies on cross reactions with veterinary disease must be performed.

(IFAT, dot-ELISA) and ELISA detected 9.7% and 4.3% of nonspecific antibodies respectively, at the threshold of the cut-off, in the control serum samples from Brazil. Curiously ELISA detected high titres of nonspecific antibodies in 4 of 12 (33%) of the serum samples from London UK, that is an all the samples from female dogs. Absolute specificity using control negative sera is seldom reported, but some authors registered high indices of cross-reactions, and low sensitivities when ELISA was compared with dot-ELISA, (Pappas *et al.*, 1984a,b). On the other hand Evans *et al.* (1990) justify the 50% more positive dogs by ELISA than IFAT as truly infected dogs.

9.7.4.2 Sensitivity

9.7.4.2.1 Serum samples

IFAT, dot-ELISA, ELISA and DAT showed percentage sensitivities of 98.2%, 98.2%, 97.9% and 80% respectively when serum samples were used and the RS considered as parasitologically positive dogs, but when the RS was based on parasitology/reference serology (as defined in the **Material and Methods**), the sensitivity of DAT was reduced to 60%.

According to El-Harith (1986) DAT possesses high specificity and sensitivity, but in our results not only was it less sensitive than IFAT, dot-ELISA and ELISA but also 20% of the parasitologically positive dogs were negative. The DAT antigen used was manufactured exactly as the author recommended, and a local strain was used. Batch variability is, however, a persistent problem with DAT antigen and it is possible that this is the cause of our low sensitivities. Indeed, the same group of serum samples (pilot study I) were analysed in parallel by El-Harith, the results obtained were more compatible with IFAT, dot-ELISA and ELISA, reinforcing his published characteristics of the DAT serological test.

These results confirm that this technique is difficult to standardise because antigen batches vary and reccability cannot be guaranteed, even with the personal

skill of the author in antigen production some batches fail (El-Harith, personal communication). Considering that DAT antigen cost \$1,600 per litre and up to 10.000 duplicate tests are possible, the cost of each is about \$ 0.16.

Low sensitivity and high negative predictive values for the DAT have been reported by Zijlstra *et al.* (1991, 1992). Similar results was observed by El-Toum *et al.* (1992). We cannot recommend DAT as the test of choice for diagnosis of canine VL.

IFAT and dot-ELISA, the serological tests that were most sensitive, were negative in 3 of 115 (2.6%) of the parasitologically positive dogs or in other words, 3 of 45 (6.6%) serologically negative dogs were parasitologically positive. Evans *et al.* (1990) reported that 16.6% of the IFAT negative dogs were parasitologically positive. It is not known from these observations on natural infections if these seronegative dogs that are parasitologically positive are recent infections or asymptomatic dogs that would recover from infection. (Lanotte *et al.*, 1979). The experimental infections (below) however clearly demonstrated that serology commonly failed to detect many recent infections, long after infection had been demonstrated parasitologically. In a few dogs seroreversion was associated with recovery.

The presence of high levels of antibodies in dogs was shown to be associated with to chance of demonstrating the parasite. Similar results were also reported by Abranches *et al.* (1991). The transmissibility study and experimental study confirmed that high seropositivity was an indicator of high skin parasitaemia and high transmissibility to sandflies.

9.7.4.2.2 Eluates from blood spots

The routine of collecting blood on filter paper began with Nussenzweig *et al.* (1957) and Alencar *et al.* (1974) using the CFT as the serological method.

Thus it was possible not only to encompass large numbers of endemic areas in the field but also to transport the samples easily by mail.

In our results loss of sensitivity of between 11.4% and 20% was registered when eluted blood samples from filter paper were used in the comparative serological diagnosis. Although Coutinho *et al.* (1985) found 95% of sensitivity their result was obtained from a comparative study with parasitologically positive dogs as RS and using as a cut-off 1:40 dilution. Loss of sensitivity for eluted blood in IFAT and ELISA has also been reported by Evans *et al.* (1990). The blood spots on filter paper used in our pilot study were preserved in good condition and maintained at 4°C until performing the test. Large losses in sensitivity could occur however when samples collected in the field go through different temperature variations and transfer to ideal conditions is delayed.

Another factor which was shown to reduce the sensitivity of the IFAT was the use of an antigen which was supplied routinely to the parasitology laboratory from the UFPI. The eluted blood samples showed 14.2% greater sensitivity when the antigen used was manufactured from an autochthonous strain from the endemic area of Teresina.

In conclusion, the total loss of sensitivity was 28.4% with eluted blood samples and without antigen from a local strain.

The protective immunity against leishmaniasis is almost wholly T cell mediated (Liew, 1990). Cabral *et al.* (1992) found a high rate of infection in asymptomatic dogs using lymphocyte proliferation assays. As these dogs had a cell mediated response they presumably had been infected but were resistant to disease. It is not known if they were parasitologically positive. We did not attempt to monitor progress to a cell mediated immune response in our experimental cohort (below) but lymphocyte preparations were made and stored at one time point.

Similarly, we have not examined antibody responses to particular antigens (Carrera *et al.*, 1996).

9.8 Transmissibility

Lu.longipalpis were easily infected experimentally from naturally infected dogs when amastigotes were disseminated in the skin. The percentage of infected sandflies after xenodiagnosis on a naturally infected dog in some cases reached 78%. This percentage is greater than the experimental rate of infection found in Bahia (29%).

Results clearly demonstrated that *Lu.longipalpis* can be infected on skin with no apparent lesion and on asymptomatic dogs. Similar observations were reported by Adler & Theodor (1932, 1935). Furthermore, transmissibility to sandflies was clearly associated with skin positivity, but not bone marrow positivity, and skin and bone marrow positivity were disassociated. No evidence was found, from parasitological examinations or membrane feeding experiments, that blood itself, rather than skin, was the primary source of infection.

Although Deane (1956) obtained 15% of positive sandflies by xenodiagnosis on untreated symptomatic human VL, we need to know how many sandflies are likely to become infected on a symptomatic patient before treatment, in the domestic environment and whether this acts as a source of new human VL.

In India *L. donovani*, is easily found in peripheral blood and the transmission is human_sandfly_human, but in contrast *L.chagasi/infantum* are said to be rarely found in human blood.

Treated human cases do not provide a source of infection to sandflies (Deane, 1956) and preliminary observations in Teresina (C. H. Costa and R. Siddiqui, personal communication) suggest that asymptomatic human VL is not a significant source of sandfly infection.

Preliminary observations (not included in this report), demonstrated that *Lu.whitmani* is highly sensitive to infection when xenodiagnoses were performed on a naturally infected dog, but it is not yet known if this species can transmit *L.chagasi*.

9.9 Experimental infection

Transmission to dogs by sandfly bites were reported by Rioux *et al.* (1979) who showed that *L. infantum* can be transmitted to a dog by exposure to a single sandfly bite, and Lainson *et al.* (1990) who infected one fox *C.thous* by bites from 2 laboratory-bred *Lu.longipalpis*. The seroconversion in this animal was noted at 5 weeks (IFAT 1:1,280). An interesting detail from the experiment of Lainson *et al.* (1990) is that xenodiagnoses on the body of this animal were positive only 15 weeks after the infected sandflies had fed on the head of the fox. Amastigotes were demonstrated by blood-agar cultures from skin, and hamsters inoculated with suspensions of bone marrow, liver and spleen also were also positive.

Experimental infections in dogs were also produced by Oliveira *et al.* (1993) but by intravenous inoculation. Six of 8 (75%) dogs inoculated intravenously with a suspension of amastigotes become positive parasitologically and clinically. Another group of animals inoculated consecutively intraperitoneally with high numbers of promastigotes were always asymptomatic and parasitologically negative during 28 months of observations. The author compared this result with data from Alencar (1959) who suggested that the greater part of naturally infected dogs are asymptomatic. The conclusions of Oliveira *et al.* (1993) are doubtful, however, because firstly, there may have been a loss of infectivity from serial subculture in the laboratory and presence of metacyclic forms was not monitored or established by control and secondly, because intraperitoneal inoculation is an artificial route of infection (Evans 1989a). Paranhos *et al.* (1993)

demonstrated that 10^8 promastigotes from cultures of *L. chagasi* can infect dogs. These promastigotes were previously showed to be highly infective to hamsters but 2×10^5 promastigotes mixed with sandfly salivary gland extract failed to infect dogs. Titus & Ribeiro (1988) reported the enhanced infectivity of promastigotes when they were mixed with sandfly salivary gland extract.

As far as we are aware, our study of experimental transmission is the first in which infection was transferred to dogs by the bite of experimentally infected sandflies. Some features of this experiment were surprising. Although the two pilot animals showed fairly rapid progression to VL, many other animals in the cohort developed apparently self-curing lesions typical of those described for cutaneous leishmaniasis. The long-term fate of these animals is not yet certain, some remain serologically and parasitologically positive, whilst others that have reverted to seronegativity may presumably make a full recovery. As long as resources allow these animals are being followed since they are unique and the nature of their immune response may also be of interest. It is likely that a higher proportion of dogs in households may progress to VL due to restricted diet or malnutrition, which are known to influence the outcome of VL infections, and yet were not a factor in the experimental cohort as food and water was given freely.

Of fundamental importance from this experiment is the observation that serological positivity was not a reliable indicator of the transmissibility of the infection. Possibly the cutaneous nature of the lesion in some way diminishes the ability of the parasite to trigger a host antibody response, just as lower antibody responses are seen in cutaneous leishmaniasis due to *L. braziliensis*. Antibody responses were, however, consistently associated with a systemic infection, as indicated by parasitological positivity of bone marrow. As the infection was easily transmitted from skin lesions, or the normal skin of asymptomatic animals, it seems a forlorn hope that serological surveys and culling of dogs can prevent

endemic/epidemic VL. Other authors (Molina *et al.*, 1994a) have also shown that asymptomatic dogs can be highly infective to sandflies. Indeed, the epidemic in Teresina was not prevented by these measures over many years, although the efficiency of their implementation is in doubt due to fluctuating resources and the inevitable time lag between obtaining results of serology and the tracing and killing of infected animals.

9.10 Treatment

Pentavalent antimonials are well-tolerated in dogs but are not a suitable treatment for canine VL. Animals with clinical signs usually relapse rapidly after a course of antimonial therapy. Repeated treatment carries the hazard of maintaining a reservoir for the propagation of new infections and, more importantly, of selecting *Leishmania* populations that are less susceptible to treatment, or have become resistant to drugs that are first line therapeutic agents for human VL (Gramiccia *et al.*, 1992; Grogl *et al.*, 1992). Failure of antimonial therapy for canine VL may in part be due to the more rapid elimination of antimonials in dogs as compared to humans, even when administered by the subcutaneous route (Tassi *et al.*, 1994). Aminosidine has become an important drug for treatment of human VL, especially in combination with pentavalent antimonials, with excellent cure rates in patients who are not immunocompromised by AIDS. The only side-effects recorded in humans is minor loss of hearing range, and this occurs rarely (Olliaro and Bryceson, 1993). The mode of action of aminosidine is inhibition of protein synthesis by attachment to the small ribosomal subunit. Doses recommended for treatment of human infections, whether bacterial or protozoal are between 10 mg/kg and 15 mg/kg, once or twice daily for up to ten days, with intervals of twenty days between repeated treatment cycles. Treatment is not advisable if there is impaired renal or cochleovestibular function. Possible side-effects are nephrotoxicity, neuro-muscular damage and weight loss. The therapeutic window between efficacy and toxicity is said to be

more narrow for dogs. We have tested the ability of aminosidine to produce clinical improvement and clinical cure in canine VL

Three groups of animals were treated. In the first 20 mg/kg for fifteen days produced dramatic clinical improvement but all three animals eventually relapsed with progressive canine VL. There were no side-effects, all signs being consistent with the disease progression, except that the unhealthy coat was shed prior to replacement with fresh growth of healthy hair. Clinical improvement was associated with apparent clearance of infection, such that it was not detectable by skin biopsy or bone marrow aspiration, but it returned to both sites during the relapses.

A four-fold increase in dose and extended treatment produced severe side-effects that included loss of appetite, weight loss, evidence of nephrotoxicity, and neuro-toxicity affecting hearing, vision and sense of smell. Only one dog in this group was cured, but with impaired vision.

An intermediate dose of 40 mg/kg for thirty days was more successful in that three of twelve (25 %) dogs survived for more than four years with no evidence of *L. chagasi* infection. Nevertheless two animals died during or just after treatment and others either did not clear their infection or relapsed at variable intervals. Possible adverse effects were still seen, principally loss of hearing and vision, but, as they did not arise until long periods after treatment had ended, it is doubtful that they are attributable to the drug treatment.

The clinical condition of dogs prior to treatment is shown in Table 7.1. It might be expected that dogs with the most severe VL, termed here symptomatic, would be most likely to suffer side-effects and die during or just after treatment, possibly in part due to the (kidney) damage caused by antigen release and deposition of complexes in the kidneys. Similarly, animals not in the terminal phase of the disease (oligosymptomatic) should be best able to withstand treatment and have the best chances of clinical cure. Table 7.1 is not particularly informative on the first of

these predictions but and the second is clearly apparent: all clinically cured dogs were initially considered to be oligosymptomatic.

As far as we are aware clinical cure canine VL with proven skin and bone marrow infections and overt clinical signs of disease has rarely been achieved. Nevertheless, aminosidine treatment clearly has no value at present as a routine control measure against canine VL in endemic areas such as Teresina: it is not a satisfactory alternative to the humane destruction of dogs. For highly prized, irreplaceable pedigree stock, it could be used to save selected animals, assuming that rigorous measures were taken to prevent transmission to sandflies from treatment failures. Ineffective treatment carries the risk of generating organisms resistant to aminosidine, for which three resistance mechanisms have now been proposed. In *Leishmania* resistance apparently depends on increased production of ribosomal RNA but not on enzymic inactivation of the drug or base substitutions in the small ribosomal subunit. Indeed, since our work was done, Poli *et al.* (1997) have published a study on aminosidine treatment of canine VL, in which, surprisingly, treated dogs were only followed up for 60 days. Poli *et al.* therefore allowed no time for detection of relapses, and the suggestion that aminosidine might be used for routine treatment on the basis of their data may be dangerous and must be regarded with extreme caution. Furthermore all the dogs treated by Poli *et al.* remained parasitologically positive.

Achievement of clinical recovery and limited cure suggest that further trials with aminosidine may be worthwhile, for example with slow release formulations or drug combinations. Combined aminosidine and antimony therapy is said to enhance the persistence of high serum concentrations of antimony, which may allow reduction of therapeutic doses and increased intervals between administrations but concomitant elevation of levels in skin, presumably crucial to cure of canine VL, has not been investigated (Belloli *et al.*, 1995). Kidney lesions in canine VL due to glomerular and tubular damage from deposition of immunoglobulins is well known

(Mancianti *et al.*, 1989) and is likely to be precipitated by antigen release during aminosidine treatment. Supportive therapy might be used to reduce the side-effects of aminosidine in dogs.

Whilst we cannot exclude efficacy of buparvaquone with higher doses than those used in this project, which have had limited success in mice, or with more prolonged treatment or improved delivery, the buparvaquone trial was not encouraging. We conclude that buparvaquone at the doses used here has no role in the treatment of symptomatic canine VL and appears to exacerbate progression of disease.

9.11 Vector control

Our studies of vector control were not complete but allowed some preliminary conclusions. Thus it was clear that occasional ultra-low volume spraying of domestic animal pens that were highly infested with sandflies only had a temporary or transitory effect on sandfly numbers. It seemed more effective in one case to remove the pig from the animal pen as sandfly numbers declined rapidly when the pig was sold. In contrast residual application of pyrethroid insecticides appeared to be a highly effective means of killing all sandflies exposed for relatively short periods to sprayed surfaces, for at least four months, and probably for much longer, after the insecticide had been used. This results was supported by the laboratory tests on pyrethroids. Although more extensive studies are required, it seems clear that sandfly control with safe agents must be a primary means of preventing epidemic outbreaks by dramatically reducing vector numbers.

A new mechanism of control with pyrethroids involves either topical application to dogs, or using pyrethroid treated dog collars to reduce vector attack. Killick Kendrick *et al.* (1997) have very promising results with pyrethroid impregnated collars, which reduced sandfly biting rates by up to 95% for at least 34 weeks. This method is due to be tested in field trials in Brazil (Killick Kendrick, personal communication).

9.12 Concluding discussion

It is apparent that the successful control of VL by culling dogs must depend on the diagnosis of canine VL at an early stage, even though animals with the highest antibody titers have higher parasite burdens and are more infective, because the dog is such a highly infective source to the sandfly vector. Serology, which was the most efficient method of diagnosis, failed to detect all the infected dogs. The failure rate, of 6.6% (for serum samples) in comparison with parasitology, and the long time to seroconversion in experimental infections suggest that a substantial proportion of infected dogs must be missed, and perhaps at least twice the 6.6% level because parasitology itself has a low (approximately 50%) sensitivity for RS+ve dogs. Serology with filter paper eluates are generally used for which the failure rate in comparison with serology on serum was much higher (14.3%). These data from naturally infected dogs therefore indicate that, at best, about 85% and 70% of the infected dogs that are examined will be removed by serology with serum and filter paper samples respectively. The high proportion of seronegative dogs in the population suggests that many early infections may be missed. It is not surprising therefore that serological surveillance of dogs did not prevent a further epidemic outbreak in Teresina. The data on naturally infected dogs were amply confirmed and extended by the experimental study. Seronegative dogs that are parasitologically positive and infective to sandflies were commonplace.

Complex mathematical models have been proposed for canine VL, in particular to estimate basic case reproduction number (R_0). Our data shows that assumptions (Hasibeder *et al* 1992) that there is a latent period after an infective bite when the dog is not infective but serologically positive and that the asymptomatic and non-infective periods are identical are untenable. Our study indicates that a low R_0 will maintain the endemic even during serological surveillance and killing of seropositive dogs.

Nevertheless, seropositive animals were most likely to be parasitologically positive and infective to sandflies. Furthermore, antibody titer rose with increasing parasitological positivity and increasing infectivity to sandflies. Culling of seropositive dogs therefore removed the most infective animals but would have no hope alone of stopping an epidemic or continuing endemic transmission. The transmissibility data indicate that dog skin infections are the overwhelming source of infectivity to sandflies. Formal, *epidemiological*, proof of the link between canine and human infections, although not shown here and difficult to achieve, is still a feasible and worthwhile endeavour. Alternative, serological or molecular biological methods might have a role in surveillance and control for canine VL but only if they are much more sensitive than present procedures, low-cost, and suitable for field use with a virtually instant result.

Aminosidine has been shown not to be the answer for treatment of canine VL. Although limited cure was obtained, side-effects were severe. New treatments are worthy of investigation as a 'single-shot' eradicated treatment would be valuable.

In conclusion, improved control of the vector, with pyrethroid insecticides, must be the priority and developed (in part based on better knowledge of the ecology and seasonal distribution of sandflies). New methods to protect dogs from sandfly attack could contribute to control. The most important relatively unexplored approach to control of canine VL is the development of a reliable, heat stable, vaccine that could be administered with the annual rabies vaccination campaigns that occur in many endemic areas of VL. Future research on canine VL should therefore be directed, in order of priority, to (a) improved implementation of available insecticide control methods, (b) vaccine development, (c) methods for long-term protection of dogs against sandfly attack, (d) rapid, simple diagnosis of all infected animals.

CHAPTER 10: CONCLUSIONS

1. The majority (77%) of human cases of VL in Teresina was found in children under ten years of age.
2. Comparisons of incidence by sex and age demonstrated that adult VL predominated in males.
3. Increase in incidence of human VL in Teresina was shown to be seasonal.
4. No relationship could be shown between records of seropositive dogs per district and the number of cases of human VL per district. This relationship may be confounded by the low sensitivity of the serology, or by the time lag between abundance of parasitologically positive dogs and any subsequent increase in human cases.
5. The agent of VL in Teresina is *L. chagasi* (*L. infantum*). No phenotypic diversity was found but further comparisons of phenotypic and genotypic diversity are required with isolates from both dogs and humans.
6. Large numbers of sandflies were found in pigsties and chicken houses.
7. Prevalence of natural infection in sandflies was particularly high when flies were captured from kennels where there were dogs with disseminated cutaneous infections.
8. The *L. donovani*-specific probe was applicable to determining prevalence rates of *L. chagasi* infection in wild caught sandflies.

9. Culling of seropositive dogs and sporadic spraying failed to prevent a new urban epidemic of VL in Teresina, approximately ten years after the previous epidemic.
10. Symptomology was not sufficiently sensitive to diagnose the presence, or absence, of canine VL.
11. Parasitology was less sensitive than serology for the diagnosis of canine VL. No parasitological test showed more than 60% sensitivity in comparison with reference standard (RS) criteria.
12. The IFAT and dot-ELISA tests were the most sensitive of the serological assays. The DAT test was highly specific but lacked sensitivity.
13. The chance of demonstrating parasites in canine VL increased with the serological titre.
14. Parasitologically positive dogs could be found among those that were serologically negative.
15. *Lu. longipalpis* could be very readily infected with *L. chagasi* by feeding on dogs with canine VL.
16. Transmissibility of *L. chagasi* from dog to sandflies was associated with amastigote infection in the skin.
17. No evidence was found that blood, rather than skin, was the primary source of infection for *Lu. longipalpis*.
18. Altered skin of symptomatic dogs was more infective than normal skin of symptomatic animals.

19. Symptomatic dogs were more infective than asymptomatic animals, yet asymptomatic animals, with normal skin, were still infective to large numbers of sandflies. Asymptomatic animals cannot therefore be excluded as a significant reservoir of infection.
20. Transmission of experimental canine *L. chagasi* infection was demonstrated by single infective sandfly bite.
21. Many infected dogs developed discrete, self-curing, cutaneous lesions, typical of cutaneous leishmaniasis.
22. Seroconversion with the most sensitive of the available assays was not a reliable indicator of presence of infection or of infectivity to sandflies. Bone marrow positivity was only seen in dogs that were serologically positive.
23. Apparent recovery from *L. chagasi* infection was seen, and serological reversion, although the long-term fate of such dogs remains to be determined.
24. Aminosidine, dependent on dose, duration of treatment and clinical status of the infected animal, was shown to be capable of producing clinical recovery and clinical cure in a small proportion of infected dogs. Aminosidine treatment cannot, however, be recommended as a systematic control strategy against canine VL in endemic areas.
25. Buparvaquone, with the doses and duration of treatment tested, was not an effective treatment for canine VL.

26. Focal single application of ultra-low volume pyrethroid insecticide to domestic animal pens highly infested with *Lu. longipalpis* was not an effective measure for vector control.
27. Pyrethroid insecticides retained high residual activity against *Lu. longipalpis* for several months when sprayed on the walls of domestic animal pens.
28. Laboratory comparisons confirmed the efficacy of pyrethroid insecticides for killing *Lu. longipalpis* and suggested that lambda cyhalothrine (ICON) was the most effective of three pyrethroid insecticides at short exposure times.
29. Overall, this project has confirmed the fundamental role of the dog as a reservoir host of epidemic VL in Teresina, given a new perspective on present and future strategies for control, emphasised the importance, in conjunction with treatment of human VL, of vector control, and explained why culling of seropositive dogs is likely to have limited impact.

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Appendix I

2-Mercaptoethanol 0.1M

2-Mercaptoethanol.....3.00 ml
Foetal calf serum (FCS).....0.42 ml
Saline solution.....39.00 ml

Antibody solution

Anti-digoxigenin alkaline phosphatase
(Boehringer Mannheim, Cat.mp/1093274) 1:10.000
TBS/3% milk powder 0,5% Tween 20 (v/v)

Chemiluminescent substrate solution

Lumiphos (Spray) Cambridge Research
Biochemicals Cat.SU-07-200

Citrate saline solution (pH 7.4)

Na₃ citrate.....14.70 g
NaCl.....8.70 g
Distilled water to.....1000.00 ml

Coating buffer solution x10 - (CB, pH 9.6)

Na₂CO₃.....7.95 g
NaHCO₃.....16.75 g
NaN₃.....1.00 g
Distilled water to.....500.00 ml

Denaturing solution

1.5M NaCl / 0.5M NaOH

Enzyme stabilizers

a.

Reagent A. dithiothreitol (DTT).....0.0308 g
Reagent B. e-aminocaproic acid(ACA)..0.0262 g

b.

EDTA 200 mM solution
EDTA dihydrate7.445 g
Distilled water.....50.00 ml

c.

Add 1.00 ml of the EDTA solution to reagent A, shaking to dissolved. Add this solution to reagent

B. This is the stock stabilizer solution and can be kept at 4° C for one week. Dilute 0,1 ml of the stock solution in 10 of distilled water just before use.

Giemsa stock solution

Methanol.....60.00 ml
Glycerol.....75.00 ml
Giemsa powder.....1.00 g
Incubate for 24 hr at 57° C and filter with paper (Whatman No.1), storing in dark bottle at room temperature. Dilute 50 ul/ml PBS before use.

Glycerol/sodium azide

Glycerol.....500.00 ml
NaN₃.....0.20 g

Haemin stock solution

Haemin powder.....0.0025g
NaHO.....0.20 g
Distilled water.....500.00 ml

Locke's solution

NaCl.....9.20 g
CaCl₂.....0.24 g
NaHCO₃.....0.15 g
KCl.....0.42 g
D-Glucose.....1.00 g
Distilled water up to.....1000.00 ml

Locke's/16% glycerol

Locke's solution.....84.00 ml
Glycerol.....16.00 ml
D-glucose.....0.50 g

Medium ('Difco' Blood agar medium)

'Bacto' Blood agar base (Difco).....4.00 g
Distilled water.....100.00 ml
Defibrinated rabbit blood.....15.00 ml

Neutralising solution

1.5M NaCl/0.5M tris pH 7.2/1mM EDTA

Phosphate buffer saline 10x - (PBS, pH 7.2)

NaCl.....400.00 g
KH₂PO₄.....10.00 g
Na₂HPO₄ - 12 H₂O.....144.00 g
KCl.....10.00 g
Distilled water.....5000.00 ml

Phosphate buffer saline /10% glycerol (v/v)

PBS solution 1x90.00 ml
Glycerol.....10.00 ml

Phosphate buffer saline / 0.05% Tween (PBST)

PBS solution 1x.....99.95 ml
Tween 20.....0.05 ml

Phosphate buffer saline / 0.05% Tween /2% milk(PBST/M)

PBST
solution.....100.00 ml
Marvel Dried Skimmed milk (low fat)...2.00 g

Phosphate citrate buffer (pH 5.5)

a. Solution A. citric acid.....21.01 g
distilled water.....1000.00 ml
Solution B. Na₂HPO₄- 12 H₂O35.00 g
distilled water.....1000.00 ml

b. Solution A.....485.00 ml
Solution B.....515.00 ml
Stored at -20 C until use.

Polyoxyethylenesorbitan monolaurate

(Tween 20) pH (1% solution):approx. 6.0
(Sigma Chemical Co. cat. #P5927)

Prehybridisation solution

Formamid 50% (v/v)
SDS (sodium dodecyle sulphate) 0.5% (w/v)
N-lauroylsarcosine 0.1% (w/v)
Dried skimmed milk powder 3% (w/v)
Boiled sonicated herring sperm DNA 100 ug/ml
in 5 x SSC

Sulphuric acid 2.5M

H₂SO₄.....13.30 ml
Distilled water.....86.70 ml

Tris-Buffer solution (TBS)

NaCl.....8.00 g
KCl.....0.20 g
Tris base.....3.00 g
Distilled water up to.....1000.00 ml

Tris-Buffer solution /2% Milk (TBSM)

TBS solution.....100.00 ml
Marvel dried skimmed milk.....2.00 g

Trypsin solution

Difco 1:250 Trypsin.....0.40 g
Locke's solution.....100.00 ml

Wash solution 1

SDS 0.1%
N-lauroylsarcosine 0.02%
in 2 x SSC

Wash solution 2

SDS 0.1%
N-lauroylsarcosine 0.02%
in 0.01 x SSC

Wash solution 3

TBS solution.....99.50 ml
Tween
20.....0.50 ml

Enzymes	Tank buffer	Gel dil	Time (min)	Volt/ plate	Substrate	Visualization method
E.C.3.1.1.1. ES	B	1:9	150	300	4-methylumbelliferyl butyrate 1 mg dissolved in 200 ul acetone	UV, filter paper
E.C.3.2.2.2. NH	C	1:4	120	200	inosine 20 mg	MTT 1.0 ml PMS 1.0 ml Agar 16.83 ml
E.C.1.1.1.44 6PGD	B	1:9	150	300	6-Phosphate gluconate (10 mg/ml) 1.0 ml	MTT 1.0 ml PMS 1.0 ml Agar 17.6 ml
E.C.1.15.1.1 SOD	E	1:9	150	300	Riboflavin 0.6 mg	NBT (5 mg/ml) 1.0 ml Agar 20.0 ml
E.C.5.3.1.8 MPI	F	1:4	150	300	Mannose-6-phosphate 10 mg	MTT 1.0 ml PMS 1.0 ml Agar 16.83 ml

Electrode buffers:

B - 0.1M Tris, 0.1M maleic acid, 0.001M Magnesium acetate, 0.001M EDTA, pH 7.4

C - 0.1M Tris, 0.1M maleic acid, 0.001M Magnesium acetate, 0.001M EDTA, pH 8.0

E - 0.08M Disodium hydrogen phosphate, 0.0189M Sodium dihydrogen phosphate, pH 7.4

F - 0.1M Tris, 0.01M Sodium dihydrogen phosphate, pH 7.6

MTT = methylthiazole tetrazolium, 5mg/ml

MPS = phenazine methosulphate, 2mg/ml

Appendix III

Dog number	IFAT	dot-ELISA	ELISA	DAT
14	1600	3200	1600	25600
27	3200	51200	1600	12800
32	800	3200	400	
38	1600	800	1600	25600
39	200	12800		
41	3200	3200	400	
43	6400	800	1600	6400
44		200		
45	200			
46		200		
47	6400	12800	6400	102400
50	200			
52	800	51200		
61		3200	400	
67		102400		
75	200	3200		
81		3200		
90	1600	200	1600	102400
92	400	800		
95	1600	800	1600	
107	3200	800	1600	
108	6400	3200	1600	25600
120	3200	3200	1600	
133	6400	12800	102400	25600
134	800	800	400	
138	800	3200	1600	12800
143	6400	3200	1600	102400
157	200	200	400	
164	800	3200	400	
167	800	3200	400	25600

Appendix III

Dog number	IFAT	dot-ELISA	ELISA	DAT
170	200			
175	200	200		
179	6400	12800	6400	25600
181	200	800	6400	
201	400	200		
213	200		400	
214	200			
500	200			
506	200	200		
507	200	800	1600	
508		200		
509		800		
510	200		400	
521	200		1600	
524	200			
525	200			
528	200			
532	800	200	1600	
533	200		1600	
536	200	200		
537	400	800	1600	
538	400	200	1600	
548	800			
553	800			
560		200		
565	200	200	1600	
687	800	200		
593	200			
595	3200	3200	800	12800
609	200			

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Visceral Leishmaniasis in Teresina, State of Piauí, Brazil: Preliminary Observations on the Detection and Transmissibility of Canine and Sandfly Infections

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A *Leishmania donovani*-complex specific DNA probe was used to confirm the widespread dissemination of amastigotes in apparently normal skin of dogs with canine visceral leishmaniasis. When *Lutzomyia longipalpis* were fed on abnormal skin of five naturally infected dogs 57 of 163 (35%) flies became infected: four of 65 flies (6%) became infected when fed on apparently normal skin. The bite of a single sandfly that had fed seven days previously on a naturally infected dog transmitted the infection to a young dog from a non-endemic area. Within 22 days a lesion had developed at the site of the infective bite (inner ear): 98 days after infection organisms had not disseminated throughout the skin, bone marrow, spleen or liver and the animal was still serologically negative by indirect immunofluorescence and dot-enzyme-linked immunosorbent assay. When fed *Lu. longipalpis* were captured from a kennel with a sick dog known to be infected, 33 out of 49 (67%) of flies contained promastigotes. In contrast only two infections were detected among more than 200 sandflies captured in houses. These observations confirm the ease of transmissibility of *L. chagasi* from dog to sandfly to dog in Teresina. It is likely that canine VL is the major source of human VL by the transmission route dog-sandfly-human. The *Lmet2* DNA probe was a useful epidemiological tool for detecting *L. chagasi* in sandflies.

Key words: canine visceral leishmaniasis - *Lutzomyia longipalpis* - xenodiagnosis - experimental transmission - DNA probe - chemiluminescence

Human visceral leishmaniasis (VL) due to *Leishmania chagasi* is endemic to the semi-arid northeastern region of Brazil: canine VL is also widespread and infection rates of more than 20% have been recorded in dogs. The anthropophilic sandfly vector is *Lutzomyia longipalpis*. VL was first reported from State of Piauí in 1934 but in the 1980s there was a large outbreak in the State capital, Teresina, and the incidence of human VL has begun to rise again in 1992/93 (Costa et al. 1990, Tavares et al. 1993)

Traditionally the detection of *L. chagasi* in infected dogs and sandflies has depended on

parasitological methods with the aid of serology to identify those dogs most likely to be infected. In the long term DNA probe technology is likely to provide additional tools for the rapid identification of infectious agents in large numbers of samples. The introduction of sensitive chemiluminescent detection procedures as an alternative to radiolabelling means that DNA probes will become more widely available (Wilson et al. 1992). An *L. donovani* complex-specific probe (*Lmet2*; Howard et al. 1991) has been developed as a tool to help identify the *L. donovani*-complex in sandflies and in samples derived from mammalian hosts (Howard et al. 1992, Gebre-Michael et al. 1993). This probe has been used successfully in the laboratory with chemiluminescence (Wilson et al. 1992) and this has suggested the production of DNA probe kits for *Leishmania* (McNerney et al. 1994).

The skin is a major site of *L. chagasi* infection in dogs that have overt symptomatic VL and yet infection rates in wild-caught sandflies are

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though less frequently, confirming the disseminated nature of the cutaneous infection in canine VL (Table). At day 5 after feeding, when gut contents and residual blood meals from dissected flies were transferred to membranes and probed, around 80% of microscopically positive flies were also probe-positive (Fig. 2, chemiluminescent probe). We have subsequently shown that protease treatment of blood-contaminated samples can restore sensitivity (McNerney et al. 1994) but chemiluminescent signals are still not necessarily obtained with such samples for flies in which very low numbers of organisms are seen by microscopy. At day 7, when there was less residual blood in the flies, 24 flies that were positive by dissection and microscopy were also positive with the DNA probe (Fig. 2, radiolabelled probe). The sensitivity of the Lmet2 probe with and without protocols to improve sensitivity for contaminated blood samples is discussed elsewhere (McNerney et al. 1994).

Transmissibility - Four sandflies, fed seven days previously on a naturally infected dog, were

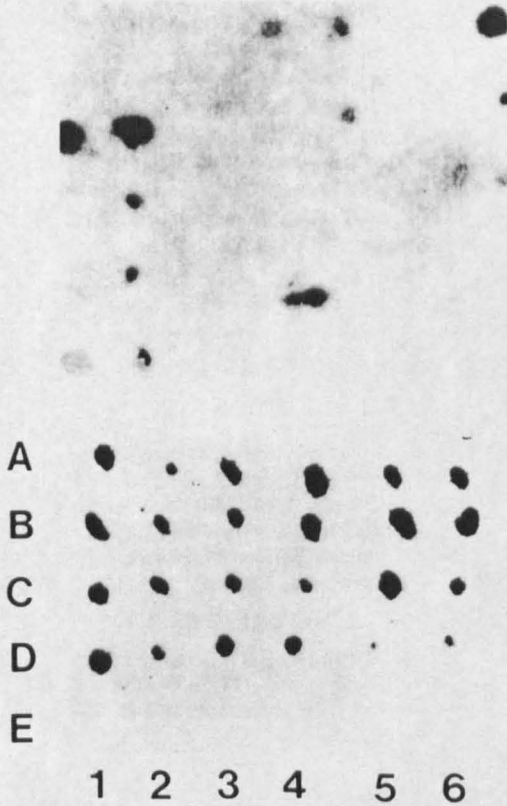


Fig. 2: Top - *Lutzomyia longipalpis* dissected, examined by microscopy, transferred to nylon membrane and subsequently probed with Lmet2, chemiluminescent detection. Bottom-*Lu. longipalpis* processed as above and probed with radiolabelled Lmet2: 24 microscopically positive flies were also probe-positive and negative controls (E1-6) were negative.

TABLE

Promastigote infection rates in *Lutzomyia longipalpis* 5-7 days after capture in kennels or after feeding on five naturally infected dogs

Source	Flies +ve	Flies -ve	%+ve
Xenodiagnosis (lesion)	57	106	35
Xenodiagnosis (lesion free)	4	61	6
Kennel (infected dog)	33	16	67
Kennel (several dogs)	2	16	11
Total	96	199	

re-fed on a young dog from a non-endemic area: one of the four flies was shown subsequently by dissection and microscopy to have been infected at the time of the second feed. Within 22 days a lesion was present at the site of the bite (left inner ear). At 98 days after the infective feed the lesion had grown considerably, but apparently remained restricted to the inner ear. Interestingly, serology (indirect immunofluorescence and dot-enzyme-linked immunosorbent assay) remained negative 98 days after infection and no amastigotes were found in bone marrow aspirates or spleen and liver biopsies. Other work has suggested that there may be a long prepatent period for seropositivity in canine VL (Ashford & Bettini 1987, Dye et al. 1993). To check whether the cutaneous infection had disseminated away from the original site of infection, xenodiagnoses with colony-bred sandflies were performed on three separate occasions and on each occasion flies were fed at three sites - the left inner ear (on the lesion), the right inner ear and the abdomen. For flies fed on the left ear 48 out of 73 flies (66%) became infected (12/17, 23/39 and 13/17 on each occasion respectively). None of 108 and 79 flies fed respectively on the right ear and abdomen became infected, indicating that the infection had not yet disseminated from the original lesion. Clearly no general conclusions can be drawn from this one animal as to whether such ease of transmissibility dog-sandfly-dog is commonplace in Teresina but this would accord with the high prevalence of canine VL in the city: further experimental studies would clarify the course of infection in dogs and allow selection of the most appropriate means of parasitological and serological diagnosis.

L. chagasi in wild-caught *Lu. longipalpis* - Dissection and microscopy of fed flies captured in a kennel with a sick dog subsequently shown to be infected demonstrated that a high proportion of such flies had promastigote infections (67%, Table). Infected flies were also found in a communal kennel with approximately 20 dogs which may or may not have been infected (Table). The Lmet2 DNA probe facilitates the

screening of large numbers of sandflies for *L. donovani* complex infections and other studies have shown that sensitivity compares favourably with microscopy (Gebre-Michael et al. 1993). We have as yet examined a relatively small number (~200) wild-caught peridomestic and domestic *Lu. longipalpis* from Teresina by this method. Two infections were detected, both from sandflies captured in houses, confirming the potential usefulness of this procedure (Fig. 3).

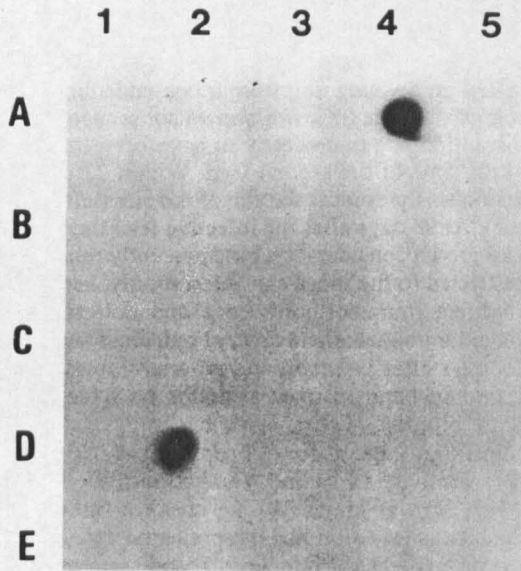


Fig. 3: two (A4, D2) of a series of approximately 200 wild-caught domestic *Lutzomyia longipalpis* were probe-positive, Lmet2 radiolabelled (composite Figure).

Overall these preliminary observations confirm the ease with which *Lu. longipalpis* can acquire infection from dogs with canine VL and suggest that the infection is highly transmissible dog-sandfly-dog. Similarly Rioux et al. (1979) have shown that *L. infantum* can be transmitted to a dog by a single sandfly. It is less certain that dog-sandfly-human is the main source of human VL (Evans et al. 1992, Tavares et al. 1993) but experimental and circumstantial evidence suggest that this transmission route is much more important than human-sandfly-human transmission. In the Mediterranean form of human VL due to *L. infantum* and in human VL in Brazil blood parasitaemias are reported to be low (Ashford & Bettini 1987, Lainson & Shaw 1987) and Deane (1956) could only demonstrate blood parasitaemias in four of 43 symptomatic patients. Although sandflies can be infected by feeding on VL patients in Brazil and the Mediterranean region, Deane (1956) found that only four of 14 cases of human VL were infective to sandflies, whereas 12 out of 16 dogs were infective. Like

humans, dogs have low numbers of circulating *L. infantum* or *L. chagasi*, but their efficiency as a source of infection is thought to be due to the abundant amastigotes in the skin. Amastigotes can be found in biopsies of apparently normal skin from patients with VL, but this is much less commonplace than in dogs. Thus Deane (1956) reported that five of 27 patients were positive by skin biopsy, and only one had abundant amastigotes, whereas 38 of 49 dogs were biopsy positive. Nevertheless in a recent study in State of Ceará, Vasconcelos et al. (1993) were able to recover *L. chagasi* from seven of 18 patients with proven kala-azar by culture of skin biopsies on Difco blood-agar. In contrast to *L. chagasi/infantum*, in Indian kala-azar due to *L. donovani*, large numbers of amastigotes can be found in the peripheral blood, many clinically ill patients can be shown to be parasitaemic and the infection is highly transmissible from humans to sandflies.

The Lmet2 DNA probe has proved to be a useful tool, especially for detecting infections in sandflies. The value of the probe in its present form for the routine diagnosis of canine VL is uncertain: it seems unlikely to replace Giemsa staining of skin biopsy impression or of bone marrow, spleen or popliteal lymph node aspirates except, perhaps, with further simplified procedures, if large numbers of animals are to be examined as part of widespread control programmes. Several rapid serological procedures are available, which are likely to be more practical for field use, although further comparative experimental studies are needed to determine how frequently they become positive early in the infection.

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generally reported to be rather low. The dog is considered to be the major reservoir of human disease but the presence of an infected dog is apparently not necessarily associated with the occurrence of human VL in a family: this has led to the suggestion that human-sandfly-human transmission may be an important source of human infections, although there are other explanations of the observed distribution of human VL (Evans et al. 1992, Tavares et al. 1993).

Here, we describe preliminary work with the Lmet2 DNA probe on the distribution of *L. chagasi* infection in naturally infected dogs, on the transmissibility of such infections and on the detection of *L. chagasi* in infected sandflies.

MATERIALS AND METHODS

Dogs suspected of infection with *L. chagasi* were either referred to us following a positive indirect immunofluorescent antibody test (IFAT) or were strays that would have been destroyed. Parasitological examination was by microscopy of Giemsa-stained skin impression smears and sternal bone marrow aspirates using standard methods. *L. chagasi* isolates were cultured on Difco blood-agar medium (Miles et al. 1981). Colony-bred *Lu. longipalpis* were used for xenodiagnoses. Peridomestic or domestic *Lu. longipalpis* were caught in the suburbs of Teresina either by hand using suction or battery operated 'pooters' or in CDC light traps. Xenodiagnosis flies or wild-caught flies were dissected and examined microscopically.

The Lmet2 DNA probe, either radiolabelled or with chemiluminescent detection, was used to screen tissue samples from dogs and to examine sandflies for the presence of *L. chagasi*. The basis of this method is described by Wilson et al. (1992) and full details of protocols are given elsewhere (Howard et al. 1991, McNerney et al. 1994). Briefly, skin biopsies from dogs were pre-blotted onto filter paper to remove excessive blood and then touch-blotted onto a nylon membrane (Hybond N, Amersham). Dissected sandflies were transferred to nylon membranes or undissected flies were squash-blotted directly on the nylon membrane by rolling a plastic or pre-soaked wooden tooth pick onto the abdomen of the fly (Gebre-Michael et al. 1993). Radiolabelled probe was produced as described by Howard et al. (1991, 1992) and digoxigenin (Boehringer Mannheim) labelled probe was produced by polymerase chain reaction (PCR) (Wilson et al. 1992).

RESULTS AND DISCUSSION

L. chagasi in symptomatic and asymptomatic dogs - Giemsa-staining of skin biopsy impression smears revealed abundant amastigotes in abnormal skin of 60% of dogs (55 of 92) with

symptomatic visceral leishmaniasis. Intensive microscopy of impression smears from apparently unchanged areas of skin could detect amastigotes in some samples. Amastigotes could also be found in skin of more than 30% of dogs (18 of 57) that were IFAT positive but apparently healthy, for example, by microscopy of skin biopsies taken from the slightly raised edge of the external ear. Around 40% of symptomatic dogs (38 of 92) had parasitologically positive sternal bone marrow aspirates by microscopy of single samples (Vexenat et al. unpublished).

The radiolabelled Lmet2 DNA probe (Fig. 1) and Lmet2 with chemiluminescent detection (not shown, McNerney et al. 1994) were able to detect amastigotes in the skin of infected dogs, although we have not yet systematically compared probe sensitivity with parasitological and serological means of diagnosis. The probe also confirmed the widespread dissemination of amastigotes in the skin, even in skin that was apparently normal: post mortem tissue samples of one animal were probe positive with skin from the right and left outer ears (but not the inner ears), the nose, adjacent to the mouth and eyes, the right and left thorax, the right and left thigh and the scrotum; positive internal organs were the spleen, liver, lymph node, intestine, heart and testicle but not the lung, kidney and pancreas. Samples from the post mortem of a dog, which had to be destroyed for other reasons in a non-endemic area, were negative (Fig. 1). As described elsewhere, excess blood from tissues was removed by pre-blotting as blood contamination is known to interfere with probe sensitivity (McNerney et al. 1994).

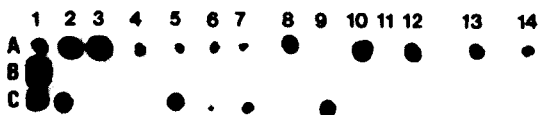


Fig 1: post mortem examination of tissue samples from a *Leishmania chagasi* infected dog with radiolabelled Lmet2 DNA probe. A: skin; 1, scrotum; 2, right thigh; 3, left thigh; 4, right thorax; 5, left thorax; 6, left eye; 7, right eye; 8, left outer ear; 9, left inner ear; 10, right outer ear; 11, right inner ear; 12, nose; 13, mouth; 14, mouth; B: positive control; C: 1, spleen; 2, liver; 3, lung; 4, kidney; 5, lymph node; 6, heart; 7, testicle; 8, pancreas; 9, intestine; A similar series of samples from the post mortem of a dog in a non-endemic area were probe-negative.

Lu. longipalpis fed on infected dogs - Xenodiagnoses were performed on dogs that had acquired natural infections of *L. chagasi* in Teresina and flies were examined 5-7 days after taking a blood meal. If flies were fed on skin with changes characteristic of canine VL they easily became infected (Table). Flies fed on apparently normal skin also became infected, al-

PRELIMINARY OBSERVATIONS ON THE DIAGNOSIS AND TRANSMISSIBILITY OF CANINE VISCERAL LEISHMANIASIS IN TERESINA, N.E. BRAZIL

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Abstract

A pilot group of 49 dogs and control groups from non-endemic areas were examined serologically for the presence of visceral leishmaniasis (VL) by direct agglutination test (DAT), indirect immunofluorescence (IFAT) enzyme-linked immunosorbent assay (ELISA) and DOT-ELISA. Results indicated that DAT is less sensitive than the other assays and that serology with filter paper blood samples is less sensitive than with serum. Promastigote infections were common in fed *Lutzomyia longipalpis* taken from a dog kennel inhabited by a dog carrying *Leishmania chagasi*. Colony-bred *Lu. longipalpis* readily acquired *L. chagasi* infection when fed on skin lesions of dogs naturally infected with *L. chagasi*: a small proportion of flies also became infected when fed on apparently normal skin. Widespread distribution of amastigotes in normal skin of asymptomatic animals was shown both by intensive microscopy and by probing skin biopsy samples with the Lmet2 *L. donovani*-complex specific DNA probe. It was demonstrated that an immunologically naive dog could be infected by a single experimentally infected sand fly. Abundant amastigotes present within the resultant lesion 22 days later were transmissible to sand flies but serology remained negative at least 45 days after the infective bite. Experimental transmission of canine VL by sand fly bite is a valuable approach for determining which diagnostic procedures are most sensitive, specific and suitable for field application in suburban households.

Introduction

The major endemic areas of visceral leishmaniasis (VL) in Brazil are in the semi-arid northeastern region. Canine VL is at least as widely distributed as the human disease and infection rates of more than 20% have been recorded in dogs. *Lutzomyia longipalpis* is the anthropophilic sand fly vector. VL has been known from Piaui State since 1934 but in the 1980s a large outbreak took place in which the principal focus was the urban area of the State capital Teresina. The incidence of symptomatic human infection peaked in 1984¹. Despite some continued intervention by serological monitoring and culling of dogs, periodic application of insecticides and the systematic treatment of human disease,

in villages in stony hills at less than 100 Km from *L. killicki* MON.8 (*L. tropica* complex) foci in neighbouring Tunisia suggest that the latter parasite might occur in Libya as well.

VL is of the infantile mediterranean type in Libya with very few adult cases. It occurs sporadically in two main northern foci in the Tripoli region and the Eastern part of the country in the whole area of the narrow strip of coastal plain from Benghazi to Tobruk, bounded by the Green Mountain to the South. These foci correspond to the most humid zones in Libya, where the annual rainfall varies from 250 to 600 mm.

It is important to cite the existence of newly described foci in the southern arid and dry saharian areas such as Oubari (Outrun, Wadi El Hala Baladiyah)(South West of Sebha) where 16 cases occurred from 1985 to 1990 according to Muhabresh (1990). In addition, other cases originating from unusual foci such as Al-Kufrah Oasis (South East) and Uzu (near the Chad border in the South East) were recently diagnosed by the pediatricians of the Children Hospital of Tripoli. The causative agent of VL in Libya is unknown. Canine leishmaniasis was reported by Dar et al. in 1987, and more recently confirmed by our team. The parasite species need to be identified properly and this is currently being carried out on 2 isolates from dogs from the Green Mountain area. Three candidate VL vectors are recorded in Libya: *P. perniciosus*, *P. longicuspis* and *P. langeroni*. Further investigation is needed for their incrimination.

In regard to vectors, little is known of the sandflies in Libya. The results of our entomological surveys during the 1991 and 1992 transmission season, using CDC light traps and sticky papers will be presented and discussed.

10,409 sandflies were collected particularly in VL and CL transmission foci. The following 21 species were identified, of which 12 species are recorded for the first time in Libya:

* *Phlebotomus* (*P.*) *papatasi* (4724), *P. bergeroti* (55), *P. Sergenti* (679), *P. alexandri* (290), *P. chabaudi* (50), *P. perniciosus* (12), *P. longicuspis* (546), *P. langeroni* (33), *P. orientalis* (or near *P. orientalis*, to be confirmed) (518), *P. tobbi* (1).

* *Sergentomyia* (*S.*) *minuta* (3131), *S. antennata* (137), *S. fallax* (193), *S. schwezi* (3), *S. bedfordi* (1), *S. cineta* (1), *S. clydei* (25), *S. adleri* (7), *S. christophersi* (1), *S. dreyfussi* (1), *S. palestinensis* (1).

DEMONSTRATION OF NATURAL *LEISHMANIA* INFECTION IN ASYMPTOMATIC DOGS IN THE ABSENCE OF SPECIFIC HUMORAL IMMUNITY

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Abstract

Asymptomatic dogs from a Kala-Azar endemic region were screened for infection status by parasitological, immunological and molecular techniques. Bone marrow was examined for the presence of parasites by NNN culture and by using the *Lmet 2* DNA probe. All the samples were negative in culture but 24 of 41 were positive as determined by the probe. Cellular and humoral immunity were detected by T cell proliferation assays and IFAT respectively. Specific cellular and humoral immunity were found in 20 and 26 dogs respectively out of a total of 41 dogs examined. The vast majority of dogs with *Leishmania*-specific antibodies were found to be parasitologically positive using the DNA probe while almost half those that had demonstrable cellular immunity were apparently parasite free.

The observation that dogs can develop cellular immunity following natural infection clearly indicates that there is a spectrum of canine leishmaniasis similar to that observed in the human disease. The prevalence of dog leishmanial infection must also be higher than was presumed.

Introduction

Visceral leishmaniasis or Kala-Azar is a disease of the mononuclear phagocytic system causing high mortality in untreated patients¹. It is endemic in all southern European countries including Portugal^{2,3}, where the High Douro river valley region, in the north of the country, is the main endemic focus of disease⁴. Wild canidae and rats are the natural hosts for Mediterranean Kala-Azar but domestic dogs are the main reservoir of the disease⁵.

Human leishmaniasis is associated with high titers of anti-*Leishmania* antibodies and no specific cellular immunity. However, the vast majority of infections are asymptomatic and these demonstrate skin test reactivity to *Leishmania* antigens^{6,3}. As in man, the disease in dogs is normally associated with high titers of anti-*Leishmania* antibodies⁷ but until recently there have been few studies on *Leishmania*-specific cellular immunity in dogs. However, in a preliminary survey we identified asymptomatic dogs with specific cellular

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between the acquisition of infection and conversion to seropositivity in dogs, although Corredor et al.⁷ reported a mean seroconversion time of 4.4 months in sentinel animals. A comparison of serology, parasitology and sensitive DNA-based detection in a cohort of animals experimentally infected by sand fly bite should allow a definitive conclusion to be drawn as to which procedure is most sensitive, specific and suitable for field application in suburban households.

Acknowledgements

We thank the Centre for Zoonoses in Teresina, particularly Dr. Vania Carvalho, for their collaboration and support. This research was supported by the Brazilian Ministry of Health, the Overseas Development Administration (UK) Programme Grant on Appropriate Technology, an EEC Science and Technology for Development contract and the WHO special Programme for Research and Training in Tropical Diseases.

We thank Dr. Richard Ward for providing some of the *Lu. longipalpis* used in xenodiagnosis. We thank Cesar Cuba Cuba (University of Brasilia) and Carlos H.M. Costa (Hospital for Infectious Diseases, Teresina) for helping to initiate collaboration between the Ministry of Health in Teresina and the London School of Hygiene and Tropical Medicine.

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incidence is again rising in 1992/93. The city is growing rapidly due to immigration from rural areas and VL is particularly common in the expanding suburban slums. One aspect of our research interests in Teresina concerns the comparison of serological, parasitological and DNA-based methods for the diagnosis of canine VL. Here we summarize preliminary results from work initiated under the auspices of the World Health Organization (1992-1993) and an EEC STD3 contract (1993-1995). A fuller account of these observations and of experiments in progress will be published elsewhere.

Materials and methods

A pilot group of 49 dogs was examined by direct agglutination test (DAT), indirect immunofluorescence (IFAT), enzyme-linked immunosorbent assay (ELISA) and DOT ELISA. These dogs were either strays that would have been destroyed or were dogs referred to us following a positive serological screening by (IFAT). Rates of *L. chagasi* infection cannot therefore be taken as indicative of the overall prevalence in the dog population of Teresina. Both serum and filter paper blood spots were used for serology. The following additional samples were also collected for future comparisons: blood spots on glass fibre discs - for examination by polymerase chain reaction (PCR) amplification of *Leishmania* DNA; white blood cells on nylon membranes after purification by centrifugation (Lymphoprep) - for probing with the Lmet2 *L. donovani*-complex specific DNA probe (McNerney et al., this volume); bone marrow samples from sternal puncture aspirates - for Giemsa staining, culture, PCR amplification or DNA probing, and skin biopsies from the external ear - for Giemsa staining and DNA-based detection of *Leishmania*. The DAT test was performed using the modified procedure described by El Harith et al.² with agglutination at 1:6400 serum dilution regarded as positive. IFAT was performed with acetone-fixed, cultured promastigotes using fluorescein (DTAF)-conjugated rabbit anti-dog IgG (H+L, Jackson) with fluorescence at 1:100 regarded as positive. ELISA was performed with sonicated promastigotes at an equivalent of 10^6 parasites per well on Immulon 2 plates (Dynatech) using peroxidase-conjugated rabbit anti-dog IgG (H+L, Jackson) with a visual coloured product at 1:200 regarded as positive. The antigen for DOT ELISA was supernatant from sonicated promastigotes dot-blotted onto nitrocellulose membranes at a concentration of 6 mg protein/ml and subsequently developed by a protein A-peroxidase (Sigma P8651) ELISA with visual product at 1:200 regarded as positive. Ten control sera from English beagles and 50 control sera from a non-endemic area (Brasilia) are being used to validate the serological assays. No positive DATs were found with control sera. Occasional borderline positives were seen with IFAT (1:100), ELISA (1:200) and dot-ELISA (1:200). Sand flies were captured in the suburbs of Teresina in CDC light traps or by hand using battery operated or suction 'pooters'. Colony-bred *Lu. longipalpis* were used for xenodiagnoses. Wild-caught flies or xenodiagnosis flies were dissected and examined microscopically and/or blotted onto membranes for probing with the Lmet2 *L. donovani*-complex specific DNA probe³.

Results and discussion

Comparative serology and parasitology

Serological and parasitological data are so far only available from the pilot series of 49 dogs, which we have used to establish protocols for methods to be applied to larger cohorts of animals. The results of DNA-based methods for the detection of infection^{4,5} are not yet available. Nevertheless some general points of interest emerge from this preliminary study.

Minimum positive titres observed with serum from dogs in Teresina were 1:200 for IFAT (3 animals), 1:400 for ELISA (3 animals) and 1:200 for DOT-ELISA (2 animals). The vast majority of end-points with positive sera were at much higher titres (6,400 - 102,400) and low titres on a single assay were frequently supported by high titres in the parallel tests or by parasitologically proven infection (not shown). Nevertheless we cannot exclude rare non-specific borderline titres among IFAT, ELISA and DOT ELISA results from Teresina (see Materials and methods).

DAT with serum or filter paper blood missed four of 20 parasitologically proven positive animals: all 20 were detected by IFAT, ELISA and DOT-ELISA with serum - one animal was missed by both IFAT and ELISA with filter paper blood (Table 1). Among the 29 parasitologically negative animals, six were positive by DAT on serum but none by DAT on filter paper blood. Between 13 and 17 of these animals were positive with serum by the other tests but only between 10 and 11 with filter paper blood samples. There was a remarkable consistency between the animals that were positive with IFAT, ELISA and DOT-ELISA. Twelve dogs were negative in all assays (Table 1).

There is at present no acceptable gold standard for the detection of *L. chagasi* infection in dogs. If microscopical detection of parasites in skin biopsy, bone marrow aspirate or cultured bone marrow aspirate is taken as the standard for comparison then the sensitivity of DAT was 75% and IFAT, ELISA and DOT-ELISAs had sensitivities of 100%. On this basis specificity of DAT was 93% and that for the other tests was very low (41%, 55% and 48% respectively).

If a combination of any three positive serological tests is taken as the standard then the sensitivities were estimated as: DAT, 61%; IFAT, 100%; ELISA, 97% and DOT-ELISA 100%. In this case specificities were: DAT, 100%; IFAT, 75%; ELISA, 94%, and DOT-ELISA 88%.

These data suggest some preliminary conclusions. Firstly, DAT is less sensitive than the other assays and this lack of sensitivity was not remedied by using lower cut off points in the test (not shown). Secondly, serology on filter paper blood is less sensitive than on serum, as noted by other authors⁶. Thirdly, IFAT, ELISA and DOT-ELISA detected all animals that could be shown to be parasitologically positive. Fourthly, compatibility between IFAT, ELISA and DOT-ELISA and the abundance of high titres, were in accord with the known poor reliability of parasitological examination.

If this first conclusion is upheld with wider studies it implies that the DAT is not suitable, even with serum, for the serological survey of dogs. A more sensitive test is required which can be used directly in suburban households on serum or whole blood.

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If this first conclusion is upheld with wider studies it implies that the DAT is not suitable, even with serum, for the serological survey of dogs. A more sensitive test is required which can be used directly in suburban households on serum or whole blood.

Table 1. Comparative serology and parasitology with sera and filter paper blood samples of 49 dogs from Teresina, Piauí State, Brazil.

	Parasitologically + VE [20] ¹		Parasitologically - VE [29]	
	Serum	Filter paper blood	Serum	Filter paper blood
DAT +VE	16	16	6	0
DAT -VE	4	4	23	29
IFAT +VE	20	19	17	12
IFAT -VE	0	1	12 ²	17
ELISA +VE	20	19	13	10
ELISA -VE	0	1	16	19
DOT-ELISA +VE	20	20	15	10
DOT-ELISA -VE	0	0	14	19

1 Parasitologically positive by either microscopy of skin biopsy or bone marrow aspirate or culture of bone marrow.

2 Negative on all tests.

Infections in wild-caught sand flies

Few *L. chagasi* infections were detected when flies captured in CDC light traps or caught by hand from in and around houses were probed with the Lmet2 *L. donovani*-complex specific DNA probe (not shown). In contrast, promastigote infections were common in fed *Lu. longipalpis* taken from a dog kennel in which there was a dog subsequently shown to be carrying *L. chagasi* (Table 2). The (suprasypharian) infections in the sand flies, which have not been tested with the Lmet2 probe, were assumed to be *L. chagasi*.

Xenodiagnoses of infected dogs

Colony-bred *Lu. longipalpis* were fed on dogs that had acquired natural infections of *L. chagasi* in Teresina. Flies were dissected 5-7 days after feeding.

Those flies that fed on skin with lesions very readily became infected, as did a small proportion of flies fed on apparently normal skin (Table 3). The widespread distribution of amastigotes throughout normal skin of asymptomatic animals could be confirmed by either intensive microscopy or by probing skin biopsy samples with the Lmet2 *L. donovani*-complex specific DNA probe (not shown). *Lu. longipalpis* thus appears to very readily acquire *L. chagasi* infection when feeding on infected dogs in Teresina either in the laboratory or in the field.

Table 2. Promastigote infection rates in fed *lutzomyia longipalpis* dissected 5-7 days after capture from dog kennels in the suburbs of Teresina, Piauí State, Brazil

KENNEL	FLIES + VE	FLIES - VE	% + VE
Dog subsequently shown infected	33	16	67
Dogs of unknown status	2	16	11
TOTALS	35	32	

Table 3. Promastigote infection rates in *lutzomyia longipalpis* dissected 5-7 days after feeding on dogs naturally infected with *Leishmania chagasi* in Teresina, Piauí State, Brazil

KENNEL	FLIES + VE	FLIES - VE	% + VE
Skin with lesions	57	106	35
Lesion free skin	4	61	6
TOTALS	61	167	

Definitive comparisons of diagnostic procedures and transmissibility could be performed with dogs from non-endemic areas exposed to infection by sand fly bite. Accordingly we have constructed a sand fly proof kennel facility at the University of Teresina. To assess whether transmission by sand fly bite was possible one of three young dogs from a non-endemic area (Brasília) was exposed to the bite of four sand flies that had fed seven days previously on a naturally infected dog. Only one of these flies was subsequently shown by dissection and microscopy to be infected. Within 22 days a small lesion containing many amastigotes had appeared at the site of the bite (inner ear) and seven out of 14 colony-bred *Lu. longipalpis* readily acquired infection when fed at the site, as again shown by dissection and microscopy seven days after feeding. Interestingly, serology remains negative 45 days after the infective bite and bone marrow aspirate and liver biopsy are also parasitologically negative. Clearly, it is impossible to draw any general conclusions from this single animal. Transmission of *L. chagasi* infection to immunologically naive dogs by the bite of experimentally infected *Lu. longipalpis* seems, however, to be a valuable experimental approach. It has been suggested that there may be a long lag-time

VISCERAL LEISHMANIASIS IN TERESINA, N. E. BRAZIL: TOWARDS A DNA PROBE KIT AND ITS ADAPTATION TO PROCESSING BLOOD-CONTAMINATED SAMPLES

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Abstract

The Lmet2 chemiluminescent DNA probe is a valuable tool for identifying parasites of the *Leishmania donovani*-complex in sand flies, dogs and human samples. Recent blood meals in sand flies or blood contamination of tissue samples inhibited probe sensitivity, whether radiolabelled or chemiluminescent detection systems were used. Treatment of membranes with protease before hybridisation restored positive signal. Alternatively samples could be lysed with protease and applied to membranes with a vacuum blotting apparatus. The Lmet2 protocol provides the basis for a DNA probe kit that is adaptable for use with a wide range of other probes.

Introduction

The detection and identification of protozoan parasites belonging to the *Leishmania donovani*-complex (*L. donovani*, *L. infantum*, *L. chagasi*) has previously relied on microscopical and immunological techniques or on the isolation and culturing of the parasite. The advent of DNA probe technology has provided exciting new research tools enabling the rapid identification of organisms in large numbers of samples. Until recently the need for radiolabelling restricted the use of DNA probes but the development of stable, sensitive alternatives allows widespread application of this technology. The relative ease with which stable labelled probes can be produced and the availability and low cost of other reagents required has encouraged us to develop a prototype 'DNA probe kit' that is suitable for use in 'low-tech' laboratories and in those countries where visceral leishmaniasis is endemic.

The Lmet2 probe¹ has been shown to be an extremely useful tool in identifying parasites of the *L. donovani*-complex in sand flies, dogs and human samples^{2,3}. Adaptation to a chemiluminescent detection system⁴ has allowed its use in a 'low-tech' laboratory in Teresina, Piaui State, Brazil, where incidence of symptomatic human visceral leishmaniasis is again rising despite previous successful interventions⁵. Here we describe the problems encountered and progress made in the development and validation of this assay, in particular the effects of whole blood contamination of samples on the sensitivity of the test.

Materials and methods

The presence of *L. donovani* genomic DNA is detected by using a DNA probe which binds preferentially to a 60 base pair repeat sequence found in the nuclear genome of organisms of the *L. donovani*-complex. Briefly, the parasite DNA is immobilized on a nylon membrane (Hybond N, Amersham). Samples may be squash-blotted or touch-blotted directly onto the membrane or pretreated and applied through a vacuum slot-blot apparatus. The Lmet2 DNA probe is produced and labelled with the hapten digoxigenin (Boehringer Mannheim) by polymerase chain reaction. Following the hybridisation step overnight at 37°C, when the labelled DNA probe binds to its target DNA, the membrane is washed to remove excess probe and 'blocked' in a solution of milk powder. The probe is detected via an alkaline phosphatase-labelled antibody (Boehringer Mannheim) against the digoxigenin label. After extensive washing to remove excess antibody the membrane is sprayed with a chemiluminescent substrate (Lumi-PhosTM, Cambridge Research Biochemicals) and then exposed to X-ray film in a lightproof cassette: if a darkroom facility is not available a 'black bag' and 'developing tank' can be used. The time of exposure depends on the sensitivity required, an initial exposure of 3 hours is recommended. The chemiluminescent signal will be maintained for up to 48 hours, if it is not convenient to expose the membrane immediately it can be stored frozen at -20°C for up to three weeks before returning it to room temperature and proceeding with the detection of the signal. A full protocol for the use of the probe is shown in the *Appendix*.

The samples used in the series of experiments described here are referred to in the Results and Figure legends.

Results and discussion

Probing sand flies

The Lmet2 chemiluminescent DNA probe has previously been shown to be capable of detecting less than 100 parasites when they are applied directly from culture to nylon membranes. The sensitivity for tissue samples and sand flies blotted directly and untreated onto membranes was subsequently assessed. A simple comparison of sensitivity for the radiolabelled and digoxigenin labelled Lmet2 probe with sand fly squash-blots is shown in Figure 1. Wild-caught *Lutzomyia longipalpis* from Para State, Brazil, kindly provided by Dr. R. Quinnell, were probed first with radioactively labelled Lmet2 probe, then the membrane was 'stripped' (50% formamide, 50% 10mM sodium phosphate pH 6.5, 65°C, 30m; wash x 2 in SSC/0.1% SDS; Appendix) and the membrane re-probed with the digoxigenin labelled probe. It is clear that one of the 36 flies on this membrane were naturally infected with parasites of the *L. donovani*-complex. Identical results were obtained by both labelling systems. The chemiluminescent result was obtained within 24 hours whereas the result with rad labelled probe required 4 days due to the long exposure time for autoradiography.

Lu. longipalpis were fed in Teresina on a dog naturally infected with *L. chagasi* to provide material for a comparison of microscopy and DNA probe detection of

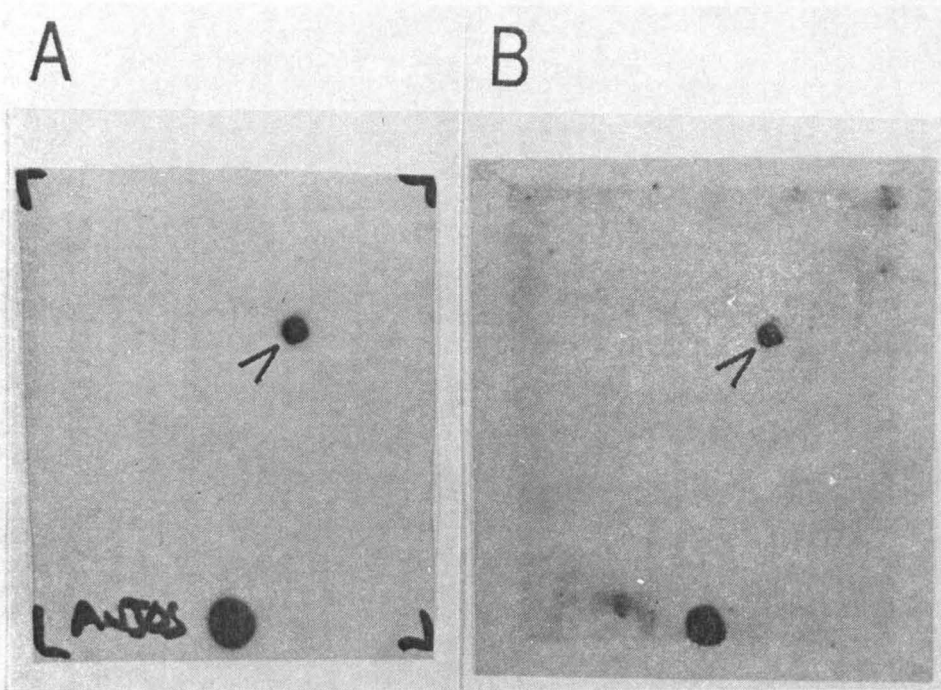


Fig. 1 : Comparison of radiolabelled and digoxigenin labelled probe for sandfly squash blots.

A. Membrane probed with ^{32}P labelled probe.

B. The membrane stripped by treatment (twice) for 30 minutes at 65°C in 50% formamide in 10mM sodium phosphate pH 6.5. and re-probed with the digoxigenin probe.

The positive control of 104 *Leishmania donovani* parasites MHOM/ET/67/HU3) can be seen at the bottom of the membrane.

The positive fly is indicated by the arrow.

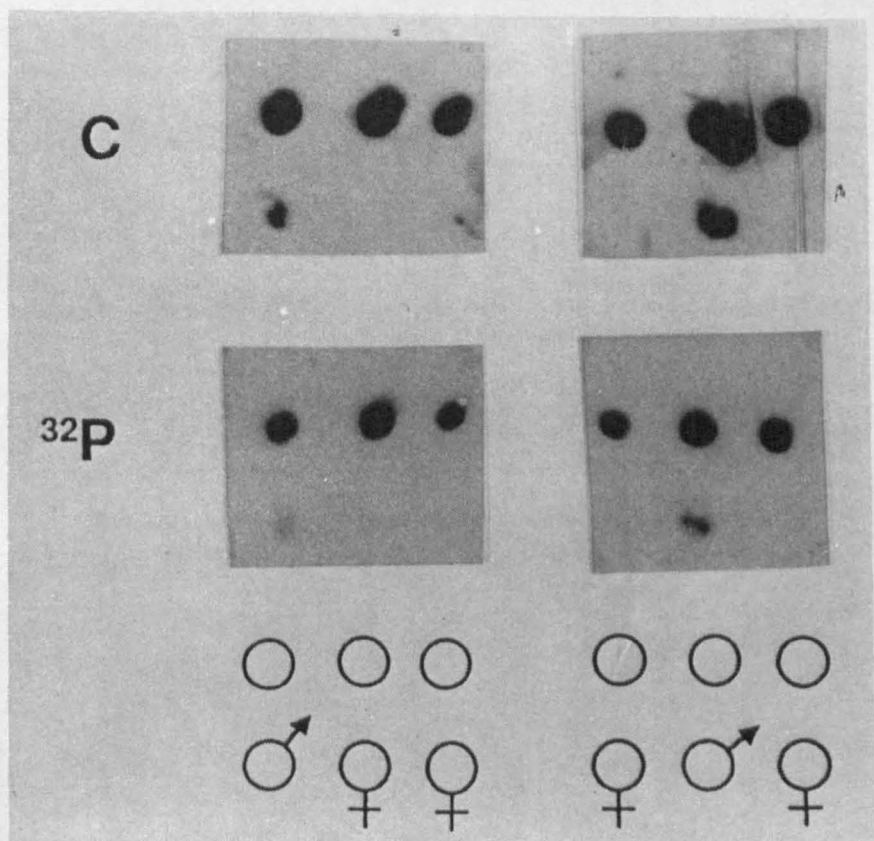


Fig. 2 : Two membranes were prepared both with triplicate samples of 10^4 *L.donovani* parasites spotted in a row at the top of the membrane. Samples of 10^4 parasites were mixed with individual male or female *Phlebotomus langeroni* flies and applied to the membrane as indicated in the diagram. The female flies had blood fed four hours previously. Both membranes were probed with the digoxigenin labelled Lmet2 probe. (Labelled C). The membranes were stripped and reprobred with radiolabelled Lmet2 probe. (Labelled 32P). The positive signal is absent from those samples which contained the female blood fed flies.

infected sand flies. Five days after feeding a proportion of the flies were dissected and the guts examined microscopically for the level of parasite infection. The dissected gut was then transferred to Hybond N membrane. A series of whole, undissected flies was also squashed directly onto the membrane.

As also reported by Gebre-Michael et al.³ for wild-caught sand flies from Ethiopia the results (not shown) indicated a good correlation between the microscopical examination and DNA probe detection of organisms in dissected flies. With the flies that were squashed whole, however, the proportion of DNA probe positive flies was less than that expected. This suggested a drop in sensitivity when whole recently blood-fed flies were squashed directly onto the membrane.

To investigate this further *Phlebotomus langeroni* male and blood-fed female (4 hours after feeding) flies were 'spiked' with 10,000 *L. donovani* parasites and then probed with Lmet2 and chemiluminescence. The results (Fig. 2) demonstrated that blood-fed female flies inhibited detection by the probe whereas positive signals were obtained from all the spiked male samples. When the membranes were re-probed with radiolabelled Lmet2 no signal was obtained from the female fly samples. Similar results (not shown) were obtained with flies fed 30 hours previously. It should be noted that the flies were squashed in a manner to ensure the maximum spread of the blood meal across the membrane in order to mimic the most serious blocking effect that could be obtained. Interestingly, the positive signal was inhibited whether the parasites were applied to the membrane before, at the same time or after the blood-fed flies were squashed onto the membrane.

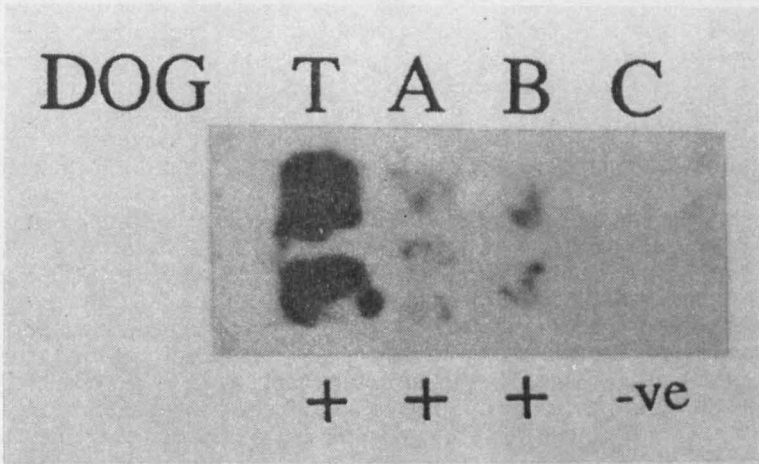
Probing tissue samples

Results with canine ear skin biopsies taken in Teresina from dogs suspected of carrying *L. chagasi* are shown in Fig. 3. These results were obtained within 24 hours of biopsy and without the use of a darkroom using the protocol summarised in the *Appendix*. Interestingly, Dog C, which had a single discrete skin ulcer, was found to be negative by both DNA probe and direct agglutination test (DAT) serology although amastigotes were seen by microscopy. This suggested the presence in Teresina of infections in dogs by *Leishmania* other than those belonging to the *L. donovani*-complex. Unfortunately this dog was not available for follow-up examination.

The potential sensitivity of the DNA probe for clinical samples such as bone marrow or splenic aspirate which may be heavily contaminated with haemoglobin, a suspected inhibitor of the chemiluminescent signal, was investigated with samples of whole blood spiked with *L. donovani* and applied directly onto Hybond N. The level of inhibition increased as the volume of blood applied to the membrane was raised; fresh untreated blood inhibited more strongly than stored buffy coat blood, and serum did not inhibit the signal (data not shown).

Improving sensitivity with blood contaminated samples

Treatment of membranes with protease before hybridisation successfully restored the positive signal, although some loss of sensitivity as compared to



DOG	MICROSCOPY	DAT	DNA
T	+ve	+ve	+ve
A	+ve	+ve	+ve
B	+ve	+ve	+ve
C	+ve	-ve	-ve

Fig. 3 : Skin biopsies taken from the ears of dogs suspected of being infected with *Leishmania chagasi* were squashed directly onto the nylon membrane for probing with digoxigenin labelled Lmet2. Samples were also taken for staining and microscopical examination and for a Direct Agglutination Test (DAT). All the animals were found positive by microscopy. Dogs T, A, and B were found positive by DNA probe and DAT. Dog C was found negative by both DNA probe and DAT (see text).

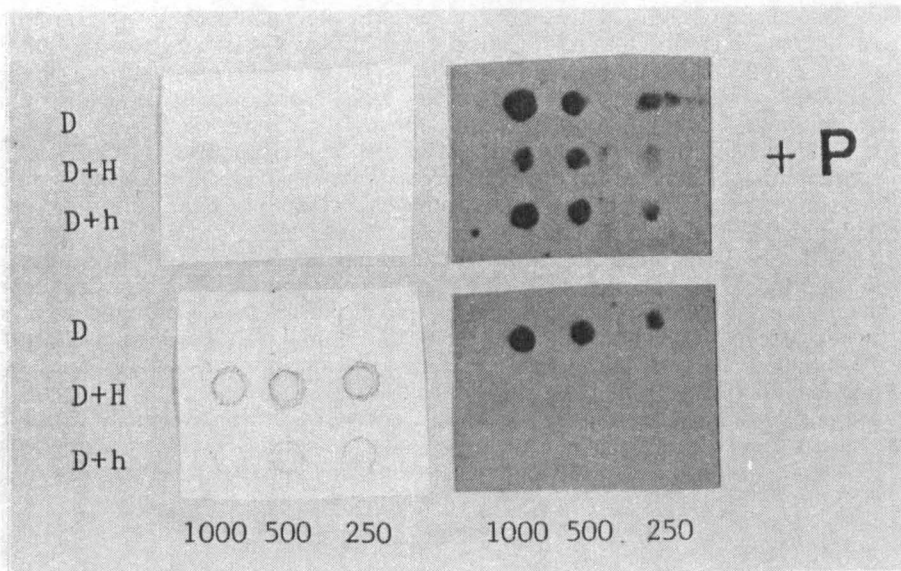


Fig. 4: Treatment of membranes with protease. Duplicate membranes were prepared with 1,000, 500 and 250 *Leishmania donovani* parasites spotted onto the membrane. The parasites were spotted in either 1 μ l tris buffered saline (D), 1 μ l fresh untreated whole blood or 1 μ l buffy coat blood. One membrane was treated with protease (+P) before they were probed with the digoxigenin labelled Lmet2 probe. The protease treated membrane gave positive signals whereas the untreated membrane did not. The signal was more readily restored in the sample mixed with stored buffy coat blood than with freshly obtained untreated blood.

blood-free samples was still observed. Results for duplicate membranes, one of which was treated for 1 hour at 37°C in 100µg/ml proteinase K (Sigma) in 10mM Tris pH 7.8, 5mM EDTA, 0.5% SDS are shown in Figure 4. Dark staining due to haemoglobin is still visible on the membrane that was not treated with protease. Signal can also be restored by treating membranes with hydrogen peroxide solution, although this is less reproducible with a wide range of samples and the concentration and time of exposure to hydrogen peroxide need to be carefully controlled to produce optimum results (data not shown). The use of protease instead of peroxide is also preferred as proteolysis may serve to remove causes of non-specific binding reported by other authors⁶.

An alternative approach to post-treatment of membranes is to lyse samples with protease and subsequently apply them to the Hybond N with a vacuum slot-blot apparatus (Millipore). This technique has been successful with a chemiluminescent DNA probe procedure for malaria parasites in human blood samples (manuscript in preparation). The use of slot-blot apparatus permits concentration of samples and therefore increases the sensitivity of the assay.

We conclude that heavy blood contamination can interfere with the sensitivity of both radiolabelled and chemiluminescent detection of the *L. donovani*-complex by the Lmet2 DNA probe but sensitivity can be largely restored by modifications to the protocol. Blood proteins appear to have a non-specific blocking effect on membranes and haemoglobin inhibits the production of the chemiluminescent signal. Recently blood-fed sand flies can be stored until the blood meal declines by digestion (7 days). Membranes carrying blood-contaminated samples can be treated with protease to remove blood proteins before the membranes are probed and positive signals are usually seen clearly even though sensitivity may not be restored to the levels obtained with blood-free samples. The most sensitive procedure with heavily blood-contaminated material appears to be pre-lysis of the samples with proteinase K and then use of a slot-blot or similar apparatus for application to membranes, although this is less convenient for field use in endemic areas.

The Lmet2 DNA probe has proved applicable to the detection of *L. donovani*-complex infections in wild-caught sand flies in Brazil and in Ethiopia³ and is useful for detecting these organisms in skin biopsy samples, or for the identification of recent isolates². The improved protocol that we have described means that the technique is also of value with material that is unavoidably blood-contaminated (bone marrow, spleen aspirates, peripheral blood). The Lmet2 protocol (*Appendix*) provides the basis for a DNA probe kit that is adaptable for use with other probes such as a *Leishmania braziliensis*-complex probe (Frame et al., in preparation) and a *Plasmodium falciparum*-specific probe.

Acknowledgments

We thank the Centre for Zoonoses in Teresina, particularly Dr. Vania Carvalho, for their collaboration and support. This research was supported by the Brazilian Ministry of Health, the Overseas Development Administration (UK) Programme Grant on Appropriate Technology, an EEC Science and Technology for Development contract and the WHO Special Programme for Research and Training in Tropical Diseases. We thank Dr. R. Quinnell for the sand fly

squash-blot shown in Fig. 1. We thank Cesar Cuba Cuba (University of Brasilia) and Dr. C.H.N. Costa (Hospital for Infectious Diseases, Teresina) for helping to initiate collaboration between the Ministry of Health in Teresina and the London School of Hygiene and Tropical Medicine.

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APPENDIX

The L. donovani -complex specific DNA probe [LEISHAID: donaprobe].

The LEISHAID donaprobe is a 58-60 base pair imperfect DNA repeat sequence that is specific to *Leishmania donovani* -complex organisms. The repeat sequence is present on at least six chromosomes of the *L. donovani* genome, of sizes ranging from 490 kb to greater than 2200 kb. Under appropriate hybridization conditions the LEISHAID: donaprobe hybridizes with equal sensitivity to *L. donovani*, *L. infantum*, *L. chagasi* and *L. donovani sensu lato* but not to other *Leishmania* species. The detection sensitivity is around 50 organisms, amastigotes or promastigotes, but may be less for organisms in mammalian tissues or sand flies.

Applications

The LEISHAID: donaprobe is applicable to the detection and identification of *L. donovani*-complex organisms in the following sources:

- a) Wild-caught or experimentally infected sand fly vectors.
- b) Touch-blot of skin biopsies from infected dogs.
- c) Primary or secondary cultures of *Leishmania* promastigotes isolated from aspirates or biopsies of bone marrow, spleen, liver and skin. Cultures can be tested with the LEISHAID: donaprobe, as long as viable *leishmanias* are present.
- d) Touch-blot or dot-blot of aspirates or biopsies from bone marrow, spleen, liver and skin provided that they are free of heavy blood contamination (see Limitations, below).

Limitations

1. In the absence of proteinase treatment (McNerney et al., above) heavy blood contamination will interfere with the performance of this test and reduce or obliterate signal. Irrespective of whether proteinase is used, recently fed sand flies should be maintained for seven days after feeding or until the obvious blood meal disappears. Sand flies can be kept in humid (but not wet) containers at 22-24°C, on a diet of sterile saturated sucrose soaked onto cotton wool. Excess blood from biopsy samples must be removed by pressing onto filter paper before the preparation of touch-blot.

2. At lowered stringencies the LEISHAID: donaprobe will give faint signals with 5×10^4 or more *L. major* and *L. mexicana*⁴. In practise this is not a limitation to probing infected sand flies or other sources of organisms providing that high numbers of promastigotes or amastigotes are prediluted and that appropriate controls are incorporated into the test series.

3. The LEISHAID: donaprobe is not intended for the sole and unequivocal diagnosis of human visceral leishmaniasis. If applied to human samples it should be used as an adjunct to diagnosis or for research purposes.

Test summary

The digoxigenin-labelled LEISHAID: donaprobe binds to target parasites immobilised on a nylon membrane. Bound probe is detected with anti-digoxigenin conjugated to alkaline phosphatase using a chemiluminescent substrate and X-ray film.

Sample preparation

1. Sand flies: Firmly squash the abdomen of sand flies onto nylon membrane using a separate disposable plastic tooth pick (cocktail stick) or other suitable implement for each sand fly. Roll the stick across the abdomen of the fly with enough force to push out the intestinal contents, but without damaging the fabric of the nylon membrane, which can lead to false positives. If wooden cocktail sticks are used they must be prewetted in 2 x SSC before use. Sand flies may be prewetted in isotonic saline (0.9% NaCl) and a trace of detergent for easy handling or can be cooled at 4°C to immobilise and then squashed dry. Fly remnants can be removed from the membrane for subsequent identification of vector species.
2. Biopsies liquid tissue samples and cultures: remove excess blood from biopsies by pressing onto filter paper with a plastic tooth pick (cocktail stick) disposable scalpel blade or sterile (flamed) forceps. Touch-blot onto the membrane. Use enough force to wet the membrane but avoid damaging the fabric of the membrane. Dot-blot liquid tissue samples or cultures onto the membrane. Note that samples that contain large numbers $5 \times 10^4/\mu\text{l}$ of promastigotes or amastigotes should be diluted prior to use (see Limitations).
3. Apply positive and negative control DNA to the membrane - 1 μl of each can be applied with a micropipette.
4. The membranes can be stored dry and a number of sample applications made before processing.

Processing of membranes

Denaturation

Denaturation and neutralisation is performed in trays overlaying the membrane on pieces of filter paper soaked in the relevant solutions.

Step 5 can be omitted for parasites and purified DNA.

1. Soak the membrane, sample side uppermost, in denaturing solution for 10 min.
2. Transfer the membrane to neutralising solution for 5 min.
3. Rinse the membrane in 2 x SSC.

4. Air dry the membrane and fix the DNA to membranes by baking at 80°C (see manufacturer's instructions) or UV cross-linking on a transilluminator (the exact dose of UV may be determined empirically with control samples) or in a machine designed for that purpose (see manufacturer's instructions).
5. Cover the membrane with proteinase K solution at 37°C and incubate for 1 hr. At this point the membranes can be rinsed in 2 x SSC and stored dried at room temperature or +4°C until hybridisation. Samples may alternatively be treated with proteinase K prior to application to membranes using a slot-blotter or similar device (Hybrislot, BRL, see McNerney et al., above).

Prehybridisation and hybridisation

Hybridisation can be performed in heat-sealed plastic bags, sealable plastic bags or in commercial roller bottles designed for use with a hybridisation oven.

The volumes in the following protocol are included as a guide and are calculated for a 50 cm² membrane processed in plastic bags.

1. Incubate the membrane in 5ml prehybridisation solution for 30 min at 37°C.
2. Pour off most of the prehybridisation solution and add 10µl of probe/ml of prehybridisation solution. The probe must be boiled for 5 min and either stored on ice prior to addition or added to the prehybridisation solution immediately. Mix and then return this solution to the membrane. Incubate at 37°C for 16 hours (overnight).

Detection

1. Remove the membrane and rinse the membrane in two 50ml changes of wash solution 1 at room temperature.
2. Wash for 15 min in three changes of wash solution 2 (500ml in total) at 37°C.
3. Rinse the membrane in 50ml TBS and then cover the membrane with 20ml of blocking solution. Leave this for 30 min at room temperature.
4. Replace the blocking solution with 10ml of antibody solution and incubate at room temperature for 30 min.
5. Wash the membrane for 30 min at room temperature in six changes of wash solution 3 (total volume 500ml).
6. Wash the membrane for 5 min in 50ml 2 x SSC.
7. Drain the membrane of excess solution and place, sample side up, on a layer of transparent plastic e.g. sealable plastic for bags, acetate sheets etc.

8. Spray the membrane sparingly with chemiluminescent substrate, cover the membrane with another layer of plastic and smooth to remove air bubbles. This should be sealed to prevent drying of the membrane.
9. Place the membrane in an autoradiography cassette or other such light tight device lined with filter paper (no intensifying screens required) and expose to X-ray film for the required exposure (2.5 - 16 hours). Multiple consecutive exposure can be made over a 24hr period.

Trouble shooting

High Background

- increase blocking step, the concentration of milk powder can be increased to 15%.
- increase the volume or number of post antibody washes (wash solution 2).
- decrease the amount of probe.
- membrane allowed to dry, keep wet throughout prehybridization and detection stages and ensure that membranes are not stuck together during processing.
- increase volumes and duration of post hybridisation washes.
- membrane contaminated, handle carefully with forceps or wear clean gloves, do not use excessive force when applying samples.
- equipment contaminated, check all containers and surfaces are clean.
- check substrate is not contaminated.

Spotty or streaked background

- antibody conjugate clumping, centrifuge briefly before taking conjugate to dilute.
- hybridisation mix not fully dissolved, make fresh stock solutions.
- filter hybridisation solutions.
- probe degraded or contaminated, try fresh aliquot.
- static discharge upon removing film - wipe plastic with anti-static cloth before loading cassette.

Low signal

- increase autoradiograph exposure time.
- check substrate has not degraded.
- check film developing.
- DNA not fixed correctly.
- hybridisation wash too stringent.
- membranes not stored dry, DNA degraded.

Solutions required

SSC 20 x stock solution	3M NaCl/0.3M Na ₃ citrate
Denaturing solution	1.5M NaCl/0.5M NaOH

Neutralising solution	1.5M NaCl/0.5M tris/pH7.2/1mM EDTA
Proteinase K solution	proteinase K type XXVIII (Sigma P4914) 100µg/ml (1 - 2 units/ml)/10 mM Tris pH7.8/5mM EDTA/0.5% (w/v) SDS
Prehybridisation solution	50% (v/v) formamide 5 x SSC 0.5% (w/v) sodium dodecyl sulphate (SDS) 0.1% (w/v) N-lauroylsarcosine 3% (w/v) dried skimmed milk powder 100µg/ml boiled sonicated herring sperm DNA
TBS 10 x stock solution	1M Tris pH7.5/1.5M NaCl
Blocking solution	3% milk powder in 1 x TBS (heat to 65°C and stir while cooling)
Antibody solution	anti-digoxigenin alkaline phosphatase (Boehringer Mannheim Cat. mp/1093274) 1:10,000 in TBS 3% milk powder 0.5% (v/v) Tween 20
Wash solution (1)	2 x SSC 0.1% sodium dodecyl sulphate (SDS) 0.02% N-lauroylsarcosine
Wash solution (2)	0.01 x SSC 0.1% sodium dodecyl sulphate (SDS) 0.02% N-lauroylsarcosine
Wash solution (3)	1 x TBS/0.5% Tween 20
Substrate	Lumiphos (Spray) Cambridge Research Biochemicals Cat. No. SU-07-200
Strip solution (1)	10mM sodium phosphate pH 6.5/50% formamid
Strip solution (2)	2 x SSC 0.5% SDS

Materials required

Nylon membrane (Amersham Hybond N recommended)

X-ray film, cassette and development facilities

Pipettes, forceps, plastic bags, washing trays, acetate or plastic sheet, water tap or oven.

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Telephone: 0044 171 92 224

CLINICAL RECOVERY AND LIMITED CURE IN CANINE VISCERAL LEISHMANIASIS TREATED WITH AMINOSIDINE (PAROMOMYCIN)

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Abstract. Three groups of three, six, and 12 dogs with parasitologically proven clinical visceral leishmaniasis (*Leishmania chagasi* infection) were treated with intramuscular aminosidine sulfate at doses of 20 mg/kg/day for 15 days; 80 mg/kg/day for 20 days, and 40 mg/kg/day for 30 days, respectively. Follow-up was by parasitologic examination of bone marrow and skin, serology using the indirect immunofluorescent antibody test, and clinical examination for signs of visceral leishmaniasis or adverse effects of treatment. In animals treated with 20 mg/kg/day, for 15 days, there was dramatic clinical improvement with disappearance of conjunctivitis, increase in appetite, weight gain, and recovery of normal skin condition and a healthy coat, but parasitologic relapse occurred between 50 and 100 days after initiation of treatment. Adverse effects were seen with treatment with 80 mg/kg/day for 20 days; three dogs died during or just after treatment, two showed temporary recovery, and one showed total clinical and parasitologic cure that was maintained for four years. Although adverse effects and relapses were seen in some dogs treated with 40 mg/kg/day for 30 days, three of 12 dogs showed complete parasitologic and clinical cure that was sustained for at least four years. Aminosidine treatment cannot be recommended as an alternative to the humane destruction of dogs for the control of canine visceral leishmaniasis because ineffective treatment may prolong carrier status or encourage development of drug resistance. This drug may be a therapeutic option if there is no danger of a dog acting as a reservoir of infection. Achievement of clinical recovery and limited cure with aminosidine suggests that further trials would be of value, possibly in combination with other anti-leishmanial drugs and with supportive measures to reduce adverse effects.

Canine visceral leishmaniasis (VL) is caused by the protozoan parasite *Leishmania infantum* in the Old World and by *Leishmania chagasi* in the New World.¹ These two *Leishmania* species are very closely related and some authorities consider them to be virtually indistinguishable.² Clinically, Old World and New World canine VL give similar presentations, causing signs such as swelling of the popliteal lymph nodes, dermatitis around the eyes and nose, disseminated alopecia, weight loss, edema, and hepatosplenomegaly.³⁻⁵

The dog is considered to be an important reservoir of infection for human VL, except in endemic areas of the Old World where the disease agent is *L. donovani*, and not *L. infantum/chagasi*. Although residual or ultra-low volume insecticide spraying may be useful for attacking the sandfly vectors, there are two principal difficulties obstructing the direct control of canine VL.

First, diagnosis of canine VL is unreliable, due to the lack of a proven, highly sensitive, specific, simple assay for antibodies that can be performed on site during household visits. Serologic screening followed by the elimination of seropositive dogs has been adopted as the primary direct method for control of canine VL in Latin America, and yet its impact on disease transmission is not clear. The alternative approach of parasitologic diagnosis requires intensive effort, and diagnostic DNA probes for field use have yet to be perfected.

Second, there is no effective drug for the treatment of canine VL. Pentavalent antimonials, which are of great value for treatment of the human disease,^{6,7} are used in some countries of the Mediterranean region to treat symptomatic canine VL or asymptomatic dogs that are serologically positive. Clinical signs of canine VL may disappear, although infec-

tions usually relapse soon after antimonial treatment is withdrawn. Treatment is often repeated every few months but such repeated use of noncurative doses of antimonials is hazardous: drug-resistant organisms⁸ are likely to arise, which may be transferred to humans, and apparently healthy infected dogs may act as a prolonged source of infection to sand flies.^{9,10} Attempts to use antimonials for the treatment of canine VL in the New World have been unsuccessful.¹¹

During the last decade there has been renewed interest in the anti-leishmanial activity of the aminoglycoside antibiotic aminosidine (paromomycin).¹² The drug is active in vitro and in vivo against various *Leishmania* species.^{13,14} Clinical studies have confirmed that it is a very useful alternative to antimonials for the treatment of human VL, although like antimonials it requires parenteral administration.¹⁵⁻¹⁷ Single-agent aminosidine treatment was found to have limited efficacy in cutaneous leishmaniasis in South America.¹⁸ Aminosidine has been used with great success in clinical trials of combination therapy with antimonials for human VL.^{15,19-21}

The aims of these experiments were to follow clinical recovery, relapse, and clinical and parasitologic cure in symptomatic canine VL treated with aminosidine.

METHODS

Study area. This study took place in Teresina, the capital of Piaui state in northeastern Brazil, where human and canine VL are endemic. There have been two epidemics of human disease, one in 1993, and a previous outbreak in the early 1980s.^{22,23} The city is located between and adjacent to two rivers, the Parnaiba and the Poty, which influence the vegetation and climate. The climate in Teresina is tropical

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with temperatures fluctuating between 22°C and 32°C. There is a rainy season between December and April (approximate cumulative rainfall = 160 mm) and a dry season from May to November (rainfall = 20 mm). Transmission of VL is also thought to be seasonal with changing vector abundance.

A new kennel facility was built at the Department of Parasitology, Federal University of Piauí such that treated dogs could be kept hygienically and separately without exposure to sandfly bites. Doors and windows were screened against sand flies with fine mesh, and the entire building was sprayed monthly with residual pyrethroid insecticide.

Selection of animal groups. A total of 21 dogs were treated. All animals had been brought to the Department of Parasitology for clinical, parasitologic, and serologic examination because the owners suspected the presence of canine VL. Dogs were parasitologically proven to be infected with *L. chagasi* before entering the treatment trial and were allocated sequentially into the experimental groups. The owners of the dogs donated the animals to the trial in the knowledge that in accordance with local public health recommendations, the only alternative was to have them destroyed.

The first group of dogs consisted of three adult Doberman pinschers. The second group of six adults was composed of two Doberman pinschers, one German shepherd, one tawny boxer, one English cocker spaniel, and one toy terrier. The third group of 12 adults was composed of three Doberman pinschers, one German shepherd, one fila Brasileiro, two toy terriers, one Irish setter, one short-haired dachshund, one bichon frise, and two of unidentified breed.

Treatment. Treatment was with aminosidine sulfate (Gabbromicina®; Farmitalia Carlo Erba, Milan, Italy) dissolved in sterile distilled water and administered by intramuscular injection, in accordance with the manufacturer's instructions. Group one was given 20 mg/kg of body weight daily for 15 days, group two 80 mg/kg for 20 days, and group three 40 mg/kg for 30 days.

The three groups were not treated simultaneously; results from group 1 were available before groups 2 and 3 were started. No specific control group of untreated animals was set up for this experiment, but during the course of this project several animals of similar clinical and parasitologic status were maintained for experiments on the transmissibility of canine VL to sandflies.^{23,24} All such animals showed progressive symptomatic canine VL, none recovered, and towards the terminal stages of the disease they were humanely killed.

Clinical, parasitologic, and serologic examination. All animals were examined clinically for signs of depilation and exfoliative dermatitis, external lesions, abnormal claws, and conjunctivitis/keratitis, and compared with the normal weight range for the breed.²⁵ Parasitologic examination was by microscopy of Giemsa-stained impression smears of skin biopsies and sternal bone marrow aspirates. All animals included in the study had amastigotes in the skin and/or bone marrow. Bone marrow samples were obtained by elevation of the head of the dog in a sitting position and sternal puncture, which was rapid and well-tolerated. Serologic examination was by the indirect immunofluorescent antibody test (IFAT) as described previously, based on comparisons of the IFAT, enzyme linked immunosorbent assay (ELISA) and the direct agglutination test.²³ Clinical, parasitologic, and sero-

logic examinations were performed prior to treatment, repeated five days after start of treatment and subsequently approximately every 15 days, or less frequently for dogs with clinical recovery and long-term survival. Impression smears of spleen and liver were examined post-mortem.

Possible adverse effects of treatment were followed by monitoring loss of appetite, weight loss, ocular changes, and lack of response to auditory or olfactory signals. True adverse effects were considered to be those occurring within one month of the end of treatment. Nevertheless, we also noted and report here any event that occurred during follow-up at any time since we could not predict long-term effects of the drug.

RESULTS

Treatment with 20 mg/kg/day for 15 days. The most striking observation from this preliminary group of three dogs was the early and dramatic clinical improvement, with the disappearance of conjunctivitis, increase in appetite, weight gain, and general improvement in skin and coat condition. All three animals were considered to be underweight prior to commencement of treatment, although there was no evidence that they had been denied food and apparent loss of appetite/weight loss was one reason why the owners had asked for the animals to be examined. The only detectable side effect was a temporary widespread loss of hair over five days during the second week of treatment and prior to replacement by a healthy coat. At the end of treatment, three animals were parasitologically negative by examination of both bone marrow and skin. Between 50 days (dogs 1 and 2) and 100 days (dog 3) after initiation of treatment, however, amastigotes were again found, first in the skin and then in the bone marrow (Figure 1). About the same time, or slightly later, symptoms of VL returned, becoming severe with weight loss, disseminated dermatitis, hair loss, and conjunctivitis. Deterioration was progressive and all three animals were killed at day 157 because there was no prospect of recovery, to prevent suffering. The dramatic weight gain, to levels typical for healthy examples of the breed, that accompanied clinical improvement, and the subsequent decrease in body weight associated with relapse are shown in Figure 2.

Treatment with 80 mg/kg/day for 20 days. In view of the relapse of all three dogs in group 1, it was decided to use a higher dose (80 mg/kg) and a slightly prolonged schedule for treatment (20 days) in a second group of animals. A third group (below) was also set up with an intermediate dose level (40 mg/kg) and a more prolonged schedule (30 days).

Dogs in the 80 mg/kg group either had severe VL (symptomatic) or less severe but obvious signs of VL (oligosymptomatic; Table 1) and all had amastigotes in both skin and bone marrow. Response to treatment was not uniform. Two dogs died within five days or one day before treatment ended, and a third dog died four days after treatment had ended. All three of these animals showed adverse effects including appetite loss, weight loss, acute dehydration (sunken eyes), loss of scent perception, and deafness; two also had keratitis. There were no detectable amastigotes in the liver or spleen post-mortem.

AMINOSIDINE AND CANINE VISCERAL LEISHMANIASIS

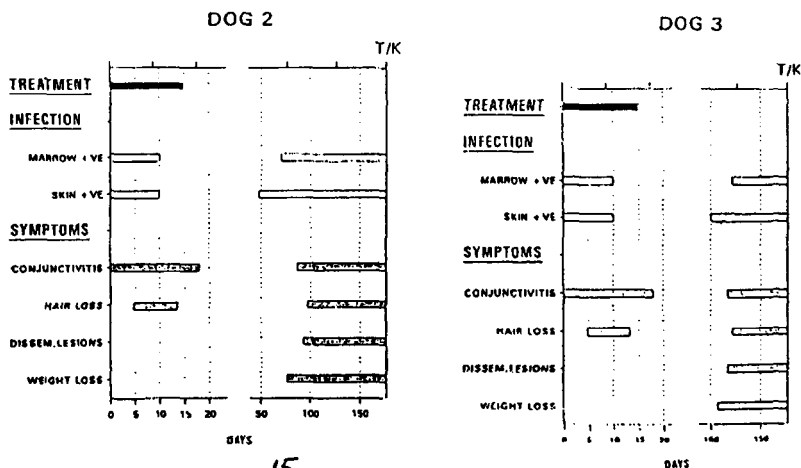


FIGURE 1. Aminosidine, 20 mg/kg/day for 15 days: relapse after a period of improvement (dogs 2 and 3). T/K = terminal phase/killed; +VE = positive; DISSEM. = disseminated.

A fourth dog survived treatment with dramatic clinical improvement, including restored appetite, weight gain, and disappearance of edema, was apparently entirely healthy apart from incipient blindness that began around day 60, but died suddenly of unknown causes. A single atypical amastigote was found post-mortem in the liver. A fifth dog also recovered dramatically, relapsed and became parasitologically positive seven months after treatment began; deafness and blindness were noted during the terminal phase of the disease. One dog in this group survived and after four years is in excellent general health, parasitologically negative, but with defective vision that arose two years after treatment.

Treatment with 40 mg/kg/day for 30 days. One dog lost weight and died before treatment was complete and a second died shortly after treatment; amastigotes were found in both animals post-mortem. Two dogs that did not clear their skin infection during treatment were killed 30 days after treatment and had amastigotes in both the liver and bone marrow post-mortem. Five dogs appeared to have cleared their infections, with weight gain and dramatic clinical improve-

ment, but relapsed between one and four months later with progressive canine VL and parasites in the skin and bone marrow. Three of the 12 dogs have survived for more than four years with no detectable *L. chagasi* infection (Figure 3).

Possible side effects in this group were weight loss in one dog that died during treatment, signs of deafness in four dogs (beginning at between days 25 and 60), and keratitis leading to partial or total blindness in six dogs (beginning at between days 28 and 500). Two of the three dogs that have survived for more than four years and are apparently totally cured of infection have no detectable side effects. The overall results of the trial in terms of clinical status before treatment and outcome of treatment are summarized in Table 1.

DISCUSSION

Pentavalent antimonials are tolerated in dogs, although there is a narrow therapeutic window, but are not a suitable treatment for canine VL. Animals with clinical signs usually relapse rapidly after a course of antimonial therapy. Repeated treatment carries the hazard of maintaining a reservoir for the propagation of new infections and, more importantly, of selecting *Leishmania* populations that are less susceptible to treatment, or have become resistant to drugs that are first line therapeutic agents for human VL.¹⁰ Failure of antimonial therapy for canine VL may in part be due to the more rapid elimination of antimonials in dogs as compared with humans, even when administered by the subcutaneous route.²⁴

Aminosidine has become an important drug for treatment of human VL, especially in combination with pentavalent antimonials, with excellent cure rates and a good record of tolerability. Possible types of toxicity are primarily ototoxicity and nephrotoxicity, but the only significant side effect

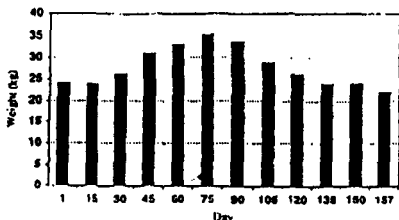


FIGURE 2. Mean weight fluctuations in three Doberman pinschers following treatment with aminosidine (20 mg/kg/day for 15 days) and in conjunction with subsequent relapse (Figure 1).

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TABLE I
 Summary of results of aminosidine treatment of canine visceral leishmaniasis

Dose	No. of dogs	Clinical condition*	Adverse effect†	Died‡	Relapsed and died	Cure§ (%)
20 mg/kg/day for 15 days	3	Symp	0/3	0/3	3/3	0/3
80 mg/kg/day for 20 days	6	Oligo 4	1/4	1/4	2/4	1/4
		Symp 2	2/2	2/2	0	0
40 mg/kg/day for 30 days	12	Oligo 7	3/7	1/7	3/7	3/7
		Symp 5	1/5	1/5	4/5	0/5

* Clinical condition before treatment: Symp = symptomatic; Oligo = oligosymptomatic.

† Adverse effects occurring within one month of the end of treatment (later clinical events were also recorded, see text and Figure 3).

‡ Died within one month of treatment.

§ Clinical and parasitologic cure.

recorded in humans treated for VL is minor loss of hearing range, and this occurs rarely.¹⁶ The mode of action of aminosidine is inhibition of protein synthesis by attachment to the small ribosomal subunit.²¹ Doses used for treatment of human VL are between 6 mg/kg and 20 mg/kg, once a day for up to 21 days alone or combined with antimony. We have tested the ability of aminosidine to produce clinical improvement and clinical and parasitologic cure in canine VL.

Three groups of animals were treated. In the first, 20 mg/kg for 15 days produced dramatic clinical improvement but all three animals eventually relapsed with progressive canine VL. There were no side effects, with all signs being consistent with the disease progression, except that the unhealthy coat was shed prior to replacement with fresh growth of healthy hair. Clinical improvement was associated with apparent clearance of infection, such that it was not detectable by skin biopsy or bone marrow aspiration, but it returned to both sites during the relapses.

A four-fold increase in dose and extended treatment produced severe side effects that included loss of appetite, weight loss, evidence of nephrotoxicity, and neurotoxicity affecting hearing, vision, and sense of smell. Only one dog in this group was cured, but it developed impaired vision almost two years after treatment.

An intermediate dose of 40 mg/kg for 30 days was more successful in that three (25%) of 12 dogs survived for more than four years with no evidence of *L. chagasi* infection. Nevertheless, two animals died during or just after treatment and others either did not clear their infection or relapsed at variable intervals. Possible late side effects were still seen, principally loss of hearing and vision, but since these did not arise until long periods after treatment had ended, the pathogenesis of such delayed toxic effects is not clear, and it is doubtful that they are attributable to the preceding aminosidine therapy. True side effects were considered to be those occurring within one month of the end of treatment (Figure 3).

The clinical condition of dogs prior to treatment is shown in Table I. It might be expected that dogs with the most severe VL, termed here symptomatic, would be most likely to suffer side effects and die during or just after treatment, possibly in part due to the damage caused by antigen release and deposition of complexes in the kidneys. Similarly, animals not in the terminal phase of the disease (oligosymptomatic) should be best able to withstand treatment and have the best chances of clinical cure. The data in Table I are not particularly informative on the first of these predictions but

the second trend is clearly apparent: all clinically cured dogs were initially considered to be oligosymptomatic.

As far as we are aware, clinical and parasitologic cure of canine VL with proven skin and bone marrow infections and overt clinical signs of disease has rarely been achieved. Although aminosidine did produce a proportion of such cures, its use clearly has no value at present as a routine control measure against canine VL in endemic areas such as Teresina: it is not a satisfactory alternative to the humane destruction of dogs. For highly prized, irreplaceable pedigree stock, it could be used to save selected animals, assuming that rigorous measures were taken to prevent transmission to sand flies from treatment failures. Ineffective treatment carries the risk of generating organisms resistant to aminosidine, for which three resistance mechanisms have now been proposed.²² *Leishmania*, resistance to aminosidine apparently depends on increased production of ribosomal RNA but not on enzymatic inactivation of the drug or base substitutions in the small ribosomal subunit.²⁷

In future studies, it would be of value to investigate the response of relapse infections to a second course of aminosidine treatment, at an early stage in the relapse, when relatively few organisms were present. Isolation of *Leishmania* pretreatment and during relapses would enable in vitro assays to determine if recrudescence organisms were less susceptible (resistant) to drug action.

Achievement of clinical recovery and cure suggest that further trials with aminosidine may be worthwhile, for example, with slow-release formulations or drug combinations. Combined aminosidine and antimony therapy is said to enhance the persistence of high serum concentrations of antimony, which may allow reduction of therapeutic doses and increased intervals between administrations, but concomitant elevation of levels in skin, presumably crucial to cure of canine VL, has not been investigated.²⁸ Kidney lesions in canine VL due to glomerular and tubular damage from deposition of immunoglobulins are well known²⁹ and are likely to be precipitated by antigen release during aminosidine treatment. Supportive therapy might be used to reduce the side effects of aminosidine in dogs.

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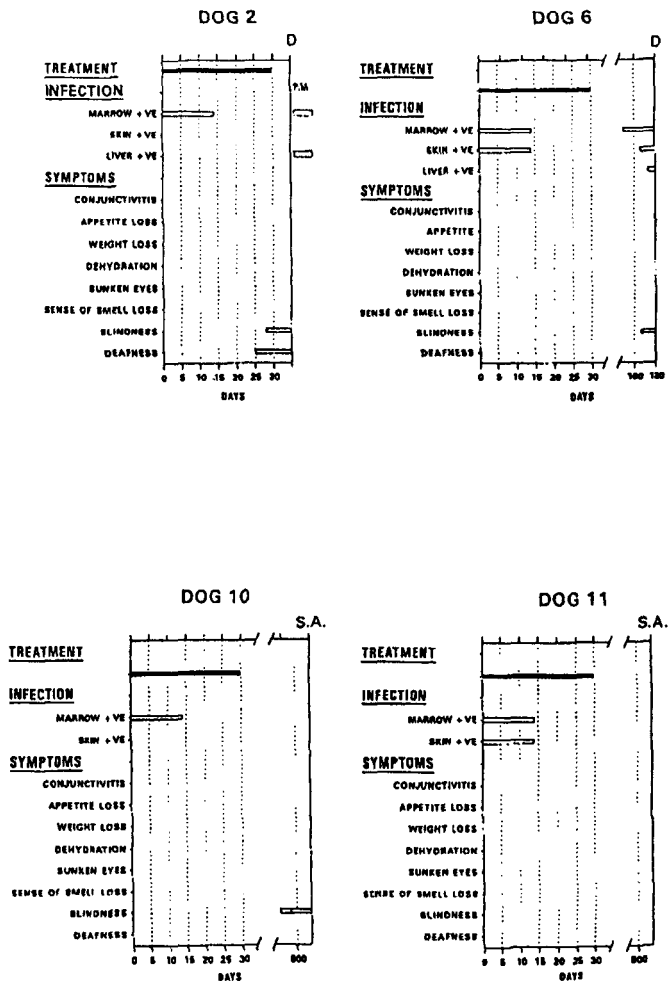


FIGURE 3. Aminosidine, 40 mg/kg/day for 30 days: dogs 2 and 6 both died in good clinical condition but were parasitologically positive. D = died; dogs 10 and 11 both parasitologically negative at 800 days of follow-up. S.A. = still alive. Late occurring blindness and/or deafness in dogs 2, 6, and 10 were not necessarily attributable to preceding aminosidine therapy (see text). +VE = positive; P.M. = post-mortem.

assist with provision of appropriate facilities; supplementary funding and aminosidine were donated by Farmitalia-Carlo Erba (now Pharmacia-Upjohn).

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Short Communication

Failure of buparvaquone (Butalex) in the treatment of canine visceral leishmaniosis

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Abstract

Buparvaquone (Butalex), a therapeutic for theileriosis, has been shown to have anti-leishmanial activity *in vitro*. Seven dogs with symptomatic, parasitologically positive, canine visceral leishmaniosis were treated with Butalex at 5 mg kg⁻¹ body weight using four doses over 12 days. Two animals showed minor clinical improvement (growth of healthy hair) but all remained parasitologically positive and disease progression was not halted.

Keywords: Dog; *Leishmania donovani*; *Leishmania infantum*–*Leishmania chagasi*; Buparvaquone; Control methods-Protozoa

Human visceral leishmaniosis (Kassai et al., 1988) (VL) caused by the *Leishmania donovani* complex is endemic in at least 62 countries, with an estimated annual incidence of more than 500 000 clinical cases per annum (de Gorgolas and Miles, 1994). In both the New and Old Worlds, where human VL is caused by *Leishmania chagasi*–*Leishmania infantum*, the dog is considered to be a significant reservoir host. In addition, canine VL is a widespread veterinary problem in the Mediterranean region, even where there is little human disease. Canine VL can be highly transmissible and dogs may be infective to sandflies before they become serologically positive (Vexenat et al., 1994).

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Frequently, dogs that are parasitologically and/or serologically positive are killed, as there is no reliable treatment. Repeated antimonial therapy is sometimes given in Mediterranean countries, but symptomatic dogs usually relapse and such treatment may select for organisms that are less susceptible or resistant, which if propagated, may complicate treatment of human VL (Gramiccia et al., 1992). Aminosidine has provided clinical improvement and limited cure in canine VL, but treatment is frequently followed by severe side-effects or relapse (Vexenat et al., 1997). An amphotericin B liposome formulation (AmBisome), highly effective in the treatment of human VL, failed to produce parasitological cure in canine VL caused by *L. infantum* (Oliva et al., 1995).

Buparvaquone (BW 720C), 2-[*trans*(4-*t*-butylcyclohexyl)-methyl]-3-hydroxy-1,4-naphthoquinone, was synthesized as part of a programme to prepare hydroxynaphthoquinones with antimalarial activity. In initial studies, it showed activity against *Plasmodium falciparum* and *Eimeria tenella*, but had exceptional activity against *Theileria* species (Hudson et al., 1985). It was subsequently developed as Butalex[®], as a therapeutic for theileriosis (Pitman–Moore, Harefield, UK) (McHardy, 1992). Buparvaquone has also been found to be a promising lead compound for the treatment of *Leishmania donovani* infections, with ED₅₀ values of between 0.12 and 0.005 µM against amastigotes in an in vitro macrophage model (Croft et al., 1992). Treatment of *Leishmania donovani* infection in the BALB/c mouse model was limited, with 62% suppression of liver amastigote numbers achieved by s.c. administration of 100 mg kg⁻¹ day⁻¹ for 5 days. However, the intrinsic anti-leishmanial activity of buparvaquone (Croft et al., 1992), together with the development of a veterinary formulation (McHardy, 1992), suggested that the potential of buparvaquone for the treatment of canine leishmaniosis should be examined.

Seven naturally infected dogs that had acquired infection in the urban endemic area of Teresina, Piauí state, Brazil, were treated with Butalex[®] (Pitman–Moore). All animals were symptomatic and parasitologically positive. Treatment was by i.m. injection into the upper rear leg, with 5 mg kg⁻¹ of body weight, on Days 0, 4, 8 and 12 (four doses, total period 12 days). The clinical and parasitological status of each animal was evaluated before, during and after treatment (Table 1).

Table 1
Treatment of seven dogs with naturally acquired symptomatic canine visceral leishmaniosis by buparvaquone (5 mg kg⁻¹, intramuscularly, four doses over 12 days)

Breed (age, sex, weight)	Clinical condition ^a (parasitological status) ^b	
	Before treatment	After treatment
(1) Mongrel (2 years, female, 18 kg)	Ear lesion (skin, b.m., liver + ve)	Lesion extended (skin, b.m., liver + ve)
(2) Basset (3 years, female, 13 kg)	Nose lesion (skin + ve)	Lesion extended (skin + ve)
(3) Mongrel (1 year, female, 7 kg)	Ear lesion (skin, b.m. + ve)	Disseminated lesions (skin, b.m. + ve)
(4) Mongrel (1 year, male, 10 kg)	Lip lesion (skin, b.m. + ve)	Hair replacement, lesion extended, second lesion (skin, b.m. + ve)
(5) Pekinese (2 years, female, 4 kg)	Lip lesion (skin + ve)	Lesion extended (skin + ve)
(6) Mongrel (3 years, female, 5 kg)	Skin lesion (skin, b.m. + ve)	Hair replacement, lesion extended and multiple (skin + ve)
(7) Mongrel (3 years, male, 6 kg)	Ear lesions (skin, b.m. + ve)	Lesions extended (skin + ve)

¹All animals died or killed between Days 41 and 85.

²b.m., Bone marrow; by microscopy of Giemsa-stained impression smears.

In two animals where there was disseminated dermatitis, minor clinical improvement was seen, with partial loss of hair and replacement by healthy hair. This improvement did not occur in all animals and was much less dramatic than that obtained with aminosidine (Vexenat et al., 1997). Single lesions deteriorated after this schedule of buparvaquone, and lesions became more widespread. Dogs died with progressive canine VL or were killed to prevent suffering.

Whereas we cannot exclude efficacy of Butalex® at higher doses or with more prolonged treatment, the results of this limited trial are not encouraging. We conclude that Butalex® has no role in the treatment of symptomatic canine VL.

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