STUDIES IN THE EPIDEMIOLOGY AND SEROEPIDEMIOLOGY OF VISCERAL LEISHMANIASIS IN IRAQ

by

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ABSTRACT

A defined population was studied over a period of 7 months to elucidate the epidemiology of visceral leishmaniasis in Iraq, using serological methods as a screening test. Seroepidemiological methods were also used in the search for a canine reservoir of infection and the tests used were studied in defined animal systems and in confirmed human cases.

A cross-sectional survey was made of the population of a defined rural area of 300 km^2, south of Baghdad. It included 19 villages with 1,171 houses and a total population of 9,889. Houses were mapped and a census completed. The 3,403 persons under 7 years of age were screened using two serological tests for visceral leishmaniasis: indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assays (ELISA). Seropositive children were fully examined clinically and by the leishmanin test. The blood picture and serum proteins were determined and, in the absence of clinical signs, follow-up was by monthly serological examination. Symptomatic children were admitted to hospital for bone marrow biopsy. Results showed a range from subclinical cases defined only by sero-conversion through to severe disease needing hospital treatment and with a high mortality.

A repeat survey of the same child population after 7 months showed serological changes following the main transmission season.

66 of the parasitologically confirmed sero-positive cases from this area and elsewhere in the endemic region were examined, and in some, monthly serology was determined at domiciliary follow-up. IFAT was found to be more sensitive than ELISA. 33% of cases of visceral leishmaniasis were found to revert to negative within 9 months of treatment.

435 hospital inpatients with a variety of diagnoses were studied to determine the specificity of tests. ELISA was found of greater specificity than IFAT. These cases included 124 clinically suspected leishmaniases of which 45 were subsequently culture-positive.

A longitudinal serological study was carried out in inbred mice of varying genetically determined susceptibility to infection. All innately susceptible mice were seropositive by day 50 and the titre continued to increase until the end of the experiment at day 130 regardless of the parasitological course of infection.

In a search for the postulated canine reservoir of visceral leishmaniasis 151 jackals and 65 dogs, largely strays, were studied parasitologically and serologically. Neither from these nor from a limited sample of rodents could the parasite be isolated, though several jackals were seropositive.

The results clarify the epidemiology of visceral leishmaniasis but do not demonstrate an animal reservoir unequivocally. They do however define criteria which any satisfactory quantitative hypothesis of transmission needs to fulfil.

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INTRODUCTION

Visceral leishmaniasis in Iraq was considered as a problem which needed more study to clarify some epidemiological aspects of the disease. Yearly, around one thousand cases were registered from children's hospitals, but real attack rates were unknown. The only method of diagnosis was parasitological and most of the cases were diagnosed on a clinical basis only and treated accordingly.

A research programme was undertaken to make an epidemiological study among a defined population over one year to find out the real attack rate of the disease, and to find the real number of children affected regardless of the severity of the clinical picture.

Also use of simpler diagnostic methods, such as the serological methods, needed exploration. Indirect immunofluorescent antibody test (IFAT) and Enzyme linked immuno sorbent assay (ELISA) were used for the diagnosis and detection of the cases.

Study on the possible animal reservoir in Iraq by trying to isolate the parasite from them and studying them serologically and epidemiologically was also undertaken. These and other matters such as a longitudinal serological follow up of infection of genetically different inbred mice with Leishmania donovani are taken up in this study.

The original focus of the work was intended to be on the use of serological methods in the search for the animal reservoir, but the need to define the epidemiological situation in man and some severe practical limitations on animal work in the defined area led to change of focus towards the human population.

CHAPTER I

REVIEW OF THE LITERATURE

In view of the epidemiological complexity of leishmaniasis and the vast literature on it (WHO Leishmaniasis reference lists, WHO/Leish/67.3 and WHO/Leish/67.4), the present literature review concentrates on the work from or directly relevant to the visceral form in Iraq, preceded by a brief historical note on the early basic work.

The published work will be reviewed under the following headings:

A. Epidemiology, which will include
   1. The epidemiological pattern of the disease in man.
   2. Vertebrate reservoirs of the infection.
   3. The insect vector.

B. Artificial infection of man and animals.

C. The human clinical picture.

D. Immunological responses in man.

E. The laboratory investigation of leishmaniasis in particular:
   1. Isolation and maintenance of the parasite.
   2. Serological procedures.
   3. Leishmanin testing.
   4. Skin windows.

A. EPIDEMIOLOGY

1. Epidemiological pattern of the disease in man.

a. Review of the routes of transmission.

In the following discussion probable routes of transmission such as oral, blood, marital and various insect vectors are discussed.

The elucidation of the mode of transmission of Leishmania donovani took a prolonged period: nearly 30 years elapsed between the discovery of the parasite in 1903 (Leishman, 1903; Donovan, 1903; Laveran and Mesnil, 1903) and the demonstration by Shortt et al. (1928, and Shortt and Swaminath 1931) that sandflies were effective vectors. Some picture of the confusion surrounding the mode of transmission can be gathered from the review by Archibald (1922) in the middle of this period.

Archibald (1922) discussed methods of transmission of visceral leishmaniasis. As to the possibility of oral route infection he referred to the successful infection of monkeys by careful feeding experiments with material containing leishmania parasites; to similar work by Basile (1920) in Europe; and to Fantham and Porter's (1916) successfully infecting some vertebrate hosts by feeding them fleas, lice and other blood-sucking insects infected with herpetomonads. He excluded the role of intestinal helminths as agents of transmission.

As to the possibility of insect vector transmission, he mentioned the work of Patton (1907, 1908) in India who claimed that bed bugs, Cimex hemiptera (= rotundatus), were the vector because the parasites could be demonstrated in experimentally-fed bugs. However, these bugs failed to transfer the infection by biting either a sterile culture medium or an animal. He also mentioned the work of Franchini (1911) who claimed, but never proved, that L. donovani can live and develop in the digestive tract of Anopheles maculipennis. Fleas, -specially the dog flea, Ctenocephalides canis, and the human flea, Pulex irritans, were also suspected vectors of the disease in the Mediterranean but proof was again lacking. Experiments with Phlebotomus flies (P.minutus) and ticks (Ornithodorus savignyi, O. moubata and Rhipicephalus

sanguineus)also yielded inconclusive evidence.

Lamborn (1955) presented evidence supporting the hypothesis that a particular group of muscid flies with blood-sucking habits (Haematophagous flies) were vectors of cutaneous leishmaniasis. He suggested a remote possibility that they may also be concerned with the transmission of visceral leishmaniasis.

Transmission by insects was subsequently discussed by Adler and Theodor (1931d). He stated that although parasites could be demonstrated in blood cultures of nearly ~~of nearly~~ all kala azar cases in the Mediterranean, they were sufficiently scarce to exclude mechanical transmission by insects through interrupted feeding. The relatively frequent occurrence of the disease in infants under twelve months suggested that transmission could occur in the absence of active interference on the part of the vertebrate host, i.e. directly through the bite of the insect (Adler and Theodor, 1931a). In spite of the high positivity of blood cultures of infected children, it is unusual to find infected macrophages in blood smears, even in heavily infected cases of kala azar (Adler and Theodor, 1935). Adler and Theodor (1931a)found that a number of infections were produced in P. papatasi with parasite concentrations of more than 400 - 800 parasites per cubic centimetre of blood. However, since this parasite concentration is never found in infected people, P. papatasi could be excluded as a carrier of the disease in Italy.

However, Adler and Theodor (1931d) concluded that since 91.7% of infected children have positive blood cultures, then infection by blood-sucking insects is not unlikely. The search for an insect vector was thus limited to blood-sucking insects which have a sterile alimentary canal and whose distribution bears some relationship to that of the disease. The blood meal contains a substance lytic for leishmania (Adler and Theodor, 1957). This factor, one of the gammaglobulins, is inactivated after ingestion in some species, e.g. Phlebotomus papatasi. In addition to blood, sandflies also ingest cells lying in the dermis outside the blood circulation, e.g. macrophages.

The Leishmania species parasitic in the blood of vertebrates show the typical anterior position in sandflies, e.g. L. tropica

in Phlebotomus papatasi and P. sergenti, L. donovani in P. argentipes. Other species, L. infantum, L. caninum, L. braziliensis and L. tarantolae, when introduced in sufficiently large numbers into P. papatasi either remain and multiply in the stomach, or, in heavy infections, ascend the cardia; in the case of L. infantum flagellates may even enter the buccal cavity and, in the case of L. tarentolae and L. canium, they may be found in the pharynx in large numbers. In no case do they show a posterior position (Adler and Theodor, 1929a).

Wenyon (1912) found sandflies which were naturally infected with leptomonads. Shortt et al. (1928) showed that the sandfly, P. argentipes, was a vector of kala-azar in India.

Swaminath et al. (1942) succeeded in infecting P. argentipes by feeding them on infected human cases and then transmitting the disease by feeding them on uninfected human volunteers.

The isolation of a strain of Leishmania from a sandfly presumed to be P. martini has been recorded in Kenya by Heisch et al. (1962). When this isolated parasite was injected into human volunteers, kala-azar was produced. P. martini was also easily infected from these human volunteers at the parasitic stage or after signs and symptoms had appeared (Minter et al., 1962 ; Manson-Bahr and Southgate, 1964). Thus P. martini is the most likely vector of kala-azar in Kenya.

Adler and Theodor (1957) stated that in the Mediterranean Phlebotomus sandflies feed on mammals whereas Sergentomyia sandflies feed mainly on lizards (geckoes). Adler and Theodor (1932) stated that P. perniciosus were infected with Leishmania by feeding on the unbroken skin of three dogs.

In the literature less frequent routes and modes of infection were described and discussed. Adler and Theodor (1931a) excluded the oral route of infection epidemiologically on the ground that the disease is rare in thickly populated urban slums where hygiene is low. Adler and Theodor (1931d) showed that family infection is rare, as are house to house infections, and the disease is rare among urban poorer classes in the centre of large cities situated in endemic foci. They concluded that the disease could not be transmitted by oral infection or by direct contact.

Forkner and Zia (1935) unsuccessfully tried to infect two human volunteers and some hamsters by repeated inoculations via oral and nasal routes using nasal secretions from patients with kala azar. Chung et al. (1948) reported that two children, four and six years of age, developed kala azar nine to ten months after having had a blood transfusion. Their mother was the donor and she had few symptoms until she was hospitalized and the parasite was isolated from her bone marrow.

In East Africa there was a case of marital transmission of leishmanial infection from the husband to the wife who developed a genital sore caused by the parasite. Sen Gupta (1962) suggested that transmission was due to the presence of parasites in the skin.

All these studies proved that the usual mode of transmission of visceral leishmaniasis is by the sandfly vector, although other routes had been described in the literature.

b. Historical review of visceral leishmaniasis in Iraq.

Kala azar was not described as an entity in the writings of the ancient civilisations of Mesopotamia. If it was present it could have been included under fevers in general. The first report of kala azar in Iraq came from Kulz (1916) who diagnosed nine cases from Baghdad by splenic puncture. The age of these cases was not reported. Contrary to later experience Kulz equated the disease with the Indian type: furthermore his report remained isolated and unconfirmed for over 30 years.

In a survey of people and dogs in Nassiryah Province, in 1918, Patton excluded the presence of the disease and suggested that the recorded cases were imported from a focus outside the country (Chadwick and Machattie, 1927). Cases were reported to the Military Health Authorities at that time (Pringle, 1956). Sprawson (1919) reported cases among military personnel coming from known foci of the disease in India. Intervals of 8 - 16 months were described between the patient leaving India and the onset of his illness in Mesopotamia. Sprawson reported that neither he nor the local doctors had seen a case of kala azar among the indigenous inhabitants. Brahmacharia (1928) was also doubtful about the presence of kala azar in Mesopotamia.

Dr. Ali Al-Hamami in 1940-41 (unpublished) carried out sternal punctures on children with signs and symptoms similar to those of kala azar but failed to find the parasite (unpublished, referred to in Prirgle, 1956).

During 1953-55 the first proven kala azar cases since the work of Kulz (1916) were reported in Iraq. Bashir (1954) and Kirchmair (1955) reported six proven cases from northern Iraq. Kirchmair (1955) stated that kala azar (or 'intestinal leishmaniasis') was first discovered in Mosul Province, northern Iraq, in 1953 but had now also been found in the vicinity of Baghdad. He reported the successful treatment with solustibosan of three infantile cases.

Tajeldin and Alousi (1954) reported four children with kala azar proven by biopsy in Baghdad. They considered the disease to be endemic in this province (Bray et al., 1967). Pringle (1956) reported a further 12 cases mainly from central Iraq. Tajeldin and Al-Hassani (1961) reported 100 parasitologically proved cases. During 1965-67 Bray and Dabbagh (1968) reported 59 parasitologically confirmed and 55 clinically diagnosed cases. For the period 1963-67 Tajeldin et al. (1969) reported 78 cases and stated that the disease was more acute, with lower relative lymphocytosis and less marked hepatosplenomegaly. Halawani and Guirges (1973) stated that kala azar had not been common in Iraq in the past but was on the increase in recent years. They went on to say that foci of the infection were found in the lowland periurban areas where there are scattered family units surrounded by fields and that the disease especially affected infants and small children.

Kala azar in Iraq could have been imported during the First World War from foci outside the country. This hypothesis is supported by the age shift to the young, the present endemicity picture and the fact that the cases reported during the First World War were all soldiers coming from countries with known foci of the disease.

The alternative hypothesis is that the disease was present in natural zoonotic foci. With the reforming of land and the increase in urban development these foci were disturbed and thus the population became more exposed. In addition, the increased number of

Health Centres and medical personnel, together with general public awareness, led to a gradual increase in the number of diagnosed cases.

c. Summary of cutaneous leishmaniasis in Iraq.

A short review of the situation of cutaneous leishmaniasis was felt necessary to be introduced at this stage to give a clear picture of the situation in regard to its importance and its relevance to the study of visceral leishmaniasis in Iraq.

Historically the first mention of cutaneous leishmaniasis in Iraq was in "Khulaset-El-Tegarib" by El-Razi published around 1500 A.D. The author states "the sore is extremely common in Baghdad and the people call it Baghdad lozenges" (Rahim and Tatar, 1966). Later Southgate (1840) described the facial disfigurement of Baghdad children which was caused by Baghdad boil. Pringle (1957) described some historical and epidemiological aspects of this disease. He reported the distribution of the disease to be mainly in crowded urban areas.

Table 1: The number of officially recorded cases of cutaneous leishmaniasis in Iraq for the period 1929-1976 according to the Statistical Compass of the same years:

Year	Number	Year	Number
1929	3190	1942	7698
1930	4444	1943	9915
1931	4601	1944	9117
1932	4580	1945	10502
1933	5888	1946	8234
1934	9046	1947	7053
1935	10799	1948	2910
1936	12069	1949	7476
1937	11262	1950	10433
1938	11759		
1939	9054	1974	426
1940	6332	1975	268
1941	7840	1976	237

It has been reported recently that both forms of cutaneous leishmaniasis occur in Iraq. Leishmania tropica minor, which causes the dry form of the disease, occurs in urban areas (Rahim and Tatar, 1966) whereas Leishmania tropica major, which causes the wet form of the disease, occurs in rural areas. The two strains were identified by isoenzyme assays (Al-Jeboori and Evans, (1980a and 1980b).

The disease used to be found in known foci in five of the 18 provinces in Iraq. These provinces are in central and northern Iraq (Mosul, Baghdad, Kerbala, Muthanna and Misan). At present it seems that some of the old foci have ceased to function and new foci are appearing.

The number of recorded cases has decreased very sharply in recent years, as shown in Table 1. This could be due to residual insecticide spraying by the Malaria Eradication Programme and/or regular city fogging with insecticides. In 1950 about 10,000 cases were recorded whereas in 1976 only 237 cases were recorded of which two were in Baghdad.

Cutaneous leishmaniasis mainly affects children and adolescents. The number of cases increases sharply in the over one year olds, reaches a peak in the two-to-nine year olds and declines in older age groups.

The disease usually appears between November and March.

The reservoir of cutaneous leishmaniasis in Iraq appears to be canine. Chadwick and Machattie (1927) found canine dermal leishmaniasis to be widely distributed and common in Iraq. Adler and Theodor (1932) reported that natural infections of dogs with L. tropica were common whereas canine kala azar did not occur. Machattie et al. (1931) also reported cutaneous leishmaniasis in a single cat.

Fifty years has now elapsed with no other positive finding among possible animal reservoirs having been reported, despite great efforts having been made (Pringle, 1957; Bray et al. 1967; Bray and Dabbagh, 1968; Tajeldin et al., 1971; Sukkar, 1974; and others).

Adler and Theodor (1929b, 1930a) showed that Phlebotomus sergenti was the vector of cutaneous leishmaniasis in Iraq. This was confirmed by Pringle (1957).

The clinical manifestations of visceral leishmaniasis in Iraq are slightly different from those in other countries. In India or Africa kala-azar may be followed by post kala-azar dermal leishmanoid; this is unknown in Iraq. Similarly the disease has not been reported to follow a dermal lesion (leishmanioma) as described by Sen Gupta (1962) and Schilirio et al. (1978).

In the past three years in which I have been working on kala azar in Iraq I have only seen one case of confirmed kala-azar in which skin lesions had developed. This was in a five year old child. These lesions were papular (about 1 cm diameter), mainly distributed on the face with a few on the chest and both arms, and were covered with fine, silvery scales. Some of the lesions had faded, leaving slight darkish discolouration of the skin. The lesions developed two to three weeks after treatment and were still apparent two years later. A biopsy was taken from a lesion and examined. The parasite was not detected in either impression smears or in cultures. Histopathological examination showed extensive chronic non-caseating granuloma with marked lymphocytic infiltration. Special stains were negative for microorganisms. Thus the lesions were not due to post kala azar dermal leishmanoid.

Not a single case of kala-azar has been reported with either an active skin lesion or with a scar from previous lesions of cutaneous leishmaniasis (Nouri and Al-Jeboori, 1973; Bray et al., 1967). The two diseases do not appear to overlap (Sukkar, 1976c).

In my work during the past three years I have not noticed any cas. of kala-azar with an ulcer or scar due to cutaneous leishmaniasis.

In summarising what had been discussed above it is possible to conclude that cutaneous leishmaniasis in Iraq has been decreasing to low levels for the past decade. This was specially the case in Baghdad province where the present study has been done. Also there is practically no case of post kala-azar dermal leishmanoid lesions reported and and no cases of visceral leishmaniasis. has a scar or an active lesion of common leishmaniasis appeared.

d. Age distribution

There are now considered to be four different nosogeographical types of visceral leishmaniasis in the world:

(a) Indian

(b) Mediterranean - Middle Asian

(c) East African

(d) American (Moskoviskij and Southgate, 1971)

Lysenko, 1971

This discussion will be restricted to the Indian and Mediterranean types. These differ mainly in the age distribution of those affected and in the existence of an animal reservoir. Moreover the Mediterranean type always occurs in an endemic state, never in epidemic outbreaks, except for the rare situation reported by Pampiglioni, (1974) of the sudden outbreak of the disease affecting a large number of people in North Italy in 1971-72 of all age groups.

In the Indian type of visceral leishmaniasis children constitute less than 25% of the cases (Chatterjee and Hagens, 1953). In the Mediterranean type of visceral leishmaniasis children are mainly affected and the disease is rare in adults. Adler and Theodor (1931a) reported that cases were rare among three - six month old infants. Adler (1940) reported 94% of cases to be under 10 years old with 80% being under five years old. He further reported (1963) 90% of cases to be under two years old.

In Iraq visceral leishmaniasis seems to be of the Mediterranean type with respect to age distribution. The youngest cases reported were four month old infants (Tajeldin and Al-Hassani, 1961; Bray et al., 1967) and a two month old infant (Nouri and Al Jeboori, 1973). Tajeldin and Al-Hassani (1961) stressed that there were no cases older than eight years of age. The cases ranged between six months and eight years old. Tajeldin et al. (1969) and Bray et al. (1967) reported two thirds of cases to be under two years old. The cases ranged between four months and six years of age. Nouri and Al-Jeboori (1973) and Halawani and Guirges (1973) reported 45%-50% of cases to be less than one year old with the disease being rarely found in children over ten years. The latest reports from the analysis of cases since 1976 showed that kala azar affects the under seven year olds. Sukkar (1976a, b and c).

Various explanations concerning age distribution in Mediterranean visceral leishmaniasis have been put forward; these included variable resistance to infection, immaturity of the immune system, acquired immunity due to previous exposures, effect of certain factors like nutrition on the state of immunity and causes due to vector habits.

Garnham (1963) discussing the immunity to protozoa confirmed that natural immunity is greatly influenced by the age of the host and the general rule is that the older the animal the less is its susceptibility to a parasite. The lack of immunity in youth is sometimes ascribed to immaturity of the body's defences, but he considered it was doubtful that this is the explanation because some pathogens may affect older animals while their young are resistant (Babesia bigemina and relative immunity of the calf; the adults are fully susceptible).

Taub (1956) found that tissue sera showed a lytic effect towards Leishmania infection which was seldom noticed in sera of children less than 5½ years old and was always present in the over seven year olds. He suggested that the presence of lytic factor in human sera corresponded to the age distribution of infection. This suggestion was not confirmed by Ben Rachid (1967) who found that all human sera had this lytic effect.

Adler (1940) stated that the disease is rare in adults, not because of immunity due to exposure to the disease in infancy, for the number of cases occurring in adults coming from non-endemic centres visiting endemic areas is few and*they are seldom attacked even after prolonged residence in endemic foci. Longo (1910) has suggested that Mediterranean adults are immune to Leishmania infection.

But this was contradicted by the reporting of adult cases getting infected after residing in endemic areas. The following are examples:

In Turkistan and the Caucasus the epidemiology resembles that found in the Mediterranean basin and differences such as the relative incidence of human and canine infections can be ascribed to differences in the bionomics of the local vectors. In part of Asiatic Russia man becomes an incidental host if he invades uninhabited territory occupied by a reservoir of infection and a vector. This

occurred in Tadzhikistan in 1950 and 1951 where outbreaks occurred in workmen opening up new territories.

Armstrong (1945) described two adult cases among soldiers coming back from North Africa. Both were proven parasitologically to have kala azar long after returning home, during the period of which they were asymptomatic, only slight fever but no toxaemia.

B.ada (1979) found a case of kala azar in a 51 year old male by bone marrow examination. He had no fever, his liver was enlarged, he had splenectomy when 7 years old after kala azar infection, he was living in an endemic area, blood picture was normal. This implies that the patient had run a subclinical course of 40 years.

During the Second World War cases of kala azar acquired in the Mediterranean region were common among British and American troops coming from non-endemic areas.

In Iraq adults were affected. Rassam and Al-Jeboori (1973) stated that kala azar in Iraq is primarily of the Mediterranean infantile type but that it occasionally affects adults. They described a parasitologically proven case of kala azar in a 22 year old adult male.

Pringle (1956) recorded two adults having kala azar, one aged 30 years, the other 50 years. Bashir (1954) described a 14 years old parasitologically proven case.

During 1979 an adult girl in her twenties was recorded to have kala azar from Mosul Province in the north of Iraq (personal communication).

Southgate and Oriedo (1967) stated that since kala azar confers lifelong immunity to reinfection, and true second attacks are virtually unknown, the at-risk population will be the newborn and immigrant non-immune.

To confirm that point was the explanation of Lainson and Strangeways-Dixon (1963) that the age of the host however does not seem to be an important factor. In man, where previous exposures can be certainly ruled out infection with either cutaneous or visceral forms may occur at any age. Thus in the Mediterranean where it is called infantile anaemia and in Brazil and China kala azar is a

disease of the young, but in India and the early Kenya outbreak (Fendall, 1953) and in the Sudan (Van Peenen and Reid, 1963) adults are infected. The paper of Southgate and Oriedo (1962) gives a reasoned analysis of the age distribution for Kenya, where newly invaded communities show a different age incidence than areas that have passed from epidemic to endemic status. Southgate (1964) discussing the degree of endemicity in Tseikuru where it was high for several years, states that the present human distribution is affected by immunity as well as by the relative exposure to infection.

Nutrition may affect the age distribution. Adler and Theodor (1931a) stressed that few cases occur among the well nourished children of the wealthier classes, who usually escape the disease even when residing in an endemic focus, which could be due to better housing.

et al.(1974)

Pampiglioni/discussed the occurrence of kala azar in the Mediterranean region and noticed that the disease is usually found in asymptomatic forms along with the typical cases; they mentioned that after famines and stress more cases occur.

One would be forced to think here at this stage about the nutritional status of children affected and the stress they have with the cessation of breast feeding and starting of eating food whether it affects or contributes to the incidence of kala azar.

Yet another explanation was discussed by Adler and Theodor (1930b). Using the Hertig apparatus they proved that Leishmania infantum can leave the biting parts of P. perniciosus during the act of biting and enter a new host only in the absence of any active interference on the part of the latter. They suggested that this accounts for the main peculiarity of Mediterranean kala azar, that is the relative frequency in infants less than 12 months of age.

Bray (1974) discussing the problem remained equivocal, stating that as it was not known if children were preferentially bitten by infected sandflies or whether older people are in some way better able to control the infection, the latter seems more likely as no one had noted any preferential feeding upon children by P. ariasi or P. major.

So probably repeated exposure of children to the disease may develop into a spectrum of clinical manifestations ranging from the typical disease with the usual signs and symptoms and severity with the high mortality to the less marked subclinical infection where the disease could pass unnoticed. This picture may be complicated by the additional stress of the nutritional status of children at the weaning time and before that.

e. Incubation period.

The incubation period could be judged from:

1. Youngest age groups reported by Adler & Theodor (1931a) suggest that incubation periods of less than 3 months are exceptional, as cases are very rare in infants of 3 - 6 months of age. Bray et al. (1967) reported the youngest case in Iraq to be four months. Nouri and Al-Jeboori (1973) registered the youngest case in Iraq to be 2 months.

2. In experimental infections of man by Swaminath et al. (1942), Adler (1964) and Manson-Bahr and Southgate (1963) the incubation period was around four months. Sprawson (1919) reported an incubation period of 8 - 16 months. Schilirio et al.(1978) reported a six year old girl who developed visceral leishmaniasis 60 days after having a cutaneous lesion which was found to be positive with Leishman-Donovan bodies.

f. Sex

The only reference about real positive findings in sex distribution came from Southgate (1974) in Kenya, where he found male adults gathering around termite hills, the microhabitat of the vector P. martini,were affected more than the women staying at home. This habit of the male population during the peak time of activity of the sandflies exposes them to the risk of biting by the vector more than the females.

However, the situation in Iraq is different due to the age affected. In Iraq children are mostly affected, most of them one year of age, so the factor of movement or habits linked with sex could be eliminated in this case.

Sex distribution in Iraq was mentioned by Tajeldine and Al-Hassani (1961) where they found after analysing 78 cases that males

predominate in 1.8 : 1 proportion. This was found also by other analyses of cases. This could be biassed statistically as the cases were selected for their studies (Sukkar 1972). Another explanation could be due to the greater attention given by parents to their male children. However, in 1976 it was found that both sexes in Iraq were equally affected (Sukkar 1976c).

g. Place

The first striking feature in the epidemiology of the disease is its focality in distribution in space. This is determined by the parasite (parasite vector host) and by environmental conditions, according to which Lysenko (1971) described three types of foci of visceral leishmaniasis: the natural foci where the transmission happens from animal to animal through the sandfly vector, the semi-synanthropic where transmission happens between animals and man through the vector, and the synanthropic where transmission happens between man to man through the vector. This situation, he stated, reflects the evolution of visceral leishmaniasis from a zoonosis to an anthroponosis.

The work of Lanotte et al. (1974) in the South of France is a good example of the microfocality of the disease in the Mediterranean area, a focality which possesses all the characteristics necessary to be classified according to Pavlovsky as natural foci.

As regards altitude, Paradiso (1926) in Sicily analysing the 1424 cases collected, found that none occurred above an altitude of 733 metres, the large majority of cases occurred between the sea level and 214 metres above sea level.

In Catania Professor Longo and his colleagues, referred to here by Adler and Theodor (1931a) have made a careful survey of the distribution of the disease which was found to be confined almost entirely to the periphery of the town, while the thickly populated centre is hardly affected.

Adler and Theodor (1931a) clarified the condition further by stating that house to house infections are not the rule, there is usually an interval of 30 metres or more between one case and another, the disease is commonest among the rural, the poorer rural or semirural population.

Coradetti (1964) discussing the knowledge about areas affected

by kala azar stated that the development of such knowledge may give rise to the wrong impression that kala azar was progressively spreading in new areas during the last sixty years, in which it is certain that the disease was indigenous, although not recognised. Another factor which affects a more precise knowledge of the actual incidence of the disease in the whole Mediterranean is the wrong conviction that kala azar only occurs in well known foci of endemicity.

In Iraq its distribution has been noted and discussed by Tajeldin and Al-Hassani (1961) and Tajeldin et al. (1969)that most of the cases come from rural areas outside towns and cities, mostly (in his experience) from the outskirts of Baghdad city and the rural areas surrounding the nearby villages and towns; city inhabitants were rarely affected.

Bray et al. (1967) discussed the distribution of the disease and found out that the distribution is in rural and periurban areas; these areas are small farmers' villages under irrigation and largely growing vegetables. The incidence is spotty, only very occasionally familial and well distributed in time and place.

Tajeldin and Al-Hassani (1961) disagreed with Kirk's (1949) result in the Sudan. They found out by analysing a series of cases that there were no definite familial infections.

Later analysis of some of the registered cases of kala azar in Iraq showed that they come from rural and semiurban areas. (Sukkar 1976c).

This distribution of infantile kala azar around Baghdad coincides with a heavy distribution of the jackal and the disease incidence in children is consistent with a feral reservoir (Bray and Dabbagh 1968).

As regards altitude, cases described by Bashir (1954) in the northern region came from altitudes below 2500 feet.

So in conclusion visceral leishmaniasis in Iraq is focal in distribution and mostly affects children living in the rural and periurban areas and is usually found in parts of the country which are considered as lowlands.

h. Time

In Iraq distribution in time shows a peak period of acute illness from December to May (Bray et al.1967) suggesting a transmission season of 4 - 6 months earlier(Halawani and Guinges) 1973)with a decrease in the number of cases during the summer months (Tajeldin et al. 1969).

Nouri and Al-Jeboori (1973) after studying the monthly distribution over the whole country found out that "There were two peaks of transmission, one in December and another in March" (see below also).

2. Vertebrate reservoir of infection.

a. Reservoirs in other countries.

To clarify the problem of transmission of the visceral leishmaniasis as regards the reservoirs an attempt was made by Moskovskij and Dukhanina in 1971 to differentiate types of leishmaniasis foci into the following:

1. Anthroponoses - man to man.
2. Zoonoses - between vertebrate and man (in Russian usage: zooanthroponosis); and this could be
   a) naturally nidalic diseases where the causative agent is harboured by wild animals;
   b) anademic diseases in which the sources of infection are synanthropic animals (domestic).
3. Anthropozoonoses - man to vertebrates.

Further they classified the disease according to the sequence of infection of man and animals in nature as follows:

1. Paraxenoses in which man and animal occupy a parallel position in the transmission cycle;
2. Metaxenoses in which man and animal are infected in sequence.

i) Man as a reservoir of visceral leishmaniasis.

In East Africa the work of Minter and Wijers (1963) proved that sandflies will not probe or feed on post kala azar dermal leishmanoid lesions but move on to normal skin. So it is unlikely on these grounds that these cases are significant as a reservoir of infection.

Southgate and Oriedo (1962) found in East Africa that human patients form a good reservoir for sandflies from 4 months after infection until diagnosis, an average period of at least 7 months. In this respect kala-azar in East Africa resembles the Indian (Swaminath et al., 1942) and South American disease and differs markedly from the Mediterranean kala azar which is not infective for sandflies. Manson-Bahr and Southgate (1964).

In the Mediterranean man can be considered quite an incidental host for L. infantum for at least two biological reasons, in the opinion of Coradetti (1964):-

1. Man is practically unable to act as a suitable host for further spreading of the parasites, as the occurrence of these in the bloodstream, although sufficient sometimes to permit cultures, is not atle to produce infection in the vectors.

2. The parasites develop in internal organs, but apparently never in the dermis.

In these conditions sandflies cannot get infection from man, that is why its distribution is sporadic in space and time. Coradetti (1964).

Adler (1964) stated that the disease (infantile kala azar) is disseminated by sandflies feeding on infected dogs and human cases are much less important as a source of infection.

ii) Canine visceral leishmaniasis.

Different studies on canine visceral leishmaniasis were made in different areas of the Mediterranean and dogs were found infected naturally. The epidemiology and pathology of canine visceral leishmaniasis were also studied. The following are examples:

Adler and Theodor (1931a) found that canine kala azar is definitely a seasonal disease. Cardamatis (1911) in Athens found a prevalence of 40% in June and 7% in January, and he concluded that canine kala azar is a seasonal disease which shows no important annual fluctuations.

In Rome the percentage of infected dogs reached the maximum of 6.2. in November 1934, but fell immediately to 0% in the

following December (Marchesi et.al., 1935). Manson-Bahr and Southgate (1964) suggested the possibility that these animals have brief periods of parasitaemia from time to time with fluctuations in their state of premunition and can infect sand-flies at these times.

Buxton (1923) conducted an examination on 156 stray dogs during 12 months in Jerusalem in Palestine; all were negative for leishmaniasis.

In 1946 Adler and Tchernomoretz treated 7 cases of canine visceral leichmaniasis in dogs in Palestine. They state that "even in cases which no parasites were found in spleen smears after intensive treatment with neostibosan and stilbene it was possible to obtain a culture from splenic juices, and parasites could be demonstrated in smears of lymphatic glands and in sections of the normal unbroken skin".

Poul and Pallas (1962) in Algeria stated that Leishmania may persist in dogs long after treatment and after symptoms have disappeared.

In Algiers and Tunis dogs were found infected. 27% of 60 stray dogs in Rome were infected. (Basile, 1910, 1913). Brahmacharia (1928) reports that Puerto (1910) examined 165 dogs in Catania and found four infected; but also in Catania, in the largest of all endemic foci. Pulvirenti (1911) found no infection in 227 dogs and Caronia and Di Giorgio (1914) found none in 1005 dogs, as reported by Adler and Theodor (1931a).

In Malta Critien (1911) examined 52 dogs; he found seven infected. In the same year he found 10 out of 83 dogs infected; Wenyon (1914) found 6 out of 46 infected; Adler and Theodor (1932) found 11 out of 100 stray dogs in Malta infected by examining the spleen.

In Athens Cadamatis (1912) found 13.8% of dogs infected.

In India no dog has been found infected. (Brahmacharia 1928).

Berberian (1959) examined 25 dogs in Kessal, a north Syrian village bordering the Sandjak province of Turkey; he found

six dogs which showed Leishmania in the skin and viscera (one was emaciated). He concluded that all were carriers of kala azar. He examined in Beirut (1935 - 1947) four typical cases of canine kala azar with visceral and cutaneous lesions; all four were pedigree dogs brought into the city from abroad. 110 mongrel dogs from the municipal pounds of Beirut were all negative for Leishmania.

There was a gradient in the morbidity of dogs from the coast to the highest points of the mountain range in France. This gradient was strongly linked to the distribution and density of the vector P.ariasi.(Lanotte et al. 1978).

Adler and Tchernomoretz (1946) stated that throughout the whole Mediterranean basin the unbroken skin of naturally infected dogs is the reservoir and, as far as present knowledge goes, the only reservoir from which sandflies of the major group infect themselves with L. infantum and transmit the disease to dogs and man. P. perniciosus could be infected with Leishmania by feeding on the unbroken skin of dogs naturally infected (Adler and Theodor, 1932) because the organisms thrive at the lower temperature of bare skin where the fly is able to bite. (Bray, 1975).

Adler and Theodor (1935) found out that infection rate in the sandfly Phlebotomus perniciosus increases progressively as the infection of the skin becomes more intense. The infection of the skin seems to be uniform; at least one infected macrophage occurred in every 1000 sq $\int^{H}$ of the skin.

The histological changes in the skin in canine kala azar can be briefly summarised as a selective infiltration of macrophages round hair follicles including the sebaceous glands, and the presence of infected macrophages in normal dermis. The latter phenomenon in the complete absence of secondary infiltration of round cells and plasma cells is the most striking characteristic of canine kala azar and differentiates it from L. tropica infection. Adler and Theodor (1932).

There are important differences between the pathology of the human and canine disease of visceral leishmaniasis. In the former, infection of the skin and keratitis is rare, while the

spleen is constantly enlarged. Adler and Theodor (1935) and Adler (1936).

Other than the skin the parasite could be found in other organs and tissues Adler and Theodor (1935). Parasites in the spleen of an infected animal may be so few as to be overlooked even after a prolonged examination of smears, although sandflies fed on the same animal become infected. Histological examination of the skin would fail to detect the infection in some animals on which sandflies P. perniciosus infected themselves to the extent of 20%. Adler and Theodor (1935).

In view of the migratory habits of infected cells and their capacity for passing through soft tissue (e.g. the conjunctiva) the discharge of infected cells in excretions (from the mouth or nasal discharges or urine) of heavily infected animals appears to be inevitable. Adler and Theodor (1935).

Marchesi et al.(1935) found the abundance of parasites present in the organs does not correspond with the macroscopical changes in the skin, and indeed when these changes are found the quantity of Leishmania present in the tissues is less.

Nicolle and Compte (1908) following their discovery in 1908 stated that a dog infected with Leishmania presented a clinical appearance and pathological changes very similar to those displayed by man.

The main symptoms recorded from naturally infected dogs are emaciation, anaemia, keratitis, scaling of the skin and depilation. Adler and Theodor (1932).

Donatien and Lestoquard (1929) have pointed out that heavily infected animals occasionally show no signs of the disease which may be discovered only at autopsy. Yakimoff and Kohl-Yakimoff (1911) in Tunis and Yakimoff (1915) in Turkestan recorded depilation in naturally infected dogs.

Chodukin and Schevtschenko (1928) recorded cutaneous ulcers, the presence of Leishman-Donovan bodies in the sebacious glands of hair follicles of apparently healthy dogs with visceral leishmaniasis in Tashkent. Strains of visceral Leishmania were isolated from a sick child and a spontaneously infected dog in

Turkmenia. Belova (1971).

Ngoka and Mutinga (1977) reported a domestic dog in Kenya with visceral leishmaniasis: the spleen was positive in culture and other stained tissues were also infected except for the blood. This warrants the statement that the dog appears to be one of the animal reservoirs of visceral leishmaniasis in Kachelba, Rift Valley, Kenya

In Turkestan apart from dogs, jackals have been found naturally infected.

Petrisheva et al.(undated) report that 33% of jackals have been shown to be positive. Symptomless infections were found in wild jackals in Tadjidsistan. Adler (1964).

Bettini et al. in Tuscany, Italy (1980) through hamster inoculations of material from suspected animals found one fox Vulpes vulpes to be positive with Leishmania when the impression smears of its viscera were negative.

(1978)
Nadim et al./reported a survey conducted in the Caspian area and north eastern part of Iran in 1970. 20 jackals and 10 foxes were shot, examination of smears from bone marrow and spleen showed the infection in a jackal (Canis aureus)and in a fox (Vulpes vulpes). Both infected animals were found in places far from any focus of cutaneous leishmaniasis, therefore the infection probably was due to L. donovani. L. donovani infection has been reported also from one dog in the Caspian area and three domestic dogs near Tehran.

iii) Leishmania in rodents.

Bettini et al. (1978) and Bettini et al. (1980) isolated three strains of Leishmania from Rattus rattus in Italy for the first time. Artificial infection of hamsters with the strain showed a parasite distribution pattern similar to that observed in typical L. donovani infection: direct impression smears of rodents' viscera were negative.

In Europe wild rodents have long been suspected of being natural reservoirs of both visceral (Hoin, 1963) and cutaneous leishmaniasis (Adler, 1962; Coradetti, 1962). L. donovani

has so far been isolated from R. rattus in Yugoslavia only. Petrovic et al. (1975). Elsewhere Leishmania has only been found in R. rattus in the Sudanese town of Malakal (Hoogstraal et al. 1963).

Haile and Lemma (1977) reported the isolation of Leishmania parasites from Arvicanthus (Nile grass rat) in Ethiopia. Biochemically it seems similar to a Leishmania strain isolated from a patient with cutaneous leishmaniasis in Sudan.

Nile grass rats Arvicanthus niloticus lutosus have been found infected in an area where human kala azar is endemic in Kenya. Manson-Bahr and Southgate (1964).

The parasites isolated from gerbils and the ground squirrel did not appear to be true human L. donovani since they produced only skin nodules and not visceral infections when inoculated into man (Manson Bahr, 1959, 1961a, 1961b).

iv) Leishmania in lizards.

Leishmania in lizards reviewed by Adler and Theodor (1929a) as follows:

1. L. ceramodactyli from Ceramodactylus doriae geckoe in the USSR and has a posterior station in sandflies.
2. L. torentale from Torentale mauritanica geckoe and has an anterior station in sandflies.
3. L. adleri from Latasia longicaudata in geckoes in Kenya and has an anterior station in sandflies.
4. L. hoogstraali in the Sudan.

L. adleri is in the promastigote stage in the lizard; all live in the blood of lizards. Garnham (1971).

Leishmania have been isolated from rodents and lizards in Kenya but apart from the data of Ngoka et al. (1977) no animal reservoir of human infection has been found; it is probable that man is the reservoir in the epidemic area. Manson-Bahr and Southgate (1964).

A large survey of leishmanial infection was carried out by E.M. Belova in the Turkmania USSR (1963-1966). A total of

A total of 3818 lizards and 9 snakes in 16 different areas, 11 species of lizards from 21 species captured were found to be carriers of Leishmania, 11 geckoes out of 56 were found to have promastigotes also. Promastigotes can survive for a long time in reptiles. Belova (1971).

Nadim et al. (1968) found Sergentomyia sintoni caught from Rhombomys opimus burrows in Khorassan area to be infected with leptomonads and they assumed that the infection could be due to lizard leishmaniasis in this area. This sandfly does not seem to play any part in the transmission of mammalian leishmaniasis in the various foci of the north eastern part of Iran.

The rare cases of experimental infection of lizards by leishmaniasis from warm blooded animals can be considered as proof of a remote genetic affinity between reptilian promastigotes and leishmaniasis of man and warm blooded animals. Promastigote strains from reptiles must be considered as non-pathogenic for warm blooded animals. A feature of reptilian infections is the extreme scarcity of parasites in blood smears and in tissue impression smears, but isolation may readily be made in culture media. Belova (1971).

Petrischeva (1971) described the main reservoir of visceral leishmaniasis foci in river valleys to be the gerbils, jackals, foxes, porcupines and possibly hedgehogs.

v) Immunological studies on animal reservoir hosts.

Dedet et al.(1973) studied canine leishmaniasis in Tunisia using a technique of immunodiffusion of canine serum against antigen extracted from cultured promastigotes of L. donovani. Division of the country into 3 bioclimatic zones (dry, semi-arid and humid) (no meteorological or ecological data are given) showed that canine leishmaniasis does not occur in the humid or very dry areas, but significant infections occur in the semi-arid, semi-humid zones (5.6% of 854 specimens examined). A study of previous prevalence rates of canine leishmaniasis in the country suggests that the endemicity of the disease has little changed in the past 60 years. The majority of cases of visceral leishmaniasis are found in the semi-arid areas where Phlebotomus perniciosus is considered to be the vector and where

the maximum canine infection is found.

Hoin et al. (1974) stated that since 1961 sporadic cases of canine leishmaniasis have been found in an area situated 30 km north of Tours and in 1971 systematic survey of dogs was carried out in that area. About ten new cases all closely located were detected by the indirect immuno-fluorescence technique. In some cases the parasites themselves could be observed and the strain was isolated. No human case of leishmaniasis has been reported from that focus yet.

Lanotte et al. (1974) stressed that systematic investigations of wild host reservoirs of leishmaniasis are still very difficult. On the contrary, the domestic host, the dog, is more accessible. After careful study of 3 immunoserological tests (complement fixation, immunodiffusion and immunofluorescence), immunofluorescence was chosen by them. A titre of 1/160 is regarded as significant for operational purposes. Figure 1 summarises the findings among animal reservoirs of visceral leishmaniasis in Asia, Africa and Europe.

b. Work done on possible animal reservoirs in Iraq.

Carnivores (Carnivora) are a common group of mammals in Iraq. Many however, particularly larger ones, were exterminated or are facing extinction. Among the canids (Canidae) the most characteristic inhabitant of the flatland is the Asiatic jackal, Canis aureus which however in all probability does not populate typical deserts and mountains (Harrison, 1968). In some regions it is so common that being active mostly at dusk and by night, it can also be seen even by day. The author had the opportunity to see with the car headlights no less than 3 - 7 jackals within 2 hours of a night trip in central Iraq. Jackals are mostly carrion-eaters and often forage near human settlements. In dry seasons they can be seen feeding on the plantations of water melons, melons and pumpkins.

Another carnivore in Iraq is the red fox (Vulpes vulpes) found all over the country. But everywhere rare is the wolf Canis lupus (Kadhim et al. 1977)

Bray (1975) discussing the epidemiology of visceral leish-

maniasis stated that in order to be an effective reservoir a host must present its parasites to the vector in a readily avail able manner. In dogs with visceral Leishmania the whole of the skin may be heavily parasitised, causing hair loss. A sandfly infected by a jackal or a fox must suck out and infect a dog to bring this parasite into domestic situations. If sandfly flight outside burrows or earths are restricted to certain times of the year, as happens in Tunisia and Iraq, and if the incubation time in the dog is greater than this period, then the dog must retain the infection for at least 9 - 10 months in order to act as domestic reservoir in the following year.

Wenyon in 1911 failed to find the disease in 111 dogs (Wenyon, 1926) from Iraq.

Patton (1918) examined a number of dogs in the Nassiryia area, none of which had the disease (reported by Chadwick and Machattie, 1927).

Chadwick and Machattie (1918) examined 120 emaciated dogs from Baghdad city without revealing Leishmania in the viscera. They also examined three dogs from Khanaquine with the same result (Chadwick and Machattie, 1927).

Tajeldin and Al-Alousi (1954) reported a survey of dogs as a possible reservoir of Leishmania in Iraq which proved negative by Mills and McCarthy in 1930 (unpublished data).

Al-Dabbagh (1954) autopsied 47 debilitated stray dogs from the eastern suburbs of the city (Baghdad); all were negative. This was reported by Pringle 1956.

An epizootic of visceral leishmaniasis was described in an imported pack of foxhounds in Baghdad by Sheriff (1957), obviously these animals are not the permanent reservoir.

In personal communication with Sheriff, he informed me that almost entirely these were locally bred; they were 50 in number and without exception male dogs became infected in their second, third or subsequent years and died. Diagnosis was made by lymph node biopsies and the direct examination or culture was positive; surrounding native dogs were negative. He suggested that infection

was by mouth and not as is generally stated by insect transmission, because the males were the only ones that bit the jackal and tore it apart (Sheriff, 1978, personal communication).

Adler and Theodor (1932) stated that in Mesopotamia where natural infections with L. tropica are common, canine kala azar does not occur.

The jackal appears to possess many of the necessary qualifications as the reservoir of the disease in Iraq, due to its reported susceptibility, wide distribution and abundance throughout the country, its ability to range widely and cross natural boundaries such as rivers and its habits which bring it close enough to habitations to make casual human infections a possibility. Furthermore the "earths" used by jackals and foxes appear to provide a highly suitable environment for sandflies and greater numbers appear to concentrate in them in daytime than in relatively smaller burrows of wild rodents. Bray et al. (1967).

The spotty non-familial distribution points to the existence of an animal reservoir. The incidence appears to indicate the existence of a feral host such as the jackal which also has a similar periurban distribution around Baghdad (this is not to say the village dog does not live an almost feral existence) and which occupies earths suitable for sandfly habitation. One present belief is that the way of life of the periurban dog wandering and sleeping on the hot flat plain may be less conducive to sandfly biting than the life of the jackal living in earths. Bray et al.(1967).

Latyshev et al. (1951) reported that the jackal is the principal reservoir of kala azar in Southern Tadjikistan where the sandfly species spectrum corresponds to that of Iraq. Wenyon (1926) recalls that in 1912 Nicolle and Blaizor proved that the jackal (Canis aureus) was susceptible to the Mediterranean strain of L. donovani. In 1956 Pringle examined two jackals, both were negative. Bray and Dabbagh (1968) examining 132 dogs, 52 rodents and 3 jackals, also failed to find any evidence of an animal reservoir.

Tajeldin et al.(1971) studied the possibility of isolating the parasite from 144 dogs, five jackals, 367 rodents and the parasite could not be found. In 1975 another 18 jackals, with 16

foxes, 6 rodents, 10 hedgehogs and 32 bats were negative for Leishmania. (Sukkar 1978).

Kadhim (1978) did examine 53 foxes and 64 jackals and no Leishmania was found.

The distribution of infantile kala azar is periurban in Baghdad (Tajeldine et al., 1969) where the dogs are not infected (Bray and Dabbagh, 1968) and it is here the jackal may be the reservoir and transmission is direct to man.

Bray and Dabbagh (1968) stated that attention must continue to be directed at rodents and even wild cats or mongooses. Rodents (in the opinion of Pringle, 1956) can get infected.

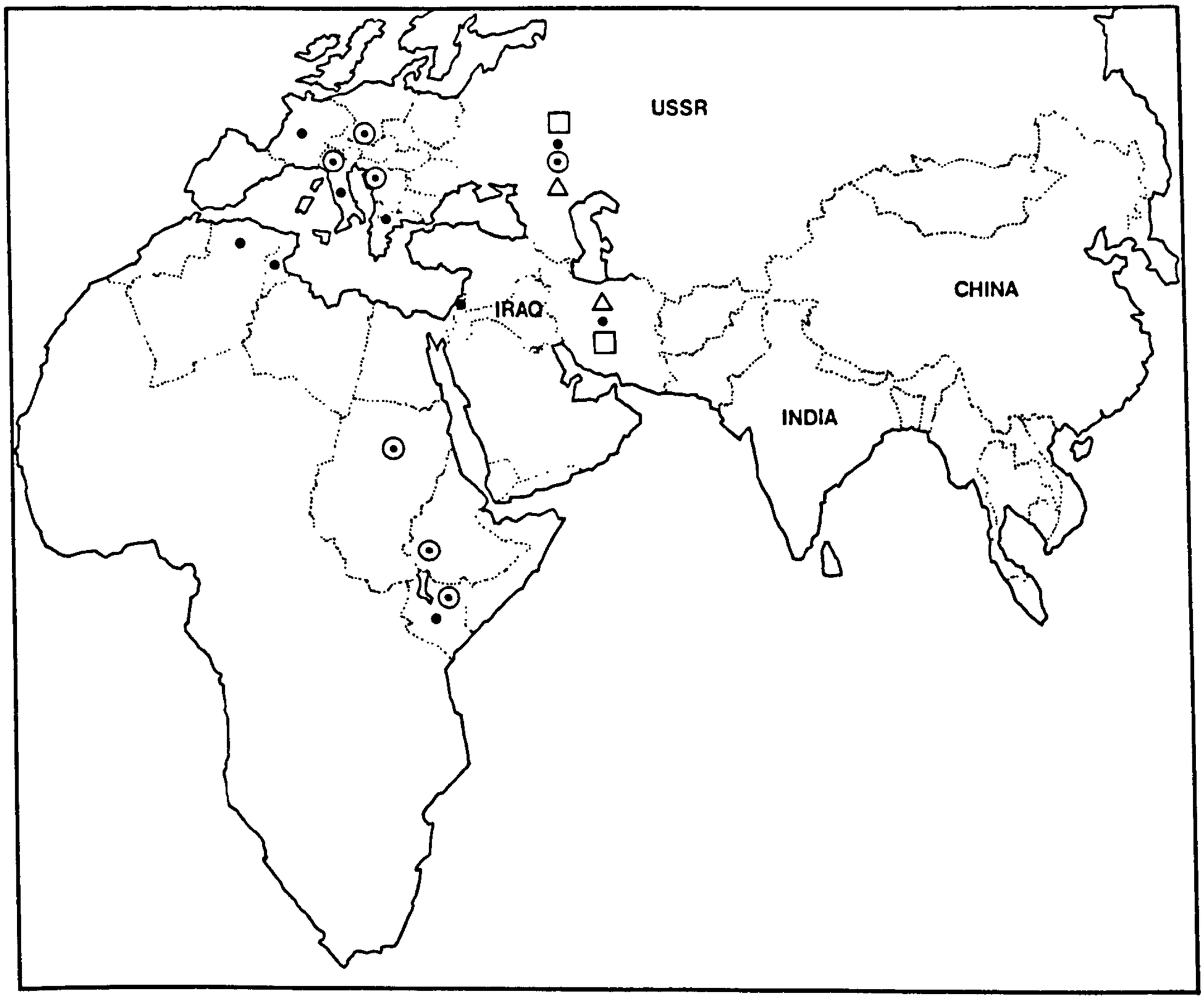
El-Adhami (1976) reported to have found natural visceral leishmaniasis in a single R. rattus which was caught in the centre of Baghdad city among 85 others, and after isolating the parasite from its blood and tissues, she injected it into a white mouse and it visceralised. Later work by Al-Jaboori and Evans (1980a and 1980b) proved that the parasite was a strain of L. tropica by isoenzyme.

Official reports stress that no animal acting as a reservoir host could be discovered until now. (Sukkar, 1976c).

Table 2. Summary of the work done on possible animal reservoirs in Iraq.

Worker	Year	Dogs	Jackals	Foxes	Rodents
Wenyon	1911	111			
Patton	1918	a number of dogs			
Chadwick and Machattie	1918	123			
Al-Dabbagh	1954	47			
Pringle	1956		2		
Bray and Dabbagh	1968	132	3		52
Tajeldine et al.	1971	144	5		367
Sukkar	1978		18	16	6
Kadhim	1978		64	53	
Total		557	92	69	425

Figure 1 Showing positive findings among animal reservoirs of visceral leishmaniasis in Asia, Africa and Europe.



3. The insect vector.

a. Studies on sandflies in other countries.

Sandflies inhabit the most diverse biotopes ranging from arid and semi-arid regions (Sudan, Iraq, Turkestan, North West India) to tropical rainforest. Adler and Theodor (1957).

This type of habitat had an effect on the focality of visceral leishmaniasis, because of the factors of their flight range, longevity and feeding.

Petrischeva (1971), Ashford et al.(1973) found out that sandflies are known to colonise jackals and porcupine holes readily and both are known reservoirs.

Adler and Theodor (1957) pointed out that the Mediterranean species of sandflies escape from the lethal atmospheric conditions into a suitable microclimate by living in rodent burrows or fissures in the soil. Young et al.(1926) first demonstrated that P.papatasi in Peshawar breeds in fissures in the ground.

Bray (1975) stressed the effect of the temperature, humidity and wind upon the flight range.

On the other hand Lewis (1971) clarified that sandflies in nature can live 2 - 3 weeks. Also Bray (1975) stated that to be a vector, the sandfly must be anthropophilic but far from exclusively so. He considers that man himself may induce the biting process by disturbing the sandflies. Adler and Theodor (1957) stressed the fact that the alimentary tract of fed and unfed sandflies is bacteriologically sterile. A chance contamination interferes with the digestion of a blood meal and is fatal to the insect.

Adler and Theodor (1931c) stated that the amount of blood ingested during a single full feed is variable, the average for P. perniciosus being 0.165 cmm. When the sandfly probes it seems able to take up tissue juice as well as blood and therefore it seems probable that it slashes and breaks cells (Adler and Theodore, 1957). This would obviously facilitate the ingestion of the intracellular leishmaniae. It also seems probable that it requires only a very few organisms to establish an infection in a sandfly (Bray 1975).

Sandflies will not probe or feed on post kala azar dermal leishmanoid lesions, but move on to normal skin (Minter and Wijers, 1963).

Adler and Theodor (1930b) using the Hertig apparatus proved that L. infantum can leave the biting mouth parts of P.perniciosus during the act of biting and enter a new host in the absence of any active interference on the part of the latter.

There is no mechanism for the ejection of flagellates as they do not invade the salivary glands. We therefore think that flagellates are simply deposited from the proboscis into the puncture wound if the distal part of the proboscis is infected.

Bray (1975) regards the process of inoculation into a vertebrate as the remaining major mystery in leishmaniasis, a lack of essential knowledge which is the main reason why a mathematical epidemiological model cannot be produced for leishmaniasis. Unless the proportion of potential deliveries of parasites into a vertebrate can be predicted, neither can relevant aspects of epidemiology.

b. Studies on sandflies in Iraq.

In Iraq Pringle (1956) discussing the problem of kala azar stated that kala azar cases seem to have originated in localities which are either periurban or in the dry, less intensively irrigated, tracts of the plain. Such a distribution of cases would suggest that P. sergenti is not the vector of kala azar in the lowlands of Iraq. Suspicion must therefore be directed towards the other anthropophilic species, P. papatasi,and the relatively rare or local P. alexandri and P. clydei, from the limited entomological studies done later on the sandflies in Iraq, P. papatasi seems the most frequent to be found in association with man and animals, so the probable vector seems to be P. papatasi. (Sukkar, 1974).

The sandflies in Jadryia and Abu-Ghraib localities were found to be absent between December and March and present later between April and November There were two peaks for the population, the first was during May - June and the second was during September - October. A low density of population was observed during June, July the peak hour of activity was found around nine o'clock at night. (Abulhab and Mahdi 1970).

Finding Leishmania promastigotes in Iraq sandflies was not recorded in the scientific literature, but personal communication with Mr. Sami Al-Mahdawi, the entomologist in the Institute of Endemic Diseases in Baghdad and with Dr. Jalil Abu Al-Hab the entomologist in the University of Baghdad, stated that they have found flagellates in sandflies, P. papatasi, situated anteriorly during the transmission season in 1974 caught from Noumaniya, an endemic area. They cultured the sandfly but it was contaminated and they did not use xenodiagnostic methods in animals. This was later reported by Sukkar in 1978.

In summary the evidence that P. papatasi is the vector of visceral leishmaniasis in Iraq is suggestive but not conclusive.

B. ARTIFICIAL INFECTION OF MAN AND ANIMALS

1. Artificial infection of animals.

That laboratory animals can be infected with Leishmania does not mean that they are the reservoirs in nature. (Adler 1964). The animals of choice for experimental purposes (hamsters and spermophils) are not as far as is known ever infected in nature. They are, however, so susceptible to infection that caution must be exercised in extending to the epidemiology of the human disease the results of experiments on these animals if misleading conclusions are to be avoided. (Adler 1940).

There is no inevitability about the inoculation of leptomonads from a heavily infected sandfly into a vertebrate host leading to disease as is the case with trypanosomes of the brucei group or sporozoites of malaria from the salivary glands of the respective vectors. There are many records of feeding experiments with heavily infected sandflies on man and susceptible animals which have given a negative result. (Adler 1964).

Stauber (1958) detected a whole range of resistance to L. donovani within the series of animal species he studied. It extends from the complete susceptibility of the hamster which eventually succumbs to the intracardial introduction of a single parasite, to the innately resistant rat and rabbits which soon dispose of even as many as several million parasites.

In terms of acquired resistance the mouse, gerbil and guinea pig are the most interesting species, because each is susceptible enough to permit some increase in parasites for a number of days after inoculation, but each later checks this increase. (Stauber 1958).

Rabbits, white rats, guinea pigs, gerbils and white mice show a wide range of innate resistance.(Heyneman 1971). In hamsters L. donovani, L. infantum, L. tropica and L. mexicana all cause visceral involvement. (Adler 1964).

The hamster never develops an efficient immunity to any strain of mammalian Leishmania to which it is susceptible. (Adler 1965). Hamsters were infected subcutaneously with L. donovani and challenged six weeks later by an intracardial route of inoculation: they showed significantly lower numbers of visceral parasites after the challenge than those not challenged as a control group. (Farrell 1976).

In 1932 Shortt and his associates succeeded in infecting only one of 32 hamsters by feeding each animal repeatedly over a long period ofstudy with the faeces of hamsters and of patients suffering from kala azar. (Shortt et al. 1932).

Killick-Kendrick et al. (1977) succeeded in transmitting cyclically L. mexicana amazonenensis in the laboratory from hamster to hamster by single bites of experimentally infected Lutzomyia longipalpis. The sandflies were denied suitable conditions for oviposition and were induced to take a second infecting meal while still gravid. In three transmissions cutaneous lesions developed rapidly on two hamsters, whereas that on the third remained small for eight months, almost disappeared and then grew to the normal large size.

The jackal infected by Dedet (1971) had a milder and longer infection than is usual in dogs. On the other hand the jackal showed little depilation.

Inoculations into mice do not give constant results even with recently isolated strains of L. tropica (Demina et al. 1968). A strain of L. tropica isolated in Baghdad from a case of oriental sore gave negative results on inoculation into the tails of four mice. (Adler and Theodor, 1929a).

A model for studying genetic control of resistance to intracellular infection using seven strains of laboratory mice, taking liver parasite burdens over 20 weeks showed some to be relatively resistant and others acutely susceptible. One of the latter group showed later a dramatic fall in parasite numbers while the other strain of the acutely susceptible maintained an immense parasite load for up to two years. (Bradley and Kirkley 1977).

The acute growth of L. donovani in 25 inbred mouse strains fell into two distinct groups: the susceptible (S) and the resistant (R). Back crossing of F_1 hybrids to R and S parents gave susceptibility ratios consistant with single gene control of acute susceptibility to visceral leishmaniasis.

The distribution of this character among inbred mouse strains does not correspond to any well-studied gene, nor does it appear to be linked to the H_2 locus (Bradley 1977).

There is some correspondence between resistance to S. typhimurium and L. donovani. (Bradley 1977, Plant and Glynn 1976).

The differences of responses to L. donovani in the R mice is not affected by thymectomy and irradiation or by more extreme attempts to reduce the T-cells (Bradley and Kirkley 1972).

Results so far do not suggest any direct relation of acute Leishmania susceptibility to Ir 1, H_2 or the ability to amount an acquired immunological response, since the gene concerned maps on Chromosome 1. A macrophage difference also controlling responses to several unrelated antigens would explain the results. (Bradley 1974).

Resistant strains of mice show much lower parasitic proliferation rates to susceptible strains. In chronic infections the rate is also reduced. Parasitised mononuclear phagocytes may undergo mitosis. No evidence of selective destruction of parasitized cells in chronic infections was found by labelling methods. (Bradley 1979).

Maggiore (1925) and Adler and Theodor (1931a) failed to infect two adults by inoculation of infected bone marrow for the first and by inoculation of L. infantum from an experimentally infected sandfly P. perniciosus for the second.

Rare cases of experimental infection of lizards by leishmaniasis from warm blooded animals can be considered as evidence of a remote genetic affinity between reptilian promastigotes and leishmaniasis of man and warm blooded animals. (Belova 1971).

2. Successful infection of man.

Swaminath et al.(1942) succeeded in infecting sandflies P. argentipes after feeding on human cases. They kept them on fruit juices until the next blood meal was taken from five human volunteers. All the five after following them up for almost one year got infected and developed kala azar.

Adler (1940) succeeded in infecting a human being, 31 years old male, a terminal case of carcinoma of the stomach, after inoculation of cultures of L. donovani. This is the first record of the successful transmission of visceral leishmaniasis in man. The incubation period was less than five months, the case had no signs of kala azar, but parasites were seen on post mortem from his liver and spleen.

Manson-Bahr et al. (1963) and Manson-Bahr and Southgate (1964) inoculated six non-immune individuals in 1962 with intradermal and sub-cutaneous inoculations of flagellate forms of human L. donovani isolated from P. argentipes by Heisch et al.(1962). Two of them developed leishmanioma without visceral involvement. Of the remaining four, one was leishmanin positive after inoculating him previously with the rodent strain of Leishmania (Manson-Bahr, 1959, 1961b). All four developed kala azar, there was a 4 months incubation period and 4 months of asymptomatic parasitaemia before the appearance of signs and symptoms.

On one occasion infection of man was achieved by a sandfly which merely probed (Bray 1975).

C. THE HUMAN CLINICAL PICTURE

Various workers have studied the types of visceral leishmaniasis in different parts of the world. Clinicoepidemiological differences were recognised by Moskovskij and Southgate (1971) which led them to their Nosological units as follows:

Nosological Unit (clinical)	Tegumentary			Visceral		
Clinicoepidemiological type	Tegumentary (West hemisphere)		Cutaneous (East hemisphere)	Mediterranean	KA	American
Clinicoepidemiological subtypes	Cutaneous	Mucocutaneous	Anthroponotic	Mediterranean	Indian	Brazilian
	Mexican Peruvian Panamanian	Espundia Pian-Bois	Zoonotic	Soviet Chinese	Sudanese Kenyan Chinese	

Changes in the body during the course of visceral leishmaniasis infection were studied.

Adler (1964) pointed out that there is a pronounced hyperglobulinaemia with an increase in globulin/albumin ratio in visceral leishmaniasis in man and dog.

The protein abnormalities in kala azar are explained by a particular reactivity of the reticulo-endothelial system produced by the intracellular parasite (Martins et al. 1969).

Some of the changes in serum proteins in kala azar are generally associated with severe infectious diseases (e.g. fall in albumin) or with tissue destruction (e.g. rise in alfa globulins), but the very large increase in gamma globulins is as yet unaccounted for, although the last may remain low in early cases (Chatterjee, 1978).

Kulkarni (1961) doing some electrophoretic studies on ten cases of kala azar found out that there was extreme hypoalbuminaemia and marked gammaglobulinaemia. This was confirmed by Martins et al.(1969) in another study, who found 78% of the cases have hyperproteinaemia, 96% hypoalbumaemia, and 100% hyperglobulinaemia. Chemical and electrophoretic techniques

demonstrated increased gammaglobulin in all patients.

But Kulkarni (1961) failed to find any direct relation between serum proteins and enlargement of liver and spleen contrary to the work of Jerkins et al. (1957). 17 out of 20 previously confirmed kala azar cases showed increased globulins and reduced albumin levels 3 - 10 years after being discharged from the hospital after being improved at that time. (Musumeci et al. 1977).

These protein changes are usually reversible with specific therapy, normal albumin concentrations are attained in a period of 2 - 4 months and globulins reached normal levels one year after beginning of treatment (Martins et al. 1969).

Both I_gG and I_gM levels were found to be statistically higher in kala azar and malaria sera compared to controls (Ghose and Chowdhury, 1977).

Martins et al. (1969) doing ultracentrifuge analysis of the serum showed a marked increase in the 7S fraction (I_gG) with normal 19S component.

I_gG and I_gM levels of 3 - 10 years previously kala azar cases were found to be still high (Mesumeci et al. 1977), probably due to successive contact with Leishmania, although evidence shows that any new challenge of infection will be dealt with in the skin and no further reticuloloendothelial system stimulation will take place. (Bryceson, 1976, quoted by Mesumeci et al. 1977).

Other workers like Chatterjea and Sen Gupta (1970) found increased levels of I_gG, also Ghose and Chowdhury (1977) and Rezai et al. (1978); this varied in persistency (Mayrick et al.1967).

I_gM levels increased as the infection progressed but fell rapidly after treatment (Chaves and Ferri, 1966).

But Ambroise-Thomas (1976) has clarified it more by stressing that I_gM is present only during the first months of the parasitic infection.

As for the blood picture, in the early stages it was found to be normal (Chatterjee, 1978), but Chatterjea and Sen Gupta (1970) noted anaemia even in early infection; they concluded that anaemia was due to defective formation and or autoimmune destruction of the red blood cells.

The latter had been suspected by Knight et al.(1967) who noted considerable sequestration in the spleen.

Woodruff et al.(1972) elaborated more fully stressing that during the active phase of kala azar the erythrocyte lifespan is shortened, erythrocytes in kala azar are destroyed in the spleen, in cases of kala azar if the spleen has been removed there was no anaemia. An autoimmune mechanism is likely to be the explanation of these observations and of the reduced erythrocyte survival in kala azar.

Chatterjee (1978) mentioned leucocytosis in early infection in Indian kala azar and attributed that to marrow irritation resulting in neutrophilia and eosinophilia.

Leucopenia was a generally observed feature (Chatterjea et al. 1970) with a relative lymphocytosis and monocytosis (Chatterjee, 1978).

Mesumeci et. al.(1978) found out that the surface radioactivity count showed (using $DF^{35}P$ di-isopropyl fluorphosphate and ^{51}Cr chromate) that the reduced granulocyte lifespan was due to pooling and probable destruction of granulocytes in the spleen and to a lesser degree in the liver.

Rezai et al. (1978) showed that T-cells in peripheral blood was reduced and B-cells in the majority of cases elevated.

Another feature of visceral leishmaniasis is prolonged clotting time noticed by Chatterjea and Sen Gupta (1970).

Bone marrow studies done by Chatterjea (1978) on 280 cases of Indian kala azar showed that bone marrow in the early stages showed different degrees of normoblastic lymphoplasia and diminished leucopoieisis. In the intermediate and later stages the marrow was found to be hypoplastic with relative lymphocytosis and monocytosis and increased macrophages.

Studies in Iraq about the signs and symptoms of visceral leishmaniasis were done by some workers. The work of Halawani and Guirges (1973) showed the clinical features of kala azar in Iraq to be similar to those found elsewhere in the Mediterranean: fever, anaemia, vomiting, diarrhoea and bronchitis were the prominent symptoms.

The following analysis of signs and symptoms of visceral

leishmaniasis in Iraq were taken from studies of large numbers of hospitalised patients (Tajeldin and Al-Hassani, 1961; Tajeldin et al. 1969; Sukkar 1976c). Fever was a presenting symptom, irrigular, remittent or intermittent (Nouri and Al-Jeboori 1973). 67% - 96% of cases had this symptom. Children may wander about with a temperature as high as 40°C, occasionally alternating pyrexia and apyexial bouts simulating undulant fever.

Progressive enlargement of the abdomen was found in 10% of cases. Hepatosplenomegaly could be roughly correlated with the duration of the fever.

Jawad (1972) states that kala azar is the most common cause of hepatosplenomegaly during childhood in Iraq. 93% of cases showed an enlarged firm liver. Average liver enlargement was 4 cm below the costal margin. All cases showed enlarged and firm spleens with the spleen average enlargement 4 cm below the costal margin.

Cole (1944) asserts that splenomegaly is not invariable and is a relatively late sign.

Cough was found in 62% of cases and diarrhoea in up to 22%.

The average haemoglobin was found to be 5.9G; it ranged from 3.6 to 10.8G. Paleness was found in 22% of patients. The white blood count ranged from 4700 to 4900 per cubic mm. In differential counts 64% - 76% of the cases had relative lymphocytosis. 17% of them had marked relative monocytosis. Tajeldin and Al-Hassani (1961) found albumin/globulin ratio disturbance as a common feature. Protein electrophoresis detected 26 out of 27 with an increase in gammaglobulin. (Tajeldin et al. 1969).

Bray et al. (1967) discussing kala azar cases in Iraq indicated that they usually show a small rise in $I_{\varepsilon}G$, but reversal of the albumin/globulin ratio is rare on these cases and occurs only in a few known chronic cases. Leucopaenia occurs in about three-quarters of the cases.

Tajeldine et al.(1969) comparing cases of 1963-1967 with previous cases stated that the degree of enlargement of the liver and spleen were less marked, relative lymphocytosis was notably diminished in incidence in the later cases and that later cases ran more acute courses than the earlier cases. Smaller infants were found to be more toxic.

D. IMMUNOLOGICAL RESPONSES IN MAN

> "The immune system is like a store with an almost unlimited stock, one ready to please any possible customer".
>
> G. Edelham

The subject of immunity in visceral leishmaniasis is complicated, and I will try to give a picture from the literature of the type of phenomena one may encounter before or during the course of infection with L. donovani in man. This account follows up the parasite beginning from inoculation of the parasite into the body and discussing the different reactions of the host towards it.

The first matter to be tackled is the innate immunity. Innate resistance in man is essentially absent. Exposed non-immunes will gradually develop the disease (visceral leishmaniasis) depending upon epidemiological factors. Children are most affected, as are newcomers of all ages who enter endemic areas (Demina et al. 1968).

While Stauber (1970) is of the opinion that the peculiar distribution of L. donovani in rodents, canids and man suggests that many mammals are innately resistant to naturally acquired infection, it is likely that many of the animals found infected in nature are sentinels of the presence of transmission of Leishmania (all species) rather than true zoonotic reservoir hosts.

Protein or pyridoxine deficiency decreased innate (early) and acquired resistance. Pantothenate deficiency at first increased host resistance, but when the deficiency was continued it later led to increased parasite burdens, probably by influencing the host's capacity to establish acquired resistance. (Stauber 1970).

Preston and Dumonde (1975) discussing immunology of leishmaniasis stated: "In studying leishmaniasis in immunological terms we view the outcome of leishmanial infection as being due to ineraction between genetically distinct populations of parasites and hosts which govern both the immunogenicity of the parasite and the immunological response of the host".

Sen Gupta (1962) summarised his views on the pathogenicity of L. donovani in man, he has the theory that the pathogenicity may be presented in different forms, it may develop a leishmanioma after the bite of an infected sandfly and visceral leishmaniasis may follow about 4 months later, or it may not be followed by kala azar (thus suggesting that infection is more widespread than the actual incidence of kala azar in an endemic

area). Or the disease may take the form of lymphadenopathy only without visceral involvement. In this case bone marrow is negative. Sometimes the infection is so insignificant that it may not be associated with general ill health. Parasites were however demonstrated in the normal looking skin in Chinese and Mediterranean kala azar, so sandfly feeding on the skin may take up the parasitised histocytes. This could be supported by the work of Manson-Bahr and Southgate (1964). They found that inoculation of live promastigotes into human beings intradermally and subcutaneously takes two paths. In non-immunes a leishmanioma develops which lasts 3 months and visceralisation takes place after 3 - 4 months. Leishmania could be isolated by culture from the leishmanioma. In immune individuals Arthus reaction with pus formation within 24 hours develops at the site of infection. No live Leishmania could be isolated by culture. (Manson-Bahr 1961).

Interactions between macrophages and leishmanial promastigotes have been extensively investigated in attempts to understand the mechanism of their entry.

Molyneux et al.(1974) stated that the actual process of infection was probably achieved by mutual cooperation between the parasite and the host resulting in phagocytosis of the parasite. This means that the intracellular Leishmania amastigote is separated from host cytoplasm by a membrane originating from the macrophage cell membrane that involutes to form the phagosome.

Pulvertaft and Hoyle (1960) described a cinematographic technique for observing this. They reported that flagellates enter monocytes only by mutual attraction.

Miller and Twohy (1967) described the in vitro stages of engulfment of promastigotes by macrophages. In this a funnel shaped protrusion gradually engulfed the parasite, flagellum first.

Zenian et al.(1979) using scanning electron microscopy found that within 5 minutes the funnel from the macrophage extended along the flagellum and reached the body of the parasite; after approximately one hour the body of the parasite was usually completely engulfed.

Chang (1979) also examined promastigote-surface interactions by scanning electron microscopy. He reported that L. donovani promastigotes mainly depended on the phagocytic activity of hamster peritoneal macrophages to gain intracellular entrance but that motility of promastigotes

and their affinity to the surface of macrophages were also important processes contributed by the parasite. He called this 'facilitated phagocytosis'.

When mouse peritoneal macrophages were infected with various species of Leishmania promastigotes, the infection rate was higher than L. donovani and L. enriettii than with L. tropica (Ardehali and Khoubyar 1978). Since Leishmania parasites show some degree of host specificity (Garnham 1971) one might expect this specificity to be reflected in the phagocytic activity of these peritoneal exudate cells.

Once Leishmania are phagocytized by macrophages they are shielded from the effects of serum antibodies and various T-cell mediators (Cohen (1975); Feldmann(1975).

Adler (1965) states that the stimulus for proliferation and differentiation of B-cells and the information essential for dictating specific determinant groups on antibodies comes from the phagocytes (including histocytes), because the antigen is absorbed by these phagocytes. In general, macrophages present antigen fragments and not intact antigen to T-cells (Benacerraf 1978).

Soluble haptemic antigens may pass continuously from the phagocytes into the bloodstream and continue to stimulate the general centres into producing ineffectual complement-fixing antibodies, non-specific gamma globulins and a massive proliferation of phagocytic cells that continue the cycle and provide media for more parasites (Heyneman, 1967).

Briefly kala azar is characterized by:

(a) high titres of specific antileishmanial antibodies
(b) production of non-specific immunoglobulins
(c) plasma cell proliferation of lesions and lymph nodes
(d) high titres of antiglobulins resembling rheumatoid factor (Preston and Dumonde, 1975).

In visceral leishmaniasis in contrast to cutaneous leishmaniasis, high titres of immunoglobulins can be detected by conventional methods (Adler 1964). Chatterjea and Sen Gupta (1970), Ferri and Chaves (1968), Irunberry et al.(1968) all reported these raised Ig levels to be almost entirely due to IgG, with occasional IgM increases. Chaves and Ferri (1966) reported increased levels of both IgG and IgM. Garnham and Humphrey (1969) stated that this increase in Ig probably contains only a

small fraction of specific antileishmania antibodies. Specific IgM are present only during the first months of the parasitic infection and do not pass across the placental barrier. Their presence in the adult presumes a recent infection and in the newborn may be regarded as congenital infection (Ambroise-Thomas, 1976).

It thus appears that the proliferating immunologically competent cells stimulated by the parasites secrete an excess of gammaglobulin, but fail to stamp the molecules with the seal of specificity (Adler, 1964), and that the large amount of gammaglobulin produced by the host during infection is not protective antibody active against Leishmania.

Taliaferro (1962) has emphasized Putnam's (1960) view that these abnormalities may be more quantitative than qualitative and that until we know much more about the many antigens inducing gammaglobulin synthesis during infection (parasite antigens, parasite-product antigens, or altered host antigens), we have little basis for calling them abnormal globulins (Stauber, 1970).

High levels of serum immunoglobulins suggest the possibility that cell mediated immune reactions may be blocked by antibody in this infection (Cohen, 1975). Fall in antibody titre in this disease is associated in time with the emergence of delayed hypersensitivity (Bryceson, 1970a).

It would appear that serum antibody implies lack of immunity (Heyneman, 1967).

There is no quantitative correlation between titre and hyperglobulinaemia (Adler, 1965). In general, serum protein changes do not affect the levels of circulating antibodies (Shaw and Voller, 1964).

When macrophages from superinfected hosts were infected with L. donovani, the parasites either failed to multiply or decreased in numbers. When serum from superinfected mice was added there was no significant change in either the rate of parasite multiplication in macrophages from normal mice or in the rate of parasite destruction in macrophages from superinfected mice (Miller and Twohy, 1969). However, Rezai et al.(1969) stated that plasma from rabbits injected with L. tropica showed an inhibitory effect on the growth of L. tropica, enriettii and donovani in vitro. This inhibitory effect was assayed by counting the viable leptomonads under the microscope using trypan blue as a vital stain.

Rezai et al.(1970) found that leishmanial growth inhibitory substance of immune rabbit serum was in the gamma globulin fraction of immunoglobulin (using immuno-electrophoresis and DEAE-cellulose chromatography). The antigen responsible for eliciting this antileishmanial activity of immune rabbit serum was located in the aquous insoluble fraction of leishmanial cells. The immune serum had no effect on the course of leishmanial infection in infected mice. Adler (1940) reported that serum from two individuals who resisted kala azar infection lysed L. donovani.

The localisation of the parasite may also affect the immune response in other ways, for example the bulk of the organisms may reside in organs where the capacity to produce antibody is not well developed, e.g. the liver (Feldmann, 1975).

The cellular reaction in visceral leishmaniasis is the same as in cutaneous leishmaniasis, i.e. proliferation of histiocytes followed by invasion by lymphocytes and plasma cells. Spontaneous cure of overt clinical disease seldom occurs in man and never in hamsters and spermophils. The immunity following cure may therefore be dependent on residual parasites, i.e. premunition (Adler 1963).

Splenomegaly and hepatomegaly in kala azar is an extreme example of a "histiocytoma" (Heyneman, 1967).

The host macrophage reaction to Leishmania is correlated neither with serum antibody titre nor with the state of immunity, nor are the latter two directly correlated (Heyneman, 1967).

Experimental evidence suggests that parasite death may be mediated by lymphocytes, macrophages and antibody cooperating in two principal mechanisms of protective immunity. First, macrophage parasitisation may promote the immunogenicity of leishmanial products, and thus the development of sensitised lymphocytes cytotoxic for macrophages bearing leishmanial antigens. Liberation of amastigotes from killed macrophages might make them available to lytic or agglutinating antibodies. Second, sensitised lymphocytes may activate macrophages by contact or by the generation of lymphokines, activated macrophages might then kill the leishmanial parasites intracellularly. In both systems of parasite killing by lymphocyte macrophage interaction, a cooperating role of antibody has been indicated. In view of the suppression of immunofluorescent antibody (presumed IgG) in thymectomised infected mice (Preston

et al. 1972), it is tempting to suggest that T-cell function is important in the generation of cooperating antibody and complement dependent lysis by antibody; and opsonization effects and lymphocyte cytotoxicity and macrophage activation. Recent work suggests that the outcome of phagosome-lysosome fusion within macrophages may determine whether intracellular micro-organisms can survive phagocytosis (Armstrong and Hart, 1971; Jones and Hirsch 1972).

Preston and Dumonde (1975), discussing the immunology of leishmaniasis during the stage of active infection in vivo noticed that macrophages cultured from mice and guinea pigs can inhibit multiplication and even kill intracellular Leishmania (Miller and Twohy, 1968; Bryceson et al., 1970; Twohy, 1971, quoted by Turk and Bryceson, 1971).

Antigen stimulated lymphocytes can release a lymphokine which activates "non specifically" certain biochemical, phagocytic and bactericidal properties of macrophages (Fowles et al. 1973).

Macrophages coated with soluble leishmanial antigen could be killed by immune lymphoid cells (Bryceson et al.1970) and these cytotoxic effects of sensitised lymphocytes were enhanced by the addition of antibody or immune complexes to the culture (Bray, personal communication, 1973), quoted by Preston and Dumonde (1975) but it was difficult repeating the experiment.

Infection with infantile kala azar appears to give some degree of protection against the diseases of childhood. Zahra-Neumann (1933) has claimed that mortality among infected children treated mainly as outpatients is less than in other children of the same age group.

There is definite antagonism between visceral leishmaniasis and malaria both in man and in hamster and this non-specific protection may be due to the relatively enormous increase in immunologically competent cells (Adler, 1963). In india, as in Sicily, it is rare to find malaria among cases of kala azar (Adler, 1965). Adler and Theodor (1935) suggested that these cases were practically immune to malaria. In Catania, Paradiso (1926) found only one case of malaria in 2000 infected children of which the majority came from endemic centres of malaria.

Kala azar appears to be a disease which depresses both the humoral (Chung and Reimann, 1930) and cell mediated (Adler, 1965) immune responses. On the other hand Adler (1954) showed that hamsters infected

with L. donovani were more resistant to infection with Plasmodium berghei than normal animals (Bray, 1975). Leishmaniasis has been shown to be one of a number of the growing group of diseases which depress the immune system. Serebryakov et al. (1972) infected subjects with L. tropica major prior to a third inoculation with diphtheria-pertussis-tetanus vaccine and showed that the subsequent immune reaction to diphtheria was suppressed. Adler (1965) obtained a prolongation of skin graft acceptance in hamsters heavily infected with L. donovani. Avitaminosis due to lack of vitamin A, a known depressor of the immune reaction, allowed metastasis in guinea pigs infected with L. enriettii when normally fed guinea pigs shows no spread of the infection (Bray, 1974).

Patients with kala azar respond very poorly to TAB vaccine (Cassimos et al. 1966). Immunosuppression may be related to antigenic competition affecting macrophage function, e.g. guinea pigs pretreated with Corynebacterium parvum or Mycobacterium tuberculosis usually develop severe lesions when exposed to L. enriettii (Bryceson et al. 1972).

Mycobacterial infections were inhibitory to the Leishmania and vice versa (Konopka et al. 1961), i.e. prior infection with L. donovani favours the host when challenged with tubercle bacilli and vice versa, suggesting important antigenic similarities between these two micro-organisms which merit further study (Stauber, 1970).

It is known that recovery from kala azar does not protect against later inoculation with L. tropica (Manson-Bahr, 1961b). Cross immunity between L. donovani and L. tropica parasites, therefore, does not exist, although Senekji's early work suggested the opposite (Senekji, 1943). There is general agreement that L. tropica does not protect against L.donovani (Manson-Bahr, 1971).

Manson-Bahr (1961b) suggested that there was complete cross immunity between rodent and all human strains of L. donovani. This was contradicted by Manson-Bahr et al. (1964) who succeeded in infecting a leishmanin positive volunteer who had been previously inoculated with a rodent strain. It was also contradicted by the trial of vaccination they carried out using the ground squirrel strain (Manson-Bahr and Southgate, 1964).

Successful treatment is apparently followed by immunity (Adler, 1965). In human visceral leishmaniasis records of spontaneous cure are rare and acquired immunity usually becomes manifest only after drug therapy

(Coradetti, 1964).

Spontaneous cure seldom occurs in man, in Adler's opinion, and never in hamsters and spermophils (Adler, 1963). Death is the usual result of untreated kala azar through an overwhelming macrophage (histiocytes) hyperplasia, i.e. the substrate in which amastigotes thrive (Heyneman, 1967). In cases of Mediterranean infantile visceral leishmaniasis relapse has been observed some months after apparent cure (Adler, 1964).

Stable immunity follows cure of visceral infection. Repeated infection in visceral leishmaniasis is unknown (Demina et al. 1968).

Preston and Dumonde (1975) suggested that the development of acquired resistance varies with individual efficiency. This was suggested by Napier's estimate that 25% of kala azar cases could heal spontaneously if left untreated (Napier, 1946). In addition chronic forms with pronounced splenomegaly have been observed where parasites were difficult to demonstrate unlike acute forms (Kirk, 1949; Napier, 1946).

Sterile (residual) immunity is not necessarily the only form of protection as non-sterile (premunition) immunity is common (Demina et al.1968).

Pugin et al. (1978) reported a case in which circulating immune complexes remained elevated two months after the disappearance of Leishmania from the bone marrow.

One can also conclude that the hamster or man, both very highly susceptible to the parasite, would not be the hosts of choice for experiments of passive transfer of immunity and that large amounts of serum would need to be transferred in such cases (Stauber, 1970).

Adler (1965) failed to transmit delayed hypersensitivity by injecting two volunteers with white blood cells from hypersensitised donors (Heyneman, 1967). Passive transfer of immunity from mother to child does not occur (Demina et al. 1968).

Antibodies may well be transferred from mother to child in the milk, but in human visceral leishmaniasis, there is no evidence that such antibodies are functionally protective (Heyneman, 1971).

Manson-Bahr and Southgate (1964) and Southgate (1967) have

reported that transient infection with L. adleri protects man against further challenge by L. adleri and L. donovani.

The protective role of frequent exposures to non-human strains of Leishmania is stressed by Southgate et al. (1967; Southgate and Manson-Bahr, 1967; Southgate, 1967).

Vaccination against L. donovani by a related non-visceralising rodent strain protects against inoculations of cultures of L. donovani but will not protect populations from natural exposure (Manson-Bahr and Southgate, 1963). But even this was contradicted by Manson-Bahr et al. (1963) who inoculated four volunteers with Leishmania parasites. One of them had been inoculated previously with the rodent strain of Leishmania and was leishmanin positive: all four developed visceral leishmaniasis.

Tabatabaii et al. (1975) described 24 cases of visceral leishmaniasis in Iran and suggested that kala azar is more prevalent than the numbers of diagnosed cases might suggest. Important factors in the high mortality rate from this infection are lack of local medical care, malnutrition, poor hygiene and lack of education.

Inapparent or non-clinical infections, equally protective against reinfection, sometimes occur in endemic foci (Demina et al. 1968).

Rezai et al.(1975) reported a natural antibody (growth inhibitory factor) against Leishmania in normal human and animal sera and attributed it to occult infection leading to immunisation, exposure to heterogenetic antigens or to genetic determination of antibody.

Cases of inapparent infection are probably instances of failure of the pathogen to spread to the major lymphocytopoietic centres (Demina et al., 1968).

In the Mediterranean young children living in close contact with infected dogs are exposed to repeated infections. As a result they develop partial immunity or premunition which protects them from infection in later life, in the opinion of Southgate and Oriedo (1967).

Pampiglioni et al. (1974) suggested that it was probable that in leishmaniasis as in most other parasitic infections (tuberculosis and leprosy) clinically evident cases form only the tip of the iceberg, there is a wide spectrum of reaction to infection, and cryptic or asymptomatic

cases form the large majority of the infections. It is also probable that it is these cryptic infections which are responsible for the high incidence of leishmanin positivity found in endemic areas of visceral leishmaniasis in Kenya and the Sudan (Manson-Bahr 1961a; Manson-Bahr and Southgate, 1964; Heynaman, 1971) and in Sicily (Pampiglioni et al.1974). The existence of self curing or cryptic infections has been suggested previously (Leishman, 1906; Napier and Krishnan 1922; Knowles et al., 1923; Napier and Das Gupta, 1930; Sen Gupta, 1947; Corkill, 1948; Berberian, 1959). The delicate immunological balance could be upset by stresses such as famine, epidemic malaria and other infections and result in the typical syndrome of visceral leishmaniasis. It has been suggested previously (Corkill, 1948) that outbreaks of visceral leishmaniasis notoriously follow famine, war, social disturbances and epidemic malaria. Such cryptic cases could form reservoirs of future outbreaks when environmental conditions were suitable as in the case of Brill's disease in epidemic typhus. (Pampiglioni et al. 1974).

Reports of asymptomatic visceral infection (Armstrong, 1945; Prata, 1957; Sen Gupta, 1962) as well as positive leishmanin test in East African and Sudanese endemic centres of visceral leishmaniasis, add to our uncertainty over the existence of a true sterile immunity. Premunition may therefore exist in many individuals and constitute a continuous source of antigen stimulation in the dermis (Heyneman, 1971).

Different reports (Manson-Bahr, 1961b; Southgate and Manson-Bahr, 1967) have revealed individuals in whom infection with Leishmania stimulate rapid acquired resistance without obvious clinical disease. Thus the prevalence of subclinical infections may determine the resistance of a population to the development of epidemics (Napier, 1946; Nadim et al. 1968)

Iversson et al.(1979) in Sao Paulo using IFAT found five asymptomatic cases among 591 sera examined from an endemic focus. One of those positives showed increased levels of serum proteins, and three of the positives showed a positive Montenegro reaction.

Pampiglioni et al.(1974) in Italy used the complement fixing antibody test and found six positive out of 655 people living in the focus. These six were subsequently positive to Leishmania. All were asymptomatic except one who had an enlarged spleen. He found Leishmania in one of the cases by liver biopsy.

Shurkina and Gorbunova (1978) doing a serological survey on

normal residents of an endemic focus using IFAT found out that there are subjects possessing antibodies towards the disease increasing in percentage with age, and they regarded this as an indirect method to confirm the existence of asymptomatic forms of infection of L. donovani among the population of foci of visceral leishmaniasis.

The number of positive leishmanin reactors is considerably higher in an endemic area than where there is no kala azar; the reason could be due to false positives, to past infections or to subclinical infections.

E. THE LABORATORY INVESTIGATION OF LEISHMANIASIS

Terminology of development stages of Trypanosomatid flagellates is adopted here according to the nomenclature of Hoare and Wallace (1966).

Rondanelli et al. (1977) used the term endomastigotes instead of amastigotes or micromastigotes.

Leishmania is a part of closely related flagellated protozoa, the Kinetoplastidia. The kinetonplast is a unique mitochondrium which has an unusually large amount of DNA localised in one region of its inner matrix and which is clearly visible at light microscope level (Honigberg et al. 1964).

The genus Leishmania belongs to the Family Trypanosomatidae (WHO/LEISH/68.7).

1. Isolation and maintenance of the parasite.

In man the leishmanial parasites could be found in the peripheral blood. In the case of Mediterranean visceral leishmaniasis parasites are very rarely found in blood smears, but 90% of the cases can be detected by sowing 2 - 3 drops of blood on Locke blood agar (Adler, 1940).

In a case of infantile kala azar acquired in Palestine it was found that cultures could be obtained by sowing 0.1 cc of blood on Locke blood agar (Adler and Theodor 1931b).

Out of a total of 36 cases (34 examined in Italy during 1930 and 2 examined in Palestine during 1929) 33 gave positive blood cultures, i.e. 91.7%.

Cannata in 1914 (quoted by Adler and Theodor 1931b) demonstrated the presence of L.D. bodies in eight cases of infantile kala azar but only after prolonged examination of a number of blood films.

Young and Van Furth (1923) in China had the experience that if blood from untreated kala azar cases is taken and most of the serum and red cells removed by centrifugation, positive cultures may be obtained in 90% of the cases. Mononuclear and polymorphs are heavier than red blood cells and go to the bottom of the centrifuge tube.

There are more parasites found in the blood in the Indian type than the Mediterranean type of kala azar (Adler and Theodor, 1931a).

Shortt et al. (1927) found that in India 78.7% of proved cases of kala azar one can see the parasites in blood smears easily. Leishmanial parasites could also be found in other places. In 1934, in China, Forkner succeeded in finding the L.D. bodies in 9 out of 15 cases in their nasal secretions, in one of them even smears from the surface of the tonsils and the saliva also contained L.D. bodies. Nasal discharge of two patients inoculated into susceptible animals produced infection in them.

In Iraq Nouri and Al-Jeboori (1973) stated that blood examination in no case revealed Leishman Donovan bodies. Nasal smears examination used previously by Tajeldine and Al-Hassani (1961) resulted in one positive out of three patients with kala azar.

Different culture media were described by different workers. Hendrick's liquid medium which included 199, Grace's insect tissue culture medium and Schneider's drosophilia medium each in combination with 30% v/v foetal calf serum. This medium is commercially available (Hendricks et al. 1978).

Steiger and Steiger (1976, 1977) described RE3 medium for culturing L. donovani using inorganic salts and 14 L-amino acids, glucose, adenosine and a mixture of 11 vitamins, and related growth factors with purified defatted albumin.

Steiger and Meshnick (1977) in a study of the nutritional requirements of promastigotes of L. donovani and L. braziliensis found that glucose was consumed most rapidly, amino acids had been consumed in varying amounts from very high glutamic acid and proline to low phenylamine.

Citri and Grossowics (1955) prepared a partially defined medium for cultivation of blood flagellates where blood was replaced by haematin, crystalline serum albumin and a series of simple growth factors. The growth was comparable to that obtained on optimal blood containing media.

Semisolid medium is described by Chang in 1947, for growing various species of Leishmania, also by Adler and Theodor (1927).

Napier (1924) discussed the change of hydrogen ion concentration in NNN media. When prepared it was pH 7.7, on day 8 it became pH 7.0, after which the hydrogen ion concentration increased so slowly that it may be looked upon as constant.

Different workers tried to modify different media to make them more simple and more yielding. Rioux et al. (1970) described the heart brain blood (rabbit and sheep) media for mass cultivation of promastigotes, using sheep blood which is easily obtained.

Dedet and Lanotte (1969) found that the potato carrot blood culture medium was least productive, the heart brain blood was best, but the NNN was preferable for conservation of strains (even up to 45 days without subculture) and general reliability, though the actual organism content was somewhat lower. The canine strain of L.donovani developed poorly in all three types of media.

Al-Jeboori (1979) described a diphasic medium lacking whole blood and using foetal calf serum to culture the parasites.

Contamination of cultures is still a problem in Iraq and diagnosis is usually made by direct examination (Sukkar, 1976c).

Rassam (1979) pointed out that the semisolid media were slightly better than the diphasic media for the primary isolation of the parasite from cases of visceral leishmaniasis. A variation of the pH from 7.2 to 7.7 or the addition of proline did not affect the results.

Maintenance of culture from first isolation was difficult. Bray was completely unable to maintain L. infantum of Baghdad in any sort of culture and addition of blood did not improve this (Bray et al. 1967).

Simpson (1968) found that the leishmania-leptomonad transformation of L. donovani occurs in 20 - 40 hours at 27°C in the absence

of cell division.

Trager (1953) found that if either stage of L. donovani on an actively growing culture is placed in any of the same culture medium and incubated at 37°C no development occurs and the organisms present die within a day or two (Christophers, 1925; Berrebi, 1936). The Leishmania form which in nature grows intracellularly at 37°C has not been obtained in culture except with surviving host cells (Weinman, 1939; Hawking, 1948). Trager (1953) incubated suspensions of Leishmania from spleen of infected hamsters incubated at 37°C after adding human erythrocyte extract and human serum, intermediate forms appeared which lasted 4 days and died whatever he did. Multiplication was confined to the first few days.

Jadin and Creemers (1967) have shown that the rosette of promastigotes found in cultures are due to a feeding process and not as has sometimes been thought, to rapid division.

Handman et al. (1974) described the method of storing promastigotes in culture medium containing 10% glycerol and kept in liquid nitrogen.

It appears that in general Leishmania spp. maintain indefinitely their infectivity for susceptible hosts by cyclical passages through sandflies.

It is also advisable to maintain strains by continuous passage through hamsters and in the case of L. enriettii through guinea pigs. (Adler, 1964).

2. Serological procedures.

The goal of these in epidemiological studies is to obtain data on the levels and patterns of antibodies to specific antigens in the sera of population groups in relation to relevant variables, and thus to contribute to the knowledge of the epidemiology of the disease (Paul and White, 1973).

Serological tests to be uscd in epidemiological surveys in developing countries must meet the following criteria:

1. Simple to perform
2. Interpretation of results free from subjectivity
3. Rapid

4. Minimal cost
5. Sensitive and specific (validity)
6. Reproducible

The enzyme linked immunosorbent assay (ELISA) and immunofluorescent antibody test (IFAT) meet most of these requirements.

Specificity is the proportion of false positive reactions among a population who have never had the infection. And the sensitivity is the proportion of false negative reactions among infected people.

There is no particular virtue in using a test that produces very high titres; the degree of reproducibility is more important for achieving comparability of the test results, and ideally a clear separation between negative and positive titres.

In areas with a low level of endemicity, a high degree of specificity of the test used is of paramount importance: if the true antibody prevalence is very low the positive test result may include a relatively large proportion of false positive reactions.

Seroepidemiological surveys need to contain serological and parasitic data in addition to other factors that may influence the occurrence of infection and disease. (Lobel and Kagan, 1978).

Problems due to cross reactivity had been worked on. Oelerich et al.(1974) reported cross reaction between sera from lepromatous leprosy, TB, trypanosomiasis and L. donovani serologically by the complement fixation test and they concluded that the double gel diffusion test proved to be the best one for identification of homologous reactivities.

Antibody has been demonstrated in sera from L. tropica and L.braziliensis infections by immunofluorescence (Oddo and Cascio, 1963). However, other attempts using this technique have failed to demonstrate circulating antibody during infection with L. tropica,L.m.mexicana and L. peruviana (Duxbury and Sadun, 1964; Quilci et al. 1968; Shaw and Voller, 1964; Bray and Lainson, 1965).

In the opinion of Adler (1964) attempts to differentiate Leishmania spp. by serological methods were the only possibility, but what is more important is the fact that antigenic composition of Leishmania is complex and a single reaction such as the complement

fixation or agglutinin cannot possibly reveal the action of the various antibodies evoked by corresponding antigens.

The work of Al-Jedoori and Evans (1980) showed that by isoenzymes one can differentiate between some different strains of the parasite. They recognised three strains out of ten isolations, one parasite was isolated and it gave two different results. This could not be linked geographically, but they suggested that it could explain the different clinical picture from the point of view of the severity of the illness referred to by Khairy and El-Hashimi in 1980.

Antigenic studies of the parasite were attempted by Le Ray et al. (1974) who showed that the specific parasite antigen includes the so called:

a) exoantigens released in the external medium (at least in vitro) of the two stages of the parasite;

b) Surface antigen;

c) Somatic antigen.

Until now the antigenic structure of Leishmania has been essentially studied at the level of culture of the promastigote form. This profile shows a complex mosaic of specific parasitic and widespread antigens, the latter present in a minority, correspond to components shared by other antigens of pathogenic microorganisms and probably by host-like antigens. (Decker-Jackson and Honigberg 1978).

Schnur et al. (1972) found that metabolic factors of Leishmania (EF) excreted in vitro by promastigotes and by amastigotes precipitate with antiserum raised against homologous promastigotes. This suggests that antigenic determinants are shared by a promastigote, by its EF and by the EF of the homologous amastigote. Excretory factor was collected during the long phase of growth of promastigote cultures 3 days after inoculation. The supernatant fluid of a culture from which promastigotes had been removed by centrifugation was filtered through millipore filters of 0.45 μm pore size and stored at -20°C.

EF of L. donovani is not dialysable in Visking membrane or Sartorius membrane filters and is unaffected by heating at 56°C for half an hour, it remains unchanged after lyophilisation and reconstitution two months later.

For seroepidemiological studies of infection in which IgM antibodies are of diagnostic value, fresh plasma samples and not absorption on filter paper should be used, because the last technique will destroy the reactivity of IgM. (Voller et al. 1976).

Iversson et al.(1929) did a seroepidemiological survey using IFAT around an autocthonous case of visceral leishmaniasis in an urban area in Sao Paulo; they found some asymptomatic cases (five in number) of ages from 4 to 16 years with 1/80 - 1/160 titres; one of them showed high serum proteins, 3 of them positive Montenegro.

Pampiglioni et al. (1974) in northern Italy made a seroepidemiological survey in an area of visceral leishmaniasis. He collected sera from the residents and examined them with IFAT and found that six indivuals showed positive titres from 1/10 - 1/80; none of them showed or developed the symptoms or the clinical syndrome (except one with splenomegaly).

a. Indirect immunofluorescent antibody test (IFAT)

Fluorescence technique is the method used in serology to detect antigen antibody reaction using a fluorescing marker like fluorescene isothiocyanate. Fluorescence antibody method is a cytochemical staining. Fluorescence is the emission of light of one colour during the time a substance is irradiated with light of another colour (Goldman, 1929).

Stoke's law: emitted photons will have less energy and therefore longer wavelengths than exciting photons. Exciting wavelength 490 nm, fluorescence wavelength is 520 nm.

Shaw and Voller (1964) described the method of fluorescence: after fixing the antigen slides the sera were added and the slides incubated for 45 minutes, washed for 15 minutes, treated with conjugate for 45 minutes, washed again for 15 minutes, mounted in 10% glycerol and examined. Kagan and Norman (1976) also described the method in detail.

One of the advantages of fluorescent antibody technique is that localisation can be accompanied on an individual cell rather than an organ basis as is the case with the use of radioactive isotopes (Tobie 1958). The major antigenic site of Leishmania as

demonstrated by the fluorescence antibody technique were the cell wall and the kinetoplast (Shaw and Voller, 1964).

For fluorescent microscopy it is important to work in a darkened room because any stray light interferes with visual observation. The actual amount of light emitted at fluorescence is of low order as compared with that of conventional microscopy.

It was found that a lamp containing a red 15 Watt fluorescent bulb properly placed near the microscope table provides sufficient light for reading but did not interfere with the microscopic observations (Tobie, 1958).

The type of antigen used is the whole parasite antigen WPA. This introduces into the test a whole "mosaic of antigens" of the parasite, this excludes the possibility of a preferential study of this or that specific antigenic fraction,and one could regret that the metabolic antigens excreted by the parasite in the body of the host and likely to be of considerable immunological importance, cannot be investigated by direct immunofluorescence (Ambroise-Thomas, 1976).

Oddo and Cascio (1963) using this indirect technique showed that serum from patients with Mediterranean visceral and cutaneous leishmaniasis reacted with culture forms of L. donovani as antigen.

Guimaraes et al. (1974) preferred to use 8-day NNN cultures of promastigotes for immunofluorescence; they stated that antigen slides could be kept at -20°C for three years without loss in antigeneity.

Shaw and Voller (1964) prepared antigen for IFAT from 10 days culture of NNN smeared on a slide and dried, immersed in 0.3 HC1 for 5 minutes followed by two washings in PBS 0.15M pH 7.2. Leishmanial forms were also used as antigen using cut edge of an infected hamster liver after blotting to wipe them on the slide, then the slide treated similarly.

Hedge et al.(1978) reported the use of an easily cultured Crithidia sp. which is sometimes used in the monoxenic culture of Entamoeba histolytica and is maintained in TTY-SB monophasic medium as described by Diamond (1968) as an antigen in IFAT for kala azar.

This antigen is also used by Lopez-Brea (1980).

Specific antibodies are produced in man following active infection with L. donovani. These antibodies are detectable in a reproducible manner by the indirect fluorescent antibody technique using as antigen leptomonad forms of L. donovani (Duxbury and Sadun, 1964).

Leishmania forms were used as antigens by Duxbury and Sadun (1964). An antigen of amastigotes, described by Shaw and Lainson (1977) for IFAT purposes, was prepared after homogenisation of a skin lesion of hamster infected with L.m. amazonensis fixed by formalin, air dried, incubated at 50°C for 30 minutes, then stored at -20°C with silica gel. They concluded that this antigen was both specific and sensitive.

But Bray and Lainson (1965) found that reactions were less strong with Leishman-Donovan bodies as slide antigens than with leptomonads.

Another method of using amastigotes as antigen was described by Herman (1965) who took Leishman-Donovan bodies from infected hamster's spleen and injected them intraperitoneally into the previously saline stimulated abdominal cavities of hamsters. Macrophages containing intracellular parasites were harvested from these hamsters and maintained in vitro on cover slips in Leighton tubes in a balanced salt solution serum medium and were used as antigen in fluorescence antibody studies.

The method of fluorescence in general was described using formalin to fix; Duxbury and Sadun (1964) used formalin for fixation of parasites, washed promastigotes fixed with acetone, also described by other workers (Quilci et al., 1968; Rioux and Golvan, 1969).

Mansueto et al.(1975) found that immunofluorescence test for visceral leishmaniasis is the better test with regard to sensitivity and specificity.

Cross reactivity is the reactivity of antibodies with an antigen other than that used for immunisation. This is due either to sharing of determinants by two antigens or to stereochemical similarity between two antigens.

The most avid antibodies (antibodies with the highest affinity) also exhibit the highest degree of cross reactivity. (Klein 1975).

Different workers tested cross reactivity with IFAT and following are some examples: Camargo and Rebouato (1969) described the cross reactivity in fluorescence between L. donovani or Chaga's serum against Leishmania or T. cruzi, also described by Shaw and Voller (1964).

Indirect immunofluorescence could not differantiate among species of Leishmania, and there was complete cross reaction with T.Cruzi (Araujo and Mayrink 1967).

Sera of 13 patients with visceral leishmaniasis were tested by IFAT by Silva and Camargo (1977) for diagnosis of Chaga's disease and positive results were obtained suggesting "group reactions" within the family Trypanosomatidae.

Shaw and Voller (1964) also found that fluorescent antibody technique revealed a group antigen antibody reaction among species and strains of Leishmania.

Other methods were used and the same results were obtained with passive haemagglutination and antitrypanosomal IgM antibodies but not with the complement fixation test.

Serum for patients with visceral leishmaniasis may cross react with T. cruzi and Mycobacterium in IFAT and indirect hemaglutination test as currently done (Zuckerman, 1975).

Araujo and Mayrink (1968) treated sera from 6 pulmonary tuberculosis patients with antigens of Leishmania donovani and they gave negative results.

L. donovani is capable of inducing an antibody which reacts with M. tuberculosis antigen, whereas the latter organism is not able to induce an antibody that can react with L. donovani and L.braziliensis antigen.

On the other hand cross reactions occurred occasionally with sera from malaria, trypanosomiasis and leprosy patients, also with sera from patients with syphilis, tuberculosis and schistosomiasis (Duxbury and Sadun, 1964, and Shaw and Voller, 1964).

Some claimed to detect circulating antibody in cutaneous leishmaniasis by fluorescent antibody technique, but it is generally accepted that antibody does not occur in simple oriental sore. While antibody was readily detected in kala azar by the use of either promastigotes or amastigotes as antigen (Bray, 1972).

In cutaneous leishmaniasis the circulating antibody level is very low, as demonstrated by Duxbury and Sadun (1964) and Araujo and Mayrink (1968).

The single-sore, cutaneous and usually self-limiting forms of leishmaniasis - Chiclero's ulcer, oriental sore, and uta - give rise to only a low circulating antibody level in sera despite the fact that they apparently cause a solid life-long immunity in most cases (Bray and Lainson, 1965).

Some of the sera from patients with proven infection with L. donovani gave a negative reaction with IFAT which could be due to deficiency in sensitivity (Duxbury and Sadun, 1964).

Mannweiler (1978) stated that the immunofluorescent test is the most sensitive one for leishmaniasis and the second best was the complement fixation. Indirect haemagglutination is unreliable.

Quilci et al.(1968) considered indirect immunofluorescence to be the most sensitive technique; they detected antibodies in 100% of kala azar cases. After treatment antibody levels fell and cell mediated immunity as indicated by delayed hypersensitivity emerged (Manson-Bahr, 1959; Chaves and Torrealba, 1967). Its shift along the spectrum was accompanied by the development of immunity to reinfection (Turk and Bryceson, 1971).

Zuckerman (1975) found that fluorescent antibody titre recedes after cure and a negative fluorescent antibody test is interpreted as signifying cure.

The method of choice for detection of antibody whether in clinical diagnosis or in survey is the fluorescent antibody test (Bray, 1975; Bray and Lainson, 1965).

Rezai et al.(1977) concluded that the technique of indirect immunofluorescence can be used in laboratory diagnosis of leishmaniasis. The fact that sera from human cases react equally well with all three

Leishmania species is an advantage in that a laboratory need not keep all species of Leishmania in culture in order to identify the leishmanial origin of questionable infections. Rezai et al. (1977) suggested the diagnostic titres to be 1/64 because the majority gave a titre of more than 1/250. This technique is also applicable in epidemiological investigations of animal infection, or animal reservoirs of Leishmania parasites.

It may be useful for investigation of subclinical infection of Leishmania organisms. There have been reports based on skin tests that subclinical infections exist in cutaneous as well as systemic leishmaniasis.

The technique is also useful for tracing the infection in experimental animals. (Rezai et al.1974).

It could also be used to advantage in epidemiological investigations of visceral leishmaniasis (Ambroise-Thomas 1976; Duxbury and Sadun, 1964), although it is group specific but this is not a serious limitation except in regions of the world where both trypanosomiasis and leishmaniasis are endemic (Shaw and Voller, 1964). Ambroise-Thomas (1976) pointed out the specificity limit of the test.

The fluorescent antibody test is proving to be of increasing value as Moskovskij and Southgate (1971) stressed, particularly as a screening method for the early diagnosis of the presymptomatic parasite carriers.

Bray and Lainson (1965) showed that while the fluorescent antibody technique might have a use in detecting visceral leishmaniasis in a field survey (as was shown by Duxbury and Sadun, 1964), it appeared that it would not be useful in any field survey of oriental sore.

IFAT was actually used in seroepidemiology to study kala azar by Iversson et al. (1979) in Sao Paulo; with this method they found some asymptomatic cases.

Shurkina (1977) found that the diagnostic value of immunofluorescence test in visceral leishmaniasis was confirmed at a titre of more than 1 in 100. She found that the level of antibody was directly related to the duration of the disease.

Shurkina and Gorbunova (1978) did a serological survey using indirect immunofluorescence test and found:

1. Within 6 - 12 months after cure from visceral leishmaniasis in about 80% of 83 cases the antibody titre declined to 1:20 or the test became negative, the maximum duration of persistence of antibody of L. donovani in convalescents was 6 years.

2. Among 1375 normal residents of visceral leishmaniasis endemic areas, the percentage of subjects posessing antibody (1:20) varied insignificantly from 0.9 - 2.8% in different foci.

3. The rate of positive tests increased in these foci in older age groups 5.4 - 7.8% and reached maximum levels in the adult population of the foci (average 13.9%).

These date may be considered to be an indirect confirmation of the existence of asymptomatic forms of infection with L. donovani among the adult population of foci of visceral leishmaniasis.

IFAT could also be used to follow up known cases of visceral leishmaniasis. Lopez-Brea (1980) followed up the titre of three kala azar cases for a period of 1 - 6 months, and the titre in one case fell from 1/320 in October to 1/40 in May the next year (in about 8 months).

b. Enzyme-linked immunosorbent assay (ELISA)

ELISA had some advantages over other methods in serology. Engval and Perlmann (1972) suggested that the use of an enzyme marker in solid phase immunoassay of antigens or antibodies should have several advantages over that of radioactive isotopes.

1. Enzyme labelled antigen or antibody can be stabilised and used for years, while isotope labelled reagents can only be used for a very limited period of time.

2. The detection of enzyme activity only requires a simple equipment if a coloured product is generated by the enzymatic reaction.

3. The sensitivity of the technique may be increased due to the catalytic nature of the marker.

The method is described in general in the Bulletin of the World Health Organisation (1976), by Voller et al.(1976), also Voller et al.(1977).

The method is described also by Roffi et al. (1980) for cutaneous leishmaniasis and by Anthony et al. (1980) for sero-diagnosis of New World leishmaniasis

Hommel (1976) used the buffers of Engval and Perlmann (1972).

A buffer (phosphate buffered saline PBS) containing a wetting agent Tween was used to prevent non-spec. .ic adsorption to the solid phase (Voller et al.1977).

In some cases it may be needed to include additional protein (e.g. albumin) to reduce background non-specific uptake of reagents to low level (Voller et al.1977).

Polystyrene plates of special formula Dynatech Lab. are best suited for coating with many proteins and lipoprotein antigens. The sensitisation can be carried out by passive adsorption in alkaline solution.

Hommel (1976) used sonicated promastigotes as antigen and adsorbed the antigen on microtitre plates. For conjugate Hommel (1976) used horseradish peroxidase as a marker enzyme, the conjugate was used at 1/400 dilution of the commercially available Miles Yeda conjugate. For substrate he used 3-3' diamino bazedine/H_2O_2 which produced brown colouration.

To date the most satisfactory peroxidase substrate has been OPD, orthophenylene diamine, because it yields a strong orange coloured product which is very soluble and is stable in the dark (Voller et al.1977).

The substrate reaction is stopped when the reference positive reaches a predetermined value (Voller et al. 1977).

Incubation at 37°C for 30 minutes for both sera and conjugate were used sometimes. The end result is expressed as adsorption on a given wavelength (Voller et al. 1976).

It was found by Voller et al.(1976) that there was poor correlation of results between ELISA and IFAT. Voller tested a group of sera, results analysed in terms of Leishmania IFAT values. It is clear that people with high Leishmania immunofluorescence antibody values are more likely to have high leishmanial micro

ELISA values, but there was not a close correlation between the two tests.

Negative wells in the plate should be colourless (Voller et al. 1977), stressing the need to include a reference positive sample on each plate.

3. Leishmanin testing.

The leishmanin test is a method for detecting sensitisation of lymphoid cells rather than circulating antibody (Demina et al. 1968).

The reaction is group specific rather than species specific (Shaw and Voller, 1964). Since the test is genus specific the leishmanial species involved is not defined by the result of the test (Zuckerman 1975).

Preparation

The leishmanin test is a skin reaction to an antigen prepared from a culture of leptomonads of various species of Leishmania (Manson-Bahr, 1961b).

Classically the test used between 1 and 10 x 10^6 flagellates of any strain of Leishmania/ml in 0.5% phenol in saline or in Coca's fluid (Bray, 1975; Manson-Bahr, 1961b).

Sergiev and Shaikina (1969) have used the supernatant from the growth of promastigotes in culture with considerable success.

Bray (1975) has suggested to standardise the test and use a known amount of protein after sonication of the parasite.

In the leishmanin test a small amount, 0.1 - 0.2 ml of an antigen is injected intradermally and the result examined after 72 hours (Manson-Bahr, 1961b).

In a positive reaction a small area of induration like a pellet of lead shot is felt in and under the skin which fades after a few days, but may in some cases persist for weeks (Manson-Bahr, 1961b).

It was found that positive leishmanin reactions were over 5 mm and reached a maximum intensity within 24 - 72 hours after injection (Manson-Bahr and Southgate, 1964).

Heyneman (1971) describing the life of the Montenegro reaction said that the ability to mount a Montenegro reaction is probably lifelong; its duration has been reported as at least 55 years (Adler, 1961), 35 years (Kellina), and 21 years (Rodjakin), the latter two were quoted by Heyneman in 1971.

However, this positive reaction may revert to negative with the passage of time (Manson-Bahr, 1961b).

Musumeci et al.(1977) skin tested some previous (3 - 10 year) cases of kala azar, discharged after being improved, and found out that they are still positive although controls are negative.

52% of previously positive Montenegro reaction due to cutaneous leishmaniasis became negative in 20 days to 7 years' time (Mayrink et al. 1976).

Extensive skin testing aided by statistical age prevalence studies might help to determine the initial onset of the disease in a community or demonstrate past epidemics. However, high infection density may mask any such interpretation (Heyneman, 1971).

Bettini et al. (1977) did leishmanin skin testing on 1285 people in the Tuscany region of Italy. In a known endemic area for leishmaniasis there was an increasing positivity with age, while in another area the curve was bell shaped, suggesting an interruption of transmission in the recent past. In all areas the positivity of males was higher than females. But it was not possible to distinguish between areas of cutaneous leishmaniasis and visceral disease.

Manson-Bahr (1961b) suggested that where over 5% of the population of an area shows a positive leishmanin reaction then kala azar is endemic in that area. It was noticed that in no areas where kala azar had occurred was there a very high leishmanin positivity rate, i.e. no areas could be termed "infected but immune" (Southgate and Oriedo, 1967).

A significant proportion of those Sudanese with no history of kala azar who were skin tested by Cahill et al.(1965) showed a positive response. As in Kenya the percentage of positives increased with age (Heyneman, 1971).

It was found that in people who did not contract the disease

the skin test was positive only after a sojourn of not less than 5 years in an endemic area (Heyneman, 1971).

The number of positive leishmanin reactors was considerably higher in an endemic area of Kenya than where there was no kala azar. These positive reactors were made up of those people who had had the disease in the past, a few true false positive reactors and some who may have had a subclinical infection (Manson-Bahr 1961b).

Iversson et al.(1979) in Sao Paulo using leishmanin found some positivity around asymptomatic cases detected by serological methods.

The Montenegro reaction is negative during the clinical course of the disease but in many cases becomes positive after successful treatment (Adler, 1965). Few cases of Mediterranean kala azar have been tested and they were negative (Adler, 1964; Rezai et al. 1978).

The absence of cell mediated immunity (CMI) in disseminated cutaneous leishmaniasis and in kala azar contrasts with the clinically effective cell mediated response which follows the majority of L. tropica infections. Since susceptible individuals show no general evidence of immune deficiency, a 'tolerogen' in the form of antigen which suppresses specific lymphocytes has been postulated to explain the observed anergy (Bryceson, 1970b); Bryceson et al. (1970).

Conditions where high titres of antibody are produced usually result in depressed CMI (Feldmann 1975).

The skin sensitivity and immunity would appear to develop 6 - 8 weeks after treatment (Manson-Bahr, 1961b).

Southgate and Oriedo (1967) in their epidemiologic study with the leishmanin test showed that there seemed to be an inverse relationship between the presence of positive skin reactions and the occurrence of cases of clinical kala azar. This indicated population immunity.

In visceral leishmaniasis and in disseminated cutaneous leishmaniasis the test remains negative throughout the period, a positive leishmanin test in visceral leishmaniasis signifies and indicates the development of cell mediated immunity (Zuckerman, 1975).

The intradermal inoculation of promastigotes cultures from lizards into mammals and man induces a positive leishmanial response

and gives rise to long-standing dermal granulomas from which living parasites can be recovered for periods of several months (Belova, 1971).

The response of persons living in an endemic area to continual exposure to animal leishmaniae may add to the complications surrounding the use of the leishmanin test (Heyneman, 1971).

The ground squirrel strain of L. donovani is dermatropic and causes dermal and not visceral lesions, and also coverts a negative leishmanin reaction to positive. (Manson-Bahr, 1961b).

Southgate in 1967 made a study on L. adleri and concluded that naturally acquired positive leishmanin reactions in Kenya may be due to transient skin infections with L. adleri and that they are associated with immunity to kala azar.

Individuals with naturally occurring positive leishmanin reactions are immune to challenge with human or rodent strains of L. donovani, just as cured kala azar patients and persons previously inoculated with rodent strains of Leishmania (Manson-Bahr and Southgate, 1964).

14 people with naturally positive leishmanin test were immune to inoculations of a rodent strain of Leishmania. Four leishmanin negative controls were infected with this strain (Southgate and Manson-Bahr, 1967).

Vaccination attempts against kala azar were taken by Manson-Bahr and Southgate (1964) of 2946 people using the strain of Leishmania isolated from Xerus rutilus and known as the Ground Squirrel strain, 50% of them were vaccinated and 50% control. After a follow up of three years it was found that the attack rates between the two groups were the same.

Halawani and Guirges (1973) used a leishmanin test (strength of 2×10^6/ml) in Iraq. 70% of kala azar cases were said to give a positive reaction when the illness had been present for six months. Leishmanin survey in two schools gave 4.7% and 5.2% positivity but the children tested were aged 5 - 10 years.

4. Skin windows.

Skin windows are used by some workers to detect macrophages coming from the blood and bone marrow or wandering about in the dermis. This technique is relatively non-traumatic and if successful in finding

infected macrophages could be employed with advantage in the diagnosis of visceral leishmaniasis. It has been used by Boggs et al. (1964), Bradley (1978) and others for cytological purposes.

CHAPTER II

SITE MATERIALS AND METHODS

A. INTRODUCTION

1. Iraq

Situated between longitudes $48^{o}45'$ and $38^{o}45'$ and between latitudes $37^{o}22'$ and $29^{o}5'$, Iraq from the geographical point of view is considered as part of the Mediterranean basin. Iran borders Iraq from the east, while Syria, Jordan and Saudi Arabia border Iraq from the west, Turkey from the north and Kuwait and the Arab gulf from the south.

Iraq is 438,446 square kilometres in area and according to the 1977 census the population of Iraq is 12,000,497, 4,354,443 of them living in the rural areas (36.3%). Details of census figures according to age and sex are shown in Table 3 and Figures 2 and 3.

The climate of Iraq is generally semi-arid, cold in the winter from November to March, and hot and dry during the summer. Temperatures may vary widely between night and day and between winter and summer, extremes of these temperatures may range from $-5^{o}C$ in winter to almost $50^{o}C$ in the summer with little rainfall, ranging from 2.5 - 30 mm monthly in the rainy season.

Administratively Iraq is divided into:

1. The northern region which includes the following provinces:
    1. Dhok
    2. Ninawa
    3. Erbil
    4. Sulaimaniya
    5. Tamin

It is relatively more cold than the rest of Iraq and topographically it is mountainous.

Table 3. Rural population of Iraq according to the 1977 Census distributed according to age and sex.

Age	Males	% of total	Females	% of total	Total
- 1	192,678	4.42	172,370	3.96	365,048
- 2	93,625	2.15	88,518	2.09	182,143
- 3	89,411	2.05	86,921	2.00	176,332
- 4	86,723	1.99	85,217	1.96	171,940
- 5	87,394	2.01	83,533	1.92	170,927
- 6	88,095	2.02	78,017	1.70	166,112
- 7	89,215	2.05	81,679	1.88	170,894
- 8	86,895	2.00	74,471	1.71	161,366
- 9	76,058	1.75	63,337	1.45	139,395
- 14	280,365	6.44	251,513	5.78	540,878
- 19	119,968	2.76	168,685	3.87	288,653
- 24	191,769	4.40	180,832	4.15	372,601
- 29	135,237	3.11	141,689	3.25	276,926
- 34	90,770	2.08	100,162	2.80	190,932
- 39	73,879	1.70	81,267	1.87	155,145
- 44	59,660	1.37	73,540	1.69	133,200
- 49	81,510	1.87	77,403	1,78	158,913
- 54	61,689	1.42	67,192	1.54	128,881
- 59	51,789	1.19	50,554	1.16	102,343
- 64	48,252	1.11	43,175	0.99	91,427
- 69	31,517	0.72	27,107	0.62	58,624
- 74	24,070	0.55	24,294	0.50	48,364
- 79	19,662	0.45	23,045	0.53	42,707
- 84	8,977	0.21	8,665	0.2	17,592
85 and over	14,096	0.32	13,866	0.32	27,967
unclassified	11,095	0.25	4,042	0.09	15,137
Total	2,203,349	50.39	2,151,094	49,87	4,354,443

Figure 2 Population pyramid of the rural areas of Iraq according to 1977 Census.

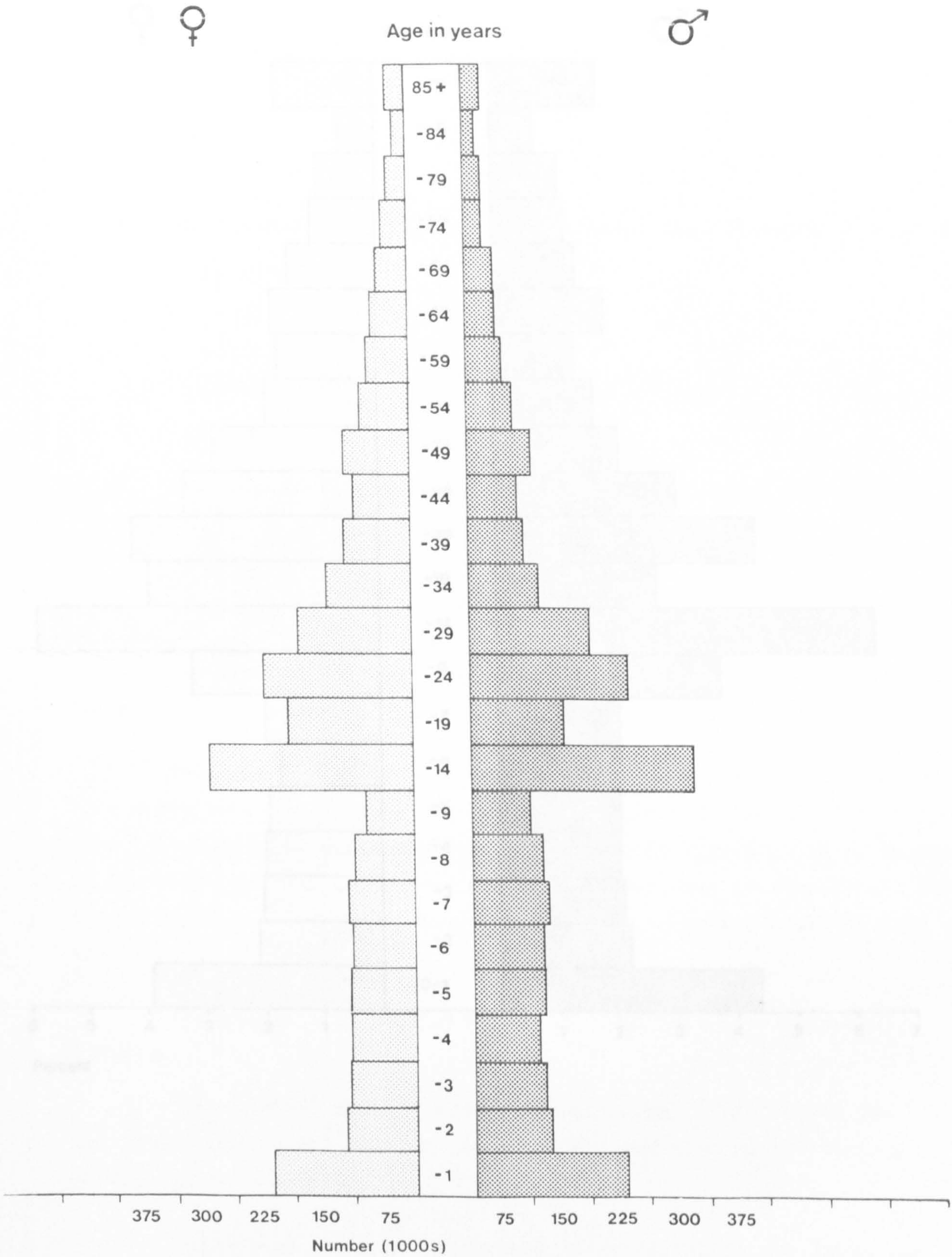
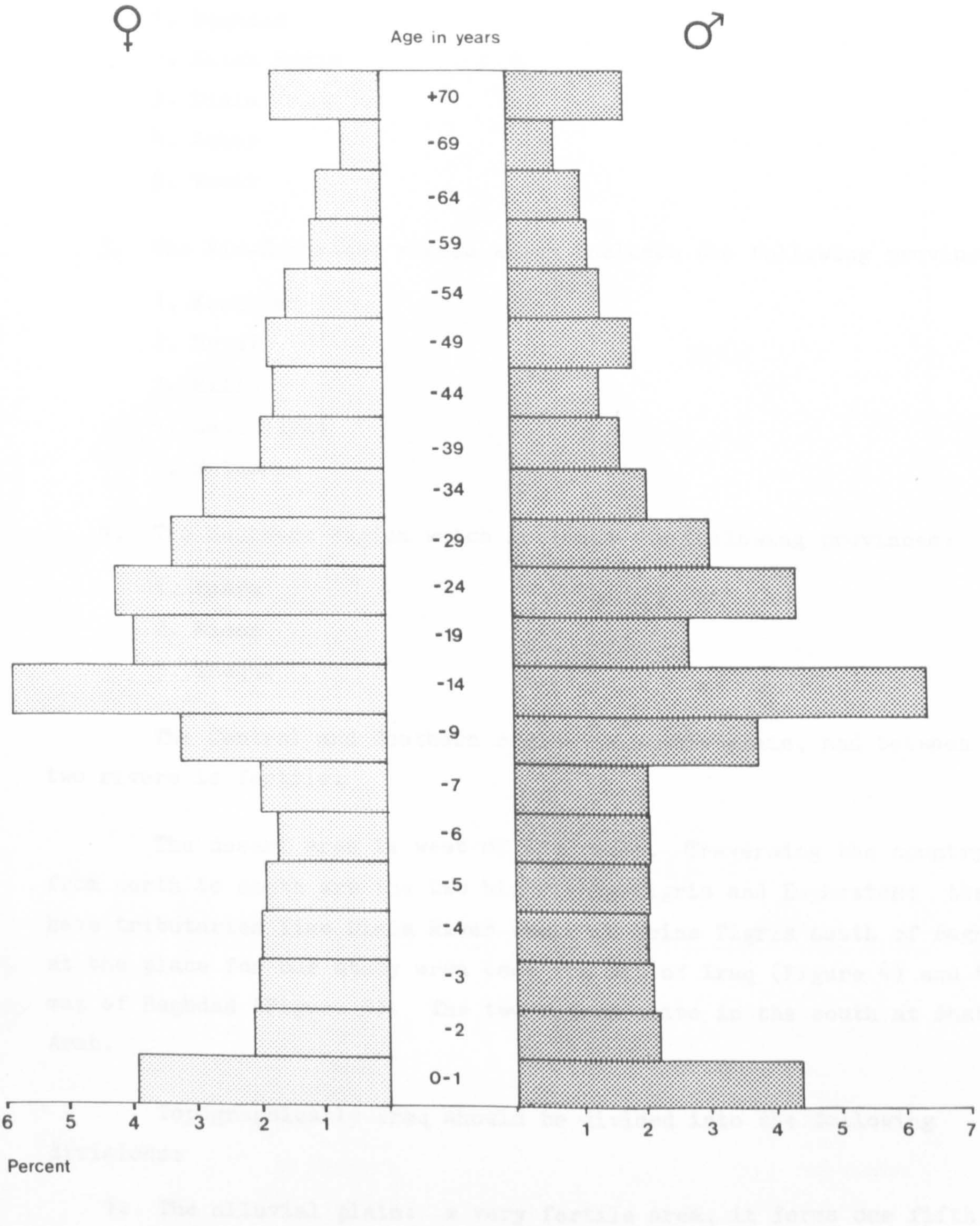


Figure 3 Population pyramid of the rural areas of Iraq according to 1977 Census.



2. The Central Region which includes the following provinces:

    1. Baghdad
    2. Salah Eddin
    3. Diala
    4. Anbar
    5. Wasit

3. The Mid-Euphrates region which includes the following provinces:

    1. Kerbala
    2. Najaf
    3. Hilla
    4. Qadissiyia
    5. Muthanna

4. The Southern Region which includes the following provinces:

    1. Basra
    2. Misan
    3. Theqar

The Central and Southern region is mostly plain, and between the two rivers is fertile.

The desert area is west of Euphrates. Traversing the country from north to south are the two big rivers Tigris and Euphrates; they have tributaries like Diala River where it joins Tigris south of Baghdad at the place for our study area (see the map of Iraq (Figure 4) and the map of Baghdad (Figure 5). The two rivers unite in the south at Shat al Arab.

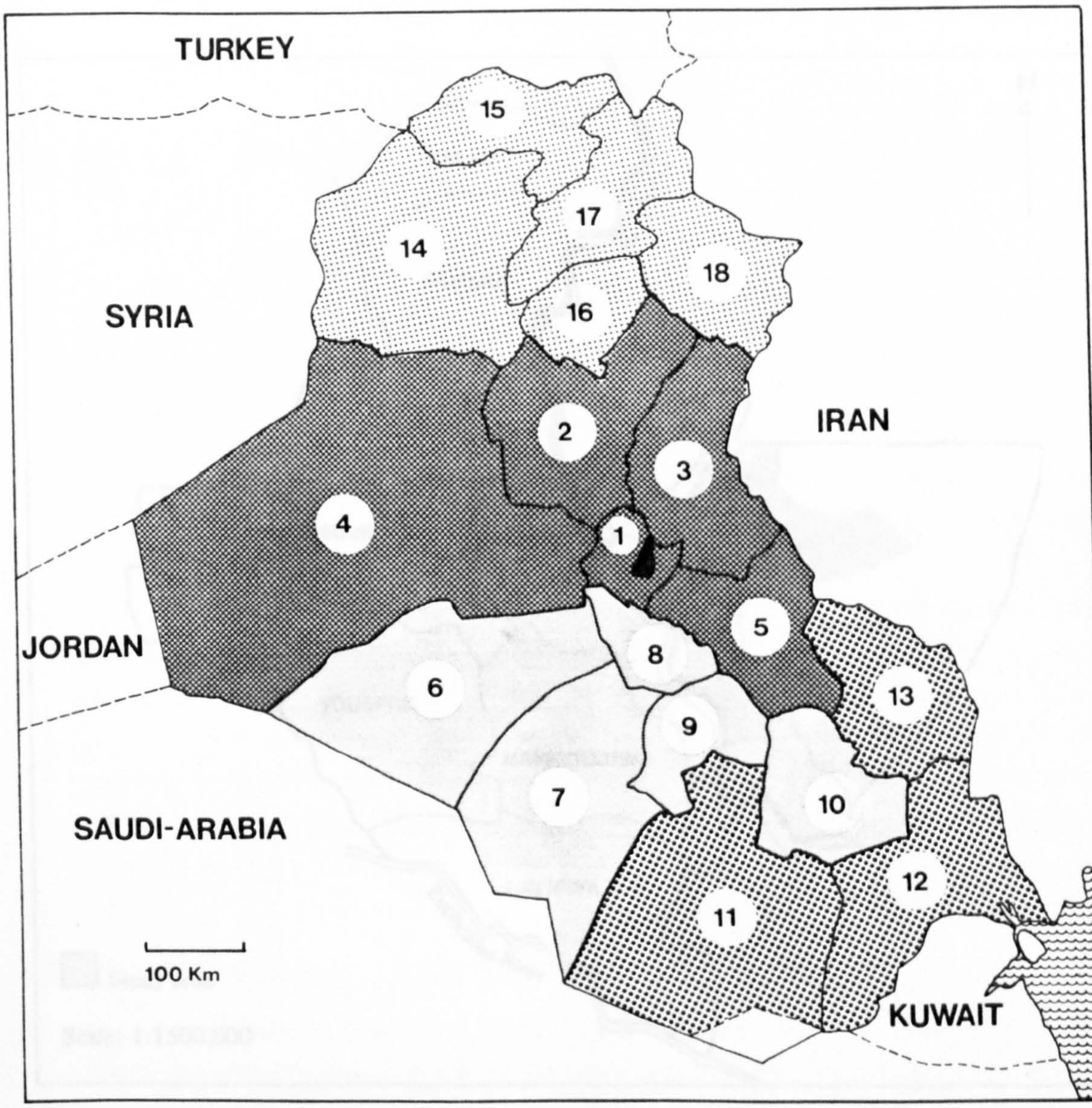
Topographically Iraq should be divided into the following divisions:

1. The alluvial plain: a very fertile area, it forms one fifth of the whole country and includes the southern and most of the central parts. It is a lowland.

2. The desert plateau: comprises three fifths of the land, and is situated in the western part of the country.

3. The mountainous region comprises one fifth of the country and is situated in the northern region.

Figure 4 Map of Iraq showing administrative divisions and the location of the study area.



The study area

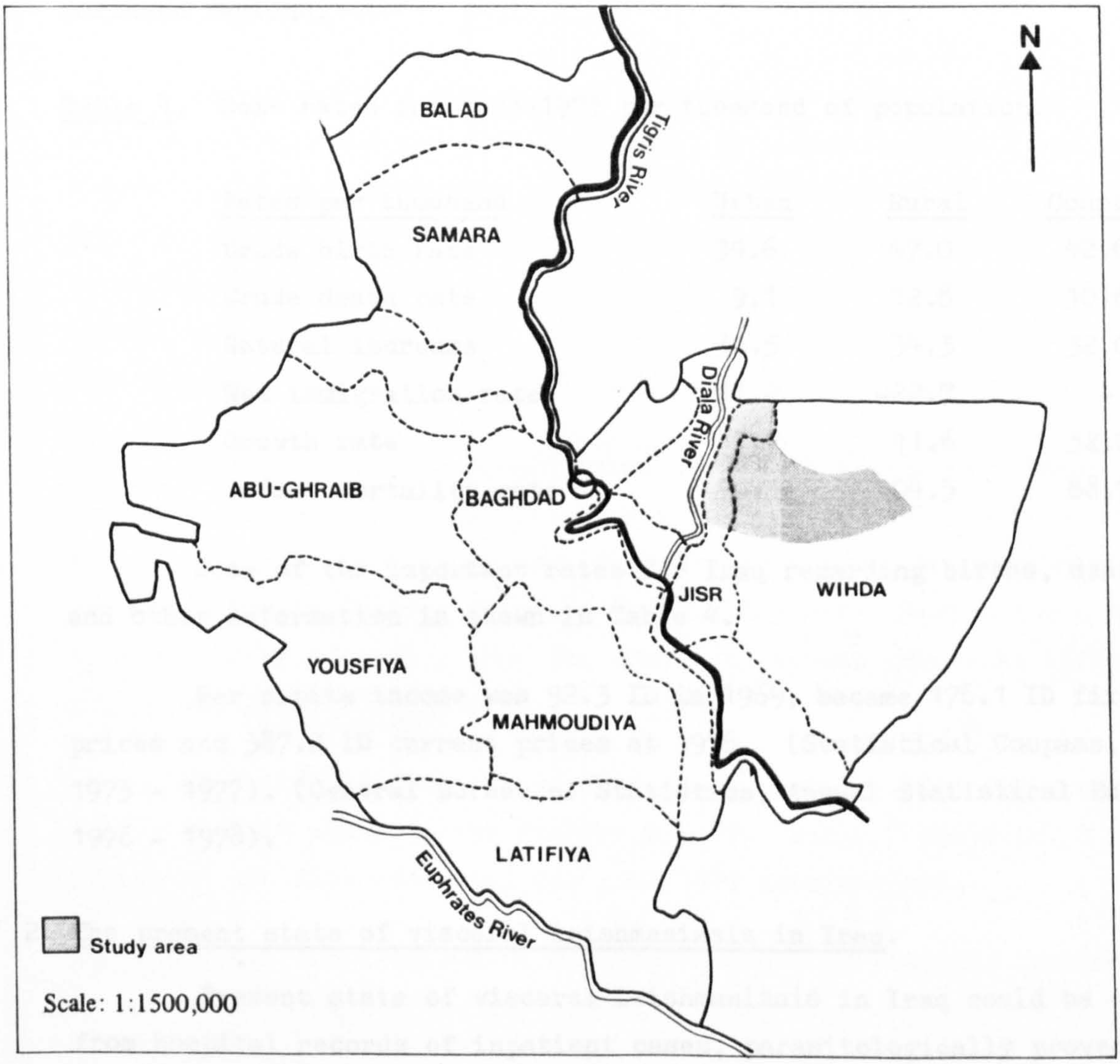
Central
1 Baghdad
2 Salaheddine
3 Diala
4 Anbar
5 Wasit

Mideuphratus
6 Kerbala
7 Najaf
8 Babylon
9 Qadissiya
10 Mathanna

Southern
11 Thequar
12 Basrah
13 Misan

Northern
14 Ninawa
15 Dhok
16 Tamim
17 Erbil
18 Sulaimaniya

Figure 5 Map of Baghdad Province showing the study area.



4. The terrain region: a transitional area between the lowlands and the high mountainous regions and situated between the central and northern regions.

Table 4. Some rates for 1973-1975 per thousand of population.

Rates per thousand	Urban	Rural	Country
Crude birth rate	39.6	47.0	42.6
Crude death rate	9.1	12.8	10.6
Natural increase	30.5	34.3	32.0
Net immigration rate	11.2	-22.7	-
Growth rate	41.7	11.6	32.0
Infant mortality rate	76.3	104.5	88.7

Some of the important rates for Iraq regarding births, deaths and other information is shown in Table 4.

Per capita income was 92.3 ID in 1969; became 176.1 ID fixed prices and 387.2 ID current prices at 1976. (Statistical Compass, 1973 - 1977), (Central Bureau of Statistics, Annual Statistical Bulletin 1976 - 1978).

2. The present state of visceral leishmaniasis in Iraq.

Present state of visceral leishmaniasis in Iraq could be elucidated from hospital records of inpatient cases, parasitologically proved or clinically suspected cases, because diagnosis sometimes depends on clinical suspicion only. The number of cases recorded as visceral leishmaniasis in Iraq since 1971 - 1979 is shown on Tables 9 and 10 and Figure 8, and they show that only a few hundreds were recorded yearly, a peak of 1691 cases were recorded in 1974; during the year 1979, 642 cases were recorded only.

The real attack rate of the disease in children is not known, moreover the incidence of the disease in children only and the rareity in adults led to two schools of thought either that the children were exposed and suffered subclinical infection which conferred them as adults with a lifelong immunity, or that children are more susceptible because of the immaturity of their immune system.

Age distribution of the disease: to have an idea about the incidence of the disease the cases recorded during 1979 were analysed and

it was found that 48% of the cases were in their first year of life. Distribution of those cases according to sex did not show significant differences, as shown in Table 6.

To demonstrate the time of recording of cases again the year 1979 cases were taken and analysed monthwise, as shown in Table 8 and in Figure 7; it was shown from that and from breaking up of the recorded cases of the year 1971 - 1979 also monthwise, that the disease was recorded round the year, no month showed cessation of the recording of cases; secondly it was shown that the recording of cases is very little during May to September rising to a peak in December to January.

To examine their geographical distribution the registered cases were studied geographically province-wise for the period 1971 - 1979, and it was shown that cases came from all over Iraq, but were more concentrated in the Central Region. The Mid Euphrates Region comes second in registering of cases, then the Southern Region, the least affected region was the Northern Region.

The distribution of cases in space province-wise for the period 1971 - 1979 is shown in the Figures 9 - 17. Table 7 shows the distribution of the disease during the year 1979 province-wise.

Table 5. Age distribution of registered cases of visceral leishmaniasis in Iraq for the year 1979.

Age	Number	Percent
- 1 year	308	47.9%
- 2 years	208	32.4%
- 3 years	64	10.0%
- 4 years	62	9.7%
Total	642	100.0%

Table 6. Sex distribution of registered cases of visceral leishmaniasis in Iraq for the year 1979.

Sex	Number	Percent
Males	333	52%
Females	309	48%
Total	642	100%

Figure 6 Distribution of visceral leishmaniasis cases for the year 1979 according to age.

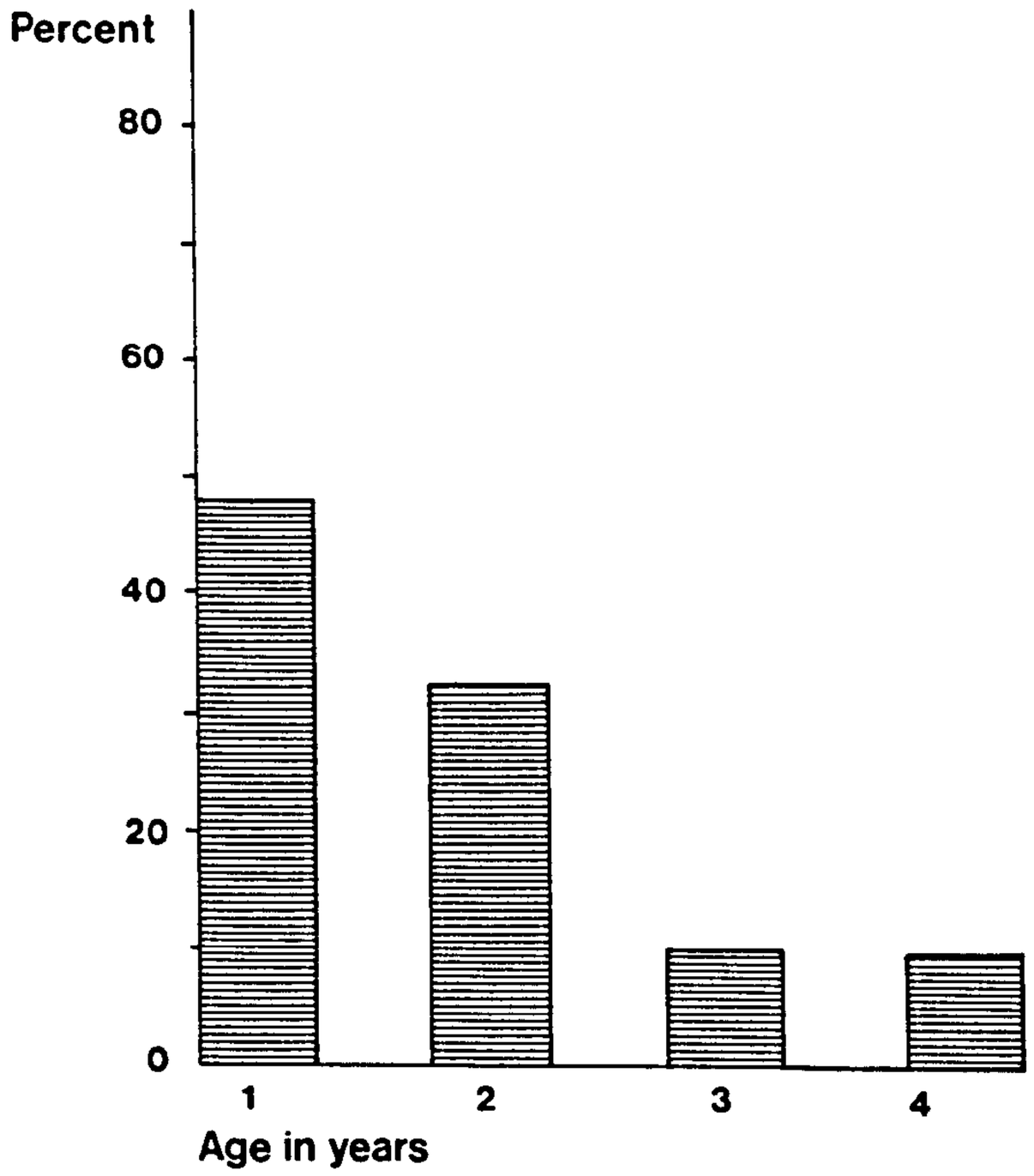


Figure 7 Distribution of visceral leishmaniasis cases for the year 1979 according to months of detection.

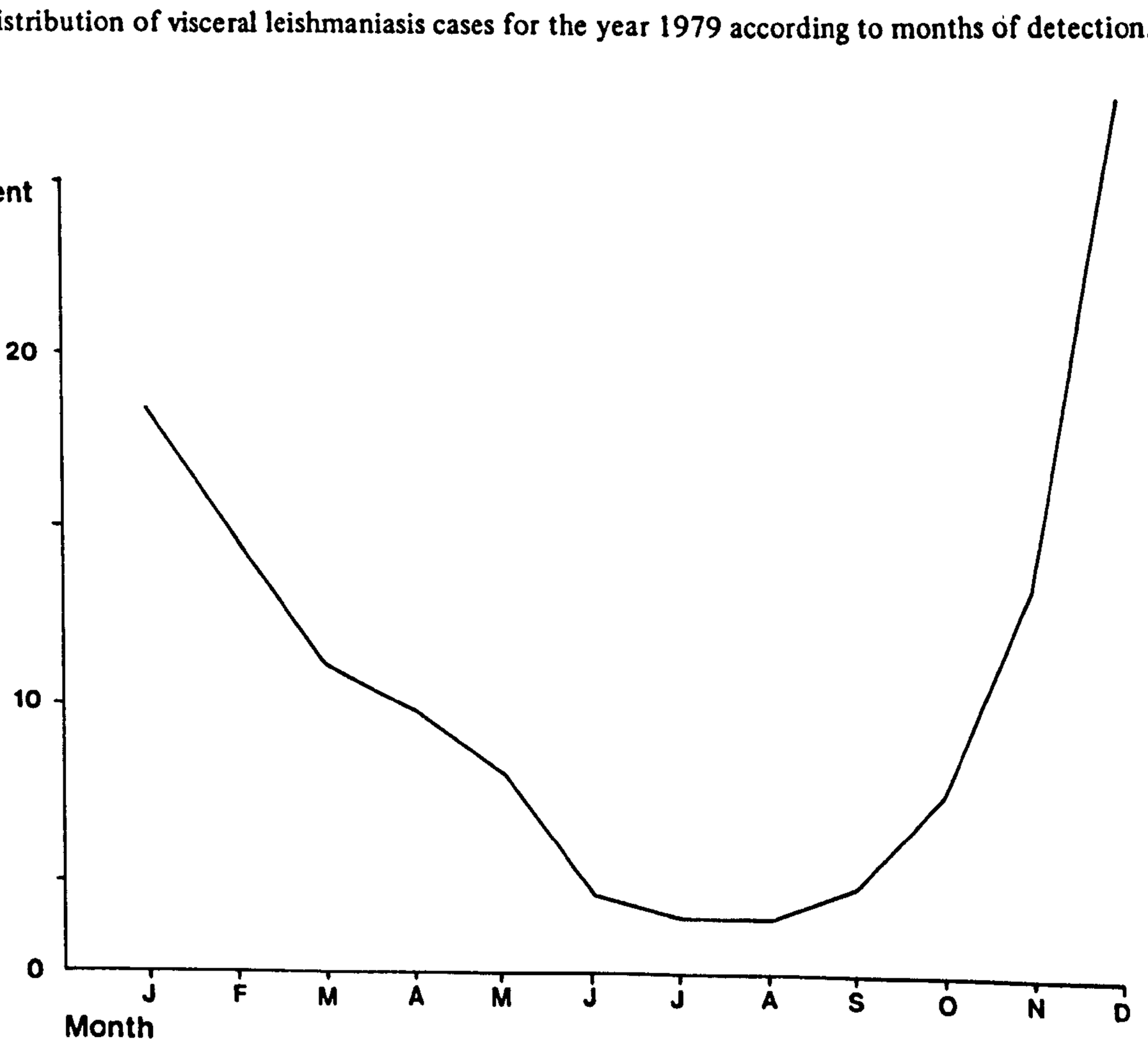


Table 7. Geographical distribution of registered cases of visceral leishmaniasis in Iraq in sequence of number of cases for the year 1979.

Province	Number of cases	Percentage
Baghdad	318	49.4%
Wasit	107	16.7%
Diala	62	9.7%
Anbar	33	5.1%
Theqar	33	5.1%
Babylon	41	6.4%
Qadisyia	20	3.1%
Karbala	3	0.5%
Salah-eldine	7	1.1%
Muthanna	8	1.2%
Misan	4	0.6%
Janaf	2	0.3%
Nineva	1	0.15%
Tamin	2	0.3%
Dhok	1	0.15%
Total	642	100.0%

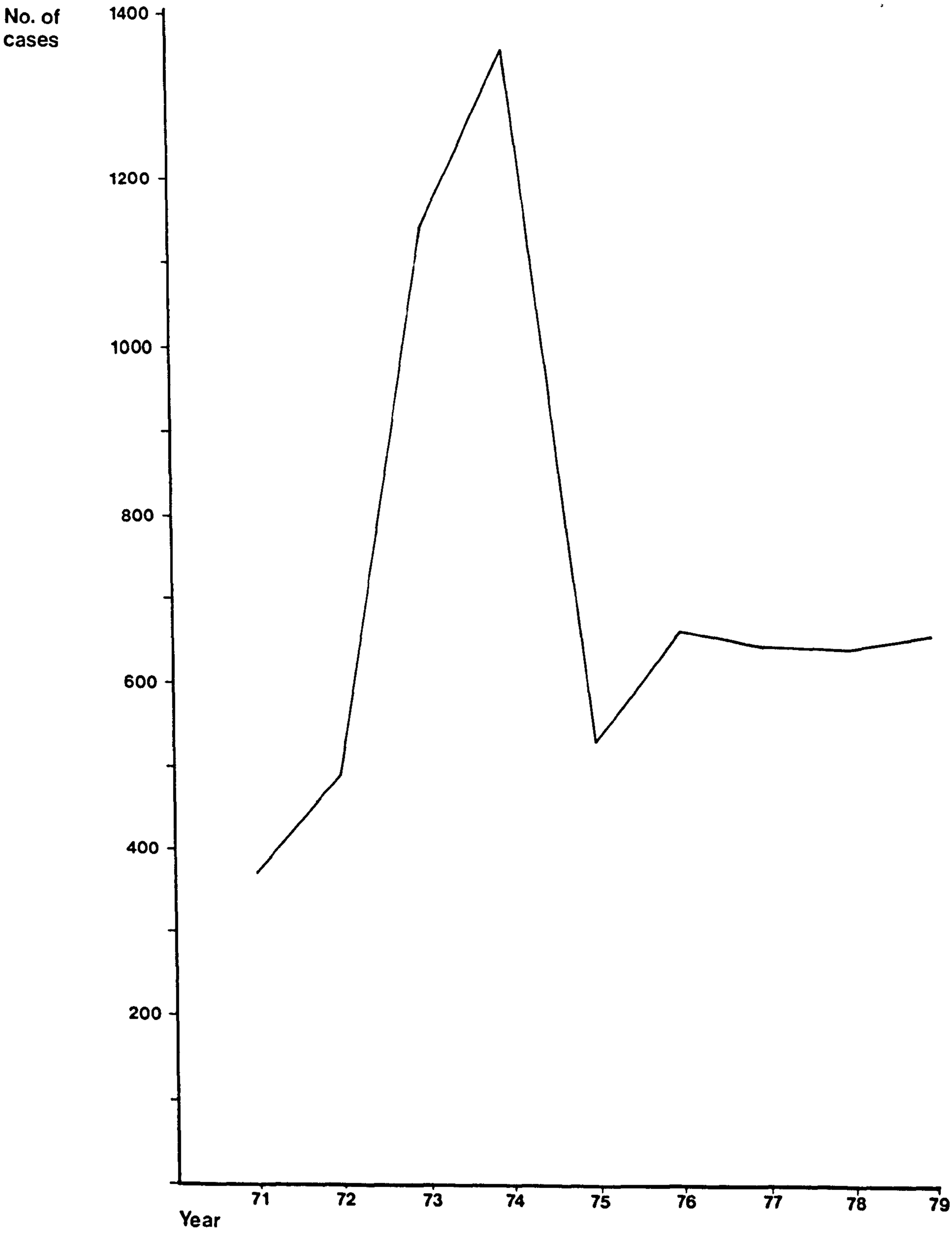
Table 8. Distribution of the 1979 registered cases of visceral leishmaniasis in Iraq according to months.

Month	Number of cases	Percentage
January	103	16.0%
February	79	12.3%
March	57	8.9%
April	49	7.6%
May	37	5.8%
June	15	2.3%
July	10	1.6%
August	10	1.6%
September	17	2.6%
October	33	5.1%
November	71	11.0%
December	161	25.1%
Total	642	100.0%

Table 9. Geographical distribution of visceral leishmaniasis cases in Iraq for the years 1971 - 1979.

Province.	1971	1972	1973	1974	1975	1976	1977	1978	1979
Dhok	2							1	1
Arbil	1				1				
Sulaimaniya		1							
Nineva			8			1	3	2	1
Tamin				1		1	9	4	2
Salah-eldin								12	7
Diala	11	15	76	226	23	27	16	56	62
Anbar	14	5	19	19	16	16	7	25	33
Baghdad	290	402	767	865	207	404	385	334	318
Wasit	18	27	130	270	88	93	67	81	107
Babylon	23	36	97	188	76	77	54	51	41
Najaf							3		2
Karbala	1		2	21	1	2	3	4	3
Qadisiya	3		1	71	74	25	12	17	20
Muthanna		1	1	1	4	2	11	2	8
Theqar	4	1	29	21	35	13	5	21	33
Maysan	2		3	6	1	6	8	2	4
Basra			1	2	2	1	4	2	
Total	569	488	1134	1691	528	668	587	635	642

Figure 8 Number of cases of visceral leishmaniasis recorded in Iraq for the period 1971-1979.



Geographical distribution of cases of visceral leishmaniasis in Iraq.

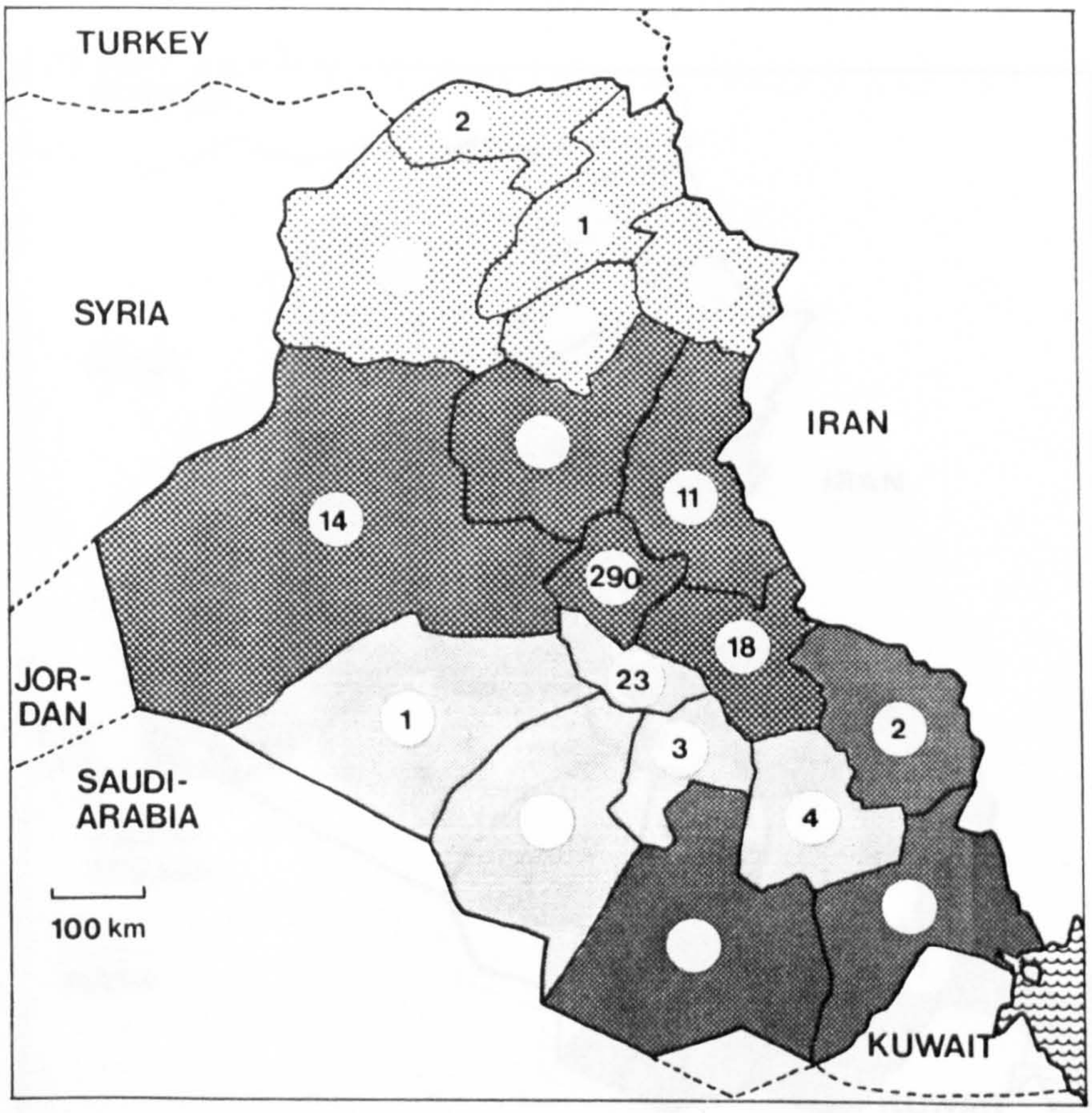


Figure 9
1971, total 369 cases

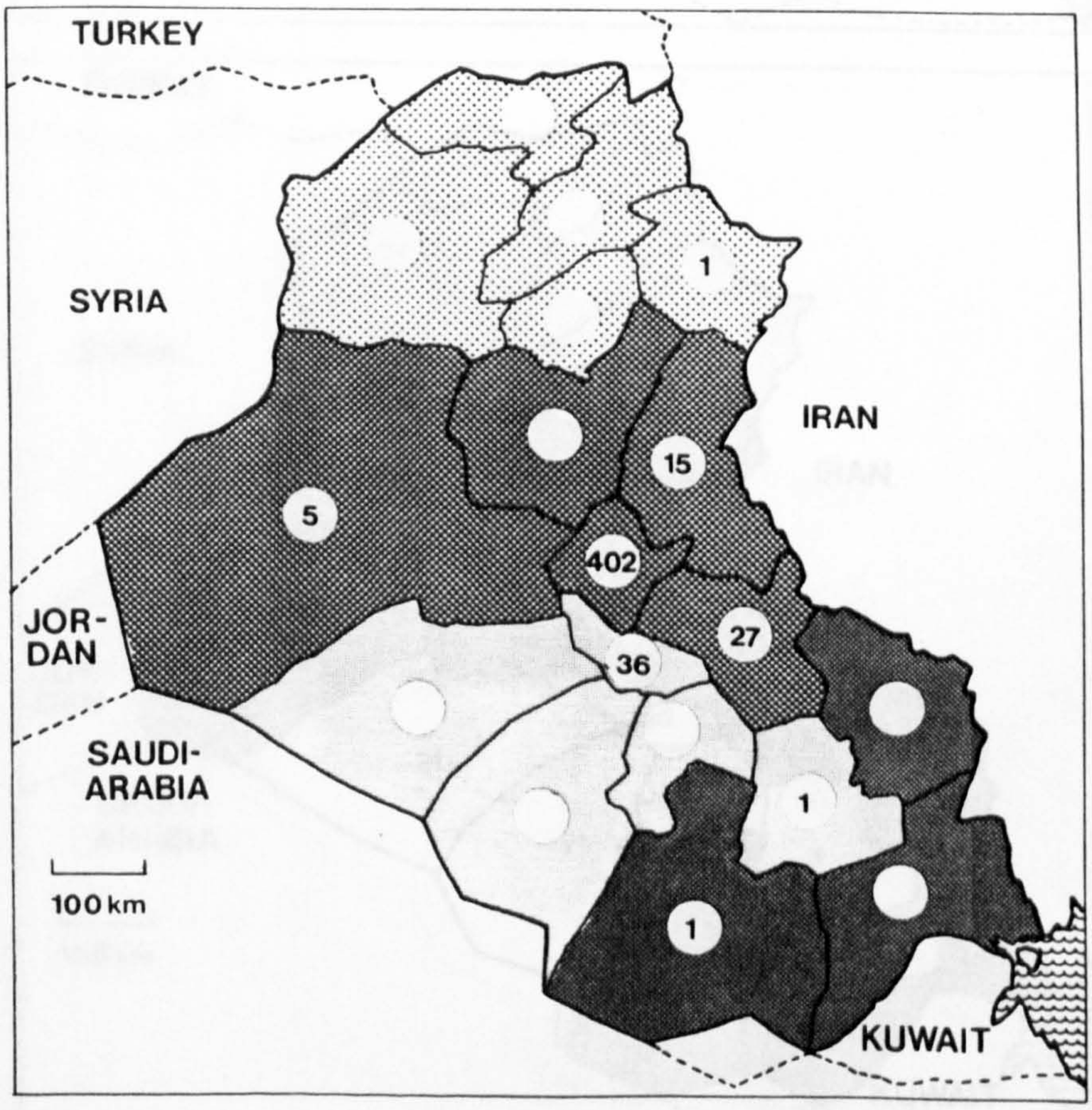


Figure 10
1972, total 488 cases

Geographical distribution of visceral leishmaniasis in Iraq.

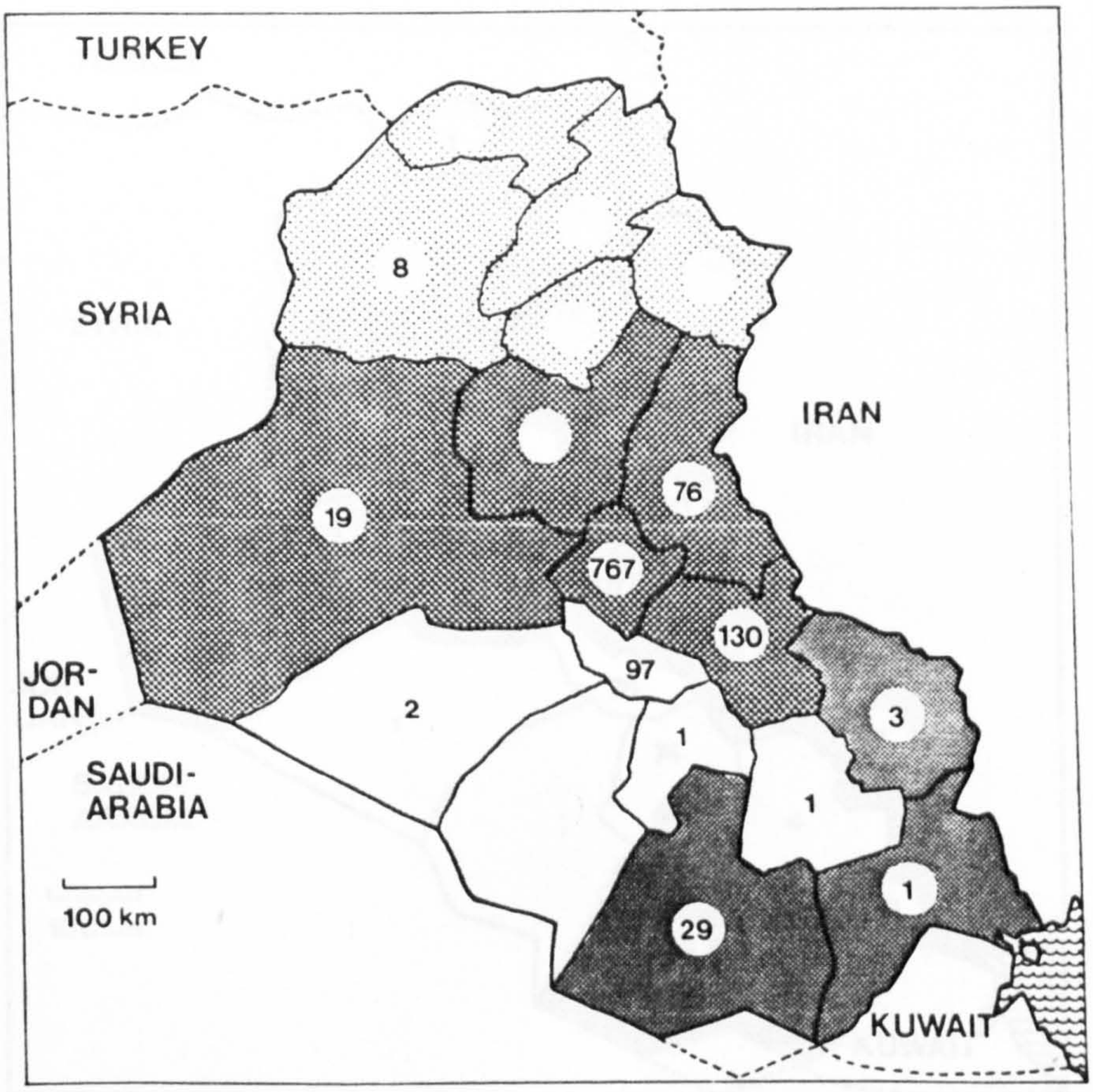


Figure 11
1973, total 1134 cases

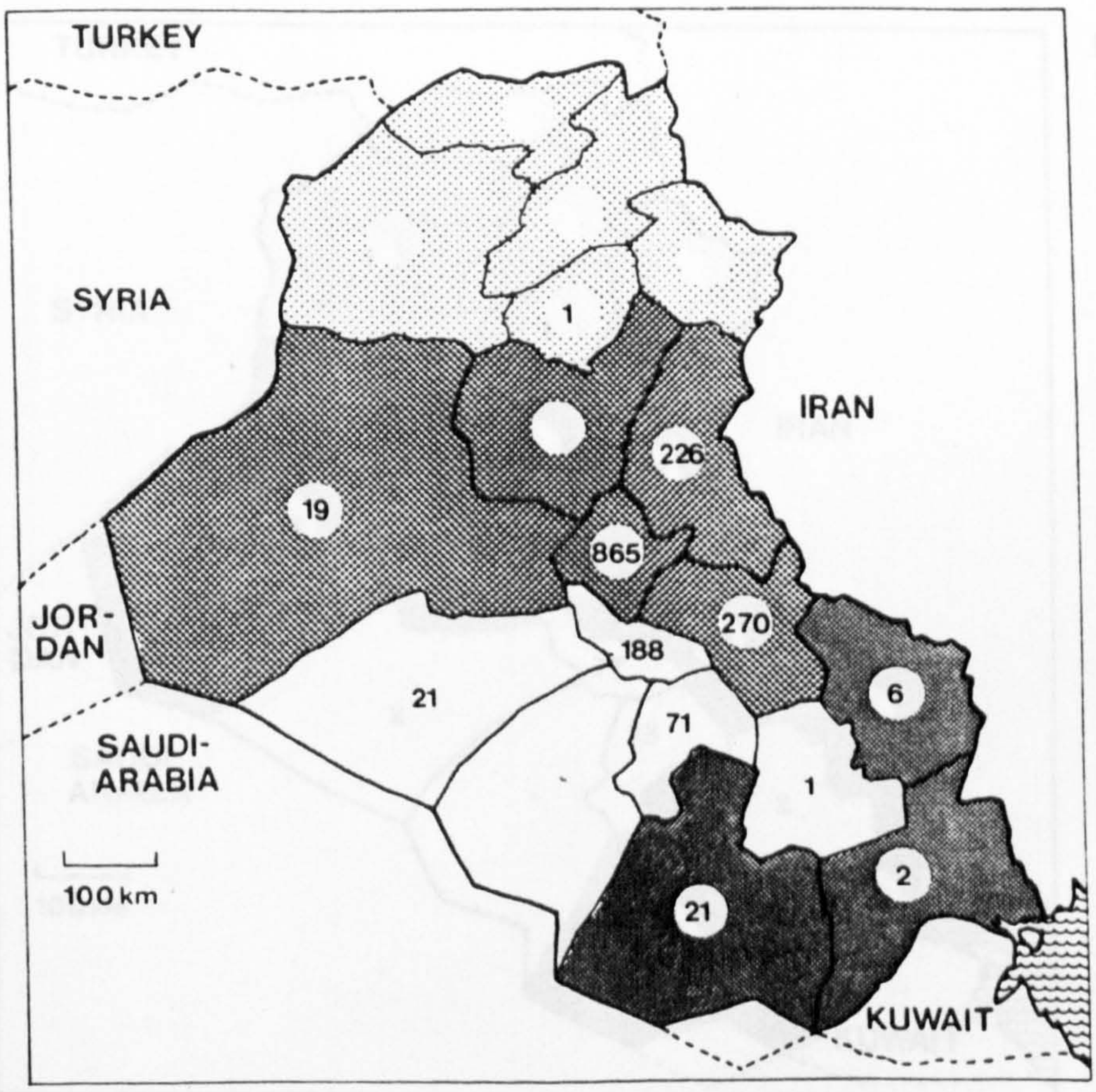


Figure 12
1974, total 1691 cases

Geographical distribution of visceral leishmaniasis in Iraq.

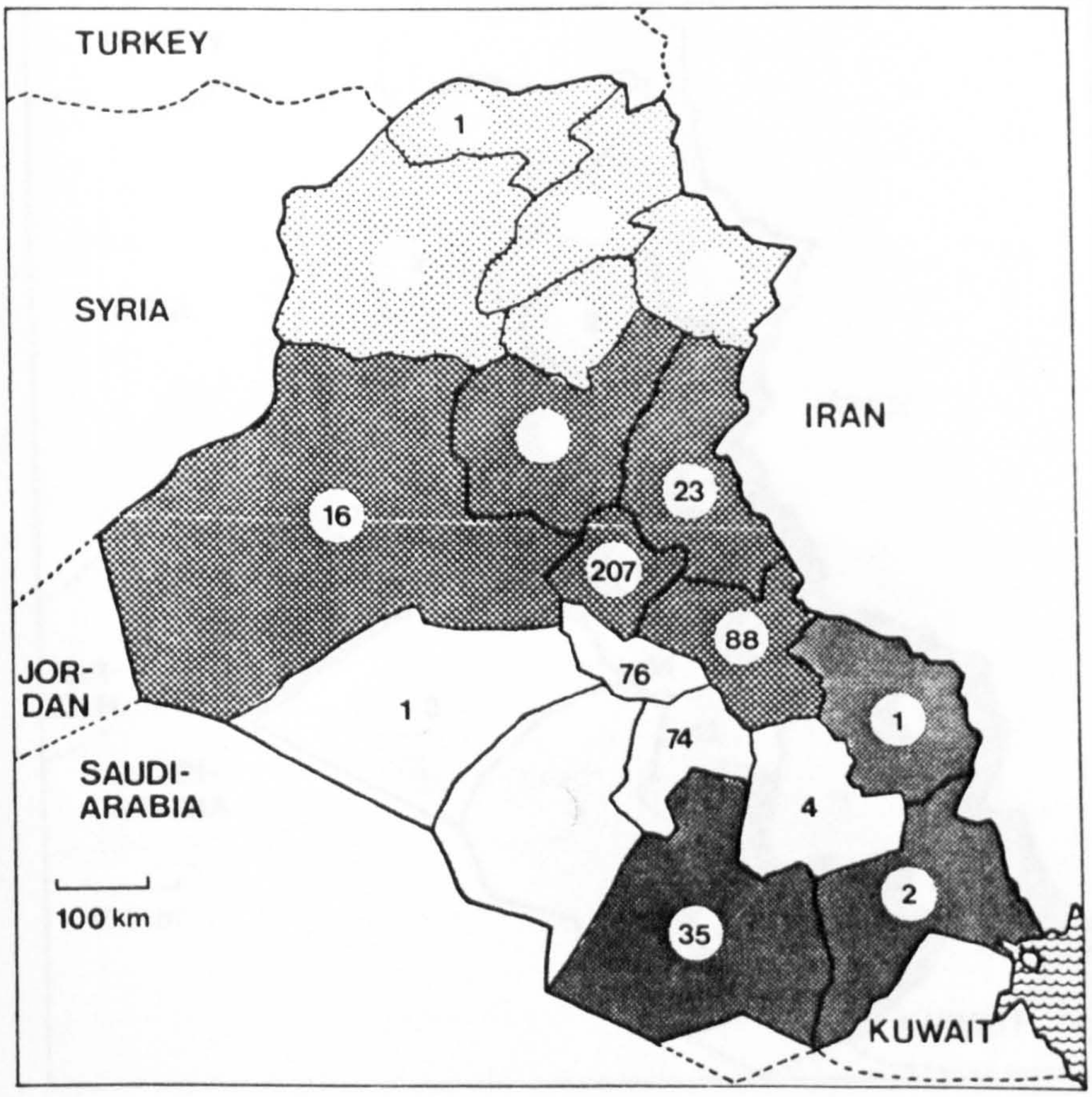


Figure 13
1975, total 528 cases

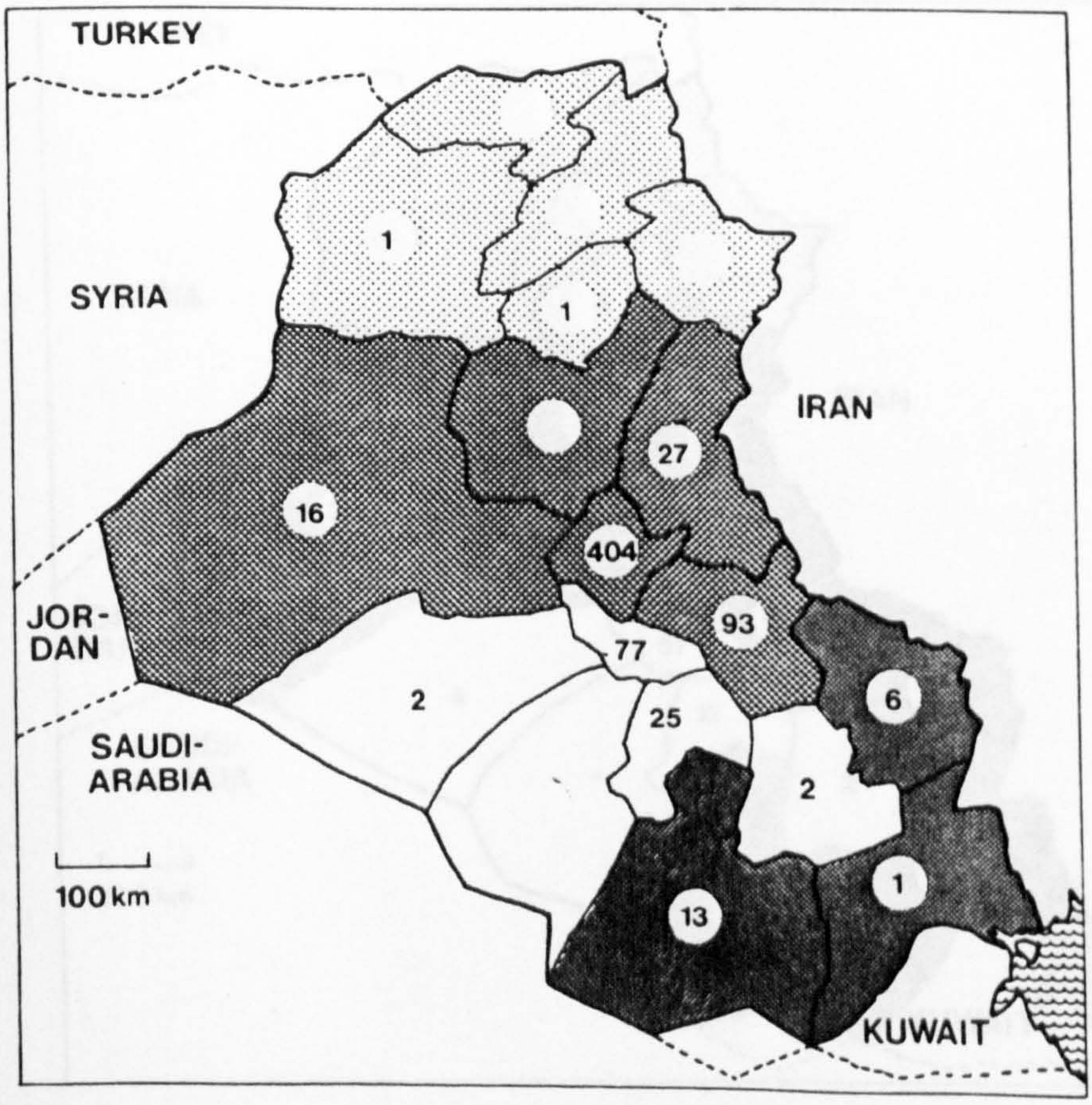


Figure 14
1976, total 688 cases

Geographical distribution of visceral leishmaniasis in Iraq.

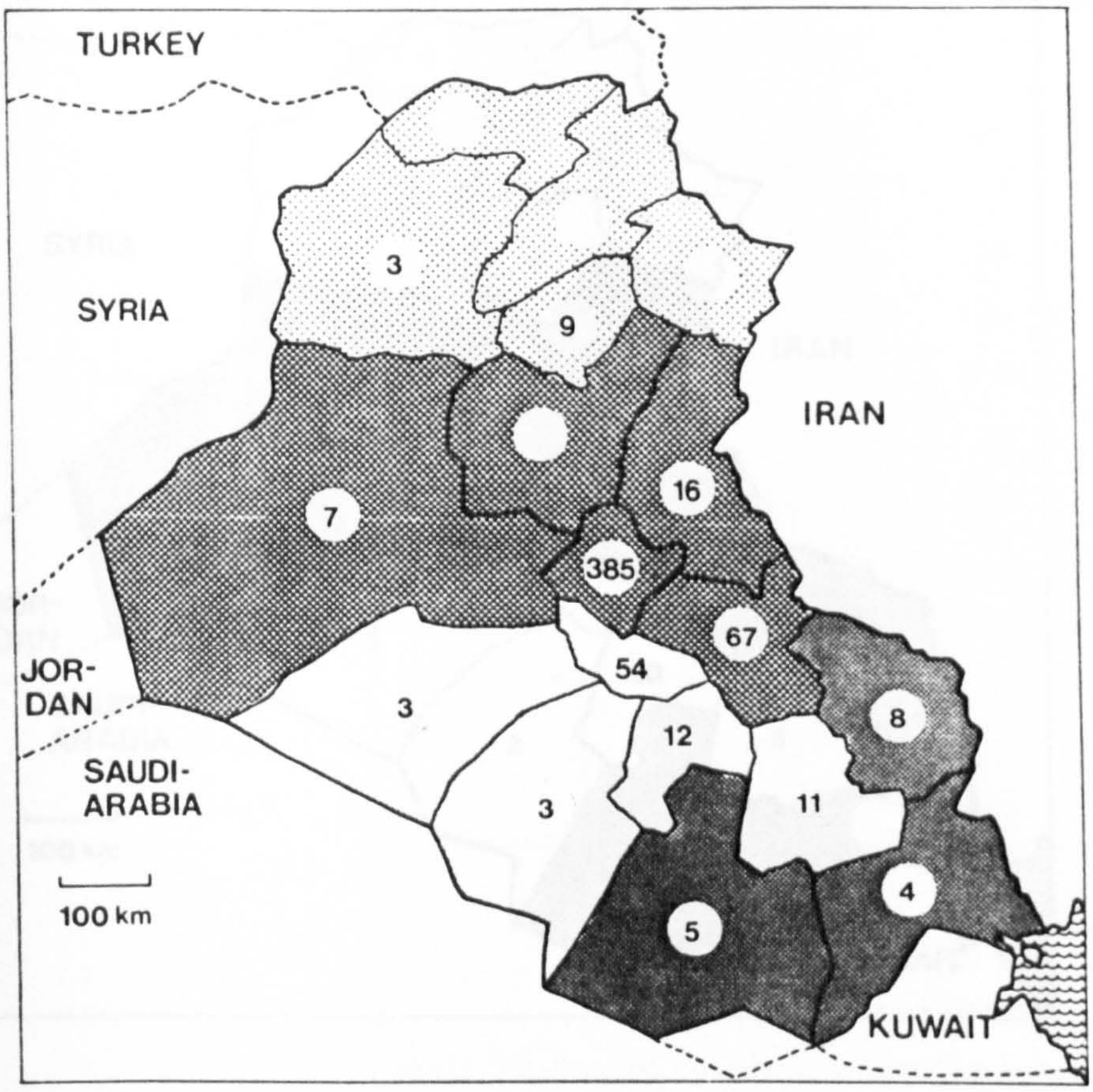


Figure 15
1977, total 587 cases

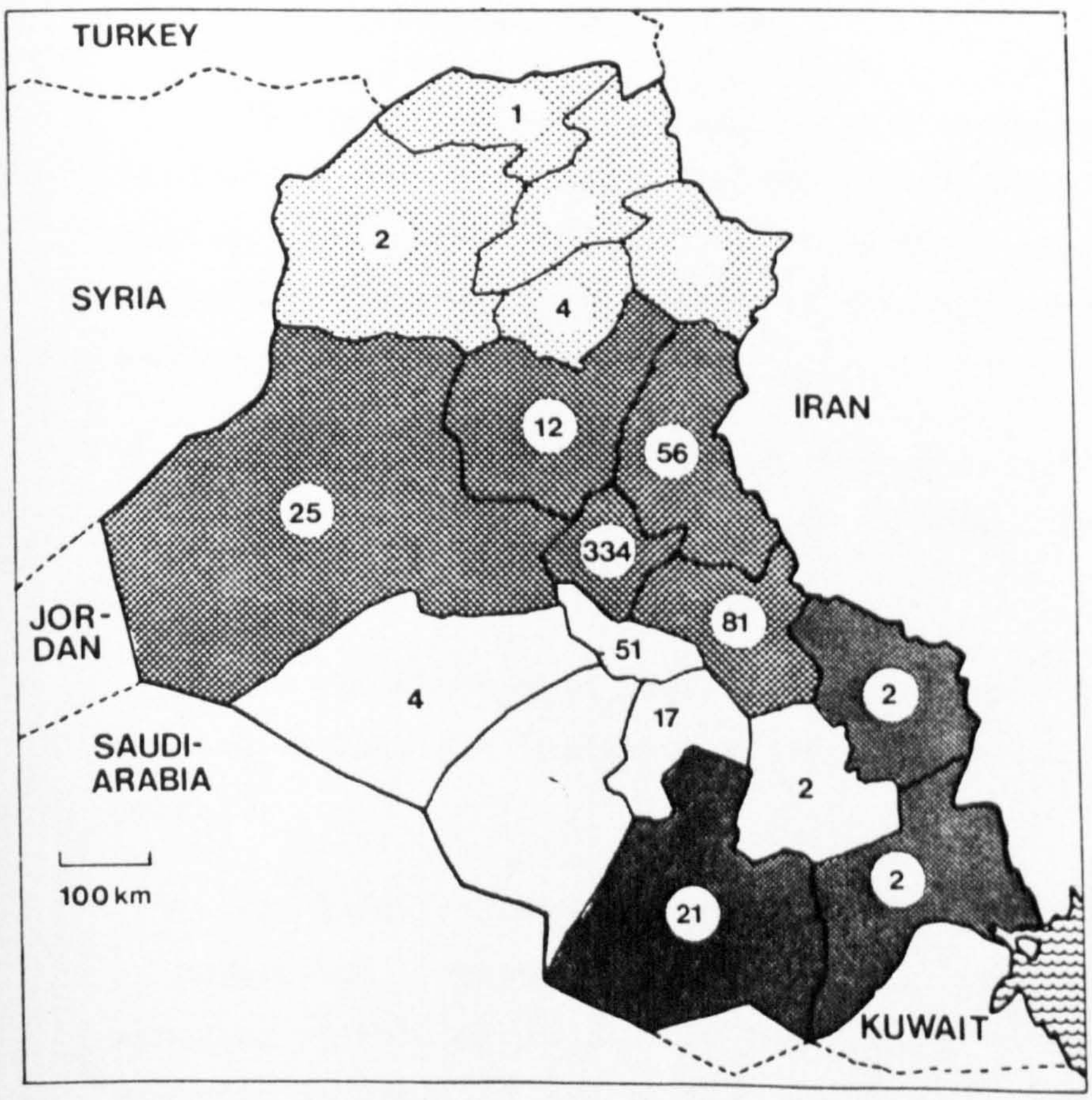


Figure 16
1978, total 635 cases

Geographical distribution of visceral leishmaniasis in Iraq.

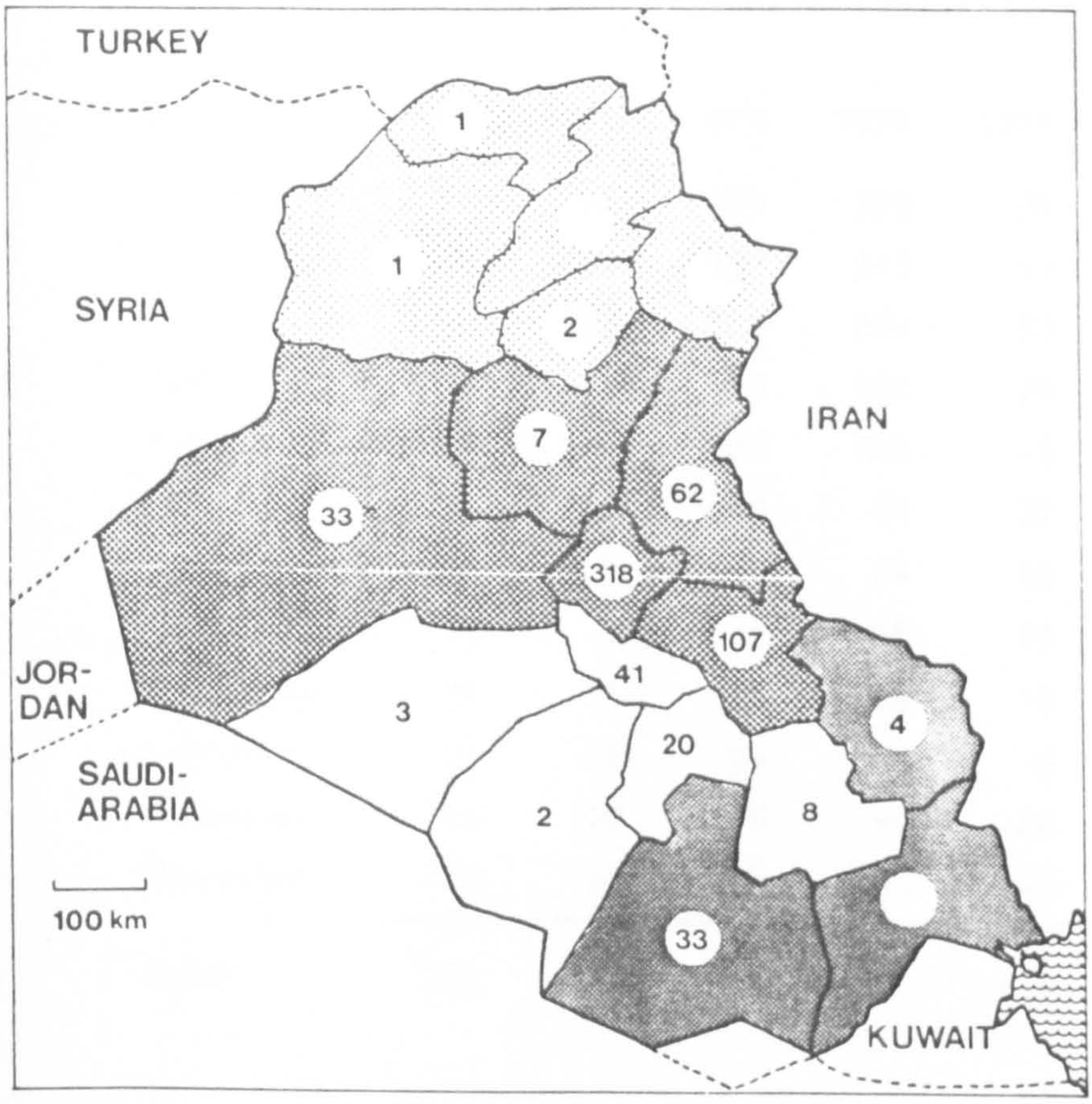


Figure 17
1979, total 642 cases

Table 10. Monthly distribution of visceral leishmaniasis cases in Iraq for the period 1971 - 1979.

Month	1971	1972	1973	1974	1975	1976	1977	1978	1979
January	34	86	174	379	94	160	94	76	103
February	57	107	137	343	69	97	69	52	79
March	58	73	123	260	83	98	38	106	57
April	32	50	85	192	74	46	36	92	49
May	18	28	72	104	43	40	63	54	37
June	15	14	47	81	27	28	41	36	15
July	16	9	59	74	22	21	52	35	10
August	15	7	18	48	19	30	39	18	10
September	10	11	26	28	13	26	20	28	17
October	17	13	54	43	9	44	24	20	33
November	42	18	105	49	26	39	14	44	71
December	55	72	234	90	49	39	97	74	161
Total	369	488	1134	1691	528	668	587	635	642

The effect of the malaria eradication programme on the incidence of visceral leishmaniasis in Iraq.

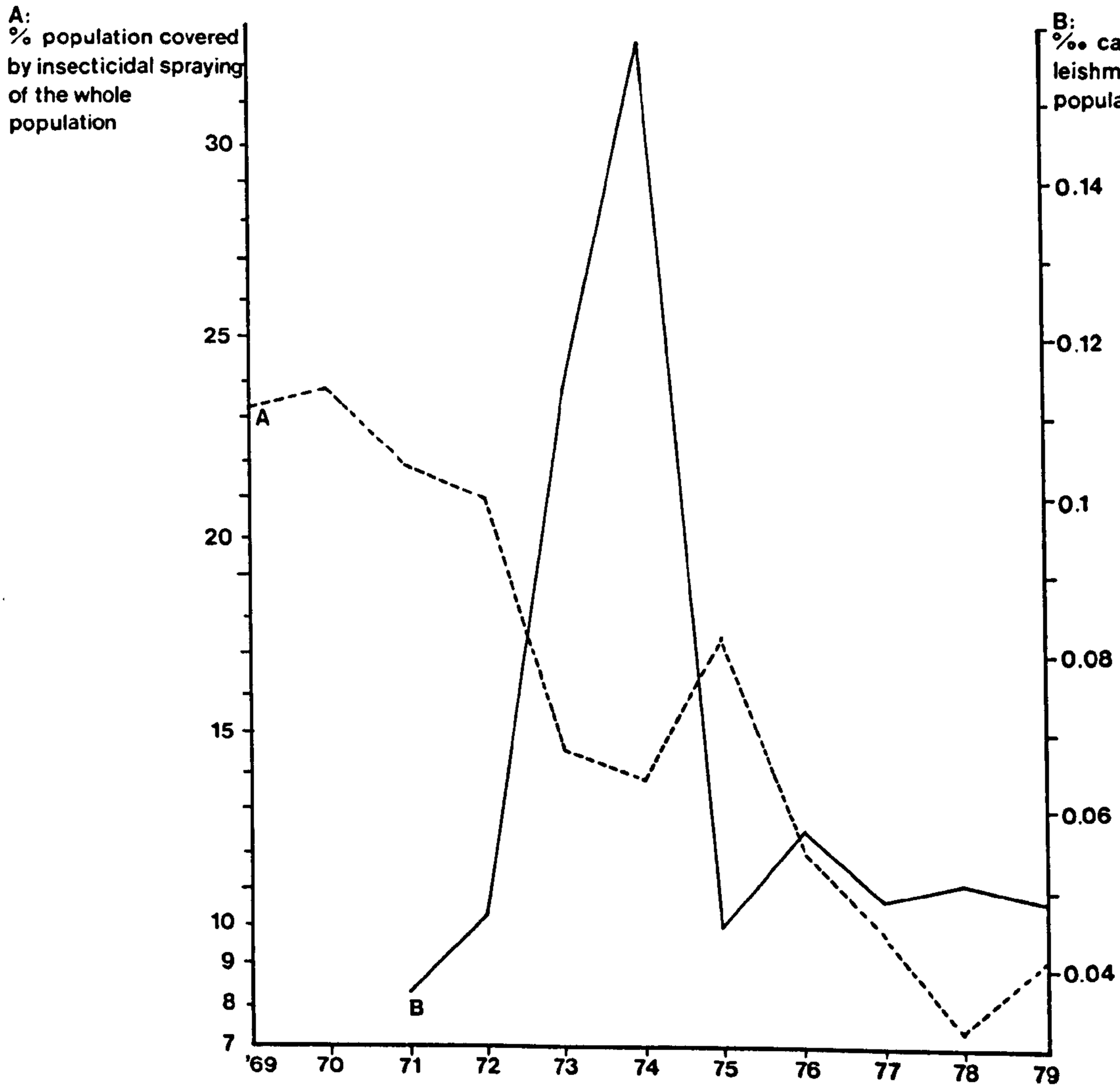
In the literature one can see few examples on this situation. Coradetti (1964) discussing incidence of visceral leishmaniasis in the Mediterranean region states that as intensive insecticide campaigns were stopped, a few years later cases of visceral leishmaniasis began to reappear.

Rezai et al. in 1977 found that with the cessation of antimalaria programmes the incidence of systemic leishmaniasis in Pars province in Iran had risen.

On the other hand Luckins et al. (1978) found that DDT spraying against sandfly was ineffective even with three cycles of spraying a year.

In Iraq, as illustrated in Table 11 and Figure 18, the percentage of population covered out of the total population by insecticidal spraying operations is plotted against the incidence of visceral leishmaniasis in Iraq for the period 1969 - 1979. This showed that a decrease in insecticidal coverage was followed by an increase in the incidence

Figure 18 Showing insecticidal coverage of the population of Iraq and the recording of cases of visceral leishmaniasis 1969-1979.



of visceral leishmaniasis, but later this was not the case because while insecticidal coverage was continually decreasing, the incidence of visceral leishmaniasis was not affected, and there was no increase as expected.

Table 11. Showing population covered by insecticidal spraying carried out by the malaria eradication programme; it shows also malaria cases and cases of visceral leishmaniasis for the period 1969 - 1979 in Iraq.

Year	Estimated pop.accor-ding to 1965 Census	% pop. covered by in-secti-cide to total pop.	No. of malaria cases	Malaria casesin 1000 pop.	No. of visceral leish-maniasis cases	VL per 1000 of total pop.
1969	9,412,690	23.1%	12,998	1.4		
1970	9,724,720	23.8	14,237	1.5		
1971	10,047,094	21.9	6,971	0.7	369	0.0367
1972	10,380,155	2.1	6,336	0.6	488	0.047
1973	10,724,257	14.6	3,783	0.4	1,134	0.111
1974	11,079,766	13.9	2,018	0.2	1,691	0.153
1975	11,447,060	17.4	14,050	1.2	528	0.046
1956	11,826,530	12.0	8,212	0.7	668	0.058
1977	12,029,760	10.0	5,069	0.4	587	0.049
1978	12,428,546	7.3	3,570	0.3	635	0.051
1979	12,840,552	9.3	3,554	0.3	642	0.049

Population estimated according to 1965 census.

Focusing on Baghdad Province only, as illustrated by Table 12 and Figure 19, we can see initially that as the insecticidal spraying coverage decreased cases of visceral leishmaniasis increased, but when spraying stopped cases of visceral leishmaniasis went to its original level, although in 1979 there was a slight increase in the number of registered cases.

It seems that the effect of insecticidal spraying operations was little on the incidence of visceral leishmaniasis.

In our study area spraying operations were stopped for many years now.

Figure 19 Showing insecticidal coverage of the rural population of Baghdad Province with the cases of visceral leishmaniasis recorded 1969-1979.

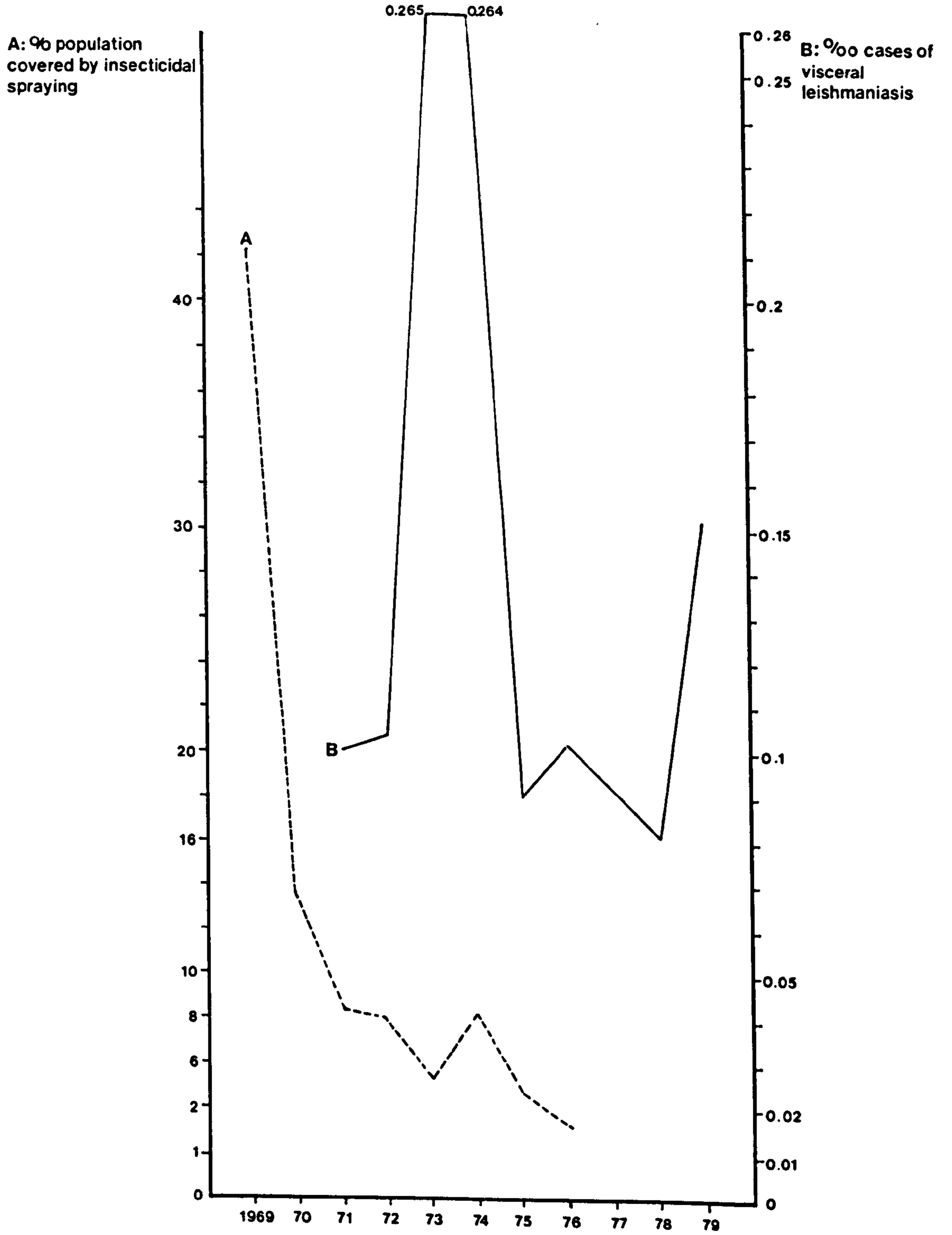


Table 12. Showing population covered by insecticidal spraying carried out by the malaria eradication program; it shows also malaria cases and cases of visceral leishmaniasis for the period 1969 - 1979 in Baghdad.

Year	Rural pop. of Baghdad Province	% coverage with insecticide	Malaria No.	cases per thousand	Visceral leishmaniasis No.	per thousand
1969	1,157,412	42.4	165	0.143		
1970	1,195,780	13.48	259	0.217		
1971	1,235,420	8.29	13	0.01	129	0.104
1972	1,276,374	8.05	6	0.0047	161	0.126
1973	1,318,686	5.2	-	-	350	0.265
1974	1,362,400	8.17	-	-	361	0.264
1975	1,407,563	4.69	6	0.0043	131	0.093
1976	1,454,224	3.27	3	0.0021	168	0.115
1977	1,502,432	-	3	0.002		
1978	1,552,238	-	10	0.0064	127	0.0818
1979	1,603,695	-	88	0.055	245	0.152

Regarding the situation of the vector, little was done in this respect.

Sukkar (1972) and Abu Al-Hab and Al-Baghdadi (1972) showed that only six sandfly species were found. These were:

Phlebotomus papatasi
P. alexandri
Sergentomyia squamipluris
S. palastenensis
S. fallax cypronotica
S. baghdadis.

Phlebotomus sergenti was not seen.
P. papatasi constituted 68% of the caught sandflies; this and because it was anthropophilic was incriminated as a vector of visceral leishmaniasis in Iraq.

From the review of literature it was seen that the suspected animal reservoir of visceral leishmaniasis was the Jackal.

The jackal as an animal lacked local study; there was no full scientific information about the distribution, the density, type of habitat, and no information about more specific matters like normal levels of serum proteins, etc.

The importance of visceral leishmaniasis in Iraq could be discussed from two points of view, both of them not very well studied and understood.

1. Attack rates of the disease were not known because the recorded cases represent the hospitalised cases and the true incidence of the disease was not known.

2. The mortality of the disease was estimated according to deaths that were registered while the child was still in hospital; true mortality rates were not known.

Jawad (1973) reported 19.7% mortality among 71 cases admitted to hospital.

Al-Nouri and Jeboori (1973) found the mortality among a smaller group of hospitalised children they studied to be 8.2%.

B. PROJECT DESIGN

The epidemiology of visceral leishmaniasis in Iraq is seen from the above review to be poorly understood, in spite of a good deal of work. Most of the work in man has been based on clinical cases in hospital, while entomological work is largely quantitative and the reservoir of infection is unknown. The present study seeks to elucidate the epidemiology of infection, with study of a defined community and also using serological techniques to extend the sensitivity with which infection may be detected.

The basic structure of the study was longitudinal follow-up of a population over a transmission season following a base-line cross-sectional prevalence survey. Seroepidemiological methods were used to explore the possible occurrence of subclinical cases and other screening procedures were also used to define a group with a high probability of having visceral leishmaniasis, who were clinically then investigated in hospital wherever possible. Conversely, children's hospital populations

were screened regularly to see if any of them had got visceral leishmaniasis and to prove that wherever possible parasitologically by a bone marrow puncture and otherwise.

The study also included some work on possible animal reservoirs. This work had to be done in different foci of infection of the disease. The aim was to use serological methods as a screening test and also to find and isolate the parasites from them by the conventional method of culture and direct examination of bone marrow and the viscera.

Some small-scale entomological studies were done to estimate the number of times a human bait is subjected for the bites of a sandfly in an endemic area, type of blood they had to feed on, the species identification and to try to isolate the promastigotes of Leishmania from their guts.

1. Experimental design.

Thus a community known to have visceral leishmaniasis was needed to make complete epidemiological studies, with some studies on possible local reservoirs and some entomological studies.

To try to make such a study has to depend on clinical and serological guidelines, so the problem of adopting seroepidemiology depends on finding a method that is:

a) sensitive
b) specific
c) reproducible and
d) easy to carry out

Two such methods were chosen and these were indirect immunofluorescent antibody test (IFAT) and the enzyme linked immunosorbent assay (ELISA), and the work with these methods began from standardising the test as much as possible and including various titrations and chequerboard studies, plus taking up the problem of specificity and sensitivity.

To put all this into field trials and to test our methods we had to go through a phase here called the pilot study.

a. Pilot study.

That included a study of the methods to be used, including IFAT and ELISA, census data collections, collection of sera, culturing etc.

All these were practised through a small field trial which included the study of a small community within a visceral leishmaniasis focus; it included also some hospital activity.

So, for the purpose of this pilot study, an area has been chosen just south of Baghdad where cases of visceral leishmaniasis had been recorded in the previous years. About 150 houses were taken, with a population of 1421 people during the period October, November and December 1978 and a census and a map was made for the area.

Collection of blood was done on all age groups and both sexes.

The pilot project has addressed the problem of sensitivity and specificity studies of the two serological tests by taking samples from the hospital and the field.

The pilot study also included studies on the possible reservoir of infection and had included an examination of a number of animals both serologically and histopathologically.

The pilot study also included some entomological activities concerning the test of the type of blood some sandflies have ingested.

The results of the pilot study led to the experimental design of the overall study.

b. Overall study.

The site selected was the same place but the sample size has been increased to about 10,000 people living in 1,171 houses and this longtitudinal survey was done on the same basis as the pilot study, except that we have taken the 0 - 7 years of age group, because the results of the pilot study showed them to be more affected serologically than the rest of the population. The study was carried out by two surveys, the first one before the usual time for the appearance of cases and the second at the time of the appearance of cases. At an interval of 7 months.Each child is followed up in the two surveys serologically by blood examination done by the two methods, IFAT and ELISA. Positive cases were followed up clinically and serologically and by other examinations like skin testing and blood pictures, WBC, differential counts and serum proteins. There were case control studies in which age and

sex standardised serologically negative children were examined clinically and for the leishmanin.

c. Logistics and constraints

i) Logistics of the study

It was found that the following had to be provided for the work to be carried out.

1. Two teams for blood collection and census work, each with a car. A team is composed of two workers, one will collect the blood, the other will take the information. Their work load was about fourhouses daily for each team. They were trained for the work during the pilot study. Their work is to carry out census information plus blood collection.

2. A follow up team which is composed of a physician and a worker. This team was assigned the tasks of physical examination and the other investigations. The team was trained also at the beginning of the study. This team was provided with a car.

3. Hospital team which is composed of a health officer and an auxiliary and to this team was assigned the work of taking blood from patients and following them up in the field after isolating the parasites from them, in addition to that it has been assigned the work of collection of sera from patients for the sake of specificity and sensitivity of the serological methods used, viz. IFAT and ELISA. This team had been trained during the pilot study and was provided with a car.

4. Reservoir team and this was composed of two medical assistants and provided with a shotgun, light projectors facilities for dissection of animals and culturing of the pasasites. This team was trained during the pilot study and was provided with a car.

5. A team of two medical assistants trained for the laboratory work by a visiting research worker from the laboratories of the Ross Institute and by me. Their work included the carrying out of the two serological procedures with the preparation of all the materials needed for the tests like

antigens, buffer, etc.

6. A draftsman for the map production.
7. An entomological team which was based in the Institute of Endemic Diseases in Baghdad.

Registers included the following:

1. The family register which is really the collection of the information gathered through the family card.
2. Animal register which is the collection of the information gathered through the animal card.
3. Blood register which included all the information about the samples of blood collected from the study area, from hospitals and other institutions, and from the animals.

ii) Logistic problems.

The collection of capillary blood and of venous blood in the follow up of seropositive children was hampered to some extent by misinterpretation by the people in spite of educational efforts, by transport problems in the rainy season, and also by internal administrative difficulties in the area unconnected with the project. The most serious consequences of the latter was that although there were no difficulties at the planning and earliest stages, it became impossible to discharge firearms in the area, so that the collection of jackals had to be abruptly transferred to other areas when it was too late to move the whole study.

2. The study area.

a. Site selections.

A rural area of around 300 square kilometres just south of Baghdad city in the central region of Iraq was chosen as the study area because cases of visceral leishmaniasis had been recorded from it in the past and it was a typical focus, it was rural in nature, near Baghdad where the laboratory was situated and from where the work was daily administered.

It is situated between latitudes $33^{o}27'$ and $33^{o}15'$ and longitudes $44^{o}32'$ and $44^{o}42'$. The boundary is the river Diala along the west side, from the other sides it is open and continuous landscape

Table 13. Monthwise distribution of the temperatures and relative humidity as measured by the meteorological station in Baghdad airport for the period 1976 - 1978.

	1976			1977			1978			Mean		
	Maximum	Minimum	R.H.	Max.	Min.	R.H.	Max.	Min.	R.H.	Max.	Min.	R.H.
January	18.4	-7.2	76	16.6	-4.6	77	17.9	-2.3	82	17.6	-4.7	78.3
February	21.2	-2.4	59	29.1	3.0	59	22.7	-1.5	53	24.3	-0.3	57.0
March	29.2	-2.6	64	29.8	1.7	49	30.6	5.2	48	29.8	1.4	53.6
April	35.5	10.1	52	35.8	9.0	44	34.6	6.0	34	35.3	8.7	43.3
May	39.2	13.8	35	41.3	13.4	30	40.1	12.0	27	40.2	13.0	30.7
June	45.0	18.8	20	45.3	18.4	22	46.0	17.8	21.5	45.4	18.3	21.0
July	45.2	18.7	18.6	45.8	18.2	21	51.5	20.5	21	47.5	19.1	20.4
August	45.7	18.0	27	47.9	19.8	23	49.4	16.6	28.5	47.0	18.1	26.2
September	42.0	15.1	31	43.5	16.2	27	44.6	15.0	29	43.4	15.4	29.0
October	38.8	9.8	41	39.3	4.5	43	38.6	11.8	34.5	38.9	8.7	39.5
November	29.8	3.0	47	26.6	3.5	58	26.7	-2.8	61	27.7	1.2	55.3
December	24.5	-0.9	68	23.0	-1.5	72	21.4	1.8	68	22.9	-0.2	69.3

Table 14. Rainfall in Baghdad (in mm) for the three years 1976 - 1978. (TR = Drops of rain or trace)

Month	1976	1977	1978	$\bar{x}$ for 3 years
January	25.3	25.3	32.7	27.8
February	24.4	24.4	9.2	10.3
March	22.7	22.7	14.0	19.8
April	22.3	22.3	TR	14.9
May	8.1	8.1	TR	5.4
June	0.1	0.1	TR	0.07
July	-	0.0	0.0	0.0
August	-	0.0	0.0	0.0
September	0.3	0.3	0.0	0.2
October	3.7	3.7	0.0	2.5
November	17.1	17.1	10.1	14.8
December	22.9	22.9	44.2	30.0
Total	146.9	146.9	110.1	134.6

Monthly $\bar{x}$ 11.2

which extends into other administrative areas and provinces.

b. Description of the area.

It is part of the alluvial plain of Iraq which is simply flat land around 0 - 200 metres above sea level and is included in the Mesopotamian part of the country which is the fertile part.

i) Road Network.

One paved road leads to the area and continues within the area traversing it from the west to the east nearly in the middle.

Other unpaved roads are present but they are cut off during the rainy season. Some houses were not connected with any kind of road and one had to walk to reach them. These were completely cut off in the rainy season.

ii) Climate (Central Bureau of Statistics, 1976, 1977, 1978)

Two main seasons could be recognised: the summer hot and dry which extends from April to September and the winter season cold and rainy beginning from November and ending in March, with very short transitional spring and autumn. Temperatures range from a maximum of around 45° - 50°C in the shade in the summer months to well below zero (up to -5°C) in the cold wintry nights, as is shown in Table 13 and Figure 20.

The pattern of the relative humidity is very low (dry) in summer and fairly high (humid) in winter.

The attached tables and graphs of the maximum and minimum temperatures and the relative humidity were the mean readings for the years 1976, 1977 and 1978.

Rainfall in the area for the last three years before the survey (1976, 1977 and 1978) was around 134.6 mm annually, the rainy season began at the end of September and continued to the beginning of June. This is illustrated in Table 14 and Figure 21.

Figure 20 Showing the mean reading according to months for maximum and minimum temperature and relative humidity 1976-1978

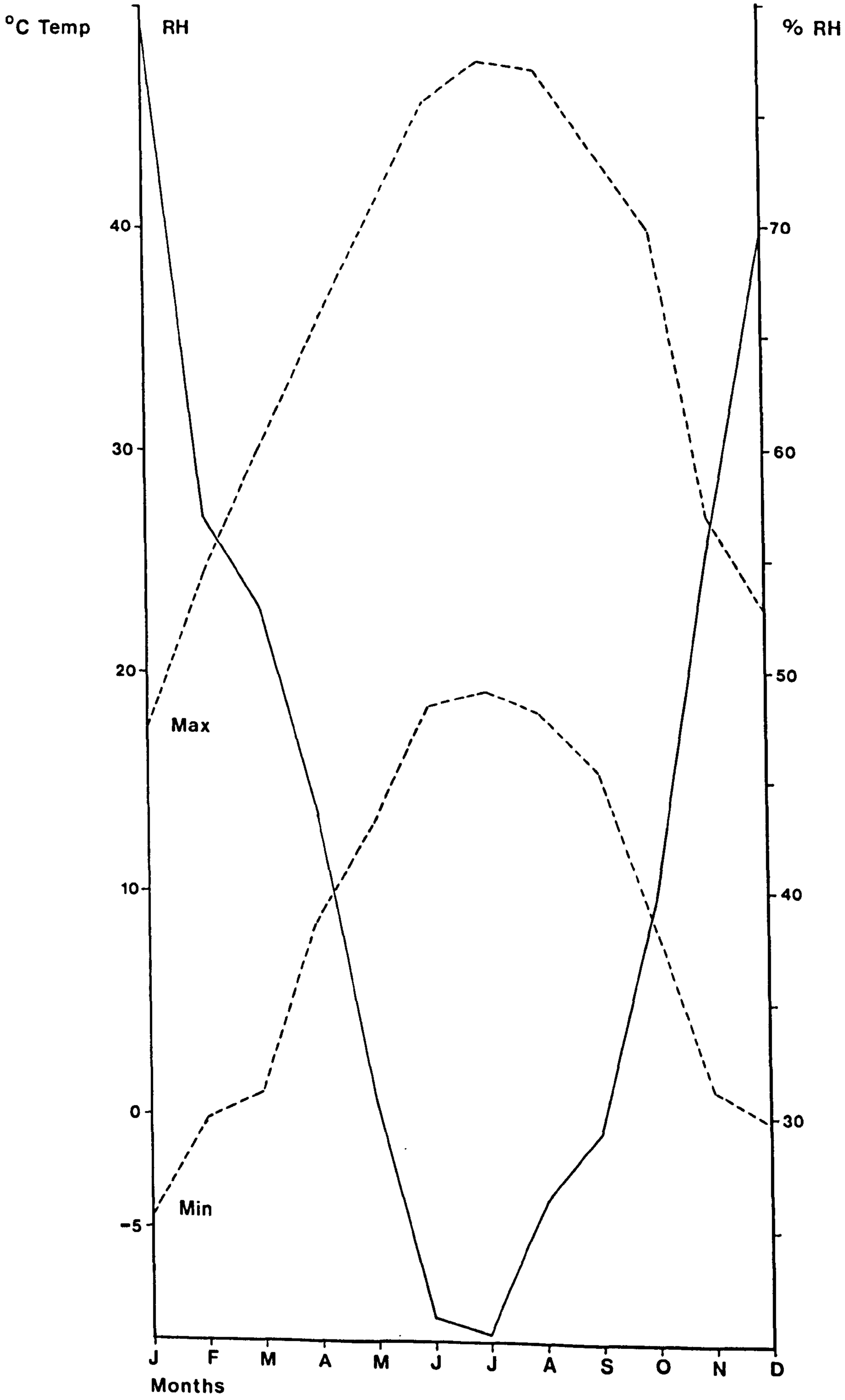
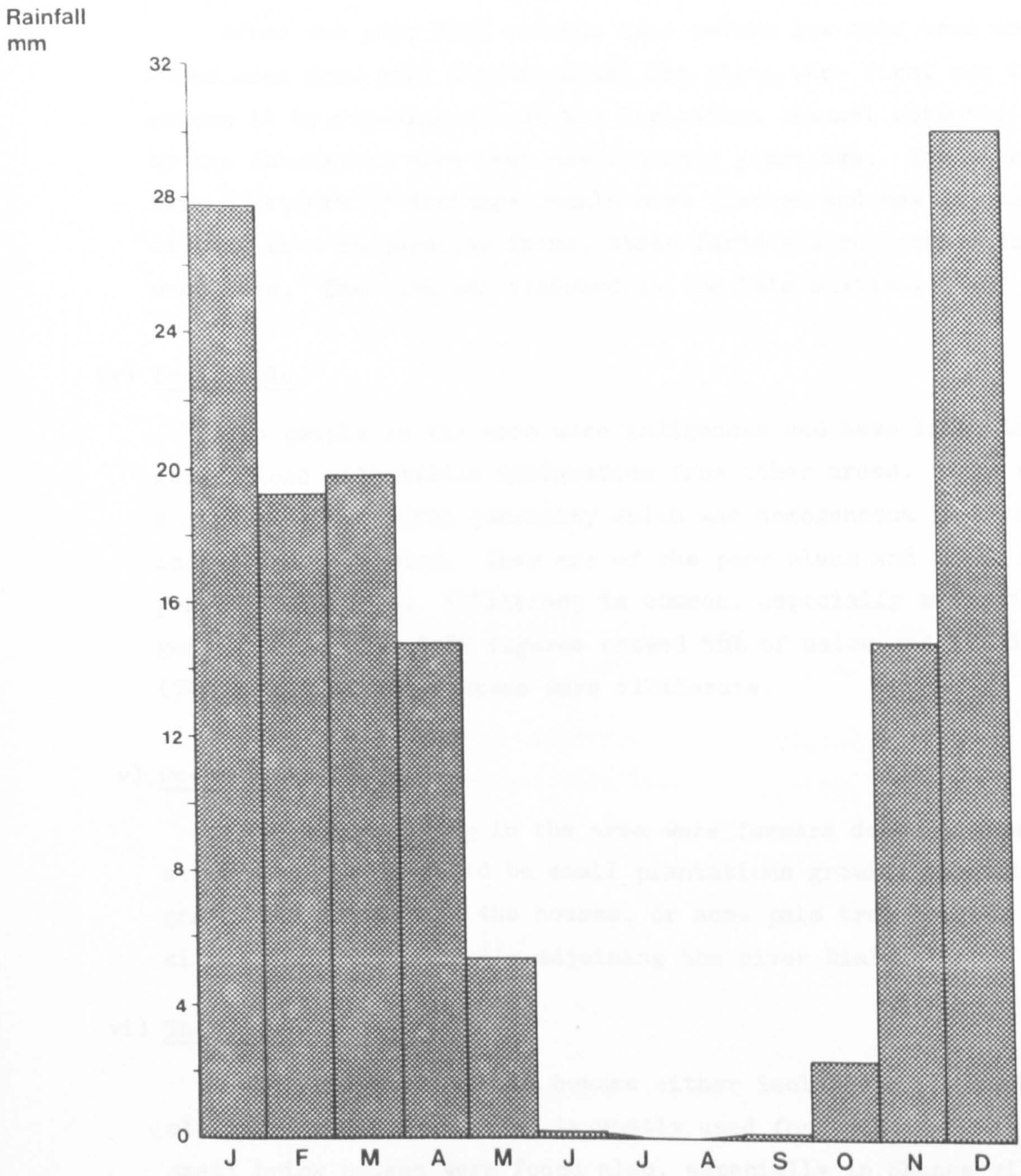


Figure 21 Rainfall in Baghdad. Mean of the years 1976-1978 according to months.



iii) Irrigation system.

After the year 1958 and the land reform law this area among other area drew more attention and the plans were first put to reform it by renewing one of the irrigation channel networks used by the Abbassyeen more than one thousand years ago. Together with this a network of drainage canals were planned and new allotments of land into cooperative farms, state farms and collective farms were made. The work was finished in the late sixties.

iv) The people

The people in the area were indigenous and have lived there for generations with little immigration from other areas. They represent a typical Iraqi rural community which was homogeneous in terms of income and otherwise. They are of the poor class and their income per capita is low; illiteracy is common, especially among older generations. The 1977 figures showed 56% of males and 93% of females (74% total) of rural areas were illiterate.

v) Occupation

The people living in the area were farmers depending on agriculture, and this could be small plantations growing vegetables and grains usually around the houses, or some palm tree gardens and citrus plantations mostly adjoining the river Diala.

vi) The houses

They live in separate houses either isolated or in small clusters or villages. Mud is mostly used for building, but new small brick houses were found also, especially in Khansa village where all the houses were made of brick. Government buildings and centres were brick-built and modern.

Replastering the walls of houses with mud was a common practice among the people; they did that usually before winter.

Building new houses and abandoning the old ones was not an unusual practice.

vii) Public services

A high voltage electricity line traverses the area from north to south and most of the houses (work is in progress) were supplied

with electricity; the use of electrical appliances like fans was not uncommon.

Water supply for the houses: chlorinated piped water supply was lacking in the area.

Newly introduced into the area was the use of water tanks provided by the municipality and chlorine was irregularly added by a local trained village man. Other water supply resources were the irrigation channels and the river Diala.

Health centres in the rural area in Iraq were of two types:

1. The primary health centre which looked after the health of 20,000 population and personnel included a physician with some health auxiliaries.

2. The secondary centre which looked after 5,000 population and the personnel included health auxiliaries but without a physician.

The services of those health centres included an outpatient clinic mostly for curative purposes with limited preventive activities.

The distribution of these centres in space was such that people can practically use them easily. The health centres function only six hours a day, six days a week.

In 1977 there were 230 primary centres and 846 secondary centres. This information was derived from the Statistical Compass 1975, 1976 and 1977. The number of doctors in service was 3,842; there were 193 hospitals with 23,374 beds (2 per 1000 of the population).

In 1975 admissions for inpatients were recorded and distributed as follows:

121,489	19.3%	due to pregnancy, labour and puerperium
48,190	15.6%	due to respiratory causes
68,851	10.96%	due to gastrointestinal causes
45,343	7.2%	due to parasitological infections
33,273	5.3%	due to genitourinary problems
34,997	5.6%	due to casualties
225,846	35.96%	due to other causes

In 1975 there were 14,925,000 visits to 2,347 outpatient clinics, i.e. around 20 visits for every clinic daily, and with a population of 12 million a 1.2 visit for each person yearly.

Our area is served by one primary centre and one secondary centre. A child with visceral leishmaniasis thus has a good opportunity to visit these centres or visit the children's hospitals directly in Baghdad city.

Visceral leishmaniasis was reported from the study area at an average of one case yearly, i.e. 1 per 10,000 population.

viii) Diseases in the area.

The following information about some of the diseases in the area is largely put to clarify the picture of cross reactivity and specificity of the serological and laboratory methods used in actual work. Anything of relevance to the actual work like other common causes of splenomegaly and hepatomegaly are discussed also.

Malaria in Baghdad was assessed through spleen surveys after the Second World War and just before the start of the Malaria Eradication Programme in 1957. The results were 1 - 6% of the Iraqi children examined in Baghdad rural areas had palpable spleens. Thus malaria was hypoendemic in this area (Pringle, 1954). The incriminated vector was Anopheles stephensi.

Malaria eradication activities in Baghdad Province started in 1957. The Province was under attack by residual insecticidal spraying using DDT 2G/square metre for two rounds. Spraying operations were discontinued until 1965 when it started again with full coverage, coverage decreased in time to 50% of population in 1970, then gradually decreased until 1976 when it stopped completely. The area was not covered by residual insecticidal spraying since 1970 - 1971 (Ossi, 1970, 1973).

Malaria cases were detected by:

1. local health centres
2. active case detection teams covering rural areas house to house, visit every month.

Blood was taken from feverish, history of fever and suspected cases.

Practically, there was no indigenous case of malaria registered in rural Baghdad since 1973, and in the study area since 1967 - 1968; hence transmission had completely stopped in our area since that time 13 - 14 years ago.

Negligible use of insecticides was practised by the agricultural department treating palm date trees against certain diseases with malathion and the very small scale use of knock down insecticides by householders.

So malaria as a cause of hepatosplenomegaly in the area is excluded.

With regard to tuberculosis, a tuberculin test was done in 1952 in Baghdad Province using 5U tuberculin PPDRT 22 : +5 mm. The results were an increasing positive tuberculin rates with age, as in Table 15.

Table 15. Tuberculin test carried out in 1952 in Baghdad Province.

Age group	Tuberculin % positivity
1 - 6 years	18.5%
7 - 14 years	42.2%
15 + years	80.1%

(Al-Fikiki, 1979)

In 1961 in Baghdad Province tuberculin testing using 1 tubercle unit PPDRT 23: + 10 mm. The results showed lower positivity rates, as in Table 16.

Table 16. Tuberculin test carried out in 1961 in Baghdad Province.

	Tuberculin % positivity	
Age group	Rural	Urban
0 - 4 years	2.2%	3.2%
5 - 9 years	6.4%	28.1%
10 - 14 years	17.7%	30.9%
15 + years	51.4%	65.4%

(Al-Fikiki, 1979)

Al-Khamese (1977) studying 4,120 children in Thawra town in Baghdad city using tuberculin test (PPDRT) in Tween 80 supplied by Agouza Laboratories Egypt) found the following results taking the 10 mm and above after 72 hours as positive, results are shown in Table 17.

Table 17. Tuberculin test carried out in 1977 in Baghdad Province.

Age group	Tuberculin % positivity
6 years	5.8%
7 - 9 years	8.19%
10 - 15 years	18.1%

In 1979 the Institute of Tuberculosis Control in Baghdad had done a study of 5,000 unvaccinated people of all age groups and found the following result with the tuberculin test as shown in Table 18.

Table 18. Tuberculin test carried out in Baghdad Province in 1979.

Size of tuberculin	Percent
0 - 4 mm	62.3%
5 - 9 mm	2.0%
positive	35.0%

(Al-Fikiki, 1979)

We have to note that BCG is compulsory for all newborns and in ten years time more than 2 millions have been vaccinated, that tuberculin positives increase with age, leaving the under seven years of age little affected, and that the trend of tuberculin positivity over the last 30 years is declining.

Six of the IFAT seropositive cases detected in the survey were tested later by tuberculin and 5 gave 0 readings: only one gave just 10 mm reading.

The area according to the official records of the Institute of Endemic Diseases was free of bilhazia (Schistosoma haematobium) but intestinal parasitic and helminthic infestations were common.

It was also free of leprosy because that disease was one of the rare diseases in Iraq. In the whole country few cases were diagnosed and recorded (less than 500 cases in Iraq) and only about 50 of them were admitted to the leprosarium in Missan Province in the southern part of Iraq.

Trypanosomiasis was not present or recorded in Iraq amongst the human inhabitants, so there were no T. cruzi or T. gambiense or any other kind of human trypanosomiasis.

Brucella cases have been detected in small numbers among the inhabitants who deal with sheep and cows grazing in the study area during a small survey using immunofluorescence techniques during 1979. The commercial Burroughs Welcome antigen was used and the Institute of Endemic Diseases - Zoonosis section could detect 22 positives out of 159 farmers (13.8% positivity rate), all of low titres (1/16), 3 of 1/32 and only one with a titre of 1/64. 18 of them were adults and only four below 7 years of age.

Oriental sore caused by Leishmania tropica was not reported from the area in the children. However, scars were seen in a few adults.

ix) Animals

Carnivores like dogs were common; they were used as guard dogs and shepherds' dogs. Raising sheep, cows and chickens was part of their daily life. Big herds of sheep are found and that means that people responsible for their grazing (mostly adults) will move for some distance from their houses for varying periods of time for the purpose of grazing.

c. Mapping

The maps that were available before the beginning of the study were:

1. Agricultural department maps which were of small scale 1/100,000, which covered the area and showed some of the irrigation and drainage system and the big villages; no details of individual houses were available and even small clusters were not plotted.

2. General maps from the Statistical Bureau which were general in

information and features and of a scale 1/50,000, again not showing single houses.

3. A few outdated sketch maps from the malaria eradication programme; this was due to the stopping of the spraying operations of residual insecticides in this area for the past ten years.

4. Aerial photographs of the strips along the river Diala also taken and surveyed some years ago and some of their information was out of date.

All these maps were different from one another in the details of the information they gave. They were also different in scales and some were out of date. None of the maps showed the distribution of single houses in the area.

A complete census had to be taken and a map produced. For that purpose the following people were needed:

1. a draughtsman for the map;
2. four workers divided into two teams for collection of census information;
3. two cars.

Timing: the work was timed to be done during the first survey and this included the general collection of information of the census and production of the map; and during the second survey for rechecking the new births, deaths, movements of people and building of new houses and other census information and finalisation of the map.

The work load was about four houses daily for the four workers. The work done included two activities: mapping and census.

Production of the map.

Aerial photograph data were transferred by a binocular instrument to sketch maps showing the location of houses and other important features. Information from these sketch maps with the information gathered from maps of the other sources was transferred to a map of 1/10,000 scale of the area specially prepared for the purpose of our study. This newly prepared large scale map (Figure 89) contains now all the information which could be gathered from the other maps.

The next step was to take the map to the field and make the

corrections on the spot during the work on the census.

Irrigation canals and drainage canals were plotted along with the other information such as roads, plantations etc. Each house was given a serial number which has nothing to do with any previous adivity in the area. Indeed this was the first time such a detailed map of the area had been prepared. The houses were not numbered previously by other services.

The houses were located exactly by using some landmarks. Each house thus a precisely known geographical location.

d. Census work

The four workers were trained on the work that included house to house visits and the following activities were done during these visits: The house was serially numbered, the house number was written on the house near the main door with aerosol spray paint. The same number was entered onto the map and then entered into the family card.

The family card included the following information:

Name of the village and the area.
The code number that was given to the house.
Information about the house: type of material it was built with.
Information about the people, which included:
    how long they have lived in the area,
    their number in each house, and
    their relation to the head of the family.
Each member is given a serial number within the family.
Their age according to the mother's information which could be
    traced back to the month of birth in those up to two years
    of age, and to the year of birth in those older. Sometimes
    the birth certificate is used also.
Their sex.
Births or deaths were registered during the seccnd survey.
The occupation of the people living in the house.
Medical history of the children in connection with visceral
    leishmaniasis; if they had had the disease before, and
    that depended on explaining the signs and symptoms to the
    mother.
Medical history in connection with cutaneous leishmaniasis of

all the inhabitants of all age groups by noticing the scars and asking the people about them.

Habits of the people, such as if they use sleeping mosquito nets or not.

Information about the animals they raise or the pet animals they keep: that includes special information about the presence of dogs, their numbers, whether any died within one year previously.

Wild animals in the area, which includes any information about the presence of jackals in the area.

FAMILY CARD

District | Village | House Number | Name of Head

Lattitudes | Longitudes

Number of people living in the house | If jackals are present in the area

Number of dogs | Dogs died last year | Chickens | Type of building material: Mud / Brick

Use of sleeping net

Serial number of family member	Age	Sex	Relation to the head	How long in the area	Date infected with visc. leish'is	Date infected with cutan's leish'is	Occupation	Clinical examination	Blood	Notes

e. Blood collection

The process of collection of blood was carried out during the two surveys of blood for the children under seven years of age and for that purpose two teams were set up, each consisting of two workers; one to collect the blood and the other to enter the information into the family card. Each team was provided with a car. These teams made a house to house visit according to the census which had been made for that purpose. So each house was visited twice: once during the first survey and the other during the second. The work load of each team was 4 houses daily. The weekends, holidays, type of roads and communications were all utilised in planning the schedules.

The first round began during March 1979 and ended on 20 September 1979, i.e. before the usual time of the appearance of cases - total days were about 200 days and total working days were around 170 days. The second survey began on 23 September 1979 and ended on 5 March 1980 - total days were 162 days and total working days around 140 days. The second survey coincided exactly with the usual time of appearance of visceral leishmaniasis in Iraq. The first survey took more time than the second because the first survey included taking and entering into the family cards all the information plus blood collection. As much as possible the same pattern of sequence of houses was visited in every survey.

For the purpose of the two surveys blood collection was made as described by Voller et al.(1976) and by Bray (1975).

Capillary blood was collected from the children in the survey in the first round during the census and clinical examination visits, and as a major part of the second round, also collected from some adults in the study area and other foci. One finger was swabbed with alcohol, dried to prevent haemolysis and then pricked with a disposable lancet (Cristalet, Gelman Hawksley Ltd., Lancing, England). The first few drops were wiped off using clean cotton wool, then 3 - 4 heparinised capillary tubes, 7 cm long each, were filled with blood. (Heparinised capillaries used were those of Gelman and Hawksley Ltd., Lancing, England). The capillaries were sealed off with Cristaseal plasticine (Hawksley and Sons Ltd., Lancing, England). It was then fixed on a small card and on it was written the serial number of the house and serial number of the child. These were immediately put in thermos flasks for transport

to the laboratory. The workers had some training in doing this during the first days of the pilot project, during 1978.

The capillaries were immediately put in the minihaematocrit centrifuge (Gelman,Hawksley, England) after noticing the numbers of the places on the disc and writing this on the same card near the serial number of the house and the child. Then the centrifuge was run for 5 minutes at 12,000g. The capillaries were taken out one by one, each capillary was cut at the junction of cells and serum, the corpuscle part was discarded, the part of the serum was sealed off again in the same way and the capillary stuck again in the same place on the card. The sample was given a code number and entered in the registers of blood. Information entered included the code number, the name, age, sex, serial number of the child and his house, and date of collection of the blood.

Then the capillaries were put in self-sealed plastic bags and they were stored at -20°C until examined within two weeks of collection.

f. Follow-up of serologically positive reactors.

When a serum sample gave a positive reaction for leishmaniasis, here defined as an indirect fluorescent antibody titre of 1/16 and more, the child concerned was visited again within 2 - 3 weeks of the serological examination by a physician and another worker, who carried out the following on the sero-positive child and the first two of them, whenever possible, on another child who had been selected as a control by the author and matched for age and sex, living nearby, and having a negative serological reaction.

a. Physical examination
b. Leishmanin testing
c. Haemoglobin estimations using Tallquist method
d. Thin blood smear collection for white blood cell differential count
e. Repeat collection of capillary blood to fill 5 - 10 capillaries
f. Venepuncture whenever possible to obtain 3 - 5 ml venous blood from the forearm using a disposable syringe; blood was kept in heparinised vials for transport to the laboratory, where serum was separated by centrifugation and kept for protein electrophoresis and immunoglobin estimation by immunodiffusion.

g. Total white cell count

h. Blood culture for leishmaniasis.

Examination of the white cell count in the field was difficult because it required all the equipment, including a microscope, with the teams. A simpler and equally reliable method was found by using 2 - 3 calibrated capillaries, emptying them into a small screw bottle in the laboratory, mixing the blood and then taking the sample (to the 0.5 mark) using a standard white cell pipette and diluting 1:20 with 2.5% v/v acetic acid. This procedure was compared with the usual one and it was found to be reliable.

C. HOSPITAL ACTIVITIES

1. Places of work

Certain hospitals in Baghdad were chosen for special activities; those hospitals were the children's hospitals which included:

1. Arab Child Hospital
2. Hospital of the Society of Child Protection
3. ALWIYA Child Hospital
4. Karama Hospital - children's wards.

These represent the hospitals which drain the endemic foci of visceral leishmaniasis around Baghdad, including the study area, and from other provinces also.

2. Method of work

A special health auxiliary person in addition to the physician was assigned to the work of the hospitals which included:

1. Routine visits to the hospitals to look for cases of visceral leishmaniasis and other cases of interest;
2. Visits according to special notifications of the presence of visceral leishmaniasis.
3. Follow-up visits in the field of parasitologically proven cases of visceral leishmaniasis.

3. Aim of work

The aim of the work was to collect blood from the following:

1. Parasitologically proven cases of visceral leishmaniasis;
2. Parasitologically unproven suspected cases of visceral leishmaniasis;
3. From non-visceral leishmaniasis cases which include diseases that may cross-react serologically with visceral leishmaniasis or diseases commonly found in the study area for the same purpose.

All this will enter into studies of sensitivity and specificity of the serological test that were applied in the study.

4. Actual work and material collected.

The following work was done:

1. Bone marrow puncture of suspected patients of visceral leishmaniasis and doing smears on microscopical slides and cultures in NNN and semisolid media. This was done by the hospital physicians and interns.

2. Blood collected in heparinised capillaries for serological examinations and by venepuncture for serum proteins, electrophoresis and immunoglobulins by immunodiffusion, for haemoglobin estimation, differential counts and white blood corpuscles count. Blood was also cultured from these patients on NNN medium and on semisolid media.

3. Information about the other tests done in the hospital, in addition to information about the exact address of the patient for follow-up purposes later.

D. WORK ON THE POSSIBLE ANIMAL RESERVOIRS OF VISCERAL LEISHMANIASIS IN IRAQ

1. Introduction

Previous work both epidemiological and pathological refers to a possibility of an animal reservoir of the disease in Iraq.

Bray (1975) excluded man as a possible reservoir of infection and he treated man as an incidental host on epidemiological grounds, so the reservoir was suspected to be an animal. Possible animals which could act as reservoirs were the carnivores and rodents.

Work on rodents all proved negative except that of Al-Edhami which isolated Leishmania from R. rattus (1976), but which proved later

to be a strain of L. tropica by Al-Jeboori and Evans (1980), after testing it with isoenzyme techniques.

Previous work on dogs (Wenyon, 1926; Chadwick and Machattie, 1972; Tajeldi[n] and Al-Alousi, 1954; Al-Dabbagh, 1954 quoted by Pringle, 1956) and others proved negative. The only positive finding in dogs was what Sheriff reported in 1957 in imported foxhounds which could not be treated as the actual reservoir of infection.

Latyshev et al.(1951) referred to the jackal as a possible reservcir in an area in USSR which has similar sandfly spectrum as Iraq. Bray (1974) excluded the dog as a possible reservoir and he concluded that transmission in Iraq was from the jackal direct to man. Work on jackals by Tajeldin et al. (1971) and Kadhim (1978) proved negative.

In conclusion, no animal reservoir has been detected in Iraq. (Sukkar, 1976).

2. Material

Work on the reservoir included the following animals:

a) jackals
b) foxes
c) dogs
d) other animals like wolves, rats, mice and mongooses.

3. Place of investigation

An overall plan was set forward to study the epidemiology of visceral leishmaniasis in the study area, but for unforeseen circumstances it was impossible to carry out the work on animals in the area, and so the work on reservoirs was done in other areas similarly known to be endemic in visceral leishmaniasis.

4. Methods

For the purpose of collecting animals the following methods were adopted:

1. A special team composed of two health auxiliaries which had been trained was provided with a shotgun and a car. Their equipment included a dissection set for animals, culture media, heparinised capillaries and tubes. Their work was mainly shooting jackals, foxes, wolves and dogs.

2. One health auxiliary was cooperating with the veterinary department shooting stray dogs in Baghdad Province. He was provided with the same equipment.

3. Mice and rats were examined after being trapped as a routine function of the Rodent Control Department in Baghdad. One health auxiliary was assigned to the work of collecting blood in heparinised capillaries and of culturing and examining the viscera, spleen and livers of the animals in the same manner that was adopted by the jackal team.

5. Actual work on jackals, dogs, foxes and wolves.

Data on jackals in Iraq and their ecology was lacking. Endemic foci of Baghdad and nearby provinces were visited by the teams at and after sunset looking for jackals. Usually a group of jackals were found near carcases and refuse places near the human dwellings. The jackal was shot and after being killed the following things were done immediately:

1. Blood from the heart was collected in heparinised test tubes and capillaries for serum protein estimation and for serological tests.
2. Cultures were made from the spleen, liver, blood, sometimes skin and lymph nodes, using semisolid (Chang, 1947) and NNN (Kagan and Norman, 1970) media on the spot, other parts of these organs were taken immediately to the laboratory and culturing was done again there. Impression smears of spleen and liver were also done in the field and in the laboratory.
3. Measurement of the jackal and weighing it.
4. Entering all this information on a special form which included giving a code number to the jackal in addition to
   a. information about the jackal itself: sex, weight, measurement
   b. information about the area in which the jackal was shot
   c. information about the serological tests and culture results and smear results.

Shooting activities included other animals like dogs, wolves and foxes. The same work was done on them as that mentioned for the jackals.

6. The actual work on rodents

This included the dissection of the animal trapped and taking biopsies of the viscera and culturing them, along with impression smears of those viscera. Blood for serological examination was also collected.

Blood from rodents was examined directly under the microscope to look for blood flagellates.

Animal Card (Dogs, Jackals, Foxes, Rodents, etc.)

1) Species

2) Code number

3) Date

4) Name of locality

5) Weight in Kilogrammes

6) Measurement tip of nose to end of tail

length of tail

beginning of tail to end of neck

7) Femus length

8) Blood collected in heparinised capillaries

in test tubes to be coagulated

9) Smear and cultures

		smear	culture
Liver	) Field		
	) Lab.		
Spleen	) Field		
	) Lab.		
B.M.	) Field		
	) Lab.		
Blood			
Skin			

10) Histopathology of cutaneous lesions

11) Others

12) Serum testing IFAT

ELISA

Serum proteins.

E. ACTIVITIES ON SANDFLIES

1. Methods

This was carried out by a special entomological team well trained on these activities.

Human baits were used to determine the number of bites a man was exposed to during one hour outside the house during the peak hour of activity of the sandfly.

Sandflies were also collected from inside different rooms by sucking them while resting on the wall. The aim was to determine their feeding state, type of blood they had ingested, to recognise the sex and the species of the parasite and to find out by direct microscopical examinations if they were infected with promastigotes.

2. Materials

Activities of sandflies included the following: On 26 October 1978, 32 fed sandflies were caught alive by a sucking tube. They were treated with chloroform and crushed on Whatman 3 chromatography paper (Reeve Angel International Limited, London), then examined later by the precipitin test to determine the type of blood they had fed on.

The collection was made from the study area and included:

10 fed sandflies collected from bedrooms
3 fed sandflies collected from bathrooms
15 fed sandflies collected from animal shelters and
4 fed sandflies collected from deserted houses.

Typing of the blood meal was kindly done by Peter Boreham of the Imperial College in London using the precipitin test.

The animal fauna as described in the study area comprised sheep, goats, cows, chickens and dogs, with wild animals like jackals and rodents.

3. Time of the work

According to Abulhab and Mahdi (1970) there were two peaks of population density of sandflies in areas around Baghdad, the main one in June and the other in September-October. The peak hour of activity was found at nine o'clock at night.

So the studies were done one during June and the other in late November, 1979. The time of the work was around nine o'clock in the evening after sunset when there was no wind or dust. The place of study was the study area.

F. LABORATORY PROCEDURES

1. Cultures

a. Materials used for culturing

A. Human

1. Bone marrow aspirates from the iliac crest of suspected cases of kala azar.
2. Blood from suspected cases in the hospital and in the field directly or after collection in heparinised capillary tubes.

B. Animals

1. Blood from the animal's heart.
2. Viscera which include the liver and spleen.
3. Bone marrow aspirates.
4. Skin snips.
5. Lymph nodes

b. Method of culture.

Under aseptic conditions in the laboratory, in the hospital and in the field, the material to be cultured was sown on suitable media for culturing the parasite, then the culture bottles were labelled and registered for the date of culturing and date of examinations in a special register. The culture bottles were then incubated at 26°C and examined afterwards.

c. Culture media used.

The culture media used in this work included the following:

NNN Medium (Kagan and Norman 1970)

composed of 6 NaCl

14 G powdered agar (Oxoid Code CM3, England)

950 ml distilled water

and all dissolved by boiling.

5 ml was put in each bottle and covered with cotton wool plugs sterilized in the autoclave at 15 lb/sq. inch for 30 minutes; aseptically 15 drops of rabbit blood were added to each tube, mixed gently and set in a sloping position; sterile screw rubber caps were fitted to each tube and incubated at 37°C overnight to exclude contamination; then stored at 4°C.

Before use 1 ml of 199 medium or Hank solution as an overlay was added.

Powdered agar used was Nutrient agar Code CM3 Oxoid, England. Formula per litre as follows:

'Lab lemco' powder (Oxoid L29)	1.0 G
Yeast extract (Oxoid L20)	2.0 G
Peptone (Oxoid L37)	5.0 G
Sodium chloride	5.0 G
Agar No. 3 (Oxoid L13)	15.0 G

pH F.4 (approximately)

<u>199 Medium</u> (Morgan, Morton and Parker, 1950)

80 ml sterilised distilled water was mixed with 10 ml medium 199 (IOX) with Earle's salt, without $NaHCO_3$, with L-Glutamine (Gibco, Biocult, Glasgow, Scotland;) $NaHCO_3$ was added until pink; 20 ml calf serum was added and penicillin 10 U/ml was added.

<u>Hank's solution with proline</u>.

Hanks and Wallace (1949) composed of the following:

KCL	0.4 G
$Na_2HPO_4.12H_2O$	0.06 G
KH_2PO_4	0.06 G
$CaCl_2.2H_2O$	0.185 G
$MgSO_4.7H_2O$	0.1 G
$MgCl_2.6H_2O$	0.1 G
NaCl	8 G
L. Proline (No. P-0380) (Hydroxy L-Proline free, Sigma Chemical Co., St. Louis, USA)	1 G
Distilled water	1 litre

pH 7.2

Modified RE III medium (Steiger and Steiger, 1976 and 1977)
composed of the following:

NaCl	8 mg
KCl	400 mg
$MgSO_4 \cdot 7H_2O$	200 mg
$Na_2HPO_4 \cdot 2H_2O$	60 mg
KH_2PO_4	60 mg
$CaCl_2$	70 mg
BME amino Acid Solution (Gibco)	10 ml
$NaHCO_3$	1 mg
Hepes	14.25 mg
Adenosine	20 mg
BME Vitamin solution (Gibco)	10 ml
Niacinamide	1 mg
D-Pantothenic acid	1 mg
Folic acid (with 1N NaOh)	9 mg
Lipoic acid (DL 6,8 Thioctic acid)	0.4 mg
Bovine albumin - fatty acid free	15 mg
Hemin (with 1N NaOH)	5 mg
Phenol red solution	1 ml
D-glucose	2 mg
L-cysteine	50 mg
L-proline	0.3 mg

made up to 1000 ml with distilled water
pH adjusted to 7.3-7.4 with 1N NaOH

Evans' medium (Evans, 1978)

composition ($litre^{-1}$)	6.8 G
Kh_2PO_4	6.8 G
Edta, disodium salt	0.4 G
NaOH	1.7 G
L-proline	1.5 G
Tryptose (Oxoid L47)	15 G
Casein hydrolysate (acid) (Oxoid L41)	10 G
Liver digest (Oxoid L27)	10 G
Blood lysate	125 ml

pH adjusted to 7.4 with NaOH

Blood lysate is prepared frcm human blood (outdated transfusion stock) from which the bulk of the plasma has been removed. It is frozen at -20°C and then thawed, the pH adjusted to 7.4 with NaOH or HC1. 20 ml of a 4% (w/w) solution of $CaCl_2$ added per litre of crude blood lysate and the mixture is allowed to clot. The mixture is shaken vigorously, transferred to a centrifuge pot and spun at approximately 20,000 g for 1 hour. The supernatant liquid is carefully decanted off the pellet and is used as the blood lysate in the above recipe. The complete medium is clarified by passage through graded nitrocellulose membrane filters (1.2, 0.8, 0.45 and 0.22 micropore sizes), sterilized by passage through a sterile 0.22 mm pore size membrane filter, dispensed into sterile bottles and stored preferably at -20°C (at 4°C medium will keep for 9 months).

Semisolid medium (Chang, 1947) composed of:

Solution A	NaCl	9.20 G
	$NaHCO_3$	0.15 G
	$CaCl_2$	0.24 G
	KCl	0.42 G
	Dextrose	1 G
	Distilled water to	1000 ml
Solution B	Agar	2.5 G
	Peptone	1.0 G
	NaCl	0.5 G
	Beef extract	0.3 G
	Distilled water to	100 ml

7 parts of Solution A + 1 part of Solution B
pH 7.4
defibrinated rabbit blood was added to make 10% v/v.

d. Contamination of cultures.

In spite of all the precautions taken to stop contamination still some developed fungus infection which was intractable. Such cultures are thrown away immediately. The use of Mycostatin at a dose of 100 U/ml killed the parasite, less than this dosage did not affect the fungus (50 U/ml).

2. Storage of parasite strains in liquid nitrogen.

Parasite strains were stored in liquid nitrogen following a method described by Handman et al.(1974). Equal volumes of promastigote culture and sterile glycerine saline (2 ml each) were mixed, then the temperature of the mixture was gradually lowered until it reached the temperature of the liquid nitrogen (- 170°C).

3. Parasite strains used.

1. Leishmania donovani Ethiopian strain L82 (Bradley and Kirkley, 1977).

2. Leishmania donovani Iraqi strain was isolated from the bone marrow of a fourteen-month-old child at the Arab Children's Hospital, Baghdad in 1977. (The child was from Swaira, Wasit province, south of Baghdad). The parasite was cultured on NNN medium (Kagan and Norman, 1970) at 26°C.

Subsequent weekly subcultures used either NNN, Evans' medium (Evans, 1978) or REIII medium (Steiger and Steiger, 1976, 1977) supplemented with 20% foetal calf serum.

4. Hamster dissection.

The hamster was killed with ether and the spleen excised aseptically. Quantitation of the parasite load was based upon the spleen impression smear method of Stauber (1958). The spleen was then homogenised in a glass grinder with medium 199 (GIBCO) containing 25% of inactivated foetal calf serum (GIBCO) and 5 u heparin (Evans Medical) per ml. The suspension was made up to 10 ml with medium 199 and centrifuged at 250 g for 5 minutes to sediment erythrocytes. The supernatant was centrifuged at 1000 g for 15 minutes. The deposit was resuspended in the same medium at a concentration of 5×10^7 parasites/ml and could be used to

(a) infect other animals,

(b) make antigen slides for IFAT,

(c) seed the parasite in culture medium.

5. Indirect immunofluorescent antibody test (IFAT)

(Shaw and Voller, 1964; Kagan and Norman, 1976).

a. Materials

Phosphate buffer saline PBS pH 7.2

Mountant glycerol

FITC conjugate Commercial Miles Yeda Ltd, UK)

Evans Blue (George Gurr Ltd, England)

Acetone (Propanone, May and Baker Ltd, Dagenham, England)

Antigen slides

Reference positive and negative sera

Dessicator with silica gel

Moist chamber incubator

Slide shaker (Luckham Ltd, UK)

Micropipettes (Jencons Scientific Ltd., UK)

Fluorescence Leitz microscope with KP540, KP500 excitation filters and K530 and K510 barrier filters.

Mountant Glycerol pH 8.6

$NaHCO_3$		0.0715 G
Na_2CO_3		0.016 G
Distilled water	to	10 ml
Glycerol	to	100 ml

Dilution of Conjugates

Conjugate used is the commercial FITC anti IgG of Miles Yeda Ltd, UK.

Aliquots of 1 : 4 PBS

	1/120	1/60	1/30
Aliquot	0.1 ml	0.1 ml	0.1 ml
PBS	2.6 ml	1.3 ml	0.650 ml
Evans Blue 1%	0.3 ml	0.15 ml	0.075 ml

Final solution of Evans Blue will be around 0.1%

1% Evans Blue

1 G Evans Blue (George Gurr Ltd, England)

100 ml distilled water.

b. Preparation of Antigen for immunofluorescence test.

i) Promastigote antigen (Oddo and Cascio, 1963; Shaw and Voller, 1964).

The promastigotes were grown in 500 ml flasks containing

250 ml REIII culture medium (Steiger and Steiger, 1976, 1977) supplemented with 20% foetal calf serum and 25,000 units of penicillin/streptomycin solution (GIBCO). The promastigotes were harvested after 7 days incubation at 26°C.

Harvested promastigotes were washed three times by centrifugation in phosphate buffered saline (PBS) pH 7.2 (3000 rpm for 15 mins) (750 G). After the last wash the cells were resuspended in PBS to give a concentration of 1-3 x 10^6 parasites/ml.

Drops of parasites were put on prepared teflon slides using a peg-applicator. The antigen slides were then air dried, wrapped in tissue paper and placed in a desiccator in a refrigerator overnight. The slides were then stored at -70°C in self-sealing polythene bags until used.

At least one antigen slide was fixed and stained with Giemsa as described by Bradley (1977) to assess the quality of the antigen. Poorly washed antigen contains a lot of unwanted background material which may fluoresce and adversely influence results.

ii) Amastigote antigens.

The L82 strain of Ethiopian Leishmania donovani was used. Details of its history and mode of preparation have been described (Bradley and Kirkley, 1977). Amastigotes were used to infect Syrian hamsters (20 x 10^7 amastigotes/ml per hamster; hamsters infected were used after having been infected for 2 - 3 months).

Impression smears.

Aseptically-excised spleen from an infected hamster was used to make impression smears for IFAT antigen slides (Shaw and Voller, 1964; Bray and Lainson, 1965).

Spleen homogenates.

Amastigotes were isolated from infected hamster spleen homogenates (see Hamster Dissection). The homogenate was used to make antigen slides as described for promastigotes (Shaw and Lainson, 1977).

Amastigote-infected macrophages.

To prepare cultures of macrophages in Carrel flasks a recently sacrificed mouse was injected intraperitoneally with 5 ml sterile medium 199. The abdomen was gently kneaded then the macrophage suspension was aspirated using a sterile syringe. The number of

macrophages was estimated by haemocytometer count and diluted to 1.0×10^5 cells/ml.

Carrel flasks were inoculated with 1.0 ml of cell suspension, stoppered and incubated overnight at $37^{o}C$.

Next day a 1.0×10^8 ml amastigote suspension was prepared from a heavily-infected hamster's spleen. The cells in the Carrel flask were washed once with fresh medium 199, then inoculated with 1.0 ml of amastigote suspension. Each flask was stoppered and incubated for 1 hour at $37^{o}C$.

After incubation the cells were again washed once, then inoculated with 1 ml fresh medium 199. Each flask was stoppered and incubated for 4 hours at $37^{o}C$. After incubation the flasks were flooded with methanol for 1 hour to fix the macrophages and to separate the Carrel flasks from the microscope slides. The slides were then wrapped in tissue paper and stored at $-70^{o}C$.

One slide of each batch was stained with Giemsa to assess the degree of infection of the macrophages.

iii) Preparation of Teflon slides.

Glass microscope slides were wiped clean with a piece of clean cloth. Into a petri dish or similar container glycerol was poured to a depth of 3 mm. The peg applicator, which consists of 16 metal rods mounted in 2 rows of 8 cn a perspex block, was dipped into the glycerol and drops were applied onto the glass slide to give a pattern of 16 well spots. The slides were then sprayed with PTFE (PTFE aerosol spray, Fisons Scientific Apparatus, Loughborough, Leics, England) from a distance of about 10 inches for a few seconds, sufficient to give a thin coat, which dries on the glass to form a chemically inert water repellant film. PTFE will not penetrate the glycerol drops. The slides were allowed to dry for a few minutes and then washed with hot water to remove the glycerol. The slides were then either blotted or placed vertically in a rack and left to dry. A pattern of wells corresponding to the drops of glycerol were left on the PTFE coated slides.

c. Method of IFAT

Antigen slides that were stored at -70°C were put quickly in a dessicator containing silica gel for 15 minutes, then they were fixed with acetone (Quilci et al. 1968; Rioux and Golvan, 1969) for 30 seconds. The slides were dried and marked and the test sera were applied (about 15 µl) of each dilution to each well, not forgetting to begin from higher dilution towards higher concentrations if using the same tip of the micropipette. The reference positive, reference negative and PBS were applied to some wells.

Slides were incubated in a moist chamber at room temperature for 30 minutes, washed with PBS for 10 minutes for three times using the slide shaker. The conjugate was applied then the slide flooded with it and incubated in the moist chamber for 30 minutes at room temperature.

The slides were washed with PBS for ten minutes three times using the shaker and each slide was dipped later in acetone to wash excess Evans Blue, washed in PBS and mounted with mountant glycerol. A cover slip was put on and the slides were examined with a fluorescent microscope.

Positive wells were defined as when the parasites in those wells were wholly fluorescing and not merely part of the parasite, and all the parasites were fluorescing in the field; fluorescence had to be uniform and fluorescence of the reference positive must reach the optimal dilution in each test.

Negative reference wells must not show any fluorescence, PBS alone with antigen similarly must show no fluorescence.

Checker boards for the optimum dilution to be used were made and it was found that final dilution of the conjugate of 1/30 showed satisfactory results.

Usual dilution of sera in PBS examined was 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, etc. in double-folds, a microtitre is usually used for dilutions.

Positivity was taken as from 1/16 and more.

6. Enzyme linked immunosorbent assay (ELISA)

a. Materials

i) Buffers used (Engvall and Perlmann, 1972; Voller et al.(1977).

1. Phosphate buffer saline (PBS) pH 7.2

NaC1	8.5 G
Na_2HPO_4 (m.v. = 141.9)	1.28 G
$NaH_2PO_4.2H_2O$	0.156 G
Distilled water	1 litre

2. Coating buffer 0.05M Carbonate pH 9.6

Na_2CO_3	1.59 G
$NaHCO_3$	2.93 G
Distilled water	1 litre

3. Incubation buffer

PBS	900 ml
Tween 20 (Sigma)	0.45 ml
Bovine serum (albumin)	1%

Tween was added to prevent non-specific adsorption to the solid phase (Voller et al. 1977). The albumin was used to reduce background IOV (Voller et al, 1977).

4. Washing solution

NaCl	45 G
Tween 20 (Sigma)	2.5 ml

Dilute to 5 litres with distilled water.

ii) Peroxidase substrate

Stock solution

100 mg of O-phenylene diamine (Sigma) was added to 10 ml of methanol (the soluble salt can be added to this same volume of distilled water). The sample was stored in the dark at $4^{o}C$.

Working solution

0.2 ml Stock solution

0.010 ml Hydrogen peroxide 6%

20 ml distilled water

iii) 8N H_2SO_4

1 ml stock H_2SO_4 sulphuric acid 1.840 (molecular weight 98.1 of H_2SO_4), 36 N

(May and Baker Ltd. Laboratory Chemical, Dagenham, England)

3.5 ml distilled water.

iv) Conjugates

Conjugate used was the commercial horseradish peroxidase conjugate Anti IgG of Miles Yeda Ltd, UK. This was aliquoted into universal tubes 50 µl in each and kept at -20°C.

v) Plates

Polystyrine microtitre plates used were the M 129 A Dynatech Laboratories, Sussex, U.K.

vi) Photometer

A Universal photometer (Vitatron, Fison MSE, Scientific Instruments, West Sussex, U.K.) was used; reading was registered at 492 nm optic density.

vii) Preparation of ELISA antigen.

The sonicated ultracentrifuged whole promastigote antigen:-

Cultures of Leishmania strains, specially those grown on RE3 medium, were taken because this medium did not have a lot of unwanted material with the promastigotes when isolating them. Washing of the promastigotes was carried out with PBS pH 7.2 at 3000 rpm (750 G) for 15 minutes. The pellet was reconstituted at each time with PBS, the last pellet was reconstituted with PBS, so that the suspension of parasites gave the highest concentration possible.

Then the parasites were sonicated using a 1/8" (3 mm) exponential probe with a(Griffin and George, England,) Sonicator. Sonication was done to the mark 10 for one minute, then the suspension was cooled in ice for five minutes. This process was repeated three times, and then the antigen was ultracentrifuged at $4^{o}C$ at 100,000 g for one hour. The precipitate was discarded and the supernatant fluid was kept to be used later as antigen.

Protein estimation of the antigen was done according to Folin-Lowry method (Lowry et al. 1951).

The new antigen was then aliquoted and kept at $-20^{o}C$.

From the experience of the writer it was found that lyophilisation and reconstitution of the antigen, especially if it was kept for some time, may have some untoward effects on the quality of the antigen and on the assay results.

Excretory Factor Antigen (Schnur et al. 1972)

Excretory factor was collected during the log phase of growth of promastigote cultures. The supernatant of a culture from which promastigotes had been removed by centrifugation was filtered through millipore filters of 0.45 um pore size and stored at $-20^{o}C$.

b. ELISA method

(Voller et al. 1975, 1976; Hommel, 1976; Voller et al. 1977; Roffi et al. 1980; Anthony et al. 1980).

200 µl of 10 µG/ml antigen in coating buffer was added to each well of the plate, the plate incubated at room temperature overnight for the antigen to be adsorbed to the solid phase.

The next day the plate was washed for three minutes with washing solution; washing was repeated three times. 200 µl of 1/100 dilution of sera in incubation buffer was added to each well and plate incubated at room temperature for two hours. Washing was repeated as before, enzyme linked anti IgG conjugate was added in 1/400 dilution in incubation buffer, 200 ul in each well, the plate was incubated at room temperature for 3 hours, then the plate was washed again and

as before, then 200 µl working solution of the orthophylene diamine substrate was added to each well and when the predetermined reading of the reference positive (after eliminating the background) had been reached (orange discolouration) then the reaction was stopped by adding 25 µl of 8N H_2SO_4 to each well, the fluid in each well was put in the photometer and read at 492 nm and the end result was expressed as absorption at that wavelength by the discolouration which had resulted, the reading was then registered.

Checkerboard tests showed that optimum dilutions to be worked with were 1/100 for the sera, 10 µg/ml for the antigen and 1/400 dilution of the conjugate.

Two main reference negatives were used and at 492 nm many readings were recorded. European negative mean reading was 0.11, the mean with two standard deviations was 0.15.

The Iraqi reference negative mean reading was 0.04 mean and two standard deviations reading was 0.07.

Reference positives were used and the readings also registered many times, a mean of the reading was taken to stop the reaction after eliminating the background of the PBS which must be as low as possible.

Testing later the bone marrow positive sera it was found that a positivity cut-off point of 0.2 was safely to be taken for the sake of specificity.

7. Serological tests for animals.

Conjugates of serological tests used for animals: antidog IgG (Miles Yeda Ltd, England) was used for dogs, jackals, foxes and wolves, both in IFAT and ELISA.

An immunodiffusion test using antidog IgG and jackal's sera showed a precipitate line similar to that made by the dog sera; the positive reference was obtained from experimentally infected dogs, the reference negative was taken from suckling dogs.

Conjugates for both tests for rats: antirat IgG and for mice, antimouse IgG were commercially available from Miles Yeda Ltd, England. Positive reference serum was obtained from the experimentally infected mice and negative sera obtained from inbred mice which had not been

infected with L. donovani or any other parasites.

8. Microimmunodiffusion Technique (described by Ouchterlony and Nilsson, 1973).

Slides were prepared as follows: Barbitone acetate buffer 0.1M, pH 8.6 was done first by dissolving 10G barbitone sodium and 6.5G sodium acetate in 64.4 ml hydrochloric acid 0.1N and diluted to 1 litre with water.

Then the gel was prepared by taking 100 ml of the reagent buffer and putting it in a flask. 1G (1%) or 2G (2%) pure agar was added and gently heated to the boiling water or just before (when the solution becomes clear), then during the heating 0.1% w/v sodium azide or 0.01% methiolate was added. Clean microscopical slides were painted with the hot solution. With a Pasteur pipette the hot solution was distributed evenly over the precoated slide (2 mm thick), slides were dried and kept in a moist chamber and incubated at $4^{o}C$ overnight. Next day the gel was perforated with another special slide with special set of hollow cylinders, the cut cylinders of gel were then removed carefully by forceps or by sucking. The slides were then kept in a moist chamber at $4^{o}C$ until used.

9. Leishmanin test.

a. Leishmanin preparation (Manson-Bahr, 1961a).

Cultures of promastigotes of L. donovani of the Iraqi strain were centrifuged under aseptic conditions at 750 g for 15 minutes, supernatant was discarded and the pellet was reconstituted, using a sterilized Pasteur pipette, with PBS that had been passed through a 0.45 um size filter. This process was repeated at least three times, then the number of promastigotes was adjusted to 5 - 10 x 10^{6}/ml and the suspension was put into a sterile rubber capped injection bottle and phenol added (0.5%). The control was prepared by using phenol 0.5% in PBS only; both were kept at $4^{o}C$ ready for use.

b. Method

0.1 - 0.2 ml of the Leishmanin preparation was injected by a 1 ml sterile and disposable hypodermic syringe (B-D Plastipak, Ireland) with a hypodermic needle of 25 G x 5/8 into the right forearm in the flexor side and 0.1 - 0.2 ml of the control prepared into

the left forearm.

Reading was done after 72 hours, the control must give no reaction; at the site of Leishmanin injection an induration was looked for and measured. If there was an induration of more than 5 mm it was considered positive (Manson-Bahr and Southgate, 1964).

Leishmanin testing was performed on serologically positive cases during the follow-up of those cases. A small leishmanin survey was done on 35 negative children as a control standardised to sex and age of the sero-positive children.

10. Skin windows.

a. Method

Method of work on skin windows is discussed by Boggs et al. (1964).

An area of skin approximately 2 x 5 mm was scraped with a No. 10 Bard-Parker blade until minute bleeding points were evident. This lesion was covered by a sterile, circular cover glass 12 mm in diameter, this cover glass was in turn covered by a piece of protective cardboard which was fixed to the skin by tape. The samples of inflammatory exudate were prepared for microscopic examination by removing the cover glass, allowing it to dry and staining it with Wright's stain.

No exogenous inflammatory stimulus was applied to this denuded area of skin. Macrophages had a tendency to clump together on the cover glass (Boggs et al. 1964).

Gange et al. (1977) also described another method of skin windows using plastic chambers (0.6 ml) containing cell attractant fluid to study neutrophil migration in sarcoidosis.

Rebuck et al. (1961) discussed the cellular constituent of the exudate from a scarified skin in time sequence the 2 - 8 hours show the presence of increasing numbers of neutrophilic leucocytes (small) a few tissue macrophages, a few haematogenous lymphocytes (10 - 12μ^{H}) and monocytes and an occasional eosinophil. At 9 hours the exudate was made up of almost equal numbers of neutrophils and lymphocytes 10 - 12μ^{H} at 12 hours predominant neutrophils small 8 - 10μ^{H}, lymphocytes 6 - 12μ^{H} and monocytes 20 - 23μ^{H} . At 14 hours degenerated

neutrophils, intact lymphocytes10 - 14$^{H}_{J}$, at 16 hours lymphocytes 15 - 16$^{H}_{J}$; at 18 hours small macrophages (histiocytes) less than 16$^{H}_{J}$; at 24 hours macrophages (histiocytes) 16 - 20$^{H}_{J}$.

So early predominance of neutrophils give way to the customary 10 - 14^{h} lymphocytic predominance and then to 16 - 24 hours massing of macrophages.

Some granulocytes and monocytes will stick to the glass even if it has been treated with silicone or paraffin wax. They are extraordinarily tenacious. But the lymphocytes appear not to stick.

Volkman and Gowans (1965) put surface coverslips on abraded skin or subcutaneous ones in rats. Polymorphs were the first cells to appear, at 4 - 24h mononuclear cells, macrophages began at 6h, abundant at 12h, predominant at 24h, the exudate macrophages are derived from the blood. It was concluded that in the rat bone marrow and to a lesser extent spleen, are major sources of the macrophages which emigrate into foci of acute non-bacterial inflammation (Volkman and Gowans, 1965).

b. Skin windows on man

In the present study skin windows were tried on human volunteers to be familiar with the procedure and the microscopical appearance. It was then tried on parasitologically proven cases of visceral leishmaniasis, and on infected inbred hamsters and mice; the aim was to get macrophages infected with Leishmania from the skin.

The method used in man was that described by Boggs et al.(1964) and by Bradley (personal communication). 5 mm sterile clear rounded cover slips were applied to an abraded area in the skin (using a blade No. 10 Bard-Parker) and fixed with cardboard and plastic, removed after 24 hours, fixed with methanol, stained with Giemsa and examined under the microscope.

c. Skin windows on animals.

For the purpose of detecting infected macrophages in the skin and subcutaneous tissue, skin windows were tried on animals. Using a similar method, described by Volkman and Gowans (1965) who found that the exudate macrophages were derived from the bone marrow.

Animals used were heavily infected hamsters and genetically susceptible mice (B10/D2, DBA/1, Balb/C)and resistant mice (CBA/Ca and C.3H) (Bradley and Kirkley, 1977). Control non-infected mice were used also. The animal was anaesthetised with ether, the skin sterilised with alcohol and a skin incision was made at the back of the mouse between the two scapulae just enough to push a 5 mm diameter clean sterile cover slip under the skin. The skin was sutured using eyeless needled suture (Ethicon Ltd, Scotland). The incision was sprayed with Nobecutane aerosol (Astra Chemicals Ltd, Watford, England).

The animals were then returned to their cages and left for 24 hours or 48 nours. The next day the animal was killed and the cover slip was looked for, dried, fixed and stained with Giemsa.

The infection state of the animal was assessed after killing it, and looking for its parasite load in the liver.

Animals used.

For the purpose of skin windows the following 17 animals and their duration of infection were used:

2 B10/D2 susceptible non-infected control mice
3 hamsters 2-3 months after their infection
1 DBA/1 susceptible mouse at day 15 after infection
2 CBA/Ca resistant mice at day 15 after infection
1 C3H resistant mouse at day 15 after infection
2 Balb/C susceptible mice at day 45 after infection
2 CBA/Ca resistant mice at day 45 after infection
2 Balb/C susceptible mice at day 60 after infection
2 CBA/Ca resistant mice at day 60 after infection.

The mice and hamsters had been infected with Leishmania donovani Ethiopian strain L82 as described by Bradley and Kirkley (1977).

G. LONGITUDINAL SEROLOGICAL STUDY OF INFECTION IN MICE

A longitudinal serological study was carried out on inbred mice of varying genetically determined susceptibility to infection (Bradley, 1974, 1977; Bradley and Kirkley, 1972, 1977).

1. Objectives

The objectives of the experiments with mice were:

1. To see whether any dramatic change in specific anti-leishmania levels accompanies the fall in parasite load in early recovery strains; and

2. to obtain a more general picture of antibody production in the mice as a model for studying possible rodent reservoir hosts in the field.

2. Material

Mice used for this work were representative of the following:

Innately susceptible mice of the two types, the susceptible cure CS7B110/Sc. Sn (here referred to as B10), Balb/C, Balb/B and Balb/K and the susceptible non-cure B10/D2; these mice were used along with the innately resistant mice C57/L. (Bradley and Kirkley, 1977, Bradley 1977).

They were infected with L. donovani Ethiopian strain as described by Bradley and Kirkley (1977).

Four experiments were carried out using in the first experiment (Exp. No. 542) 13 mice: 6 mice of B10/D2 type and 7 mice of B10 type.

In the second experiment (Exp. No. 552), 53 mice were used:

15 Balb/C
15 Balb/B
15 Balb/K
4 C57 B10/Sc. Sn.
4 C57/L

In the third experiment (Exp. No. 553) 26 mice were used:

10 of the B10/D2
12 of the B10
4 of the C57/L

In the fourth experiment (Exp. No. 554) 22 mice were used:

10 of the B10/D
10 of the B10
2 of the C57/L

So in total 114 mice were used and they were:

B10/D2 - 26 mice
B10 - 33 mice
Balb/C - 15 mice
Balb/B - 15 mice
Balb/K - 15 mice
C57/L - 10 mice

3. Method

Blood was taken from non-infected mice to be used later as reference negative in the serological tests.

An equal number of mice for each type were killed on days 15, 50, 85 and 130 to calculate their "Leishman donovan units" (LDU) as the number of parasites per 500 liver cell nuclei counted from Giemsa stained impression smears (Stauber, 1955).

Blood from these mice was taken at days 1, 15, 30, 50, 85 and 130. Serum was isolated and kept until examined by IFAT and ELISA later.

CHAPTER III

RESULTS

A. RESULTS OF THE USE OF DIFFERENT ANTIGENS IN SEROLOGICAL TESTS

Results of the work on the excretory factor (Schnur et al. 1972)

The excretory factor antigen was prepared from different culture media at their log time of multiplication and from PBS (leaving the promastigotes for 24 - 48 hours in PBS), as described by Schnur et al. (1972) and then it was treated in two different tests to determine its antigenicity against known positive sera with antileishmania antibodies. In immunodiffusion it was tested against positive sera from cases infected with L. donovani. In the ELISA method it was used as an antigen to coat microtitre ELISA plates. Both methods gave negative results even at the lowest dilutions of the excretory factor.

The best antigen used for ELISA was found to be the washed and sonicated promastigotes, and later ultra-centrifuged.

The various types of antigen for immunofluorescence were tried using positive sera which were taken from infected genetically susceptible inbred mice. Negative sera were taken from non-infected mice, positive sera from cases of visceral leishmaniasis in Iraqi children and non-infected Iraqi children were used also.

1. Impression smears from cut spleens used as antigens in IFAT gave a negative result with the positive sera and no fluorescence could be detected on the slide.

2. Amastigotes from spleen emulsion used as antigens in IFAT gave carpet fluorescence of the slide with some distinct Leishman Donovan bodies fluorescing dots, but the slide was full of background due to the other constituents of the emulsion like blood corpuscles and cells etc.

3. Intracellular amastigotes in macrophages used as antigen in IFAT. One slide was fixed and stained with Giemsa to assess the state of infection of the macrophages, other slides were treated as antigen slides in immunofluorescence using positive sera, negative sera and sera from mice infected with T. lewisii. The results show fluorescing small dots

with very small hollow centres which were the amastigotes and they were packed inside the macrophages, some were outside them. The macrophage nucleus showed red discolouration, the cytoplasm of the macrophage showed similar red discolouration, the negative sera did not elecit any kind of reaction and the sera of T. lewisii gave negative results (not like the promastigote antigen where they cross react with T. lewisii). This antigen was ideal except for certain limitations:

a. The laborious and complicated method used to prepare it with the two kinds of animals used and the process of their infection for a certain period of time with the quantitative limitations compared to the easy way a promastigote antigen was prepared, and the greater number of slides produced.

b. Higher magnifications were needed and still the promastigote fluorescence was more striking.

4. Promastigote antigen was seen to be the ideal to use from the point of view of its easiness of preparation and simple method of production.

With the promastigote the parasite was well recognised and even the flagellum was seen fluorescing.

B. HOSPITAL ACTIVITIES

For the purpose of studying sensitivity and specificity matters of the serological tests used in this study, blood was collected from:

66 cases of bone marrow positive visceral leishmaniasis in children

79 cases of bone marrow negative suspected cases of visceral leishmaniasis in children

46 children patients suspected to have visceral leishmaniasis but bone marrow was not done on them

23 cases of fever and/or hepatosplenomegaly in children who were not suspected as cases of visceral leishmaniasis

2 adult cases having the ulcerative stage of Leishmania tropica infection

23 cases of leprosy (9 tuberculous and 14 lepromatous) from the leper colony in south of Iraq; all were adults

37 cases of tuberculosis, 31 pulmonary (15 of them were open cases) and the rest were extrapulmonary, all of them were adults, except

4 children with tuberculous meningitis.

7 cases of brucellosis from adults and children diagnosed by IFAT test during a small field survey of shepherds

31 cases of parasitologically proven malaria before treating them, one was P. falciparum, all were adults.

2 cases of toxoplasmosis from adults also detected during IFAT test among pregnant women

2 cases of measles in children

7 cases of thalassemia in children

8 proved (Widal positive) typhoid cases in children

57 cases of intestinal helminths and parasites in children from field surveys, those included children infected with Ankylostoma duodenale, Ascaris lumbricoids, Hymenolepis nana, Enterobius vermicularis, Entamoeba histolytica without hepatic involvement and Giardia lamblia.

12 cases of Schistosoma mansoni amongst infected adult Egyptians

6 cases of Schistosoma haematobium amongst adults

3 Echinococcus granulosus immediately after removing the cyst

4 cases of septic meningitis in children

5 cases of renal infection in children

5 cases of respiratory infections in children

2 cases of gastroenteritis in children

2 cases of rheumatic fever in children

1 case of rheumatic arthritis in a child

1 case of tetralogy of Fallot in an infant

1 case of a child with favism

All totalled to 433 cases.

In addition two sera were available from stored specimens:

1 case of Trypanosoma gambiense in an adult

1 case of monkey Trypanosoma cruzi

C. SENSITIVITY AND SPECIFICITY OF SEROLOGICAL TESTS USED

Specificity is the proportion of false positive reactions among people who have never had the infection.

The sensitivity on the other hand is the proportion of false negative reactions among infected people (Lobel and Kagan, 1978).

1. Sensitivity test for indirect immunofluorescent antibody technique and ELISA technique.

For that purpose sera from 45 recently detected cases of visceral leishmaniasis and sera from 14 previously detected cases of versiceral leishmaniasis were used.

Those 59 sera from parasitologically proven cases of visceral leishmaniasis were tested by both techniques and the following results show that IFAT picked up 94.9% of the parasitologically proven cases of visceral leishmaniasis, titres as from 1/16 were taken as positive, negative references were used from European and from healthy Iraqi children which were not normally inhabitants of an endemic area.

For ELISA the cut-off point was taken at 0.2 reading of the sera when put in a photometer and read at 492 nm because this was found to separate the negatives from the positives with high confidence.

41
Sera/from parasitologically proven cases of visceral leishmaniasis taken, at the time of diagnosis and before commencing to treat them, and examined by ELISA, 11 cases, 26.8% were found to be negative and 30 cases were found positive.

Sensitivity of ELISA was thus found to be 73.2% of the cases of the disease.

It was possible also to take together the parasitologically proven cases of visceral leishmaniasis with the bone marrow negative suspected cases of visceral leishmaniasis and the no bone marrow clinically suspected cases of visceral leishmaniasis and treated all as suspected cases, the results of their serological examinations by both techniques are shown overleaf in Table 19 and 20.

Sera from 43 cases of visceral leishmaniasis which were proved parasitologically positive were taken irrespective of the time of diagnosis or treatment and were tested by both ELISA and IFAT and the results were plotted against each other. The results show matching results though not very well correlated, Figure 22.

Table 19. Showing the results of examinations of cases of visceral leishmaniasis, both proved and suspected, by IFAT and ELISA serological techniques.

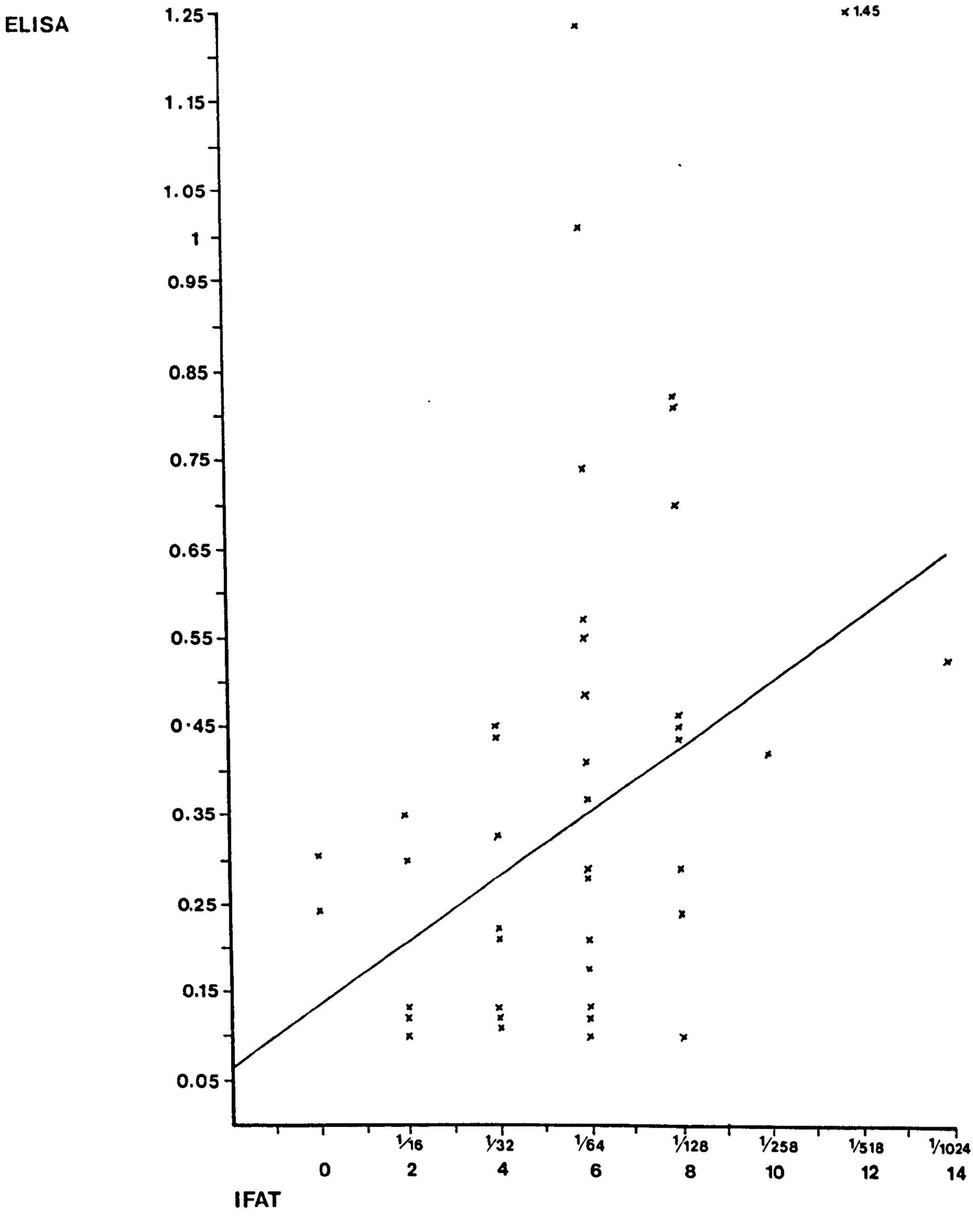
		Total cases	Negative	Percent negative of those examined	Positive	Percent positive of those examined
Bone marrow positive cases of visceral leishmaniasis	IFAT	59	3	5.1%	56	94.9%
	ELISA	41	11	26.8%	30	73.2%
Bone marrow negative suspected cases of visceral leishmaniasis	IFAT	79	25	31.6%	54	68.4%
	ELISA	52	36	69.0%	16	31.0%
No bone marrow clinically suspected cases of visceral leishmaniasis	IFAT	46	11	23.9%	35	76.1%
	ELISA	41	29	70.7%	12	29.3%
Total	IFAT	184	39	21.2%	145	78.8%
	ELISA	134	76	56.7%	58	43.3%

Table 20. Titres of 43 cases of bone marrow positive visceral leishmaniasis tested by IFAT and ELISA techniques. (x = is a score given to IFAT titre)

Name	IFAT	x	ELISA	y^2	xy
G.N.	1/128	8	0.45	0.2	3.60
Th.S.S.	1/64	6	0.74	0.55	4.44
M.J.	1/128	8	0.70	0.49	5.60
M.H.R.B.	1/64	6	0.48	0.23	2.88
A.H.A.M.	1/64	6	1.01	1.02	6.06
I.Kh.	1/128	8	0.82	0.67	6.56
I.O.	1/64	6	0.41	0.17	2.46
B.F.	1/16	2	0.13	0.016	0.26
K.M.	1/128	8	0.46	0.21	3.68
Z.Kh.	1/64	6	0.29	0.08	1.74
A.J.A.H.	1/128	8	0.82	0.67	6.56
Z.H.A.	1/32	4	0.11	0.012	0.421
M.K.O.	1/32	4	0.45	0.20	1.80
H.M.N.	1/64	6	0.10	0.01	0.60
S.T.	1/32	4	0.45	0.20	1.80
I.M.	1/32	4	0.22	0.05	0.88
I.M.	1/64	6	0.27	0.07	1.42
H.H.	1/16	2	0.10	0.01	0.20
H.S.	1/32	4	0.13	0.0169	0.52
A.H.	1/16	2	0.13	0.0169	0.26
F.Sh.	1/64	6	0.13	0.0169	0.78
S.H.	1/128	8	0.10	0.01	0.80
F.F.	1/64	6	0.37	0.1369	2.22
I.M.	1/32	4	0.13	0.0169	0.52
N.A.	1/64	6	0.12	0.0144	0.72
M.A.	1/16	2	0.35	0.1225	0.70
T.A.	1/32	4	0.33	0.1089	1.32
N.H.	vi	0	0.31	0.096	0.00
1249	1/621	6	0.57	0.32	3.42
1250	1/16	2	0.30	0.90	0.60
1251	1/64	6	0.21	0.04	1.26
1252	1/256	10	0.47	0.221	4.70
1253	1/128	8	0.24	0.058	1.92
1254	1/64	6	0.18	0.03	1.08
1255	1/128	8	0.44	0.19	3.52
1256	-vi	0	0.24	0.058	0.00
1257	1/512	12	1.45	2.10	17.4
1258	1/64	6	0.55	0.3	3.30
1259	1/128	8	0.29	0.08	2.32
B.J.	1/32	4	0.28	0.078	1.12
A.A.	1/32	4	0.21	0.04	0.84
H.M.	1/64	6	1.23	1.50	7.38
F.A.	1/1024	14	0.52	0.27	7.28

$r = 0.439$ $P_{(41,0.05)} = 0.304$ $y = 0.102 + 0.053x$

Figure 22 Showing IFAT and ELISA titres of bone marrow positive cases.



2. Specificity test for IFAT and ELISA

As for the specificity of the serological tests used, the IFAT and the ELISA, the following Table 21 shows the results of the tests performed on sera collected from different types of diseases other than visceral leishmaniasis.

3. Cross reactivity in serological methods used.

The following Tables 22 and 23 and Figures 23, 24 and 25 (graphs) show the cross reactivity of certain diseases with leishmanial antigens when examined by the IFAT and the ELISA techniques and they show the extent of this cross reactivity also in terms of titre.

All the serological tests done in this study were made blindly, sera were given code numbers and serological examination was done on them without any knowledge concerning the source of the sera.

Judging from these tables and from the graphs it will be found it will be found that a positivity of 1/32 in IFAT and a reading more than 0.2 in ELISA are to be taken as diagnostic titres of visceral leishmaniasis after taking into account the problems of cross reactivity.

So from the previous result it was shown that indirect immunofluorescent test was more sensitive, but ELISA was found to be more specific.

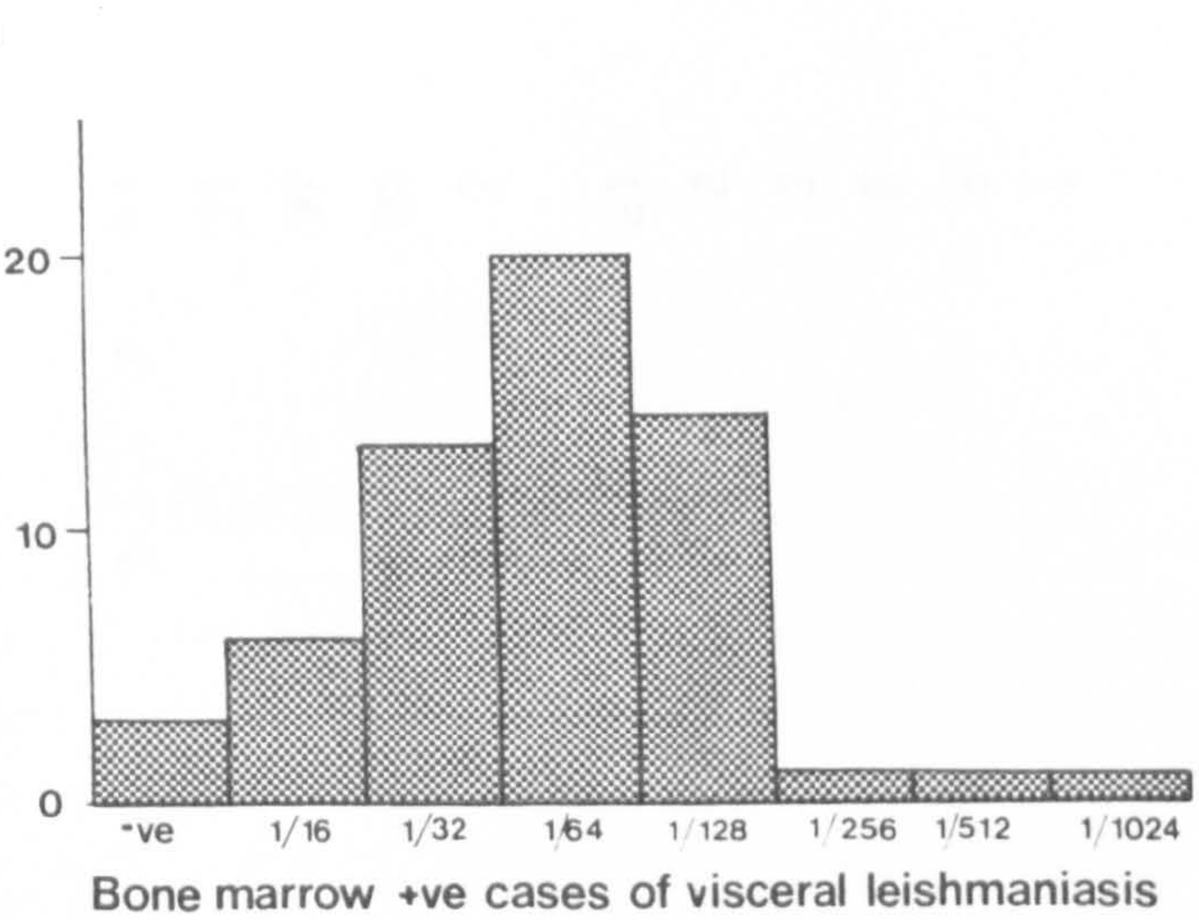
Table 21. Showing results of testing of sera collected from different diseases by IFAT and ELISA.

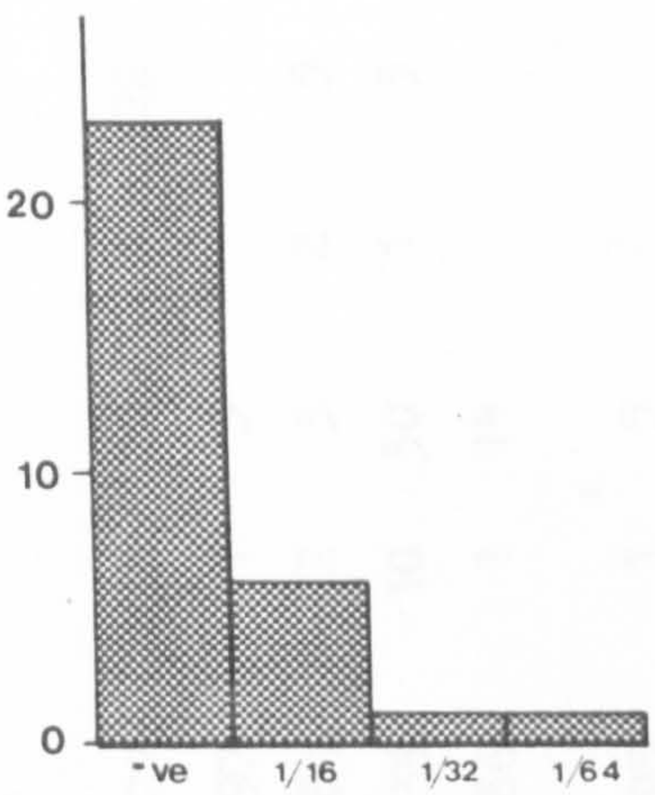
Disease	No. of cases	IFAT tested	Positive	%	ELISA tested	Positive	%
Malaria	31	31	8	26	31	1	3
Tuberculosis	37	36	16	44	37	4	11
Brucella	7	7	2	29	7	1	14
Leprosy	23	23	14	61	20	13	65
Fever and hepato splenomegaly	23	23	1	4	21	1	5
Measles	3	3	0	0	3	0	0
Thalassaemia	7	6	0	0	5	0	0
Typhoid	8	7	0	0	6	0	0
Intestinal helminths and parasites	57	57	0	0	57	0	0
Toxoplasma gondii	2	2	0	0	2	1	50
Leishmania tropica	2	2	1	50	2	1	50
Schistosoma mansoni	12	8	3	33	8	0	0
Schistosoma haematobium	6	6	1	17	6	1	17
Echinococcus granulosus	3	3	0	0	3	0	0
Trypanosoma gambiense	1	1	0	0	1	0	0
Trypanosoma cruzi	1	1	1	100	1	0	0
Septic meningitis	4	4	0	0	4	0	0
Renal infections	5	5	0	0	5	0	0
Respiratory diseases	5	5	0	0	5	0	0
Favism	1	1	0	0	1	0	0
Gastronenteritis	2	2	0	0	2	0	0
Rheumatic fever	2	2	0	0	2	0	0
Rheumatic arthritis	1	1	0	0	1	0	0
Tetralogy of Fallot	1	1	0	0	1	0	0
Total	244	237	47	20	230	23	10

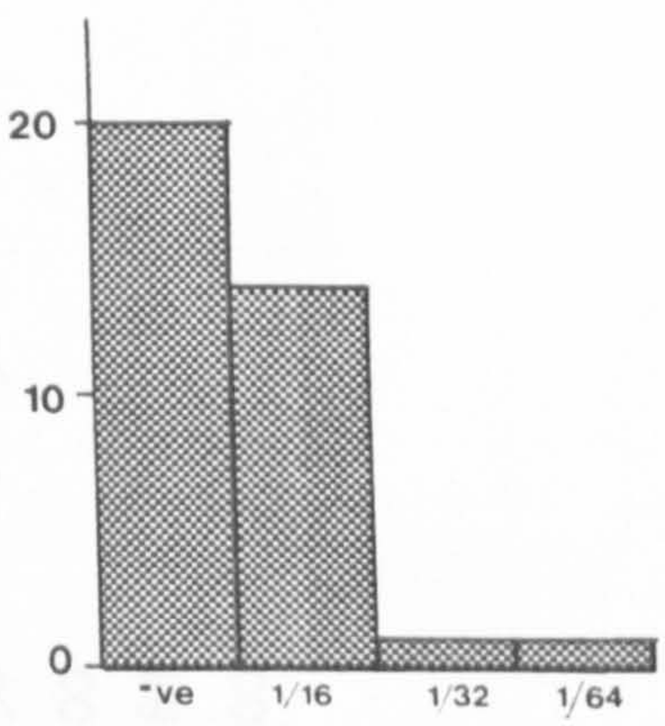
Table 22. Results of examination of sera from different diseases using leishmanial antigen in IFAT

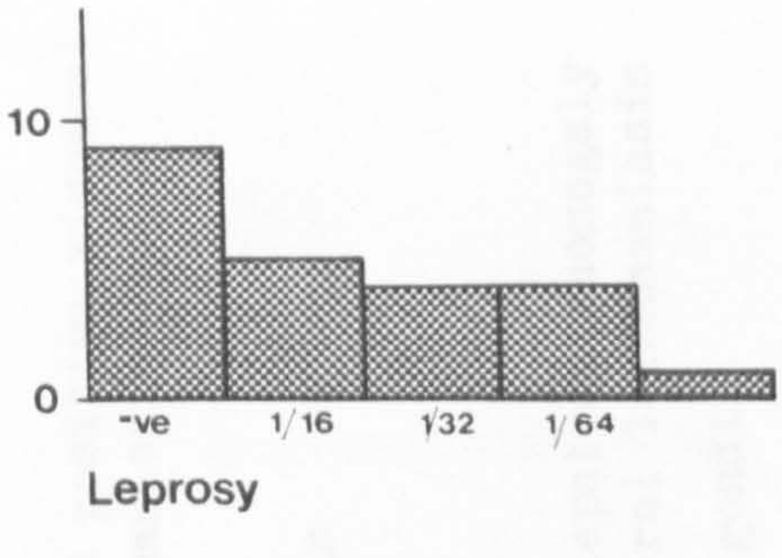
Disease	Negative	%	1/16	%	1/32	%	1/64	%	1/128	%	1/256	%	1/512	%	1/1024	%	Total
Bone marrow positive visceral leishmaniasis	3	5	6	10	13	22	20	24	14	24	1	22	1	2	1	2	59
Malaria	23	74	6	19	1	3	1	3									31
Tubercylosis	20	56	14	39	1	3	1	3									36
Leprosy	9	39	5	22	4	17	4	17	1	4							23
Brucella	5	71	2	29													7
Fever and hepatosplenomegaly not visceral leishmaniasis	22	96	1	4													23
Toxoplasma gondii	2	100															2
Leishmanin tropica	1	50	1	50													2
Schistosoma mansoni	5	66	3	33													8
Schistosoma haematobin	5	83	1	17													6
Trypanosoma cruzi									1	100							1

Figure 23 Results of examination of sera from different diseases using leishmanial antigen in IFAT.









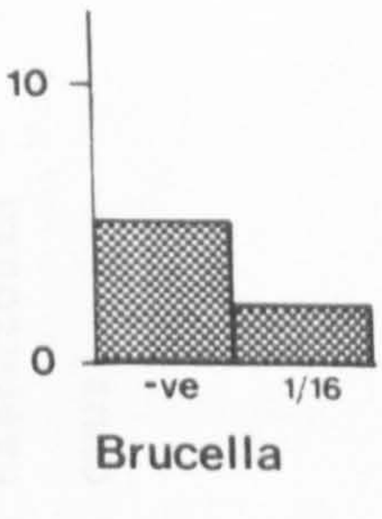


Table 23. Results of examination of sera from different diseases using leishmanial antigen in ELISA

	-0.20	%	-0.35	%	-0.50	%	-0.85	%	0.85-0.00	%	Total
Bone marrow positive visceral leishmaniasis	11	27	11	27	9	22	7	17	3	7	41
Malaria	30	97	1	3							31
Tuberculosis	33	89	2	5	2	5					37
Leprosy	7	35	10	50	1	5	1	5	1	5	20
Brucella	6	86	1	14							7
Fever and hepatosplenomegaly not visceral leishmaniasis	20	95	1	5							21
Toxoplasma gondii	1	50	1	50							2
Leishmania tropica	1	50	1	50							2
Schistosoma mansoni	8	100									8
Schistosoma haematobin	5	83	1	17							6
Trypanosoma cruzi	1	100									1

Figure 24 Results of examination of sera from different diseases using leishmanial antigen in ELISA.

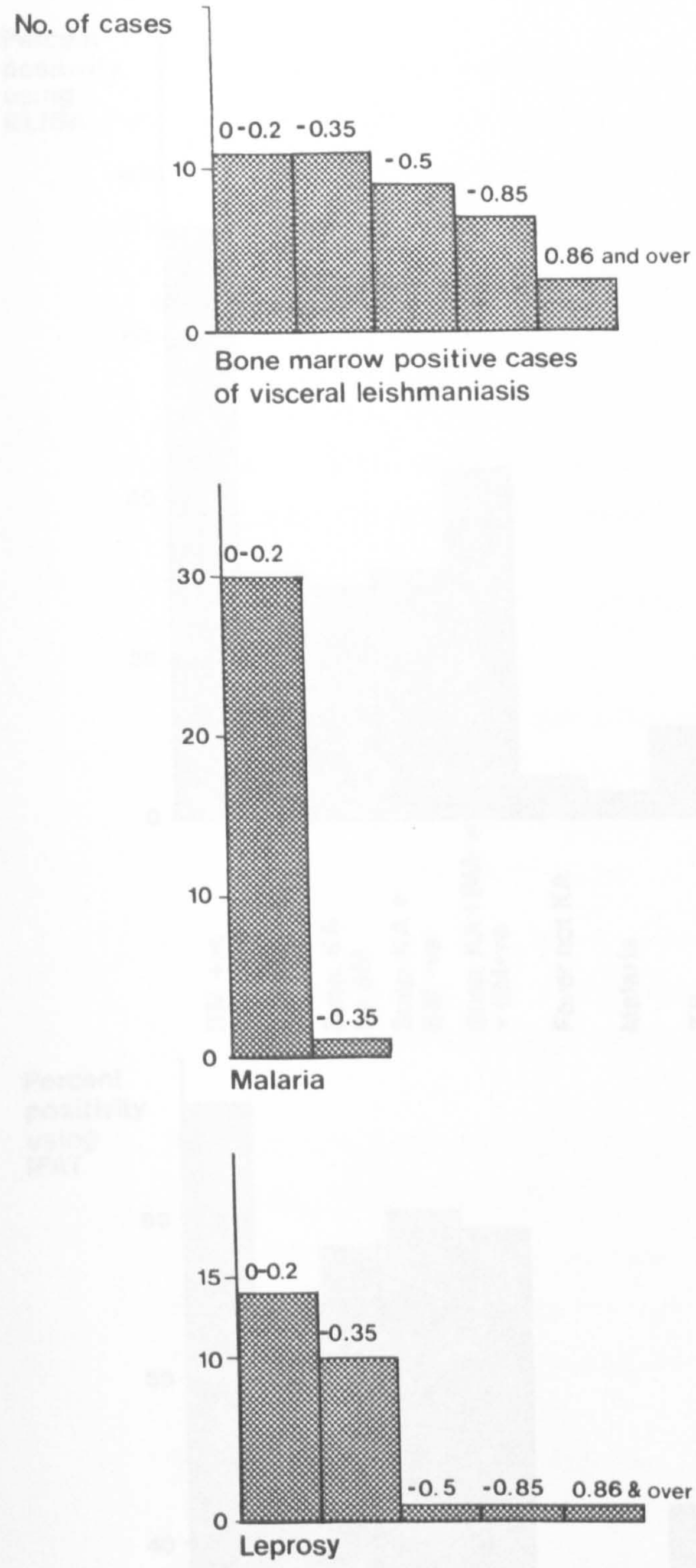
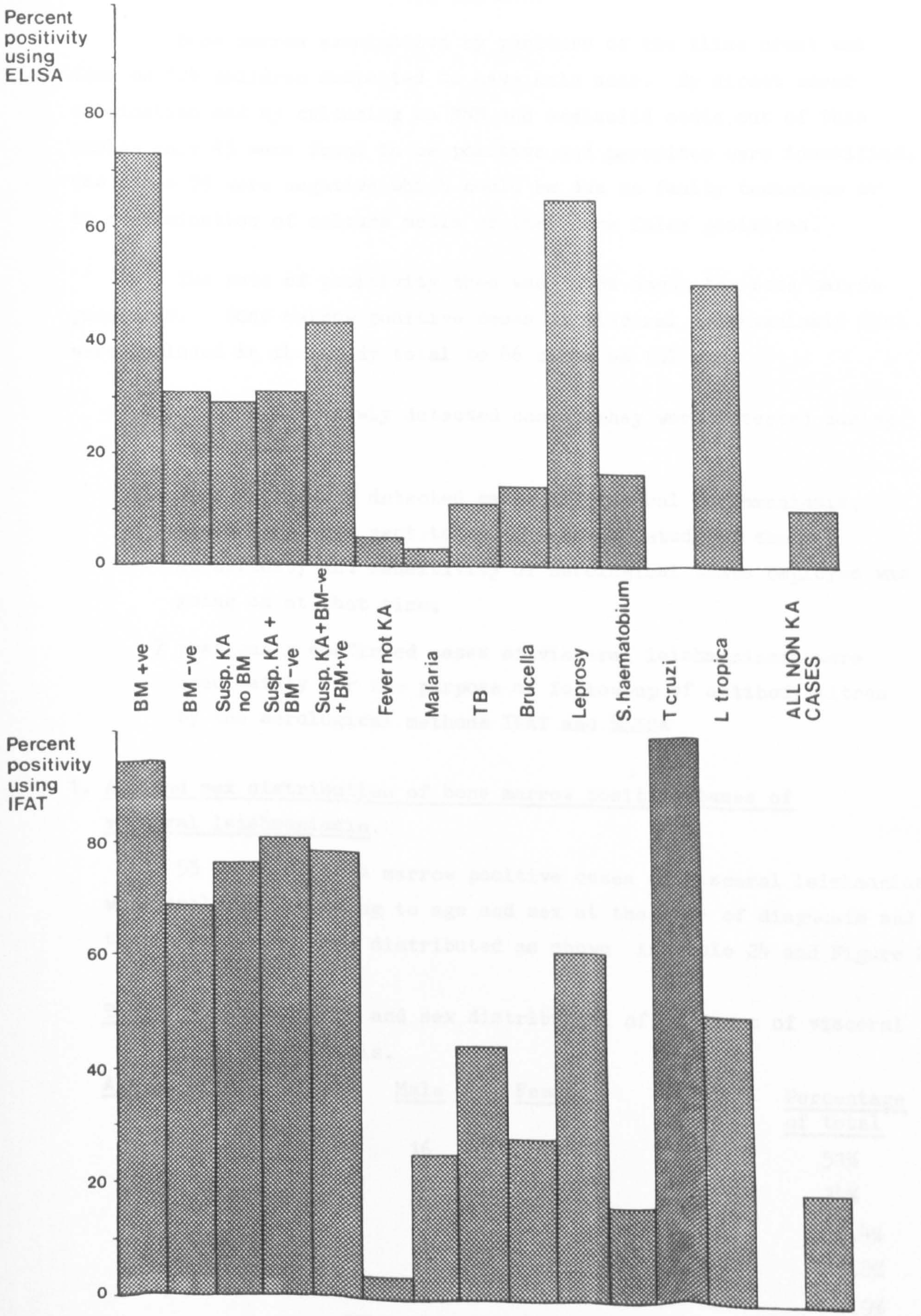


Figure 25 Showing results of examination of sera collected from different diseases.



D. RESULTS OF THE WORK ON BONE MARROW

Bone marrow examination by puncture of the iliac crest was done on 124 children suspected to have kala azar. By direct smear examination and by culturing on NNN and semisolid media out of this number only 45 were found to be positive and parasites were identified. The other 79 were negative which could be due to faulty technique or to contamination of culture media or they were false positives.

The rate of positivity then was 36.3% among the bone marrow punctures. Bone marrow positive cases of visceral leishmaniasis that were included in the study total to 66 cases as follows:

45 of them were newly detected cases, they were detected during the study;

14 were previously detected cases of visceral leishmaniasis, their sera were sent to the UK while a study of the specificity and sensitivity of serological tests employed was going on at that time;

7 previously confirmed cases of visceral leishmaniasis were used mainly for the purpose of follow-up of antibody titres by the serological methods IFAT and ELISA

1. Age and sex distribution of bone marrow positive cases of visceral leishmaniasis.

53 cases of bone marrow positive cases of visceral leishmaniasis were analysed according to age and sex at the time of diagnosis and they were found to be distributed as shown in Table 24 and Figure 26.

Table 24. Showing age and sex distribution of 53 cases of visceral leishmaniasis.

Age in years.	Male	Female	Total	Percentage of total
<1	16	11	27	51%
-2	10	8	18	34%
-3	1	4	5	9.4%
-4	1	1	2	3.8%
-5	1	-	1	1.9%
Total	29 (54.7%)	24 (45.3%)	53	

So nearly half the cases were under one year of age and more than three quarters of the patients were under 2 years of age.

There was no difference statistically between male and female number of cases (X^2 = 0.47; <0.50).

The reason why 53 were chosen is that information about their age and sex were available.

2. <u>Serological titres of bone marrow positive cases of visceral leishmaniasis studied against age</u>.

It seems that titre tends to increase with age until it reaches the third year and then it declines, in both ELISA and IFAT tests in the bone marrow positive cases of visceral leishmaniasis, as shown in Tables 25 and 26 and in Figures 27 and 28, a fact which tallies with the age distribution of the disease discussed earlier, and with the rate of reversion of serological positivity which will be discussed later.

Table 25. Results of immunofluorescence test against age in years for 53 bone marrow positive cases.

Age in years	Sex	-ve	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	Total
-1	M	1	3	4	4	4				16
	F	1		1	8		1			11
	T	2	3	5	12	4	1			27
-2	M			2	3	4		1		10
	F			2	3	3				8
	T			4	6	7		1		18
-3	M					1				1
	F				1	2			1	4
	T				1	3			1	5
-4	M		1							1
	F				1					1
	T		1		1					2
-5	M			1						1
	F									
	T			1						1
Total	M	1	4	7	7	9		1		29
	F	1		3	13	5	1		1	24
	T	2	4	10	20	14	1	1	1	53

Table 26. Results of ELISA against age in years in bone marrow positive cases.

Age in years	Sex	-0.2	-0.35	-0.50	-0.85	0.86 - over	Total
-1	M	2	4	4	1		11
	F	1		2	1		4
	T	3	4	6	2		15
-2	M	2	1	1	2		6
	F	1	1	2		2	6
	T	3	2	3	2	2	12
-3	M					1	1
	F		1	1	1		3
	T		1	1	1	1	4
-4	M	1					1
	F		1				1
	T	1	1				2
Total	M	5	5	5	3	1	19
	F	2	3	5	2	2	14
	T	7	8	10	5	3	33

Figure 26 Fifty-three bone marrow positive cases of visceral leishmaniasis distributed according to age.

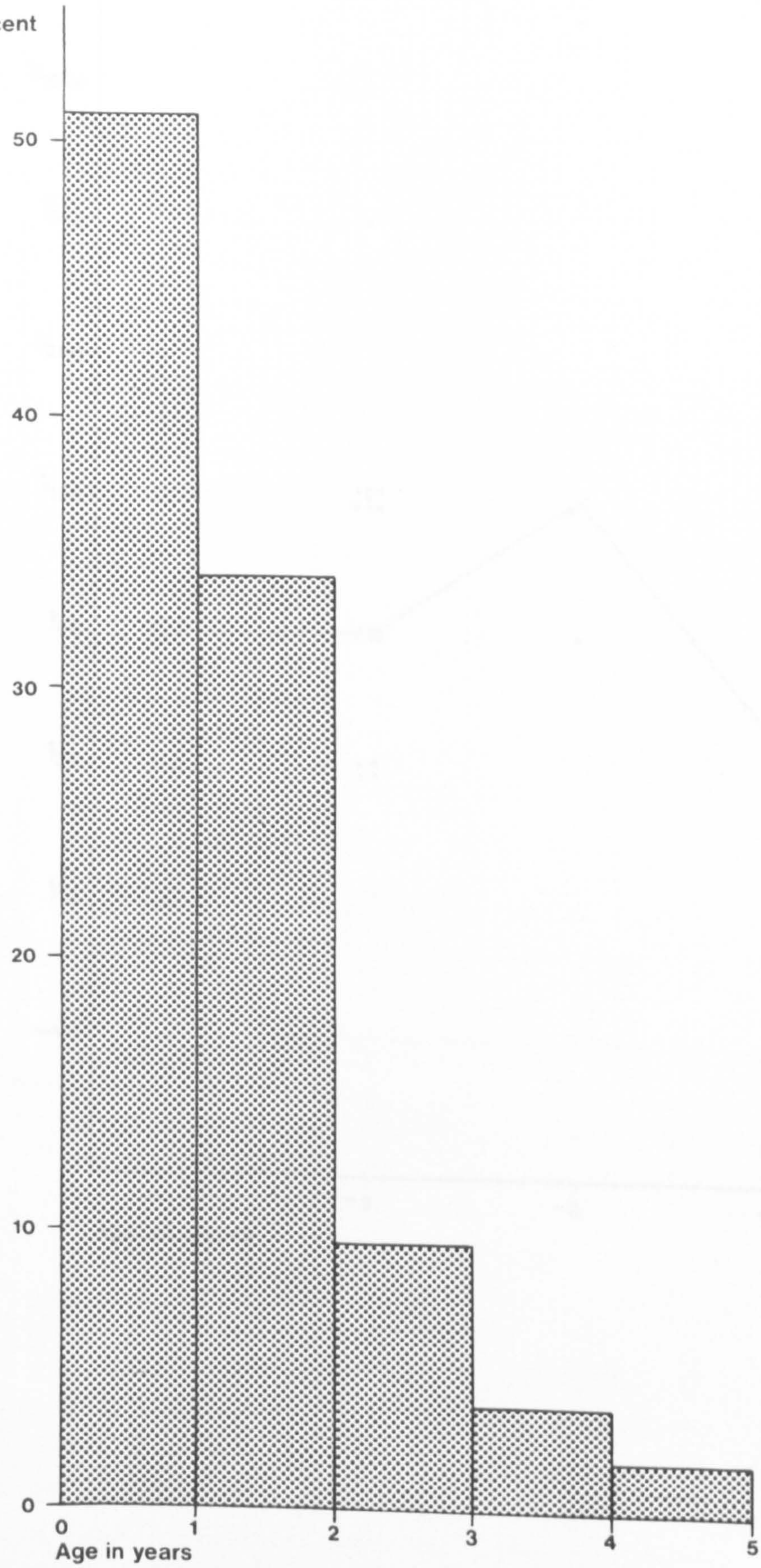


Figure 27 Distribution of IFAT titres of 53 bone marrow positive cases of visceral leishmaniasis according to age, using a median in each group.

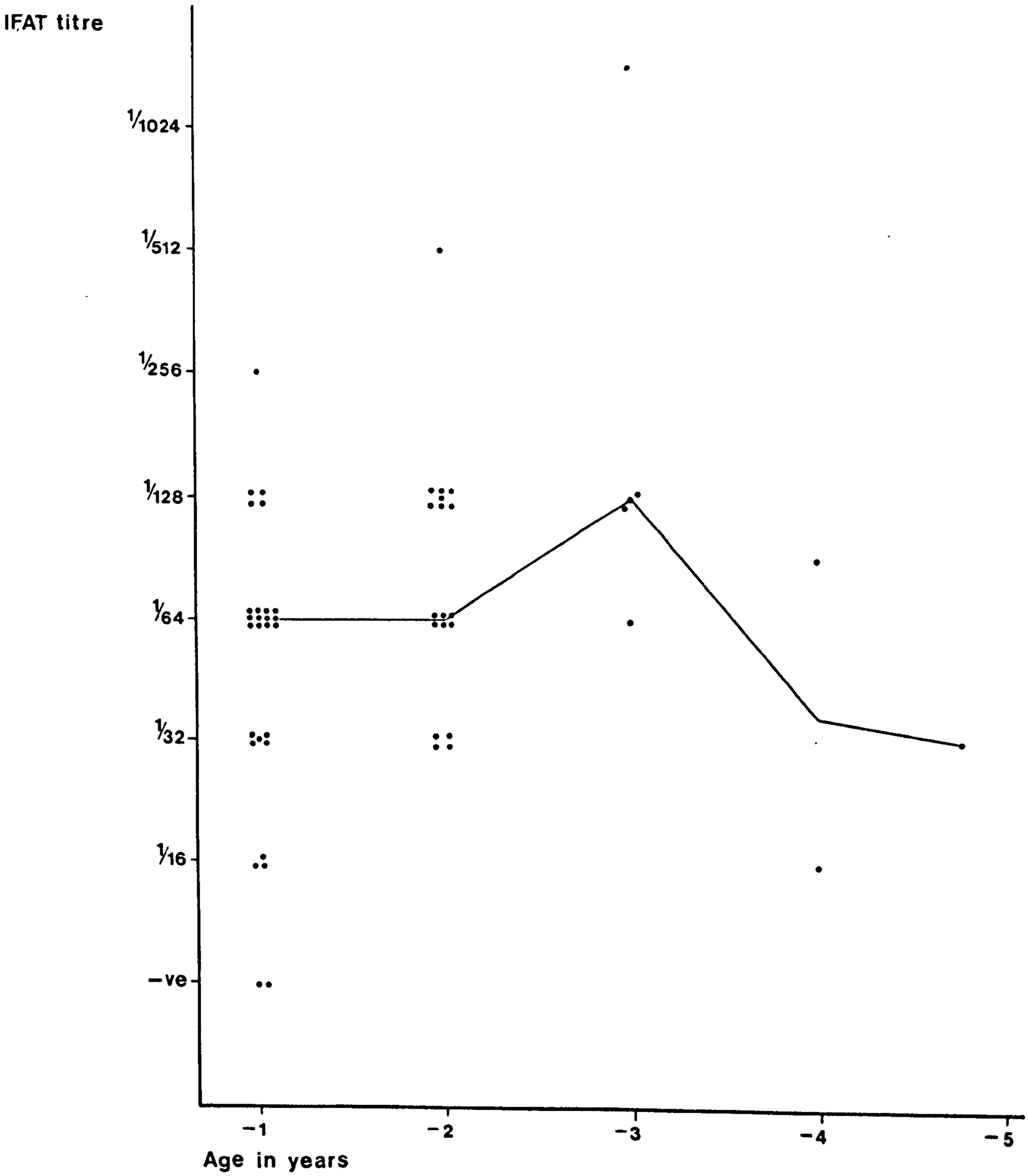
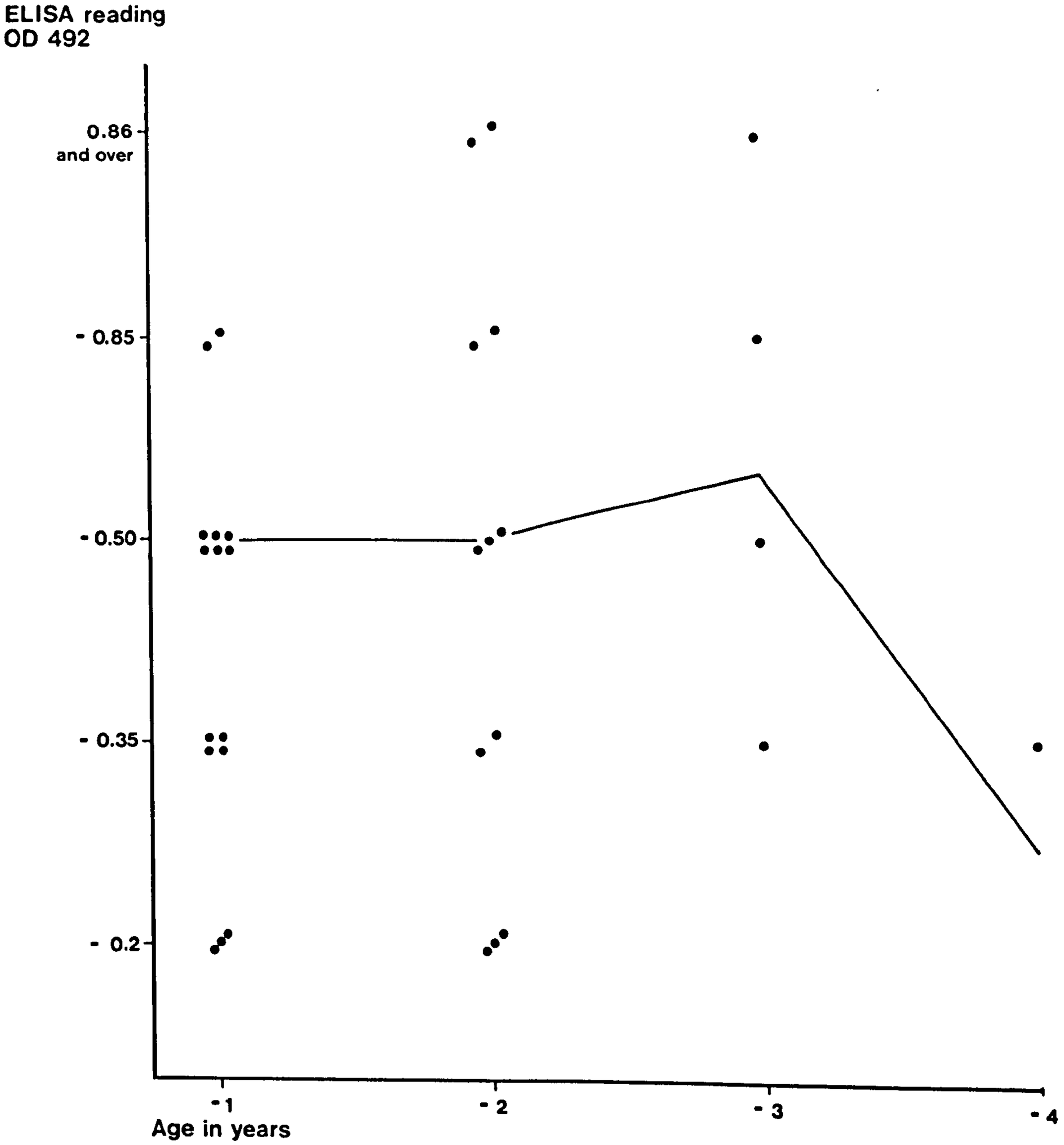


Figure 28 ELISA readings for 33 bone marrow positive cases of visceral leishmaniasis according to age, using a median in each group.



3. Follow up of parasitologically proven cases of visceral leishmaniasis.

Out of the 45 newly discovered cases 13 cases outside the area could be traced and followed up along with four cases in the area also newly discovered. Follow up could be done on another 7 previously detected cases also, so that out of 66 cases of parasitologically proven cases, 24 were followed up both for the purpose of finding the mortality and for the estimation of their level of antibody in their blood.

Follow up for the purpose of mortality was done properly on the 13 cases outside the study area, the follow up was done by monthly visit to their house, 4 out of the 13 died within 2 days to 3 months of the time of diagnosis, only one of those four was not treated with Pentostam (Wellcome Foundation Ltd., London); a mortality rate of 30.8%. Usually the parent of these children refused to readmit them again to hospitals in spite of their deteriorating condition. The recently detected 4 field cases had a much shorter time available for follow up.

For the purpose of antibody determination in the blood the whole 24 cases of visceral leishmaniasis were taken, these cases were visited regularly every month. The results were as follows:

Of the 13 cases newly detected outside the study area, three turned serologically negative during the follow up within a period of 3 - 9 months and as follows:

1 case (Z.Kh.) within 3.5 months of detection,
1 case (A.H.A.M.) within 7 months of detection, and
1 case (K.M.) within 8.5 months of detection.

The four cases that were detected in the study area were still serologically positive after a few days to three months after their detection.

Out of the seven previously detected cases, five turned serologically negative within a period of 16 months to 2 years, one turned negative after five years, the last one was still positive after five years.

So out of 24 parisitologically proven cases of visceral

leishmaniasis that have been followed up for their seropositivity:

9 (38%) turned negative

and as follows of the newly detected:

A.H.A.M. within 7 months
K.M. within 8.5 months
Z.Kh. within 3.5 months

of the old cases:

M.D.G. in 2 years
H.Th.S. in 16 months
W.M. in 2 years
I.Kh. in 2 years
A.M. in 2 years
Z.I. in 5 years.

To sum up, and as shown in Figure 29,

after 4 months	1 turned negative	11%
after 7 months	2 turned negative	22%
" 9 months	3 turned negative	33%
" 16 months	4 turned negative	44%
" 2 years	8 turned negative	89%
" 5 years	9 turned negative	100%

The rest of the 24 cases followed up showed:

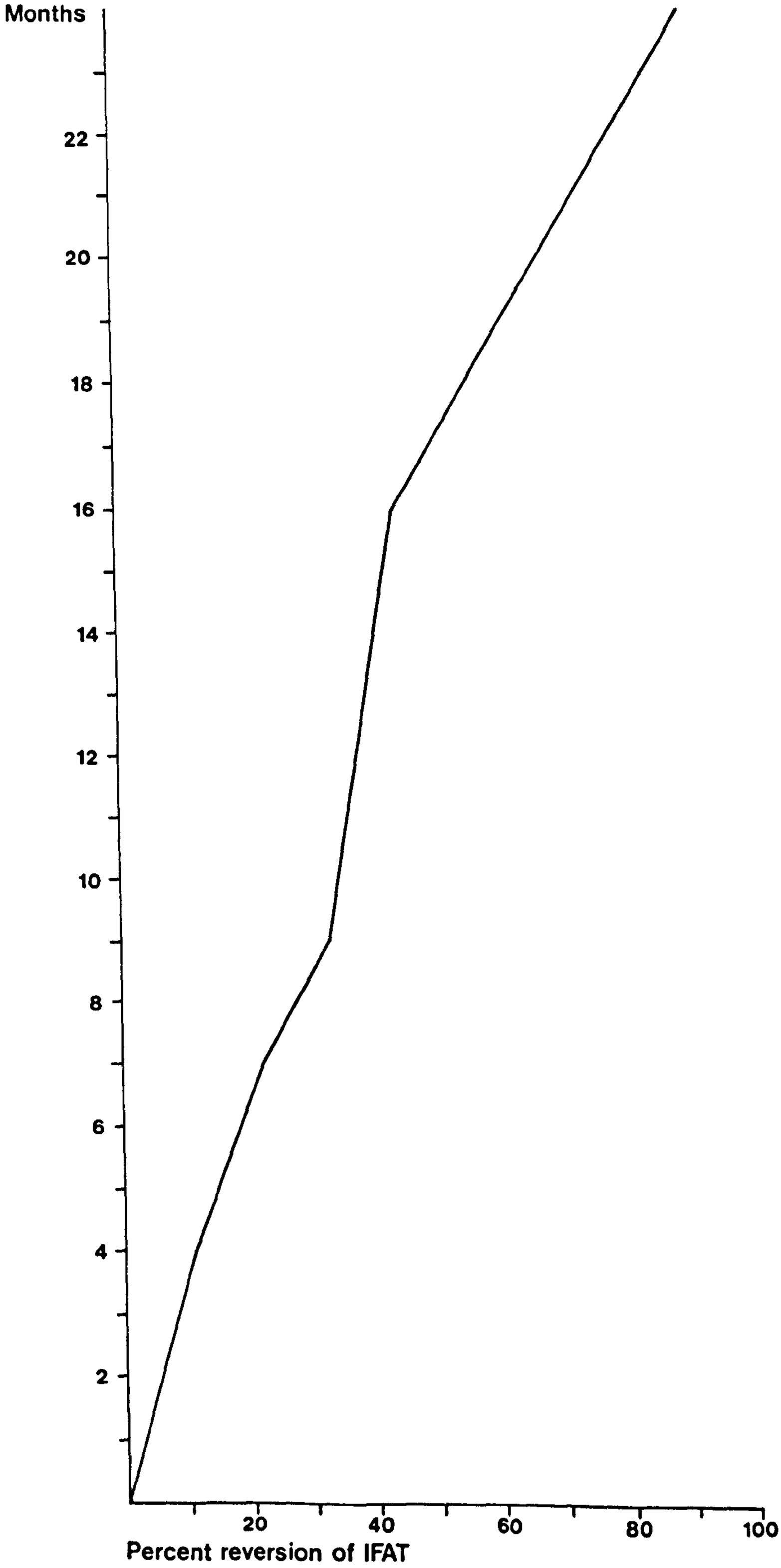
4 deaths and 11 still positive after a few days to 19 months, one case was still positive after 5 years.

4. Investigations done on the cases of visceral leishmaniasis.

Classical changes in kala azar were investigated, it was seen that all these changes were manifested in the cases analysed and as the following table and figures will show, none of these cases had scar or ulcer of oriental sore. Only one had a skin rash after treatment which was proved not to be post kala azar dermal leishmanoid. Out of the 35 serologically negative controls that had been examined clinically for their spleen and liver, only one showed spleen on deep inspiration (2.9%) compared to 88% in kala azar cases

Results of serum proteins and immunoglobines estimated show that the lgG is mostly affected, 80% had an increase in their lgG,

Figure 29 Serological follow-up of 9 parasitologically positive cases of visceral leishmaniasis.



IgM is affected to a lower degree (40%), globulin and globulin factions are raised, 83% of the cases had a disturbed A/G ratio. This is illustrated in Tables 27 and 30 and in Figures 30, 31 and 32. 71% of them had leocopenia as shown in Table 28. Results of physical examination showed that 88% of them had splen-megaly and 100% had hepatomegaly; this is illustrated in Table 29.

Table 27. Electrophoretic analysis of serum proteins and immunoglobulin values tested by immunodiffusion of six parasitologically proven cases.

Patient	IFT titre	Age	Sex	Total protein	Albumin	Globulin	α 1	α 2	β	λ	IgG	IgM
Normal value				6.8 g/100 ml	3.5-5.5	1.7-3.7	0.1-0.4	0.4-0.8	0.5-1	0.7-1.5	564-1675 mg/100 ml	53-130 mg/100 ml
Th.S.	1/64	1.2y	M	9.2	4.139	5.068	0.844	1.327	0.965	1.930	1640	82
M.J.	1/128	1.2y	M	7.4	2.259	5.141	0.536	0.536	0.644	3.417	2900	610
I.Kh.	1/128	1.4y	M	11.5	4.012	7.486	0.587	0.978	1.370	4.551	2640	736
I.O.	1/64	8m	F	11.9	4.642	7.256	0.676	0.856	0.901	4.823	2980	212
A.F.K.	1/32			7.7	3.662	4.038	0.782	1.158	0.845	1.252	1960	330
Kh.H.N.	1/32	1y	F	7.7	4.352	3.346	0.526	1.147	1.004	0.669		

Figure 30 Gamma globulin level of 6 cases of bone marrow positive cases of visceral leishmaniasis drawn against their IFAT titres.

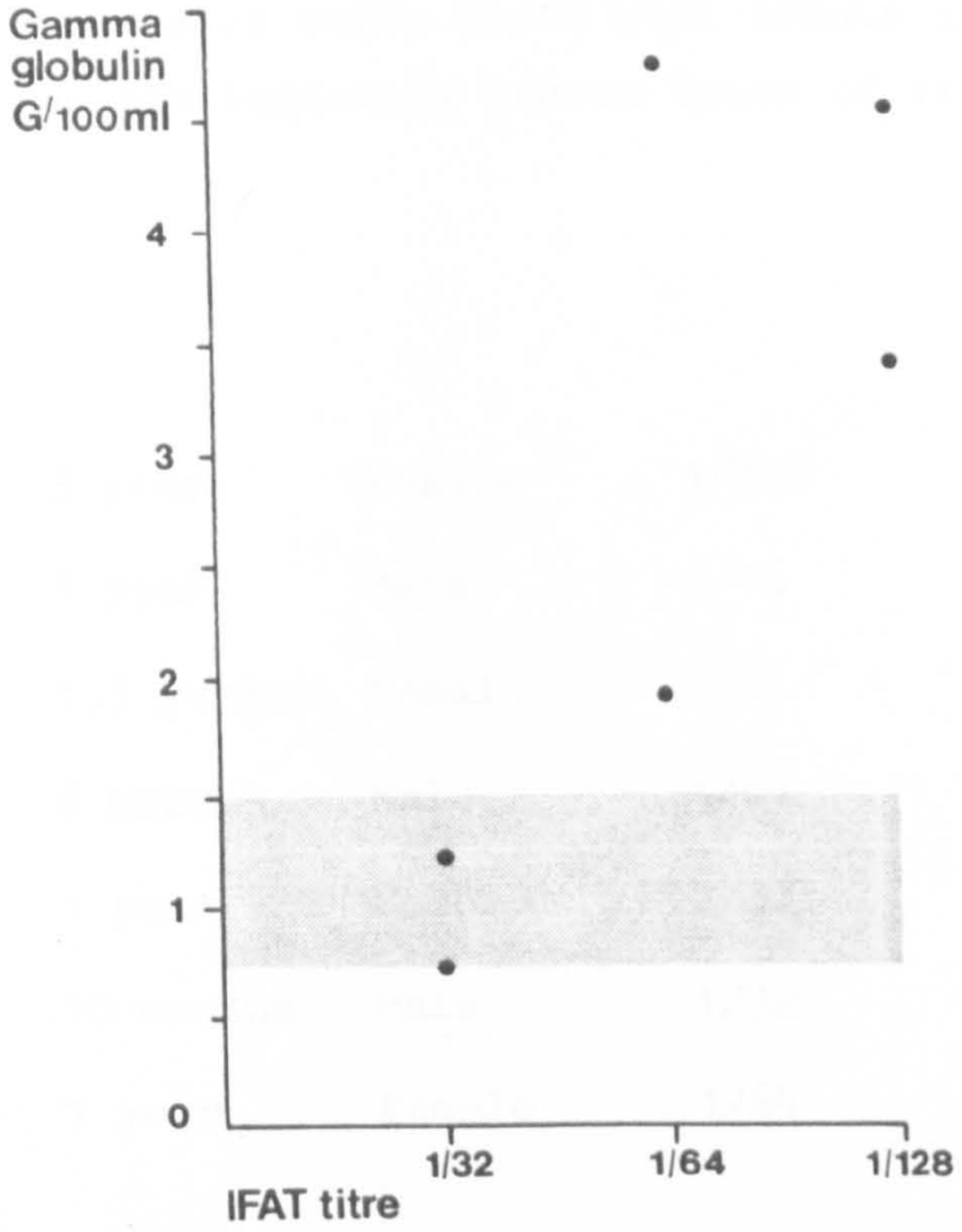


Figure 31 IgG level of 5 cases of bone marrow positive cases of visceral leishmaniasis drawn against their IFAT titres.

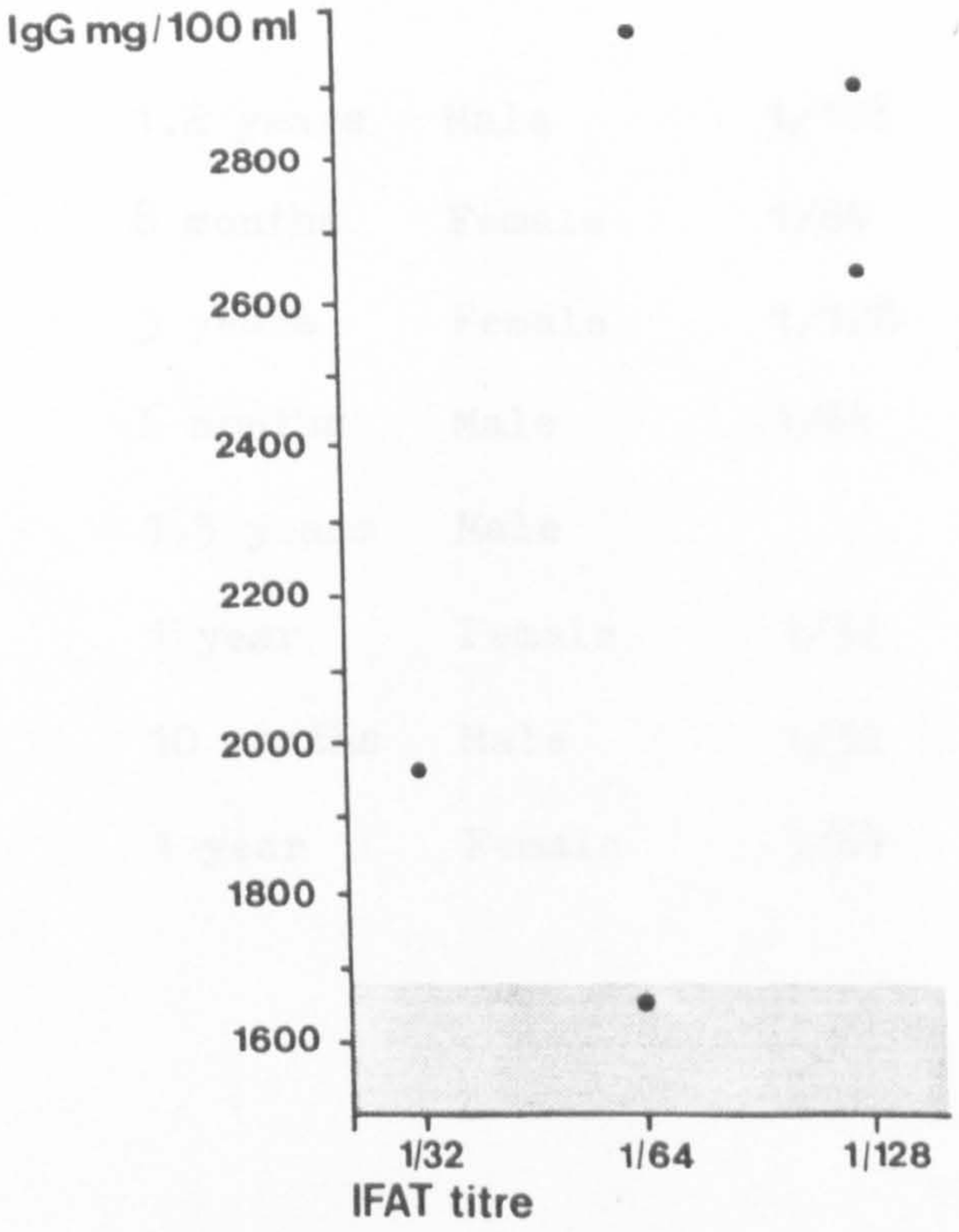


Table 28. Results of white blood cell counts in seven parasitilogically proven cases of visceral leishmaniasis.

				WBC (normal value 5000-10,000)
Z.Kh.H.	3 years	Female	1/128	4000
M.K.O.	1 year	Male	1/16	6600
Z.H.A.	1.3 years	Female	1/64	2800
H.M.N.	6 months	Male	1/64	6000
Kh.H.N.	1 year	Female	1/32	2600
S.T.	10 months	Male	1/32	2400
I.M.	1 year	Female	1/64	4400

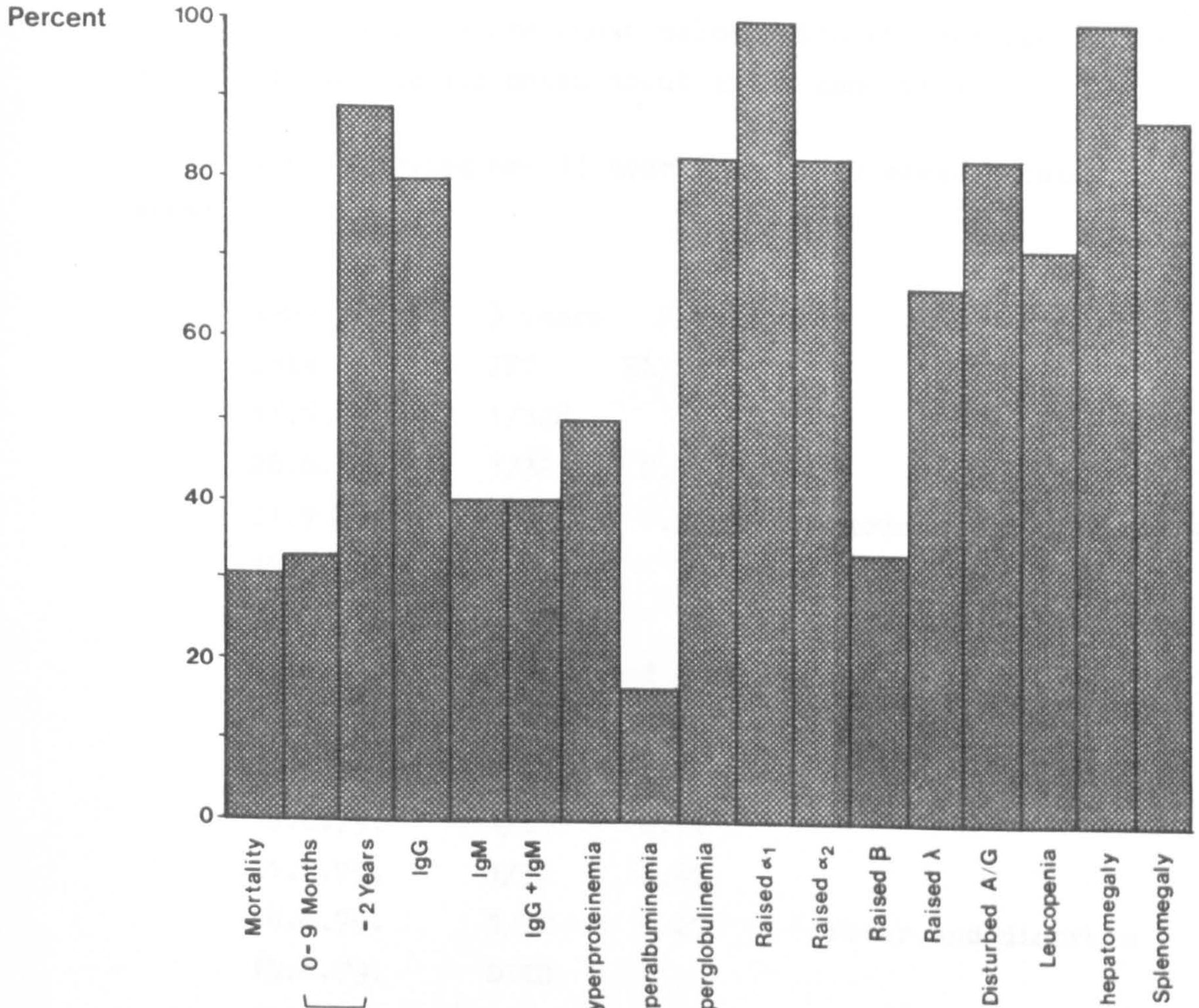
Table 29. Results of physical examinations of eight bone marrow positive cases of visceral leishmaniasis for their spleens and livers.

				Spleen	Liver
M.J.	1.2 years	Male	1/128	5 cm	3 cm
I.O.	8 months	Female	1/64	5 cm	3 cm
Z.Kh.H.	3 years	Female	1/128	6 cm	1 cm
H.M.N.	6 months	Male	1/64	3 cm	3 cm
R.S.	1.5 years	Male		4 cm	4 cm
Kh.H.N.	1 year	Female	1/32	-ve	just
S.T.	10 months	Male	1/32	3.5 cm	3 cm
I.M.	1 year	Female	1/64	2 cm	2 cm

Table 30. Summary of the abnormal findings in investigating cases of bone marrow positive visceral leishmaniasis.

	Total Examined	Raised value	
IgG	5	4	80%
IgM	5	2	40%
Both IgG and IgM	5	2	40%
Hyperproteinaemia	6	3	50%
α 1	6	6	100%
α 2	6	5	83%
β	6	2	33.3%
λ	6	4	66.6%
Globulin	6	5	83%
Albumin	6	decreased 1	16.5%
Disturbed A/G	6	5	83%
Liver	8	8	100%
Spleen	8	7	88%
Fever	8	8	100%
Leucopaenia	7	5	71.4%

Figure 32 Showing different positive findings among the bone marrow positive visceral leishmaniasis cases.



5. Details of the 66 cases of visceral leishmaniasis used in this study.

These details are shown below, with the details of their monthly follow-up and notes about their condition.

The following are 13 nearly detected cases outside the study area:

Name: G.N. 3 years Female

Date:	IFT	ELISA	
15.5.79.	1/128		
26.6.79.	1/32	0.45	
21.7.79.	1/128	0.20	Deteriorating clinically
15.8.79.	DIED		

Name: T.S.S. 1 year and 2 months Male

Date:	IFT	ELISA	
12.5.79	1/64		
18.6.79.	1/64	0.74	
21.7.79.	1/32	0.47	
14.8.79.	1/32	0.27	Fever and diarrhoe
15.8.79.	DIED		

Name: M.J. 1 year and 2 months

Date:	IFT	ELISA
17.10.78.	1/128	0.24
8. 5.79.	1/64	0.70
18.6.79.	1/32	0.44
21. 7.79.	1/64	0.58
14. 8.79.	1/64	0.27
23. 9.79.	1/32	
7.11.79.	1/64	0.35
16.12.79.	1/64	0.42
19. 1.80.	1/32	0.37
4. 3.80.	1/64	

Name: MHRB 9 months Male

Date	IFT	ELISA
10. 7.79.	1/64	0.48
24. 7.79.	1/64	
14. 8.79.	1/32	0.21
29. 9.79.	1/64	0.35
26.12.79.	1/32	0.19
3. 2.80.	1/32	0.15

Name: AHAM 2 years Female

Date	IFT	ELISA
3. 7.79.	1/64	0.37
23. 7.79.	1/64	1.01
15. 8.79.	1/16	0.27
22. 9.79.	1/16	0.26
7.11.79.	-ve	0.30
16.12.79.	1/32	0.06
2. 2.80.	-ve	
24. 2.80.	-ve	0.01

Name: I.O. 8 months Female

Date	IFT	ELISA	
18. 2.79.	1/128	0.41	
6. 5.79.	1/64	0.50	
31. 5.79.	1/512		
17.6.79.	1/128	0.47	
19. 7.79.	1/128	0.45	Splenomegaly down to umbilicus
7.10.79.	DIED		

Name: B.F. 1 year 5 months Female

Date	IFT	ELISA
12. 9.79.	1/64	0.05
12.11.79.	1/32	0.08
16.12.79.	-ve	
19. 1.80.	1/16	0.13
18. 2.80.	1/16	0.02

Name: K.M. Female

Date	IFT	ELISA
7.10.78.	1/128	0.46
19. 6.79.	-ve	0.13

Name: Z. Kh. 1 year and 3 months Male

Date	IFT	ELISA
10.11.79.	1/64	
19.11.79.	1/64	0.22
22.11.79.	1/64	
18.12.79.	1/16	
27. 1.80.	1/16	0.46
24. 2.80.	-ve	0.09

Name: A.J.A.H. 2 years Female

Date	IFT	ELISA
1.12.79.	1/128	
1.12.79.	1/256	0.82
7. 1.80.	1/64	

Name: Z.H.A. 8 months Female

Date	IFT	ELISA
5.12.79.	1/64	
17.12.79.	1/32	0.13
29. 1.80.	1/32	0.11

Name: S.H.A.H. 6 months Female

Date	IFT	ELISA
11.12.79.	1/64	
13.12.79.	DIED	

Name: I.Kh. 1 year and 4 months Male

Date	IFT	ELISA
31. 7.79.	1/128	0.82
23. 9.79.	1/32	0.75
7.11.79.	1/64	0.45
16.12.79.	1/64	
19. 1.80.	1/16	0.87
18. 2.80.	1/32	0.11

The other 28 cases of visceral leishmaniasis outside the study area:

Name	Age	Sex	Date	IFT	ELISA	
A.F.		F	14. 5.79.	1/32		
I.M.	3y	F	19.10.78.	1/64	0.27	
H.H.	4y	M	4.11.78.	1/16	0.10	
H.S.	1y 1m	M	5.11.78.	1/32	0.13	
A.H.	1y	M	14.11.78.	1/16	0.13	DIED
F.Sh.	1y	F	27.11.78.	1/64	0.13	
S.H.	2y	M	30.11.78.	1/128	0.10	
T.F.	10m	F	13.12.78.	1/64	0.37	
A.Sh.	10m	M	19.12.78.	1/128		
I.M.		F	20.12.78.	1/32	0.13	
N.A.	2y	F	23.12.78.	1/64	0.12	
Z.Kh.H.	3y	F	18.11.79.	1/128		
R.B.	1y 6m	F	12. 6.79.	1/32		DIED
S.A.	4m	M	16. 1.80.	1/16		
T.Kh.	1y	M	16. 1.80.	1/32		
Th.A.	5y	M	13. 1.80.	1/32		DIED
B.J.	1y 1m	F	6. 6.79.	1/32	0.28	
A.A.	11m	M	14. 1.79.	1/32	0.21	
N.A.A.	7m	M	31. 1.79.	1/32		
N.M.	1y 2m	M	1. 2.79.	1/128		
H.M.	1y 4m	M	1. 2.79.	1/64	1.23	
F.A.	37	F	23. 3.79.	1/1024	0.52	
K.S.	9m	F	7. 2.79.	1/64		
H.H.	1y 5m	M	23. 4.79.	1/32		
L.A.	1y 5m	F	14. 5.79.	1/128		
F.J.	27 5m	M	30. 5.79.	1/128		
Kh.H.N.	1y	F	28.12.79.	1/32		
Th.Kh.	1y	F	7. 2.80.	-ve		

4 new cases parasitologically confirmed of visceral leishmaniasis in the study area:

Name: M.K.O. 1 year (when BM +ve) Male

Date	IFT	ELISA	
10. 7.79.	-ve	0.02	
1.10.79.	-ve	0.01	
5.11.79.	1/16		BM +ve
2.12.79.	1/64	0.45	
18.12.79.	1/32	0.27	
20. 2.80.	1/32	0.09	

Name: H.M.N. 6 months (when BM +ve) Male

Date	IFT	ELISA	
7.10.79.	-ve	0.00	
13. 1.80.	1/64		BM +ve
15. 1.80.	1/32		
20. 2.80.	1/32	0.10	

Name: S.T. 10 months Male

Date	IFT	ELISA	
9.10.79.	-ve	0.04	
24. 1.80.	1/32	0.45	BM +ve
26. 2.80.	1/64	0.39	

Name: I.M. 1 year (when BM +ve) Female

Date	IFT	ELISA	
12. 6.79.	-ve	0.01	
20.10.79.	-ve	0.02	
18. 2.80.	1/64		BM +ve

7 cases previously detected in the study area

Name M.D.G. case of BM +ve kala azar 1977

Date	IFT	ELISA
2.10.78.	-ve	0.13
10. 7.78.	-ve	0.03
24. 7.79.	-ve	
15.10.79.	-ve	0.02

Name: H.Th.S. 5 years Male Case of BM +ve kala azar 1978

3.10.78.	1/32	0.19
29. 7.79.	-ve	
19. 1.80.	-ve	0.05

Name: I.M. 6 years Male Parasitologically proven case of kala azar late 1975

12.10.78.	1/32	0.22
25. 7.79.	1/32	
27. 8.79.	1/32	0.24
16. 9.79.	1/16	0.07
16.10.79.	1/16	0.39
27.10.79.	-ve	0.00
24. 2.80.	1/16	0.11

Name: W.M. 3 years Female Case of kala azar in 1977

9.10.79.	1/16	0.04
25.10.79.	-ve	0.04

Name: I.Kh. 3 years Male Case of kala azar 1977

26.11.79.	1/16	0.11
18.12.79.	-ve	0.15

Name: A.M. 4 years Male Case of kala azar in 1977

Date	IFT	ELISA
22.11.78.	1/16	
12. 7.79.	-ve	
13.10.79.	-ve	

Name: Z.I. 7 years Female History of kala azar 1975

4. 9.79.	1/16	0.17
12. 9.79.	1/16	0.35
1. 3.80.	-ve	0.06

14 cases of visceral leishmaniasis previously detected and serum taken at the time of diagnosis and kept.

Code number		262	20. 7.77.	1/16	0.35
Code number		263	20. 7.77.	1/32	0.33
Code number		264	24. 7.77.	-ve	0.31
1 year	Male	1249	29.11.77.	1/64	0.57
?	Male	1250	?	1/16	0.30
4 years	Female	1251	3.11.77.	1/64	0.21
9 months	Female	1252	12. 7.77.	1/256	0.47
11 months	Male	1253	12. 4.77.	1/128	0.24
9 months	Male	1254	26.10.77.	1/64	0.18
3 months	Male	1255	3. 8.77.	1/128	0.44
8 months	Male	1255	6. 8.77.	-ve	0.24
2 years	Male	1257	21. 8.77.	1/512	1.45
6 months	Female	1258	2.12.77.	1/64	0.55
7 months	Male	1259	15.11.77.	1/128	0.29

E. RESULTS OF MAPPING AND CENSUS

A map of 1/10,000 scale was produced, where all the houses were located and numbered and plotted (Figure 89) with other information like roads, canals both of irrigation and of drainage, poultry farms, villages, rivers, schools etc.

The map was fully scaled and longitude and latitude were indicated also.

The area was around 300 square kilometres in surface area.

There were 1171 houses, distributed in 19 villages; the houses in the villages were dispersed in space specially at the periphery of the village.

The population was 9889, 5093 males (51.5%) and 4796 females (48.5%). Children up to seven years of age were 3403 (34.4% of the whole population). The attached population pyramid of the area (Table 31 and Figures 33 and 34) indicate the age and sex distribution of the residents; one can compare it with the general population in the rural area shown in Figures 2 and 3 and in Table 3 attached.

There were 10 primary education schools for children
9 illiteracy centres for adults
2 health centres
10 poultry farms

Table 31. Results of the population census of the study area according to age and sex.

Age	Male	Percent of grand total	Females	Percent of grand total	Total
- 1	294	2.98	313	3.17	607
- 2	289	2.93	248	2.51	537
- 3	247	2.50	264	2.67	511
- 4	222	2.25	225	2.28	447
- 5	228	2.31	251	2.54	479
- 6	220	2.23	187	1.89	407
- 7	231	2.34	184	1.86	415
-10	566	5.73	429	4.34	995
-15	658	6.66	549	5.56	1207
-20	421	4.26	511	5.17	932
-25	311	3.15	335	3.39	646
-30	277	2.81	289	2.93	566
-35	231	2.34	217	2.20	448
-40	186	1.88	161	1.63	347
-45	155	1.57	157	1.50	312
-50	196	1.99	231	2.16	409
-55	90	0.91	72	0.73	162
-6C	137	2.40	78	0.79	215
-65	35	0.36	61	0.62	96
-70	45	0.46	26	0.27	71
Over	54	0.55	26	0.27	80
Total	5093	51.50	4796	48.50	9889

Table 32. Showing the size of the families in the study area.

Family size	No. of families	
0-2	115	9.8%
3-5	191	16.3%
6-10	581	49.6%
10 and over	284	24.3%
Total	1171	100 %

Figure 33 Population pyramid of the study area.

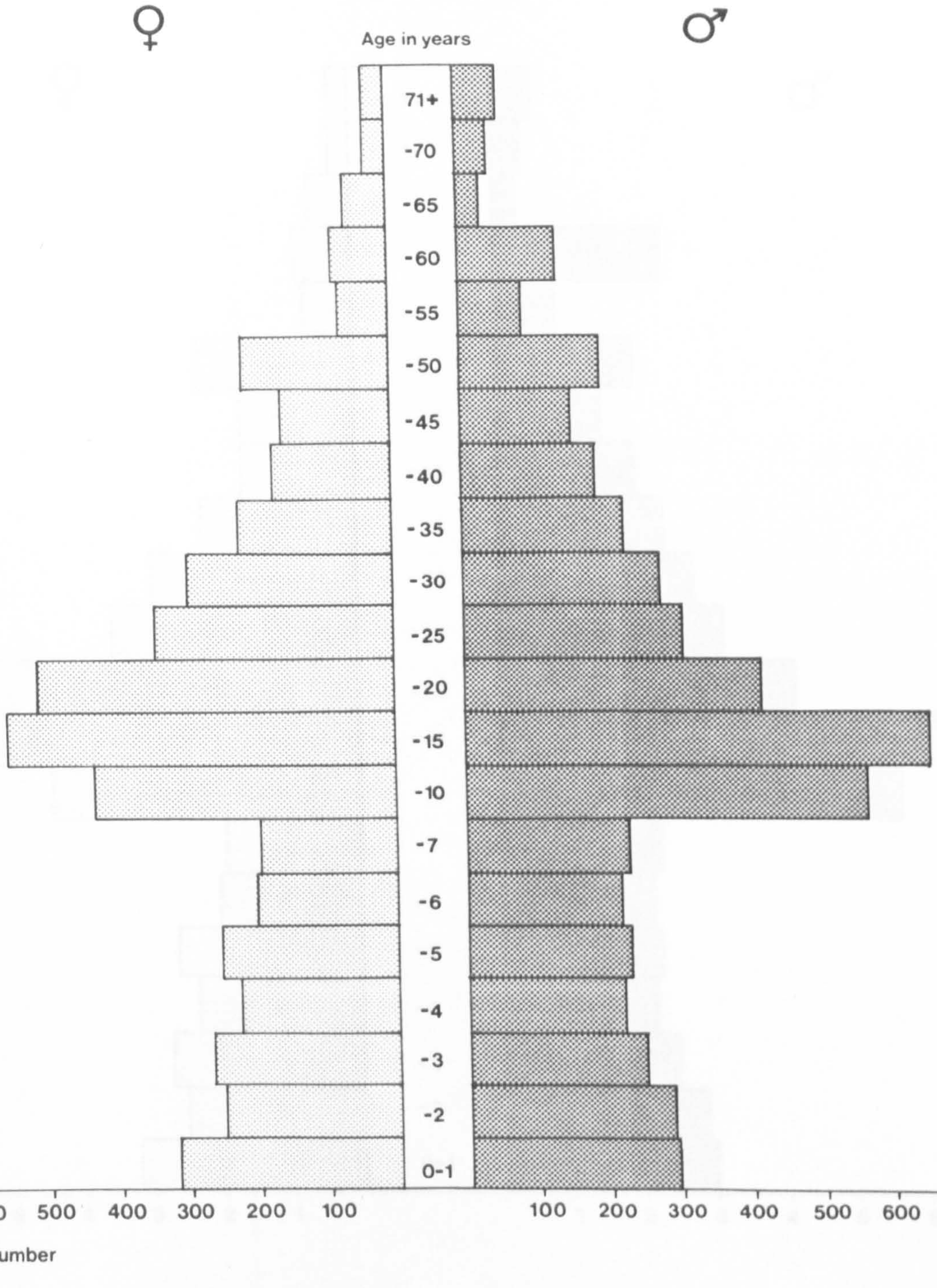


Figure 34 Population pyramid of the study area.

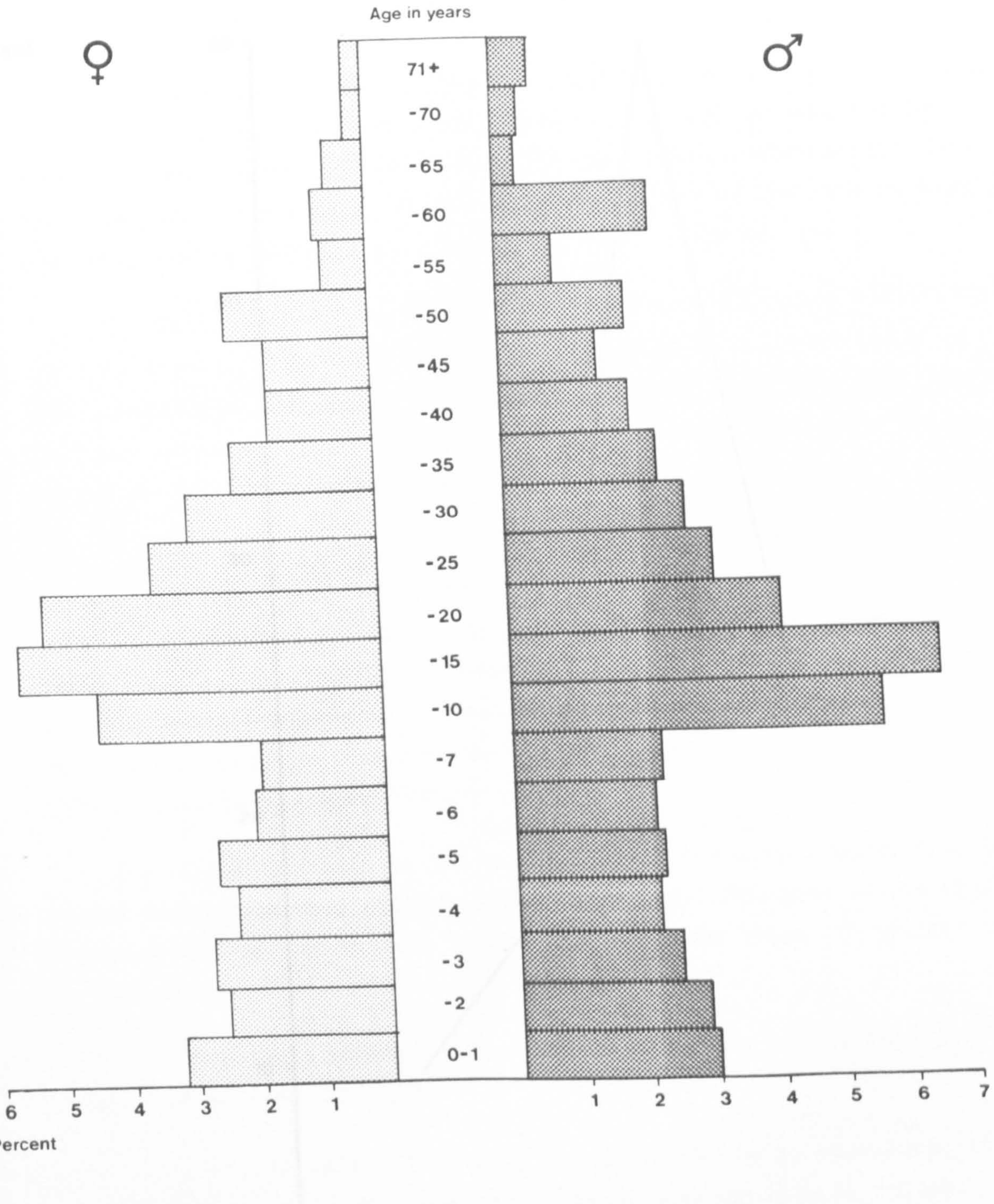
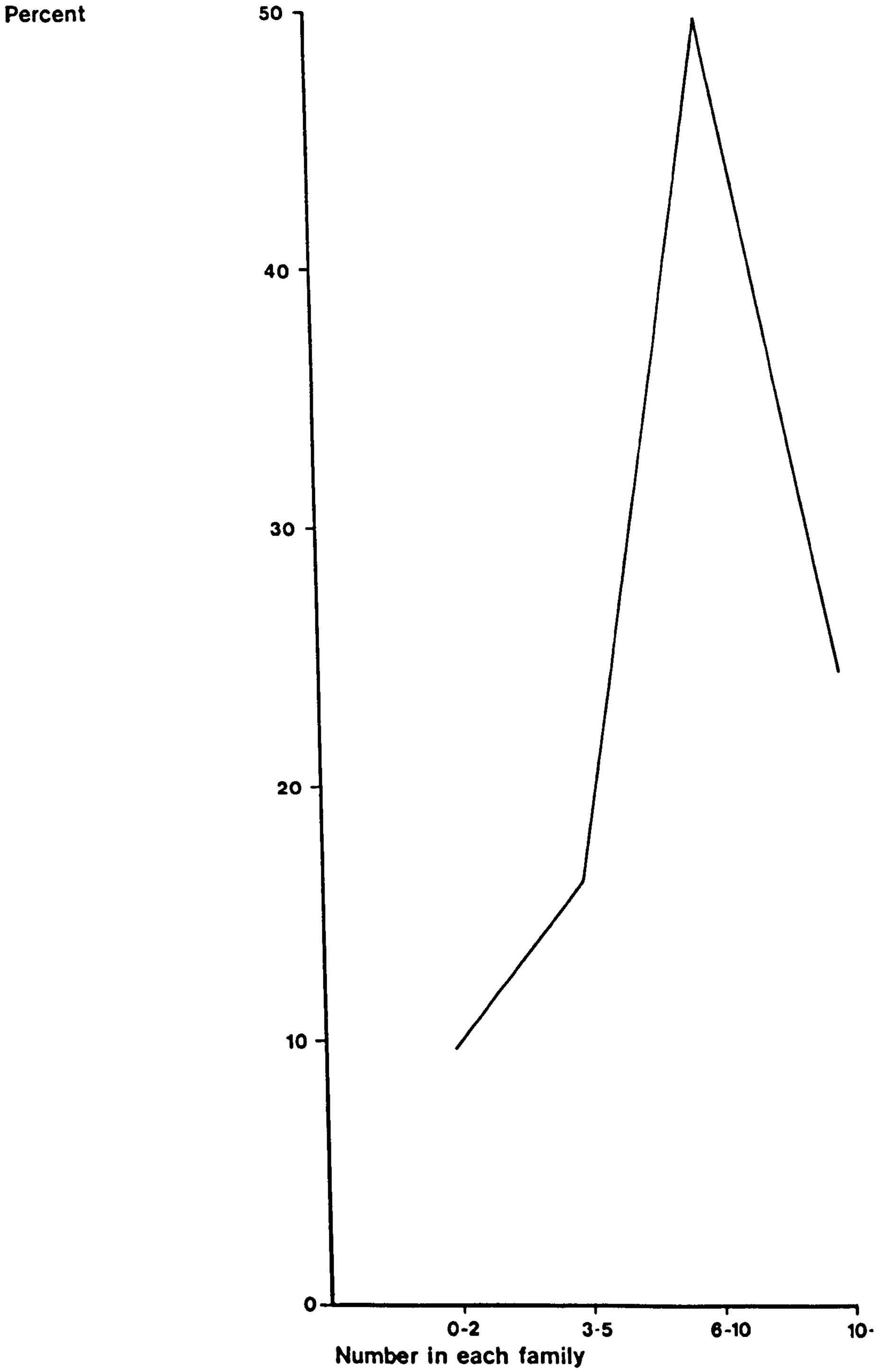


Figure 35 Size of the family.



The size of the family living in a house was usually big; as shown above about half of the houses have 6 - 10 inhabitants (Table 32 and Figure 35).

The number of new births and deaths in the area was registered during the second visit; this will cover the period between the two visits for each house and is between five and seven months depending on the household. The number of deaths in adults may be low, and is because the study was concentrating on the under seven years of age.

The number of new births registered were 83, crude birth rate for this five months period is 8.39 per thousand in our population of 9889 inhabitants, corrected to one year the crude birth rate will be 21.7 per thousand. Crude annual birth rate of rural population in Iraq was 47 per thousand for the period 1973 - 1975.

The number of deaths registered in the five months period were 10, and the crude death rate for the five months were one per thousand, crude death rate corrected to one year will be 2.4 per thousand; crude death rate per thousand in one year for the under seven years of age in the area was 5.6 (there were 8 out of 10 under seven years). Crude death rate in the rural part of Iraq was 13 per thousand for the period 1973 - 1975 (Statistical Compass 1973 - 1977).

Animals: information gathered from the agricultural department showed that there were around 10,000 sheep and 2,000 cows in the area. Chickens were raised in each house and in poultry farms (10 poultry farms in this area).

Dogs were noticed with every house; they are of the fierce type, some of them look ill.

Jackals were reported by the people to be in abundance, they usually inhabit palm date gardens, plantations and live in suitable places near drainage or water canals.

Photographs attached illustrate some aspects of topography of the area, some type of the houses, the kind of roads, channels, the Diala river, some jackal burrows and rodent burrows, and some dogs of the area which looked sick.

F. PILOT STUDY

A pilot study of a small part of the study area was done during October to December 1978 to test our methods of work and to have an idea about the population structure and their reaction in general.

The area had no census information, so a primary census and mapping was necessary, also registers were made for the inhabitants to record each activity and then blood was taken from the whole population.

The area chosen consists of 149 houses and the following Tables (33 and 34) show some characteristics about the census and size of the family. There were 571 children who are under seven years of age, 40.2% of the whole population.

The number of the under seven years covered were 202 out of 571 living amongst 1421 people in the pilot study area, a coverage rate of around 35.38%.

Out of those 202 children examined 14 showed positivity in the serological techniques (6.9%). 7 of them were males and 7 were females. The number of houses with positives were 13 (one with 2 cases), i.e. 8.7% of the houses.

IFAT titres of positives were 64.2% (9 cases had a titre of 1/16 in IFAT, 28.6% (4 cases) in the 1/32 titre, and 7.1% (1 case) in the 1/128 titre. This is illustrated in Figure 36.

Age distribution showed a peak in the 4-5 years of age with some distribution around it, as shown in Figure 37.

Mean of IFAT titre of the serologically positive cases seems to increase with age of the cases, as shown in Figure 38.

Table 33. Population census of the pilot study according to age and sex.

Age group	Male	Female	Total
0-	18	17	35
1-	123	141	264
5-	136	123	259
10-	94	81	175
15-	83	74	157
20-	38	37	75
25-	42	49	91
30-	34	27	61
35-	32	34	66
40-	19	35	54
45-	22	27	49
50-over	82	53	135
Total	723	693	1421

Table 34. Showing the size of the family in the pilot study

Size of family	No. of families	Percent
0 - 2	2	1.3%
3 - 5	23	15.4%
6 - 10	76	51.0%
11 -	48	32.2%
Total	149	100.0%

Figure 36 Showing percent distribution of IFAT titres among the IFAT seropositives in the pilot study; it also shows their sex distribution.

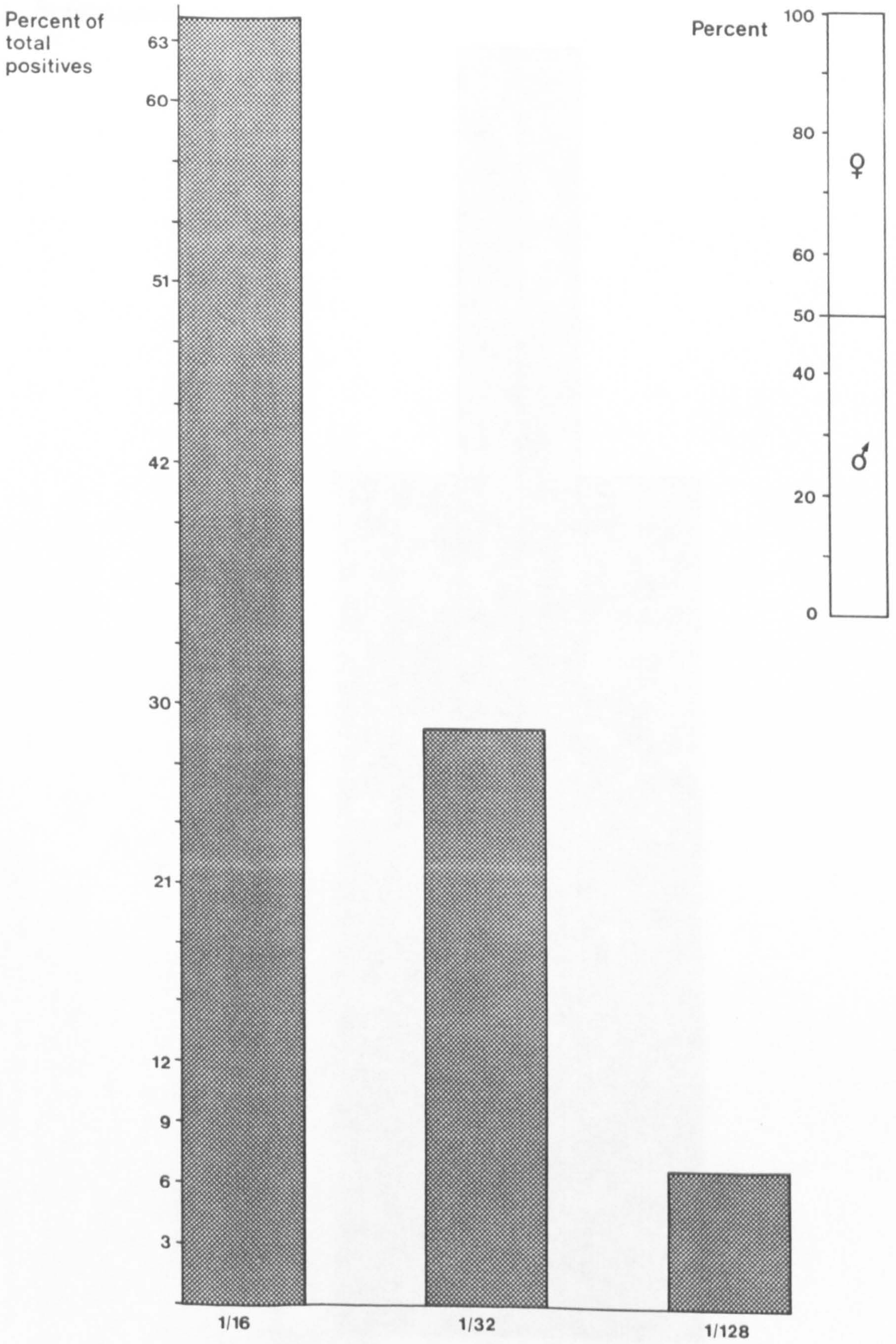


Figure 37 Age distribution of IFAT seropositives detected during the pilot study.

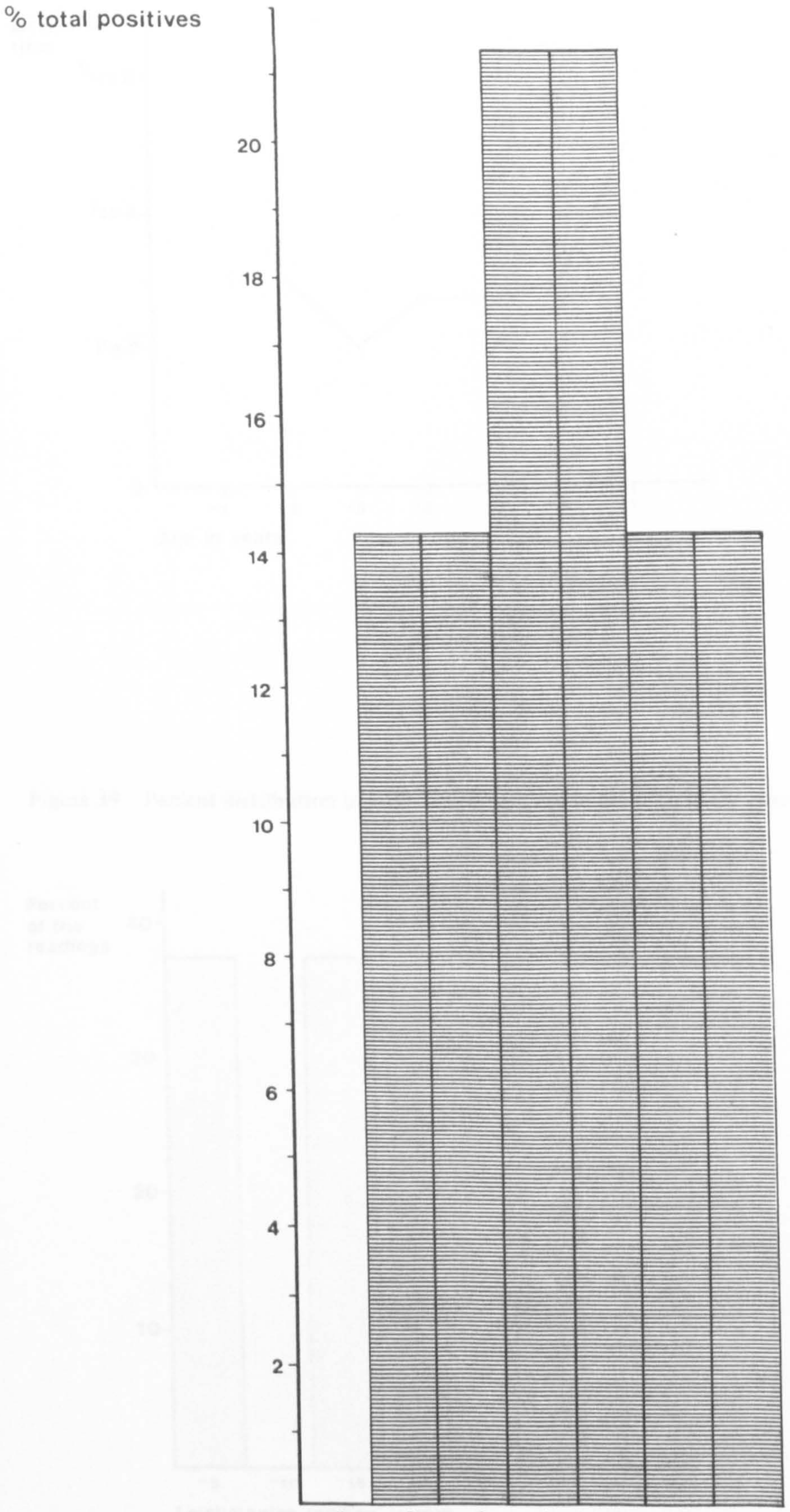


Figure 38 Showing the mean IFAT titre in each age group among the IFAT seropositives detected during the pilot study.

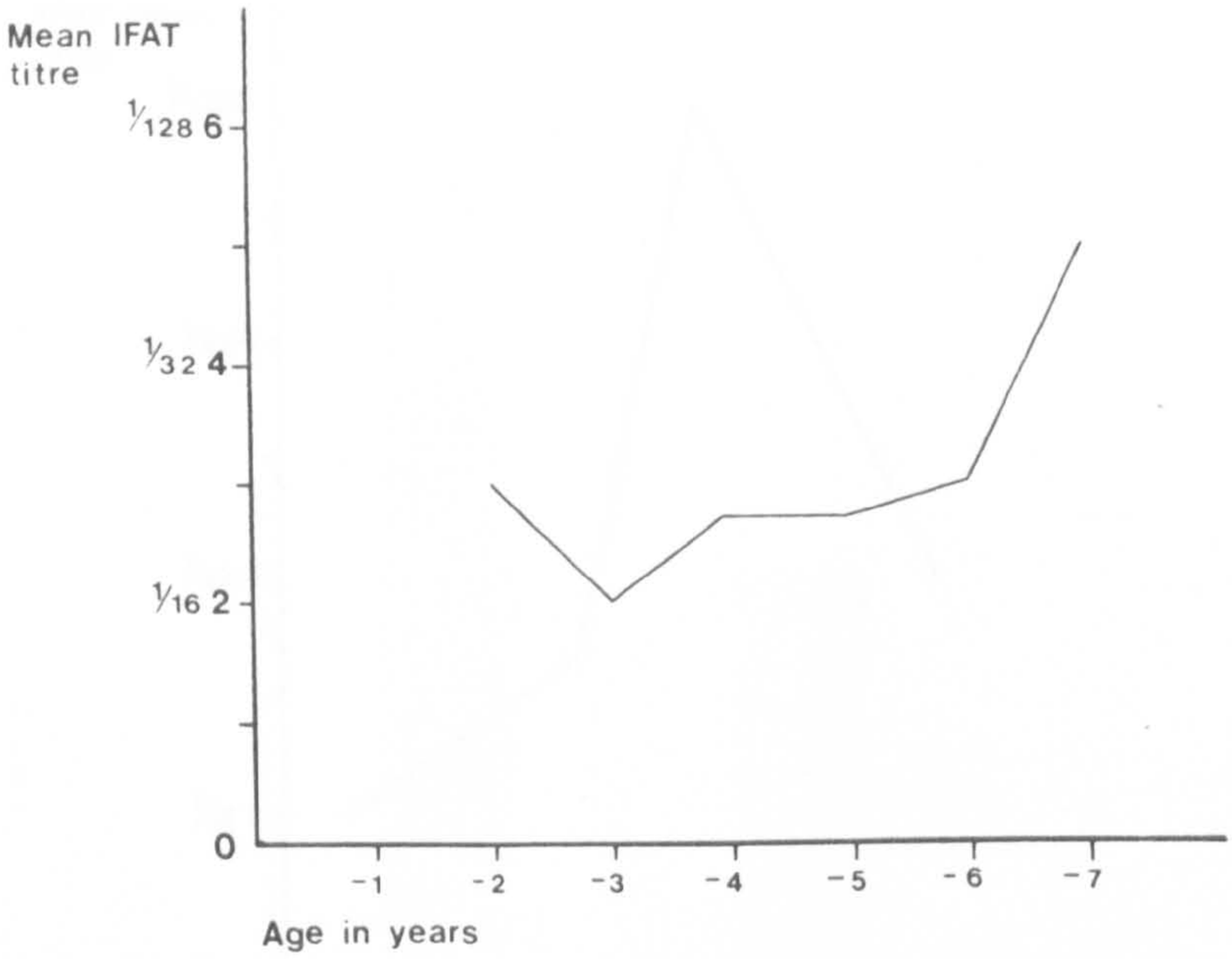


Figure 39 Percent distribution of leishmanine readings in the pilot study among IFAT seropositives.

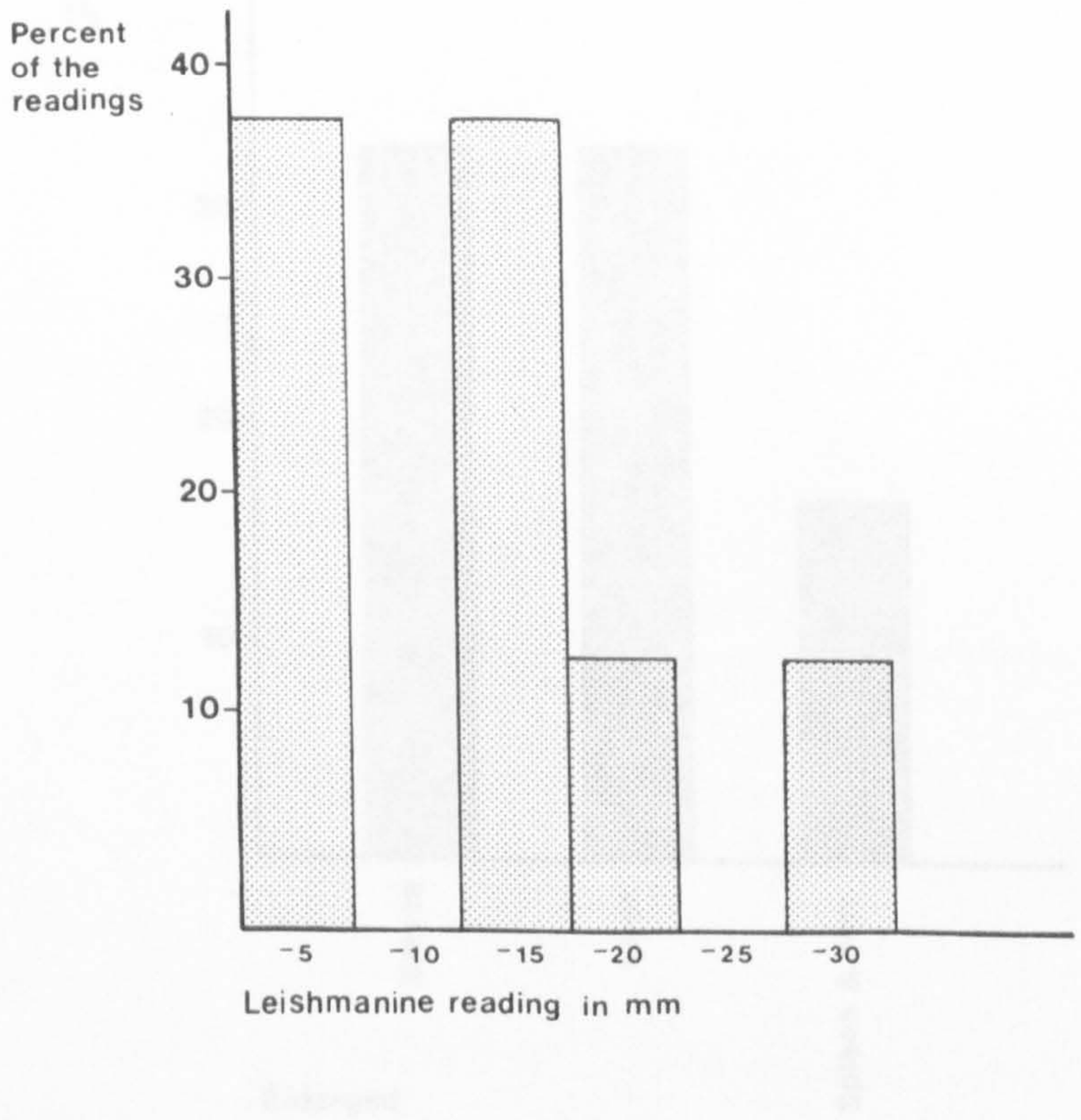


Figure 40 Showing mean IFAT titre for each group of leishmanine readings in the pilot study.

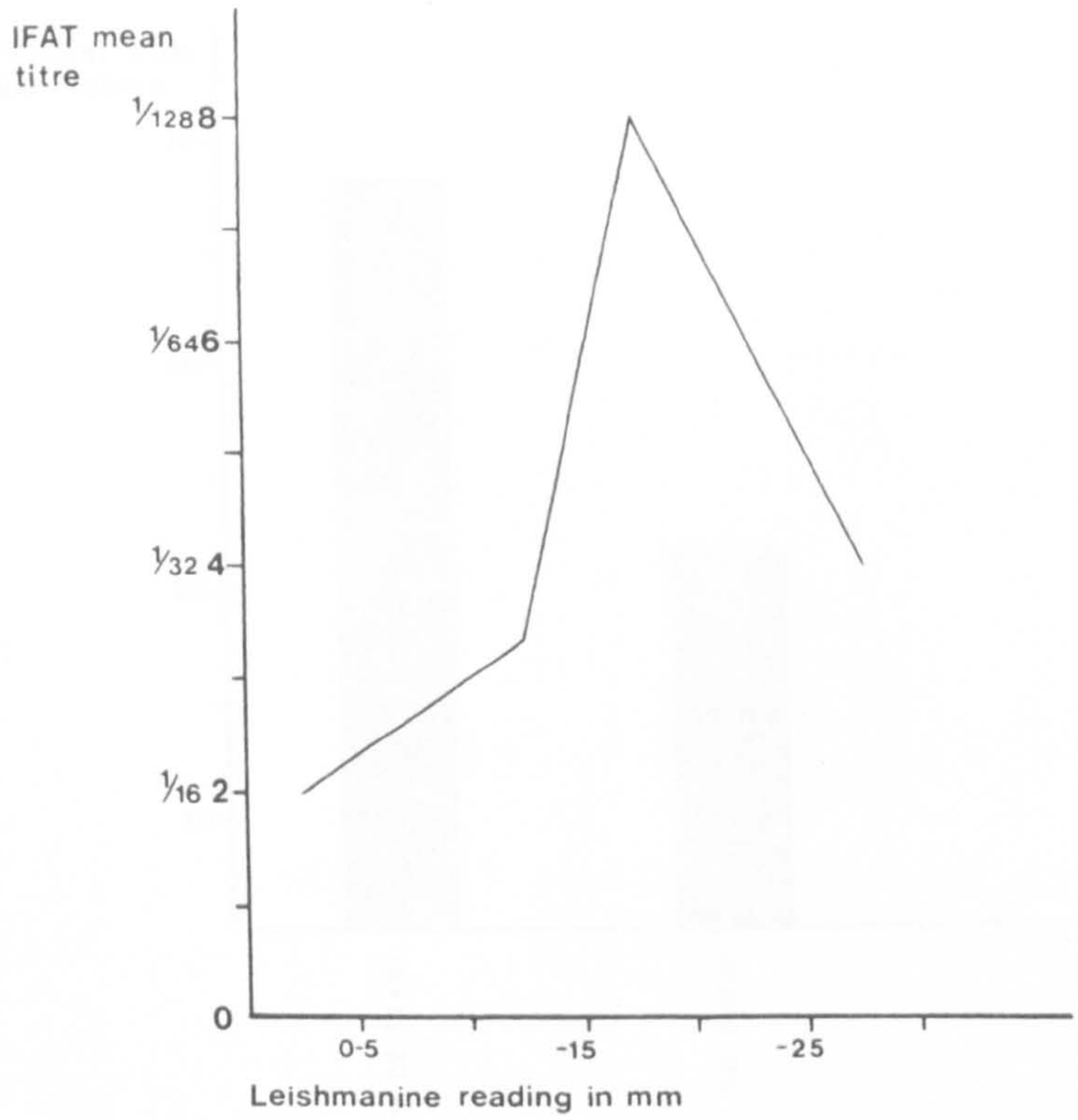


Figure 41 Splenomegaly and hepatomegaly amongst the IFAT seropositives in the pilot study.

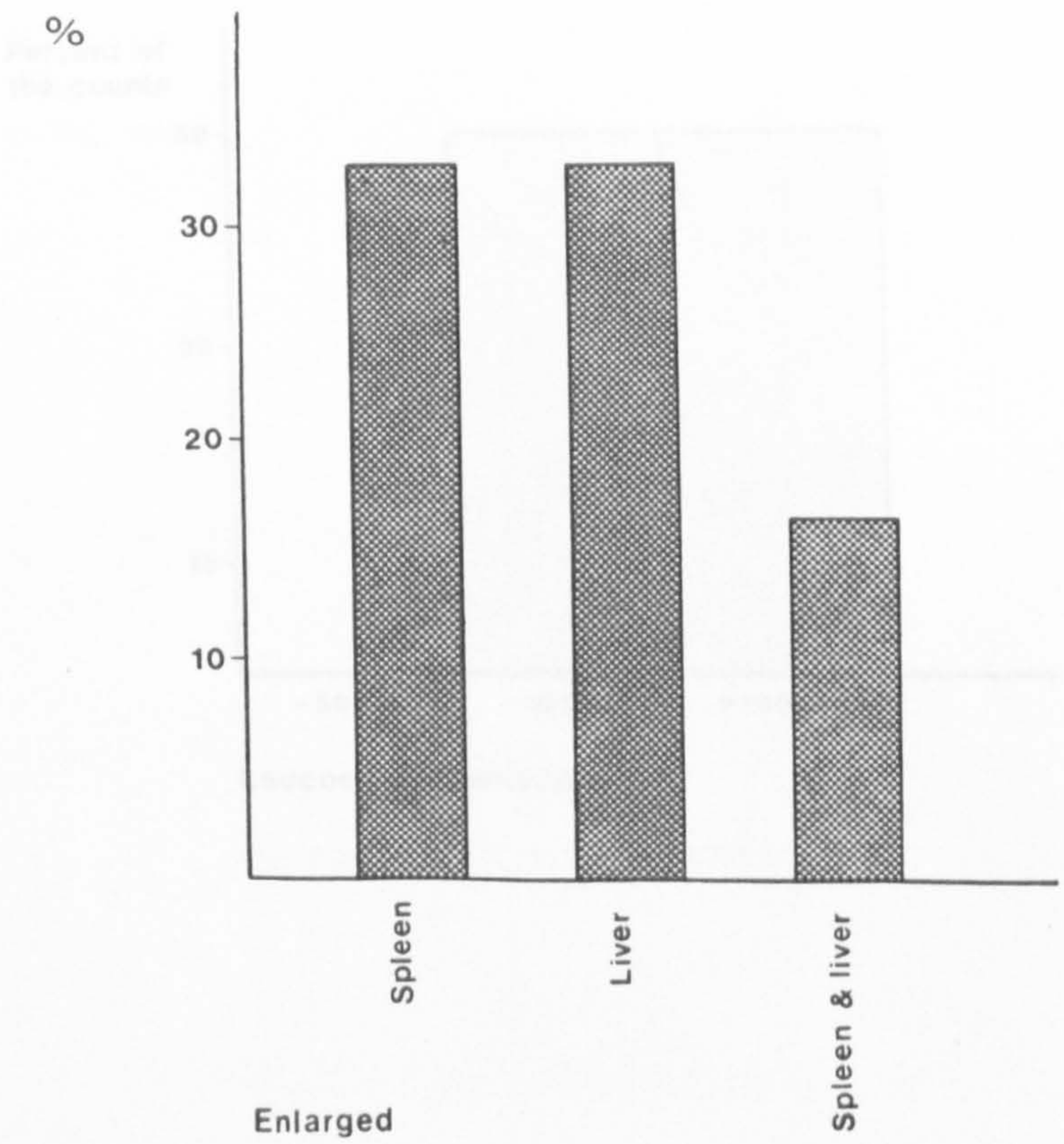


Figure 42 Fever incidence among the IFAT seropositives in the pilot study.

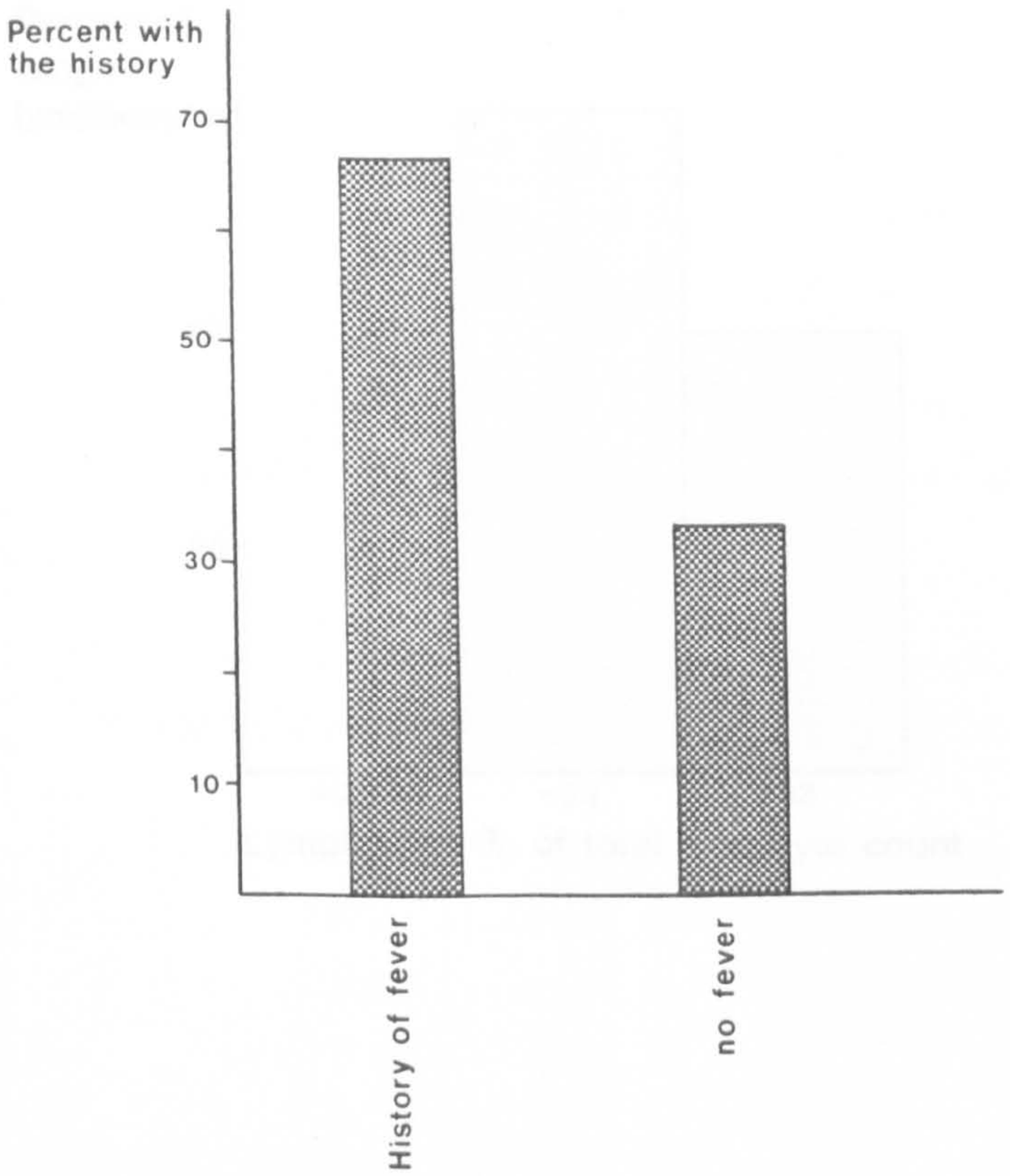


Figure 43 Results of leucocyte counts of the IFAT seropositives in the pilot study.

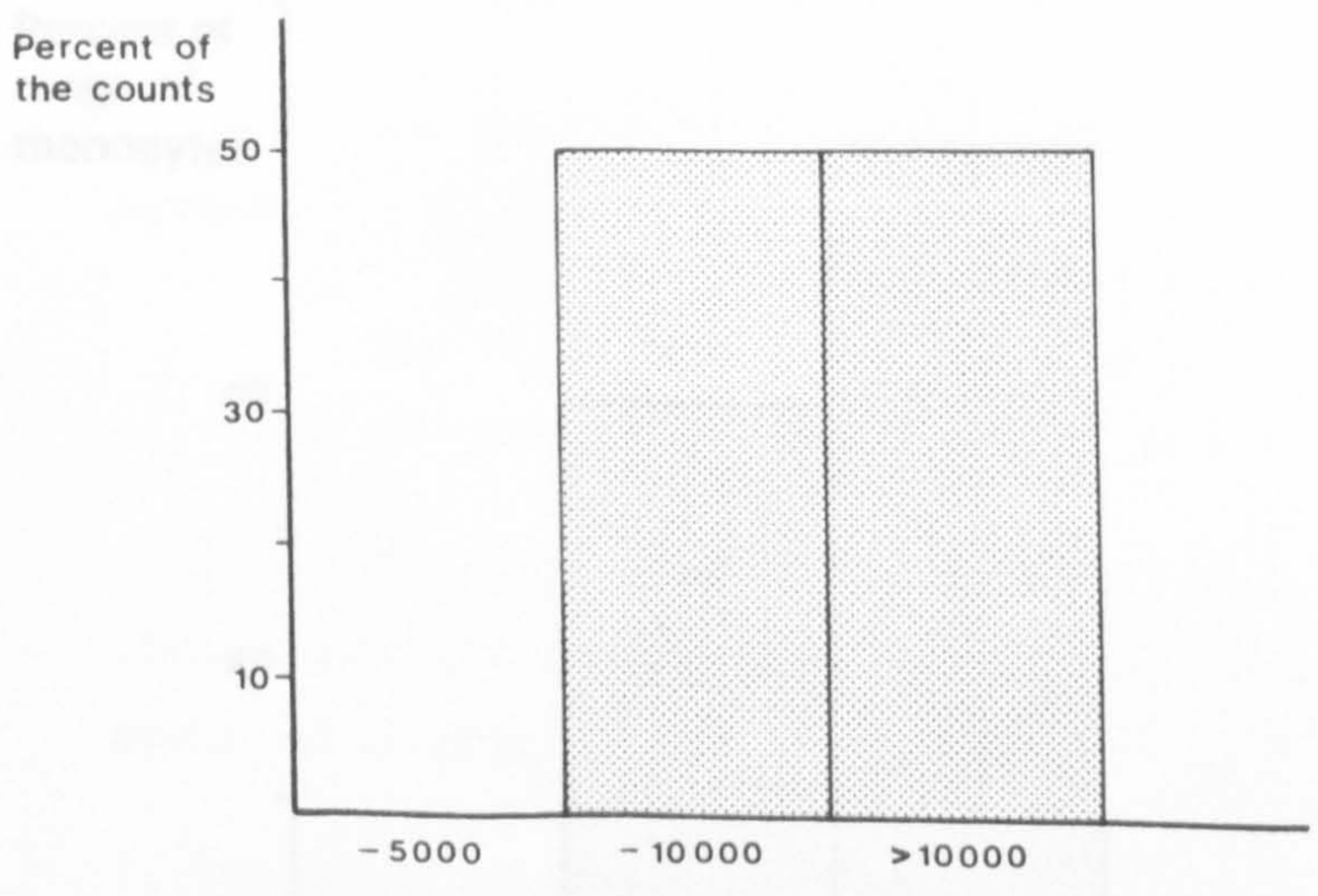


Figure 44 Results of lymphocyte estimations of the IFAT seropositives in the pilot study.

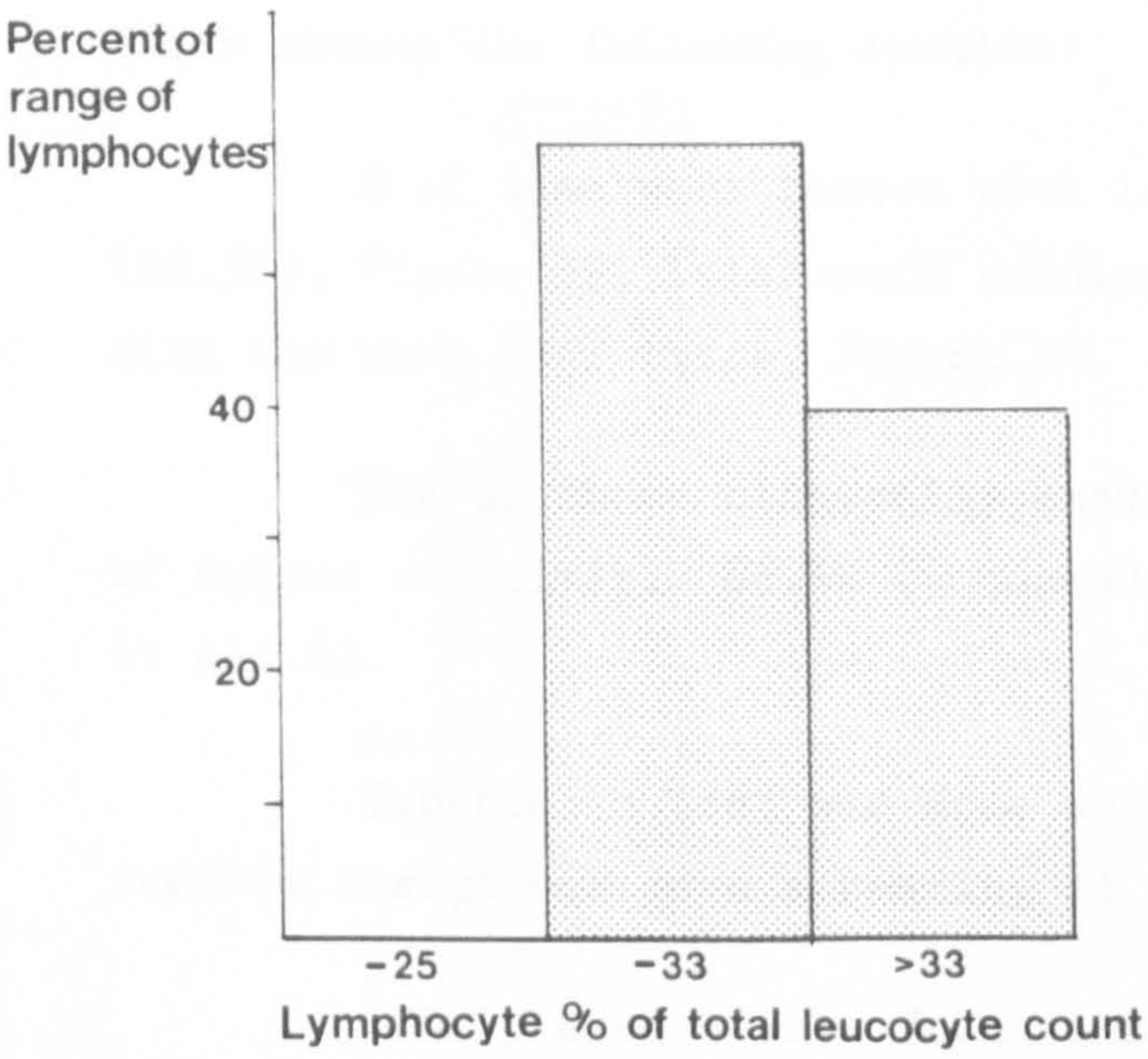
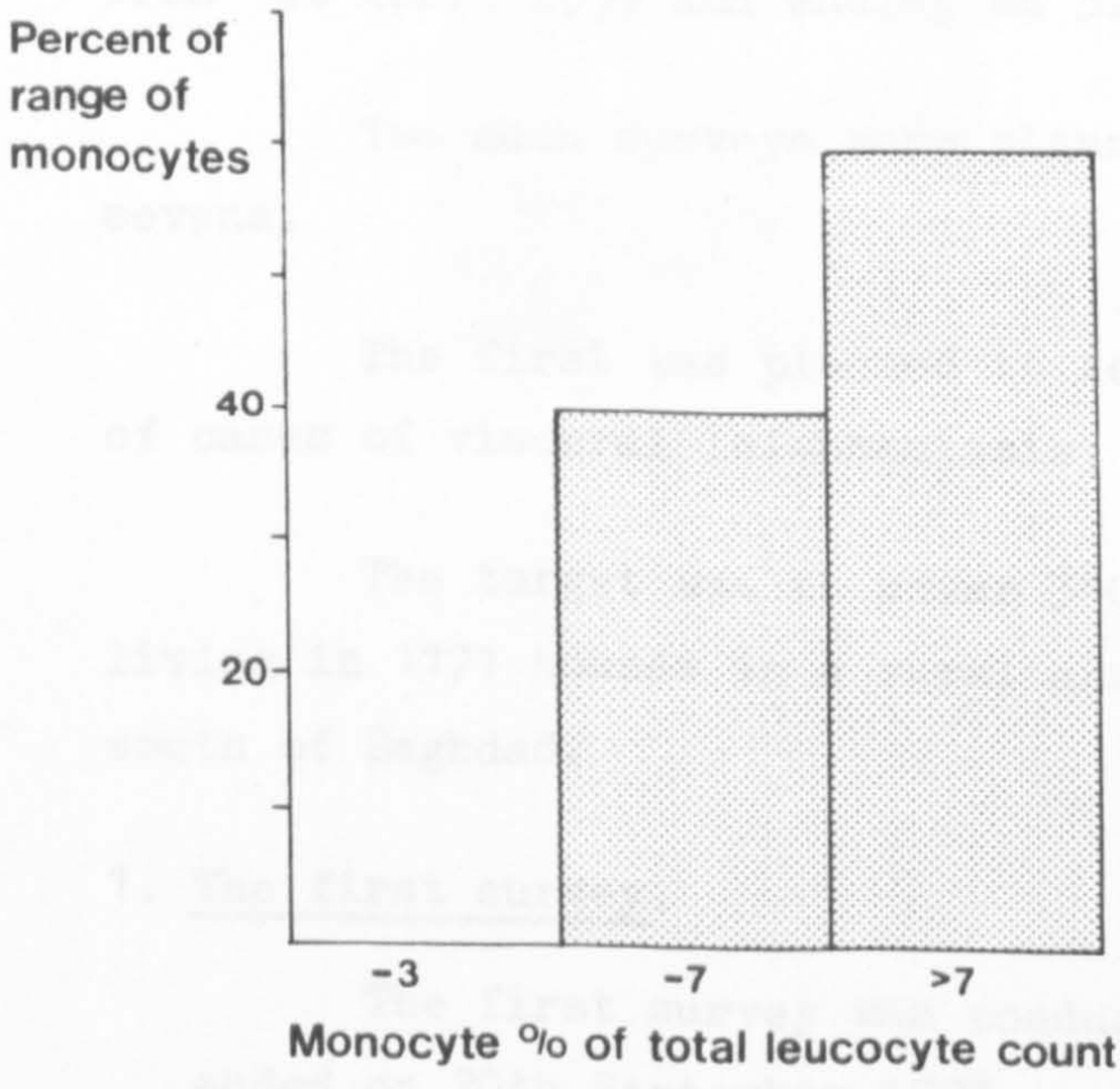


Figure 45 Results of monocyte estimations of the IFAT seropositives in the pilot study.



Later when they were followed up the serologically positive cases showed the following results:

8 of them were tested with leishmanin and 5 showed positivity (62.5%), Figure 39, leishmanin reading to some extent seems to increase with the mean IFAT titre, Figure 40.

50% of those clinically examined (6 cases) showed enlargement of spleen and liver. 66.6% (4 cases) gave a history of fever, Figures 41 and 42.

Tuberculin test was done on 6 positives, all gave negative (0) results except one gave a reading of 10 mm which is just positive.

60% (3 cases) were found anaemic.
40% (2 cases) with relative lymphocytosis, and
60% (3 cases) with relative monocytosis, Figures 43, 44 and 45.

G. FIELD SURVEYS

In the experimental design of the study, field surveys were planned to cover the under seven years of age during one whole year, beginning from 1st April 1979 and ending on 31st March 1980.

Two such surveys were planned to collect blood from the under severs.

The first was planned to be before the usual time of the appearance of cases of visceral leishmaniasis, the other during that time.

The target was to cover 3403 children of a total 9889 population living in 1171 houses in a rural area about 300 square kilometres, just south of Baghdad.

1. The first survey.

The first survey was conducted between the 1st March 1979 and ended on 20th September 1979.

The number of children covered was 2650 with a coverage rate of 77.87%.

Those samples of blood which were collected from the under seven year old children were examined by IFAT and ELISA and 98 cases were found serologically positive, a positivity rate of 3.69% to those

Figure 46 Showing percent distribution of IFAT titres among the IFAT seropositives in the first survey; it also shows their sex distribution.

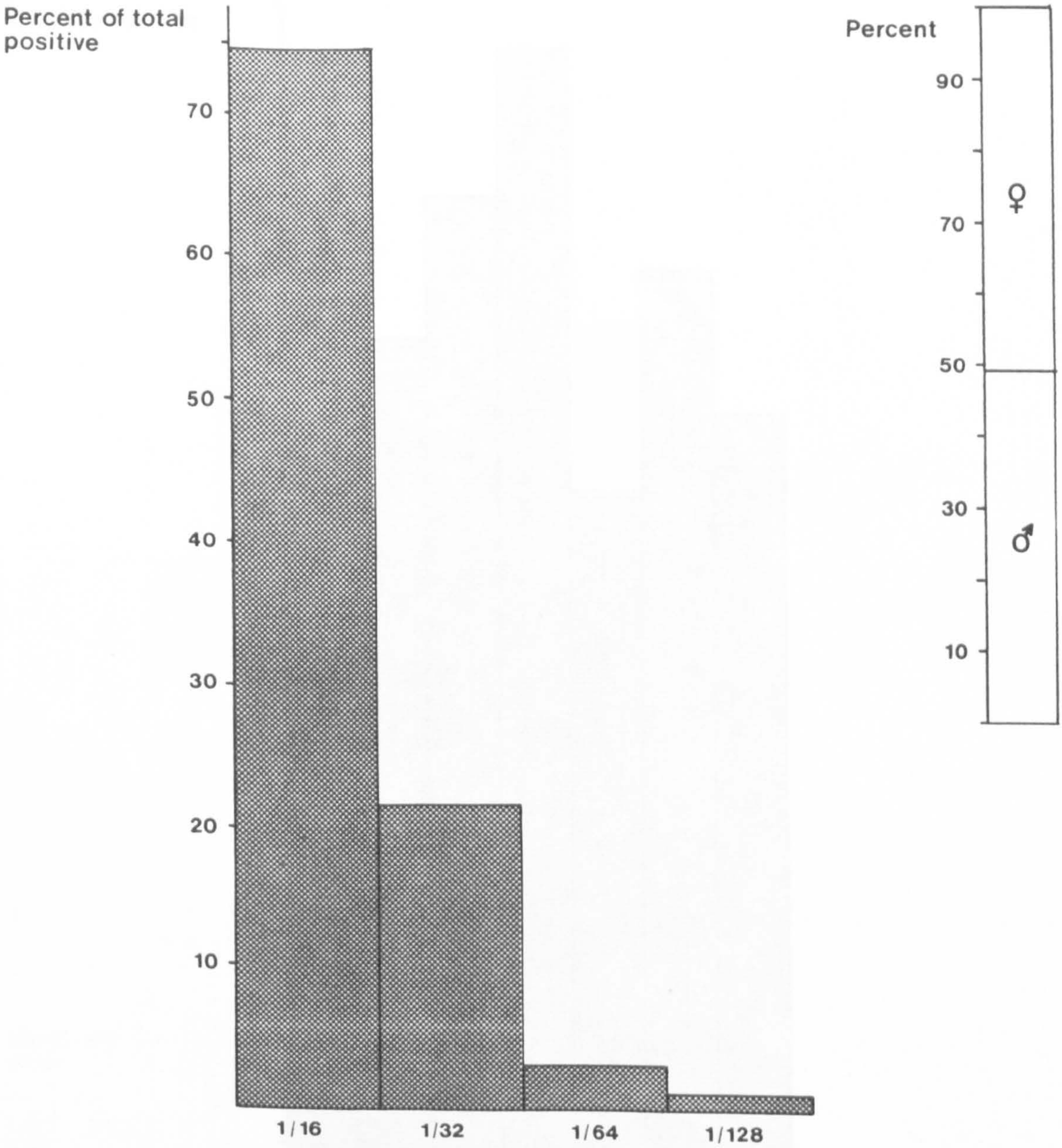


Figure 47 Age distribution of the IFAT seropositives detected during the first survey.

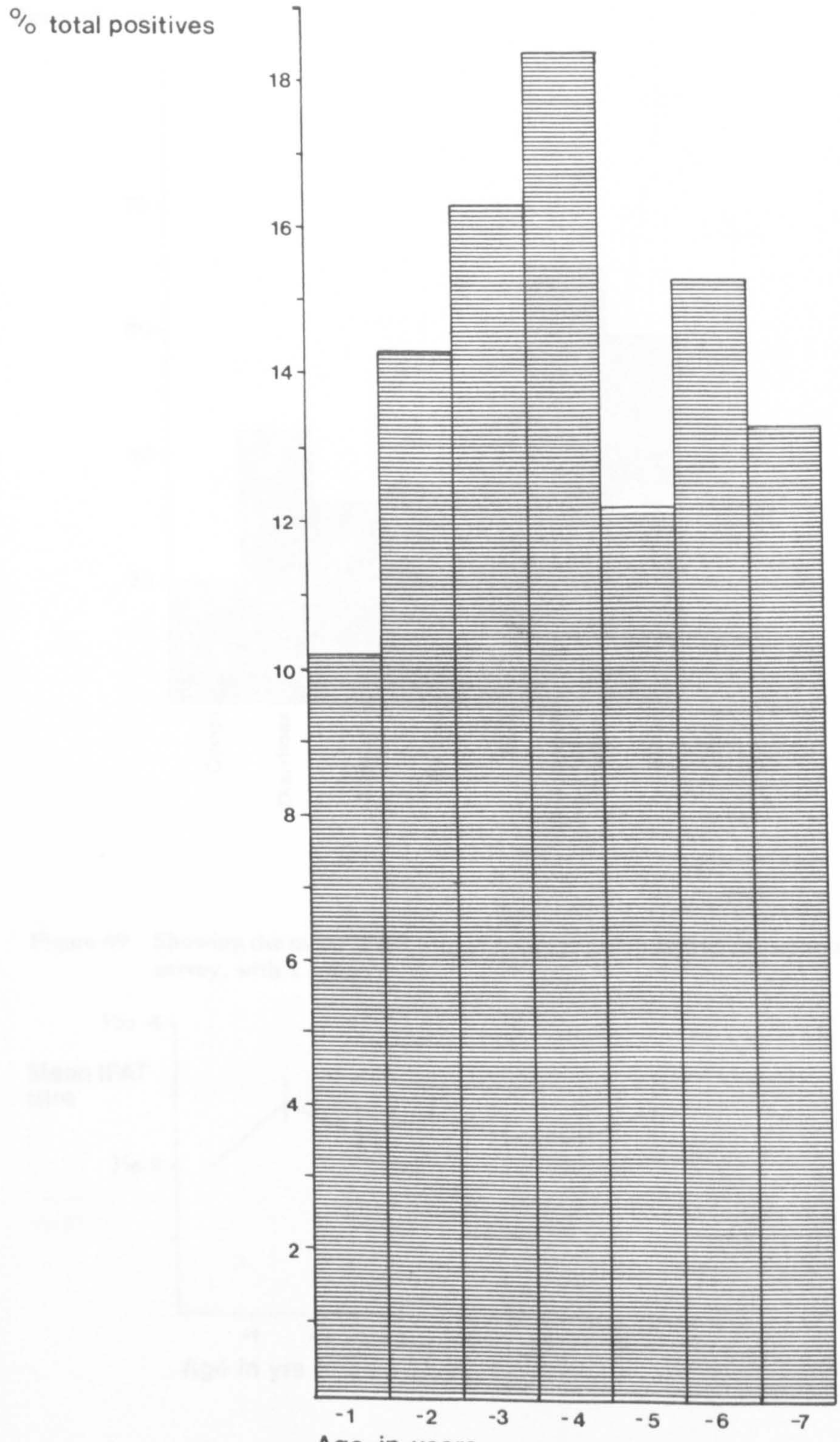


Figure 48 Positive findings among the IFAT seropositives in the first survey.

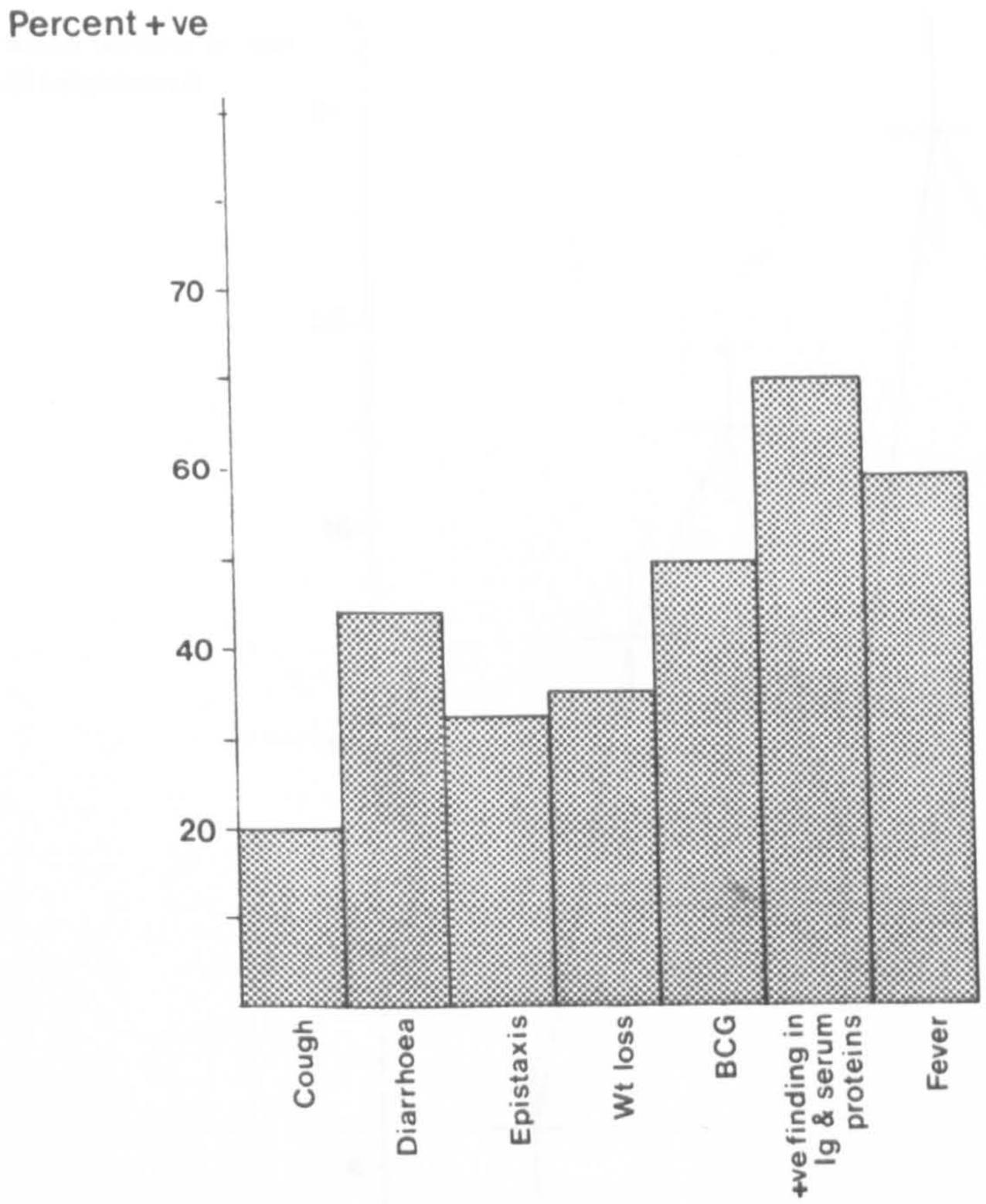


Figure 49 Showing the mean IFAT titre in each age group among the seropositives detected during the first survey, with ± 1 S.E.

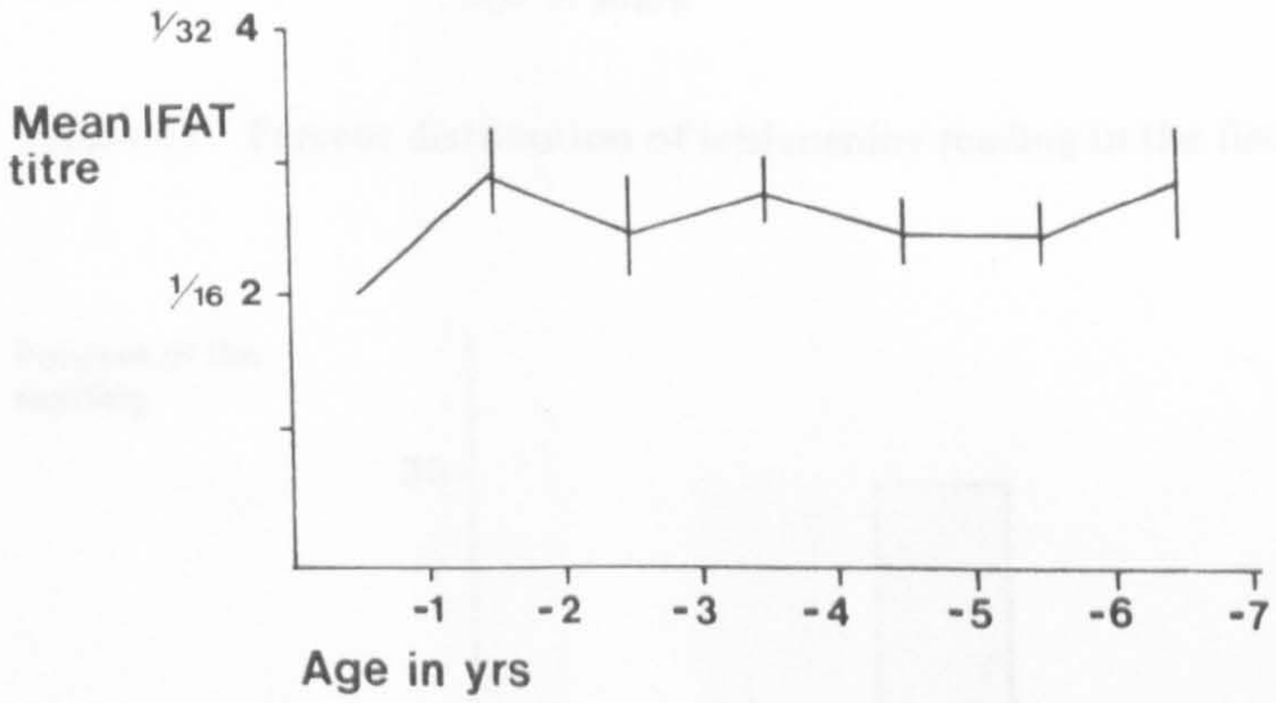


Figure 50 Showing the mean leishmanine reading ± 1 S.E. for each age group among the seropositives in the first survey.

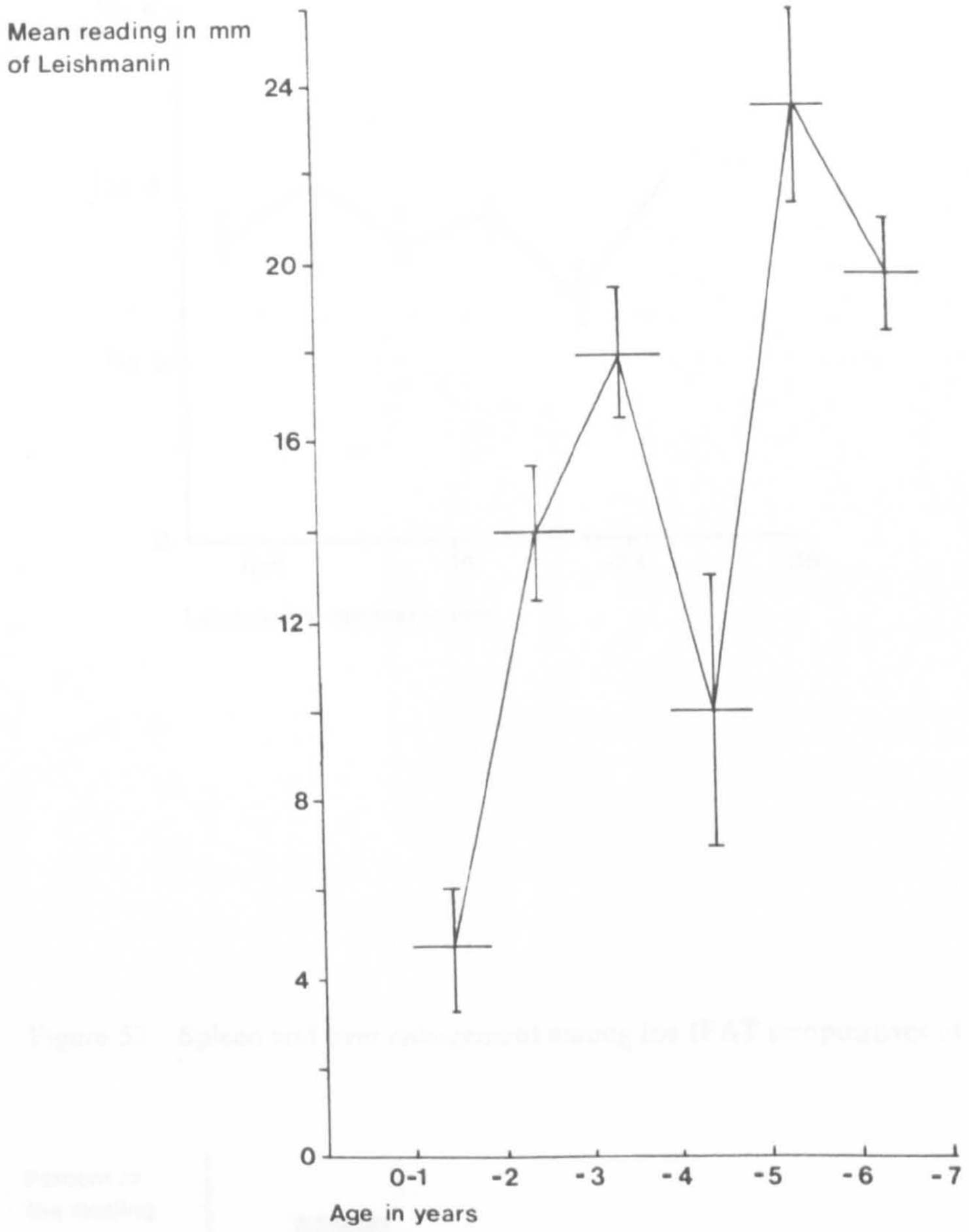


Figure 51 Percent distribution of leishmanine reading in the first survey.

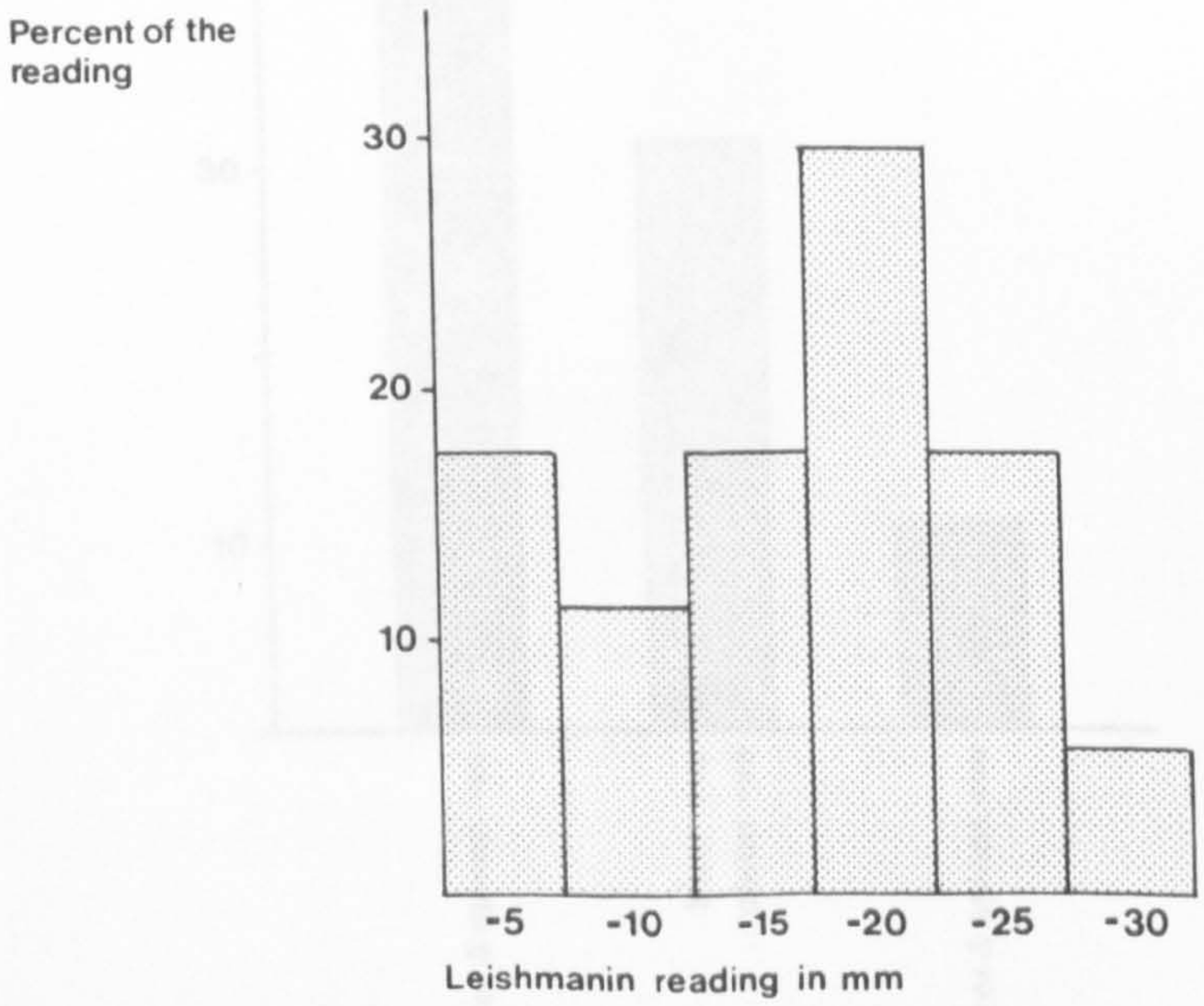


Figure 52 Showing mean IFAT titre for each group (± 1 S.E.) of leishmanine reading in the first survey.

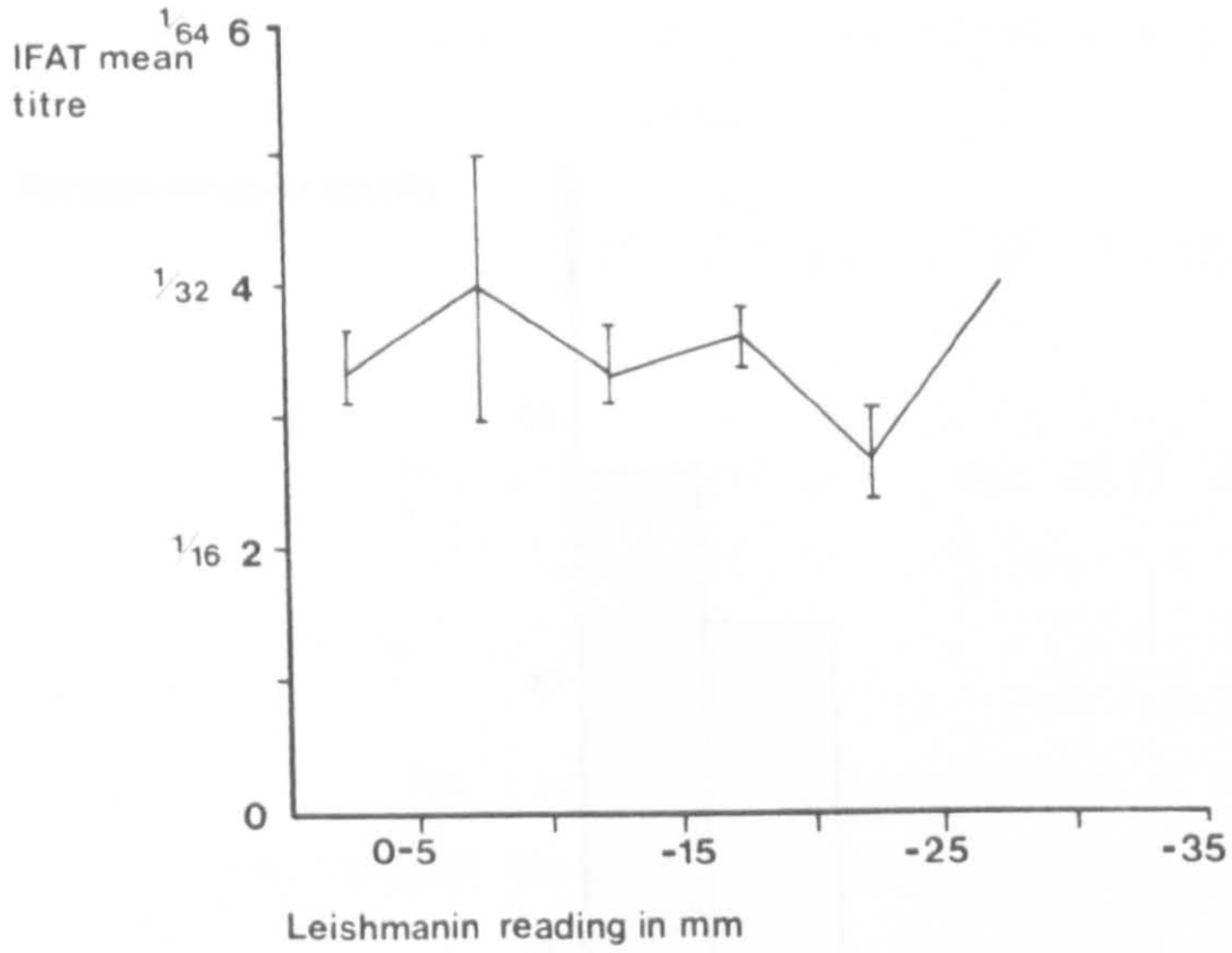


Figure 53 Spleen and liver enlargement among the IFAT seropositives of the first survey.

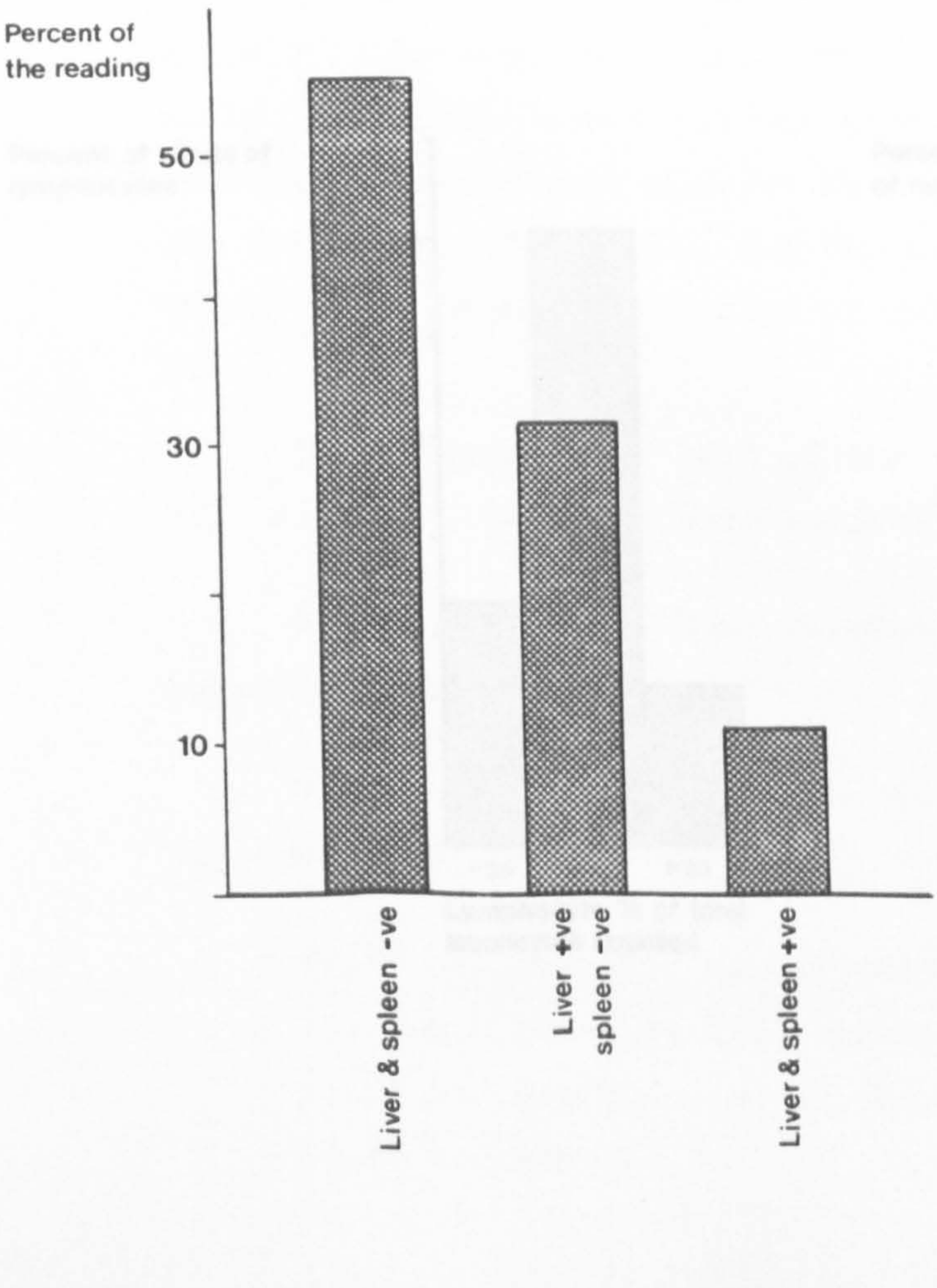


Figure 54 Results of the leucocyte counts of the IFAT seropositives in the first survey.

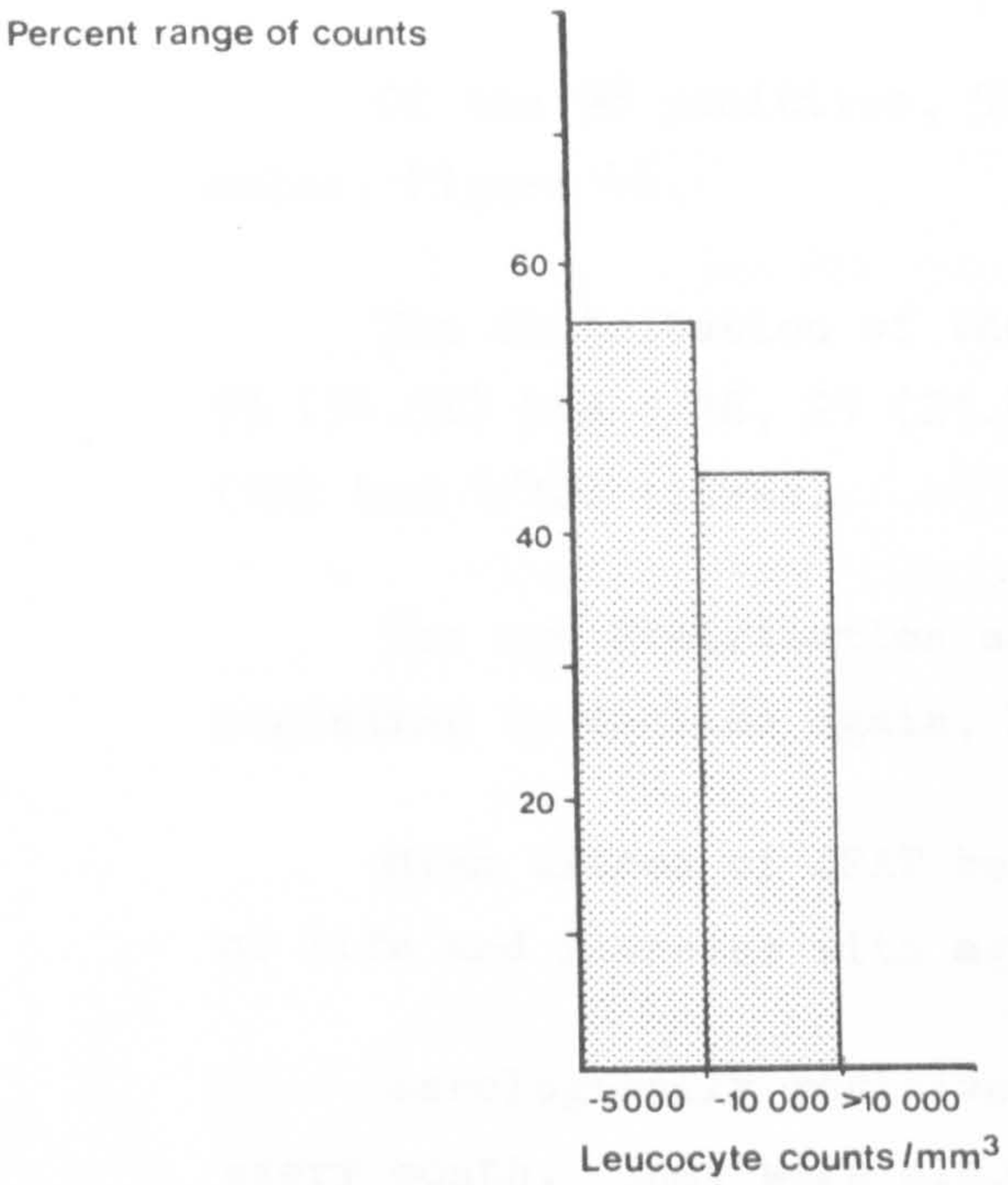
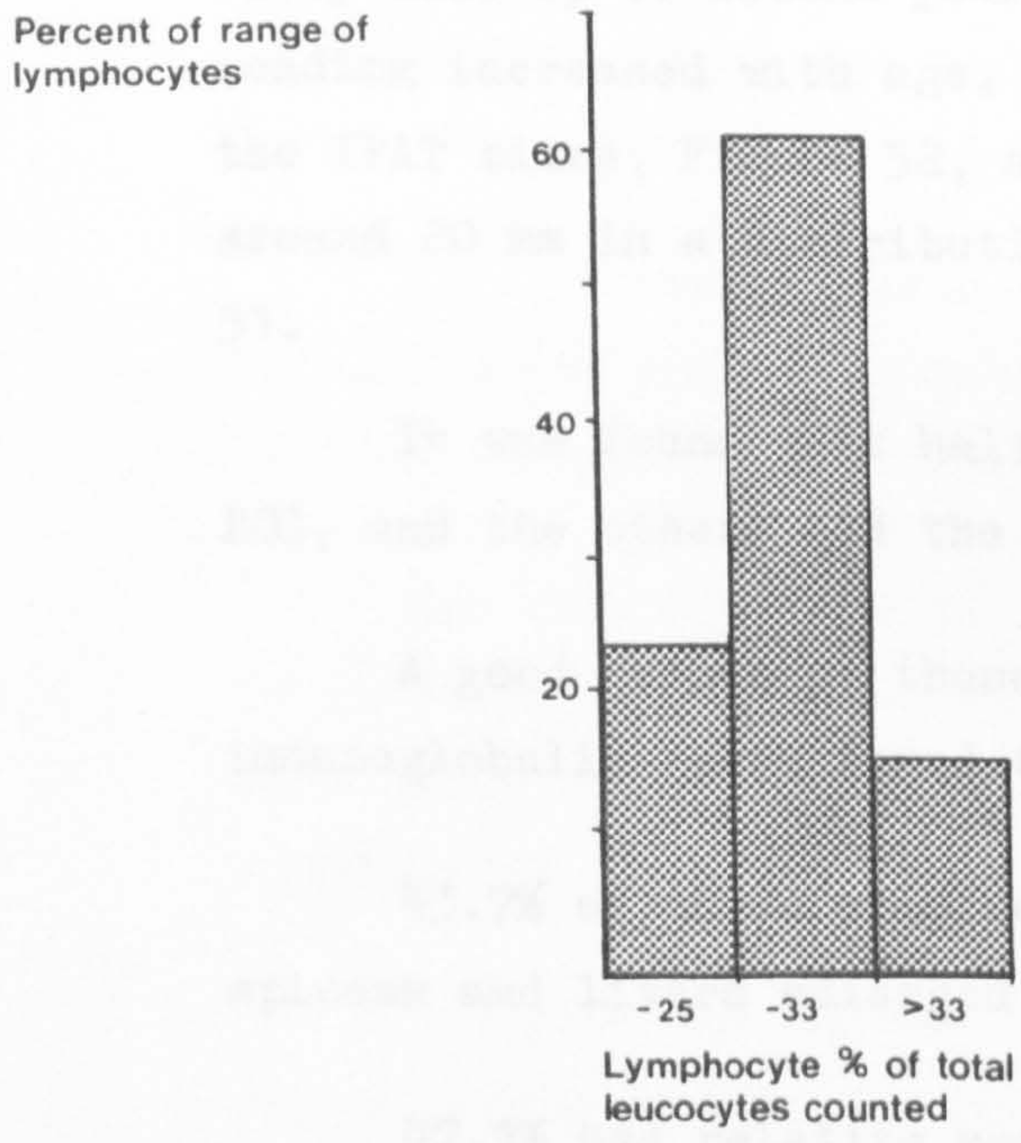
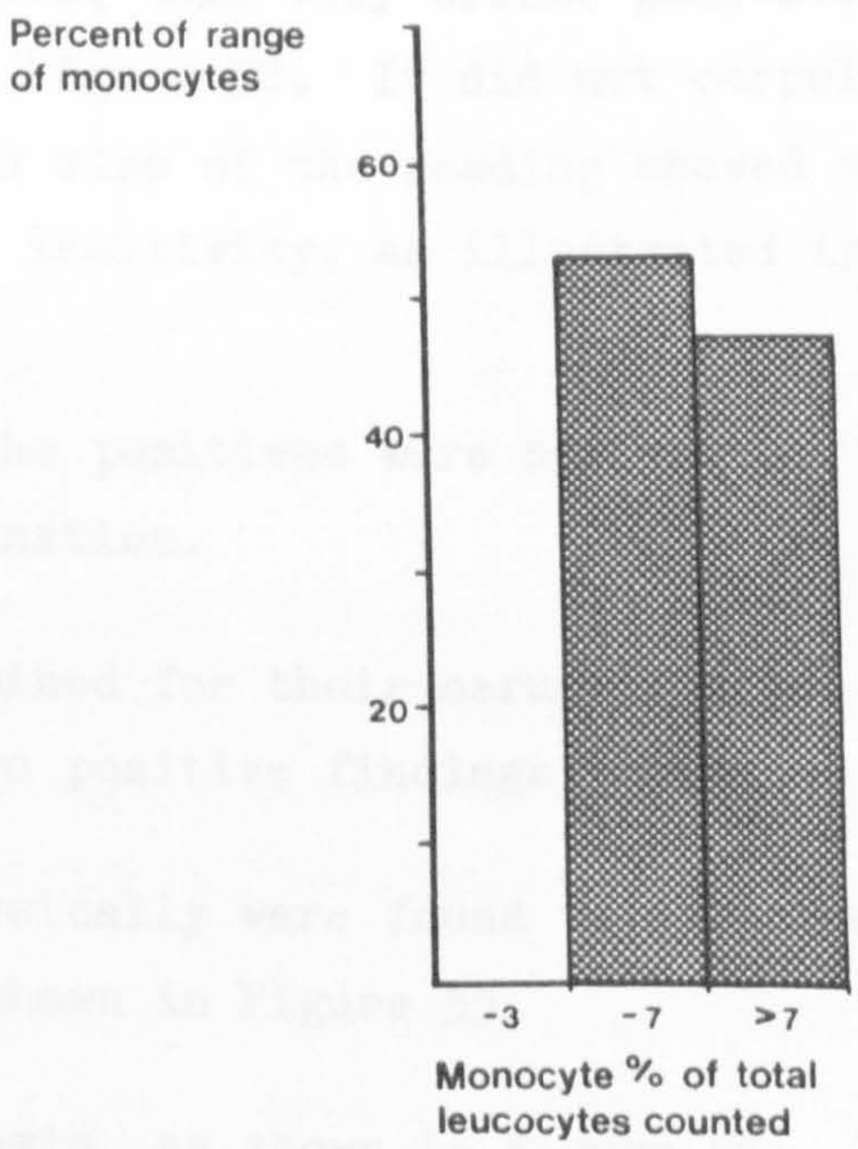


Figure 55 Results of lymphocyte estimations of the IFAT seropositives in the first survey.

Figure 56 Results of monocyte estimations of the Ifat seropositives in the first survey.





actually covered. The number of houses with infected children was 89 (9 houses with double cases), a positivity rate of 7.6% of the houses in the area.

Of the 98 positives, 50 (51.1%) were females and 48 (48.9%) were males, Figure 46.

The distribution of the IFAT titres of the positives shows that 73 (74.5%) had 1/16, 21 (21.4%) had 1/32, 3 (3.1%) had 1/64, and 1 (1%) had 1/128 titres.

The age distribution shows a curve rising till 4 years then beginning to decline again, Figure 47.

Mean titres of IFAT begin to be positive during the first year of life and increase with age, Figure 49.

Serologically positive children were followed up serologically every month. They were also examined clinically and their history was taken. Other investigations included white blood cell estimation and their differential counts, serum proteins and immunoglobulin estimations and leishmanin testing, Figure 48.

82.4% of those examined with leishmanin were positive, as seen on Table 35. Leishmanin reading was found to begin from negative at early life up to second year of life, then they become positive and reading increased with age, as in Figure 50. It did not correlate with the IFAT titre, Figure 52, and the size of the reading showed a peak around 20 mm in a distribution of positivity, as illustrated in Figure 51.

It was found that half of the positives were not vaccinated with BCG, and the others had the vaccination.

A good number of those examined for their serum proteins and immunoglobulins were found to have positive findings (70%).

43.7% of those examined physically were found to have their spleens and livers enlarged, as shown in Figure 53.

47.1% had relative monocytosis, as shown in Figure 56.
15.3% had relative lymphocytosis, as shown in Figure 55.
55.5% had leucopaenia, as shown in Figure 54, and
80.3% had anaemia.

History of cases were taken and fever was a prominent symptom for almost 60% of cases, other percentages of positivity were found for epistaxis, cough, diarrhoea and weight loss.

2. The second survey.

The second survey was conducted between 23rd September 1979 and 5th March 1980.

The number of children covered was 2981, a coverage rate of 87.6%. Those samples similarly to the first survey were examined blindly by IFAT and ELISA and 135 positives were detected, a positivity rate of 4.53%.

Of the 135 positives, 74 were males (54.8%) and 61 were females (45.2%).

IFAT titre distribution shows that 77 of them (57%) had a titre of 1/16; 44 of them (32.6%) had a titre of 1/32; and 14 of them (10.4%) had a titre of 1/64. This is illustrated in Figure 57.

Ten of those cases were hospitalised, bone marrow puncture was done on six of them, four were found to be positive and they showed the amastigotes in the smear and their cultures were positive; the other two were negative.

The remaining four, two of them refused to have a bone marrow puncture, the other two began on the schedule of treatment before having the chance to do bone marrow puncture on them.

All ten were treated as cases of visceral leishmaniasis with Pentostam (Wellcome Foundation Ltd., Lordon). Out of those ten two died in the hospital because of the disease.

The number of houses with infected children was 121 houses; this included 10 houses with double cases and two houses with three cases each. The other 109 houses had a single case each. These 121 houses constituted 10.5% of the total houses in the area.

Distribution of the serologically positive cases according to age showed a gradual increase to reach a peak at 3 years of age and then the curve began to decline again. This is shown in Figure 58.

Mean titre of IFAT against age distribution, as illustrated in Figure 66, shows a gradual increase with age generally the mean titre of positivity is higher with second survey positives than in the first survey. (This goes with the mean titre of IFAT during the whole survey shown in Figure 74).

The follow-up on serologically positive children was done and the results were as follows:

Leishmanin testing showed that 61.8% of those examined with leishmanin were positive, frequency distribution of the size of the induration shows the frequency decreasing slowly as the induration increases. This is illustrated in Figure 62.

Mean titre of IFAT with leishmanin shows more clearly than the first survey that it increases with the size of induration, as is shown in Figure 63. It also shows that the leishmanin positive reading had increased steadily with age, as illustrated in Figure 65.

Regarding previous BCG vaccination of those positive cases, it was found that only 27.8% were actually vaccinated. Nearly the same rate of positivity as in the first survey was seen here regarding serum proteins and immunoglobulins, 9 out of 13 (69.2%) of those examined for their serum proteins and immunoglobulins, as seen in Table 36 and Figure 64, showed positive findings.

97 out of 131 cases (74%) of those examined for their spleens and livers were found to have them enlarged.

43 cases out of 75 (57%) of those examined for their white blood corpuścles showed leucopaenia, as shown in Figure 59.

87 cases out of 134 (64%) reported having fever during the last few days to two months.

Most striking of all, that ten cases of the 135 (7.4%) were treated as cases of visceral leishmaniasis after admission to the hospital. Bone marrow was done on six of them and four were found positive with the parasite.

So from 3% of the 135 cases, the parasite was isolated successfully. 24.8% of those examined (31) had relative lymphocytosis, as illustrated in Figure 60. 39% (51) had anaemia. 35% had a cough. 35% had

Figure 57 Showing percent distribution of IFAT titres among the IFAT seropositives in the second survey; it also shows their sex distribution.

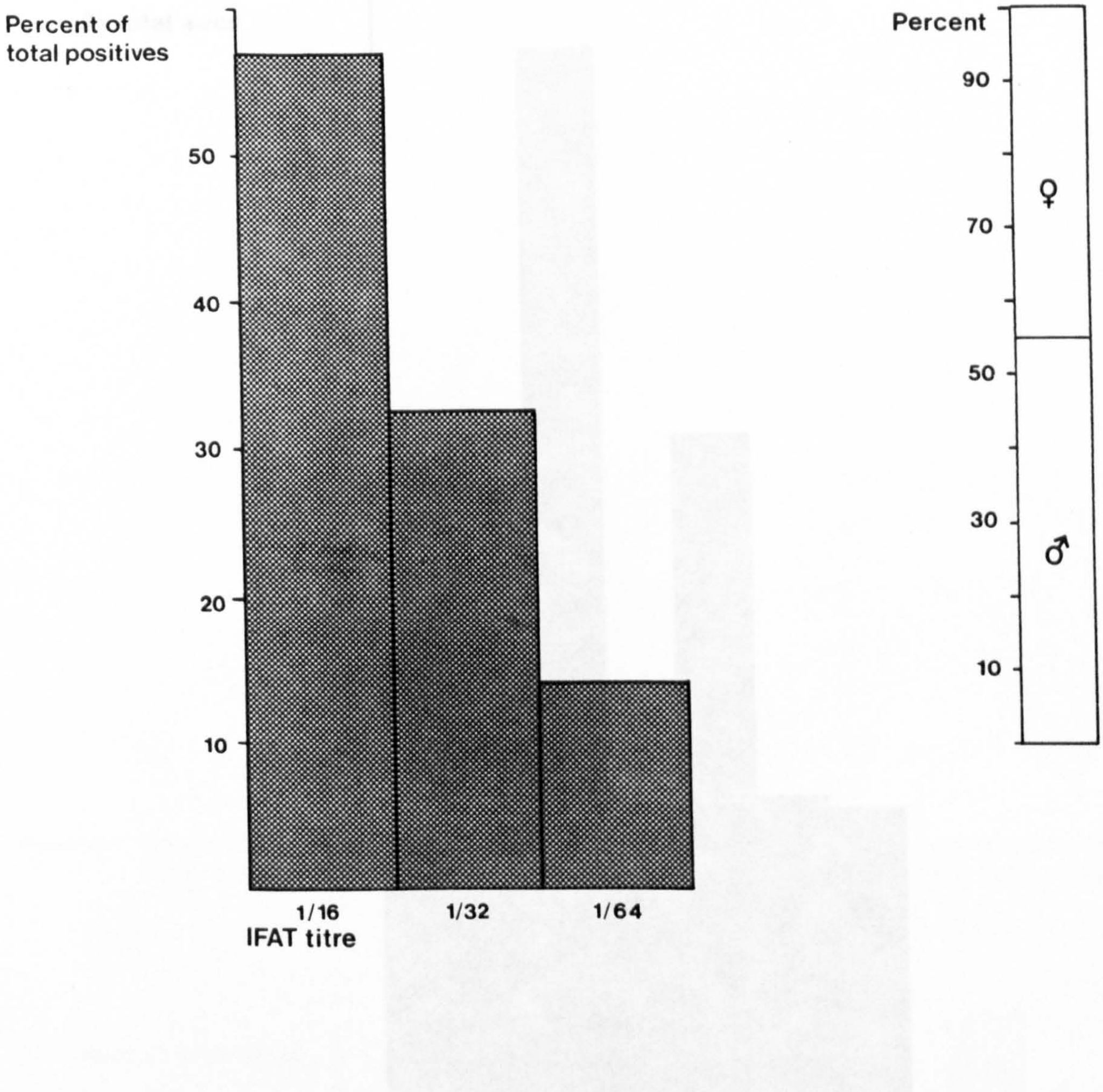


Figure 58 Age distribution of IFAT seropositives detected during the second survey.

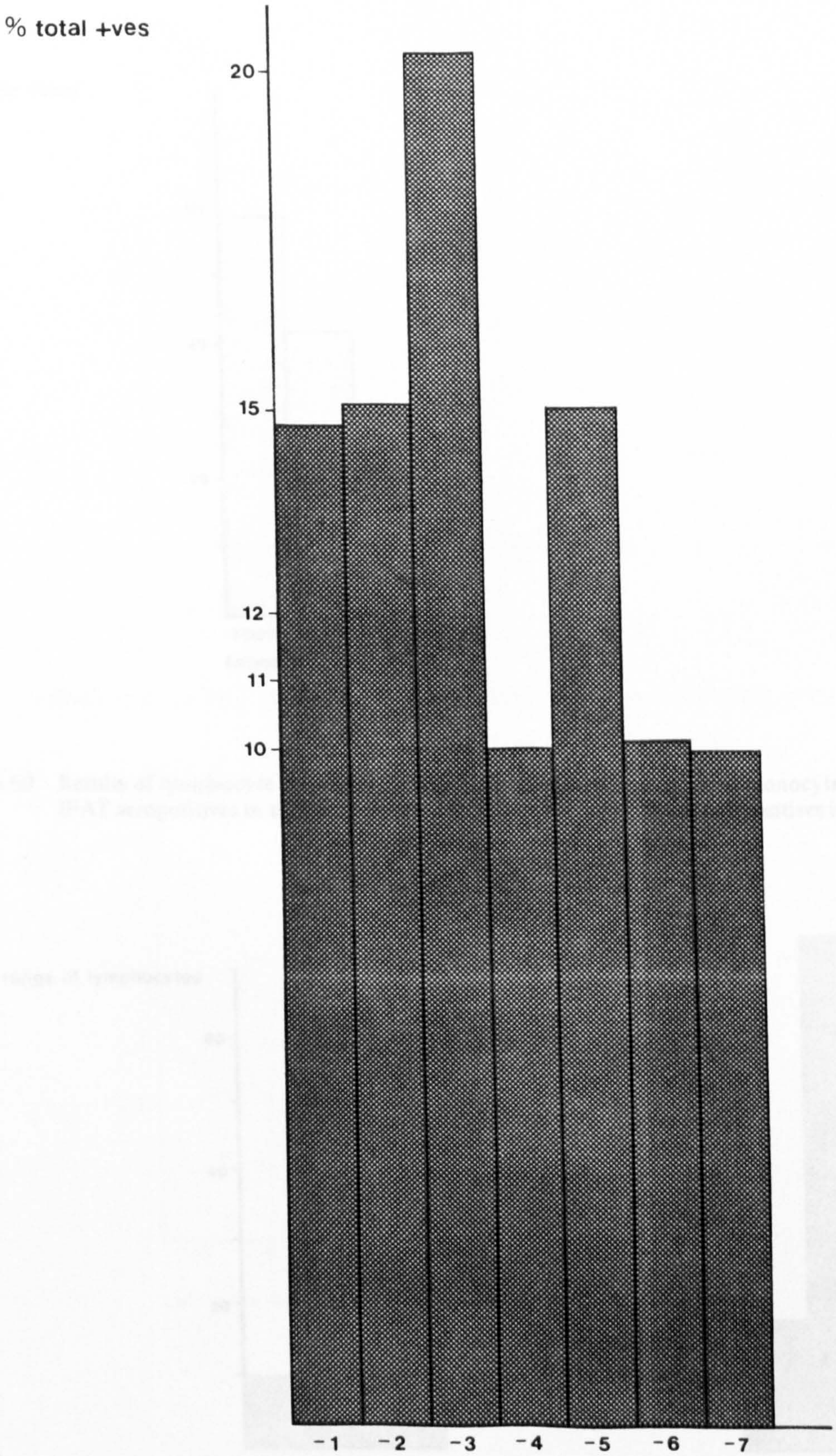


Figure 59 Results of leucocyte counts of the IFAT seropositives in the second survey.

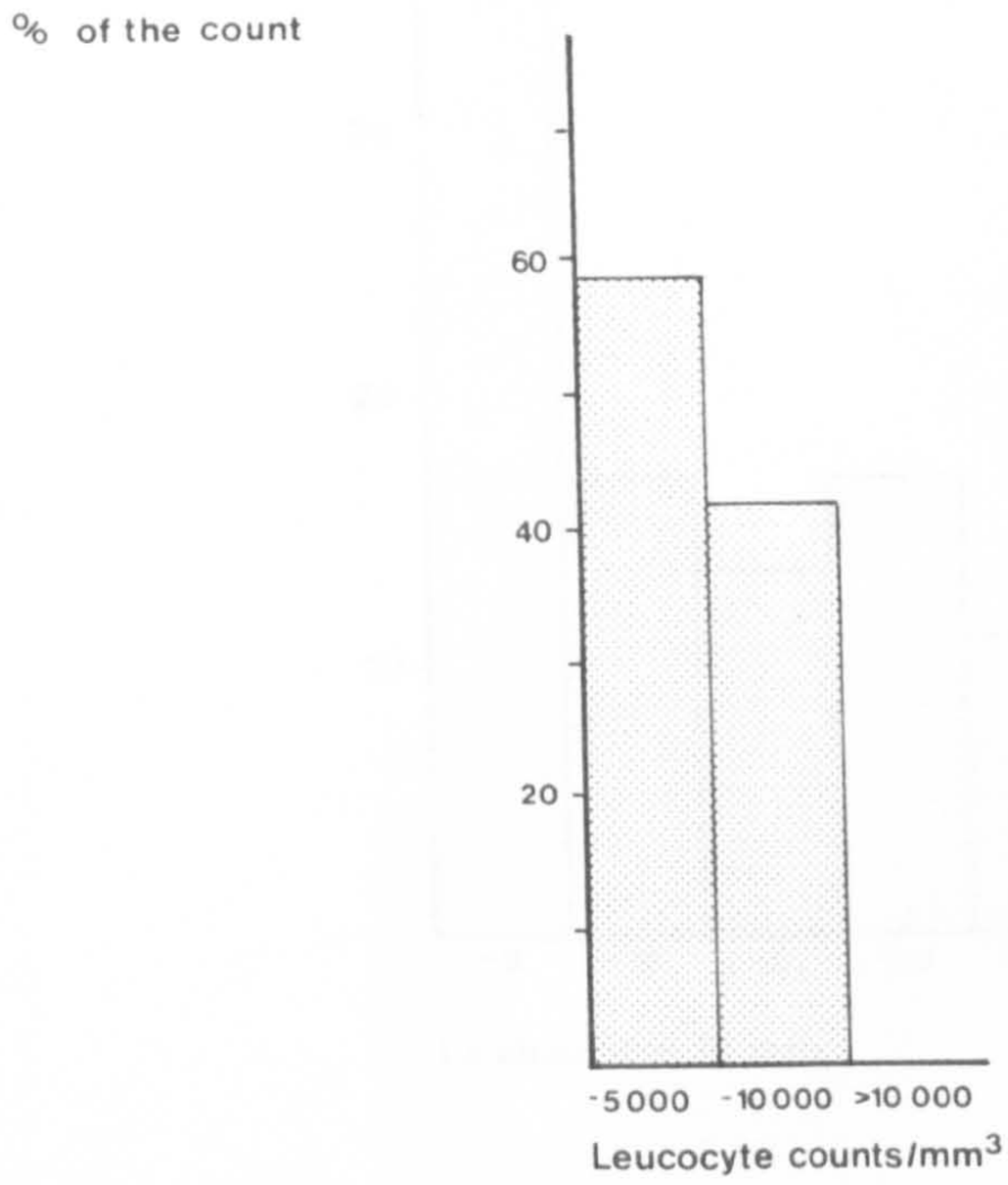


Figure 60 Results of lymphocyte estimations of the IFAT seropositives in the second survey.

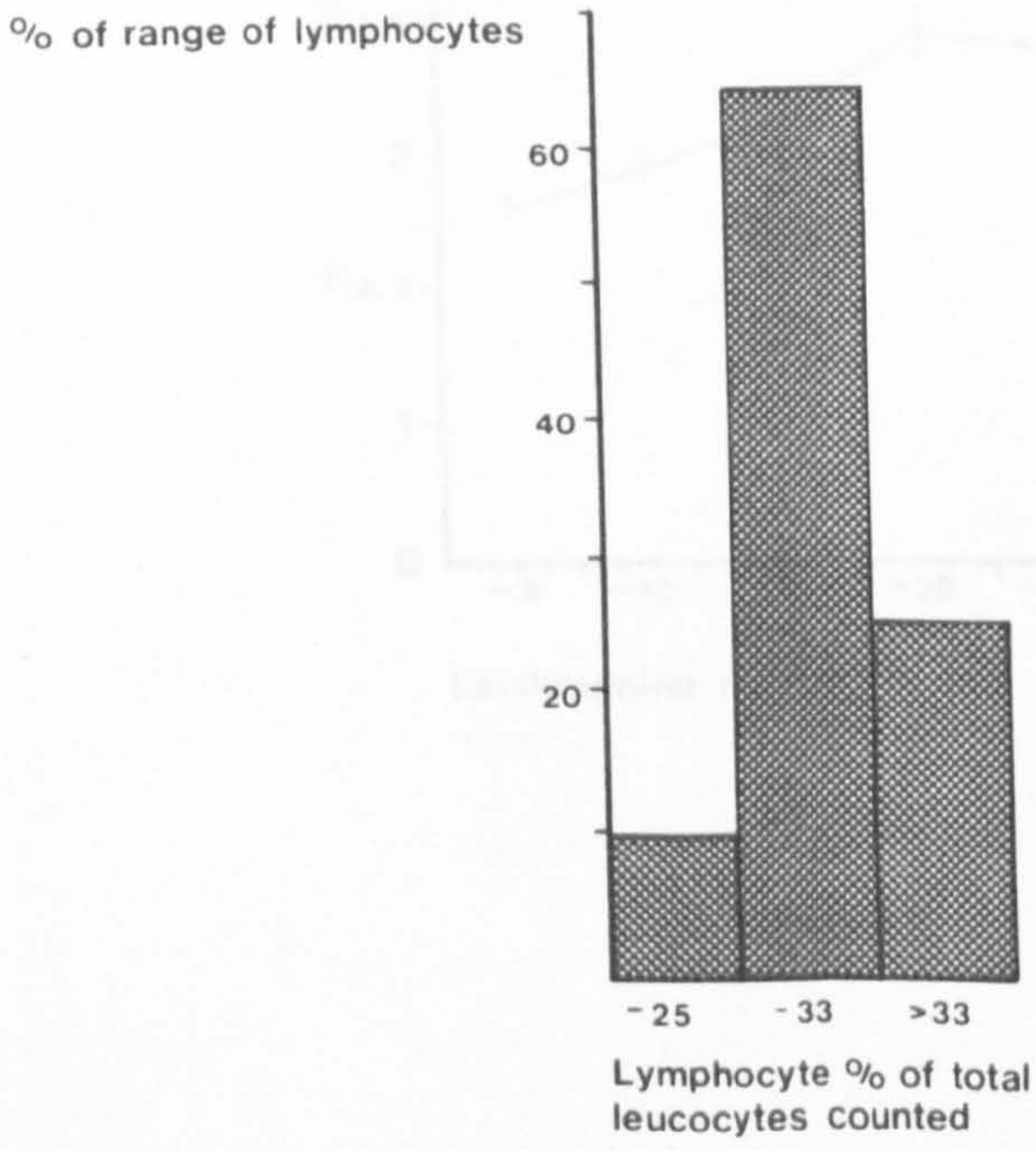


Figure 61 Results of monocyte estimations of the IFAT seropositives in the second survey.

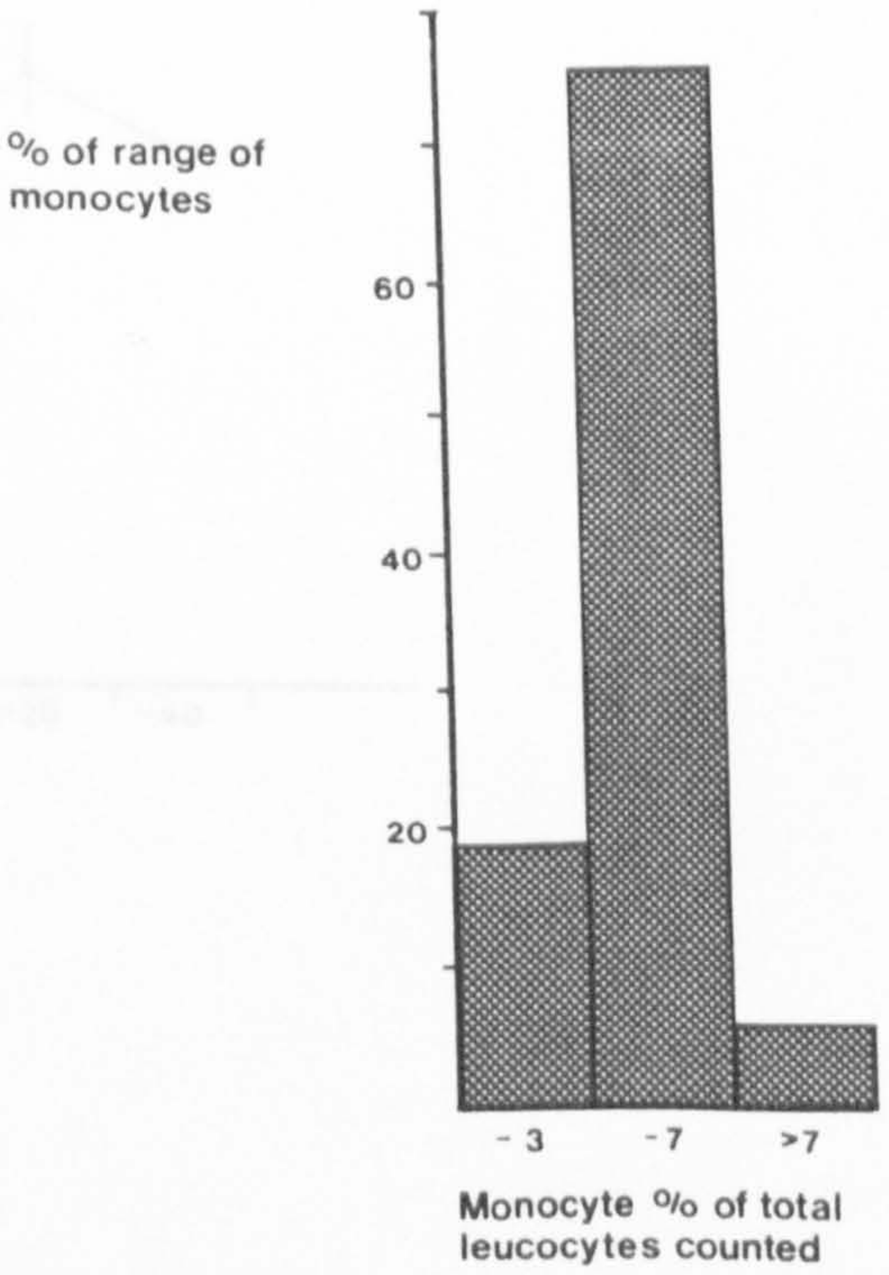


Figure 62 Percent distribution of leishmanine readings in the second survey.

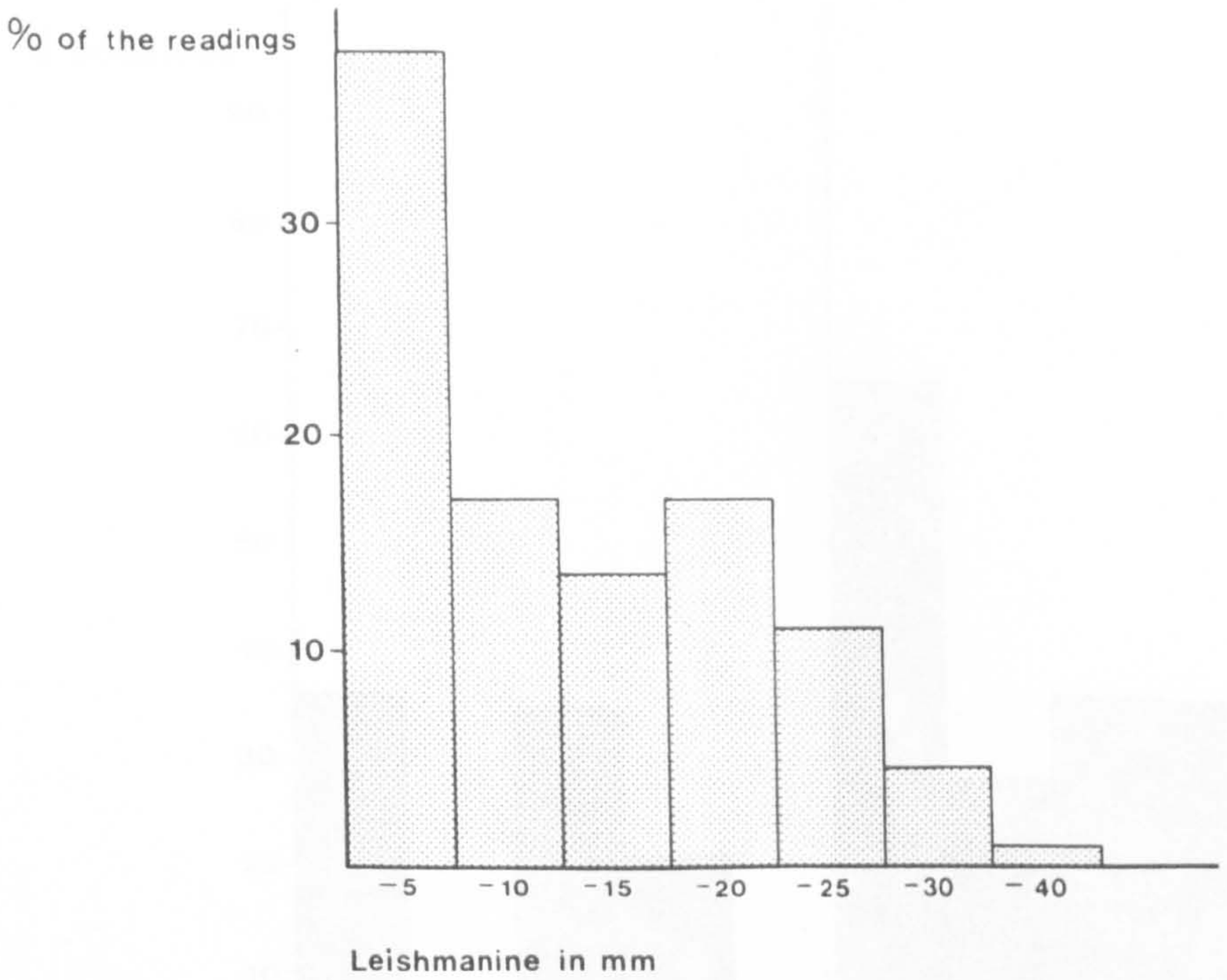


Figure 63 Showing mean IFAT (± 1 S.E.) titre for each group of leishmanine readings in the second survey.

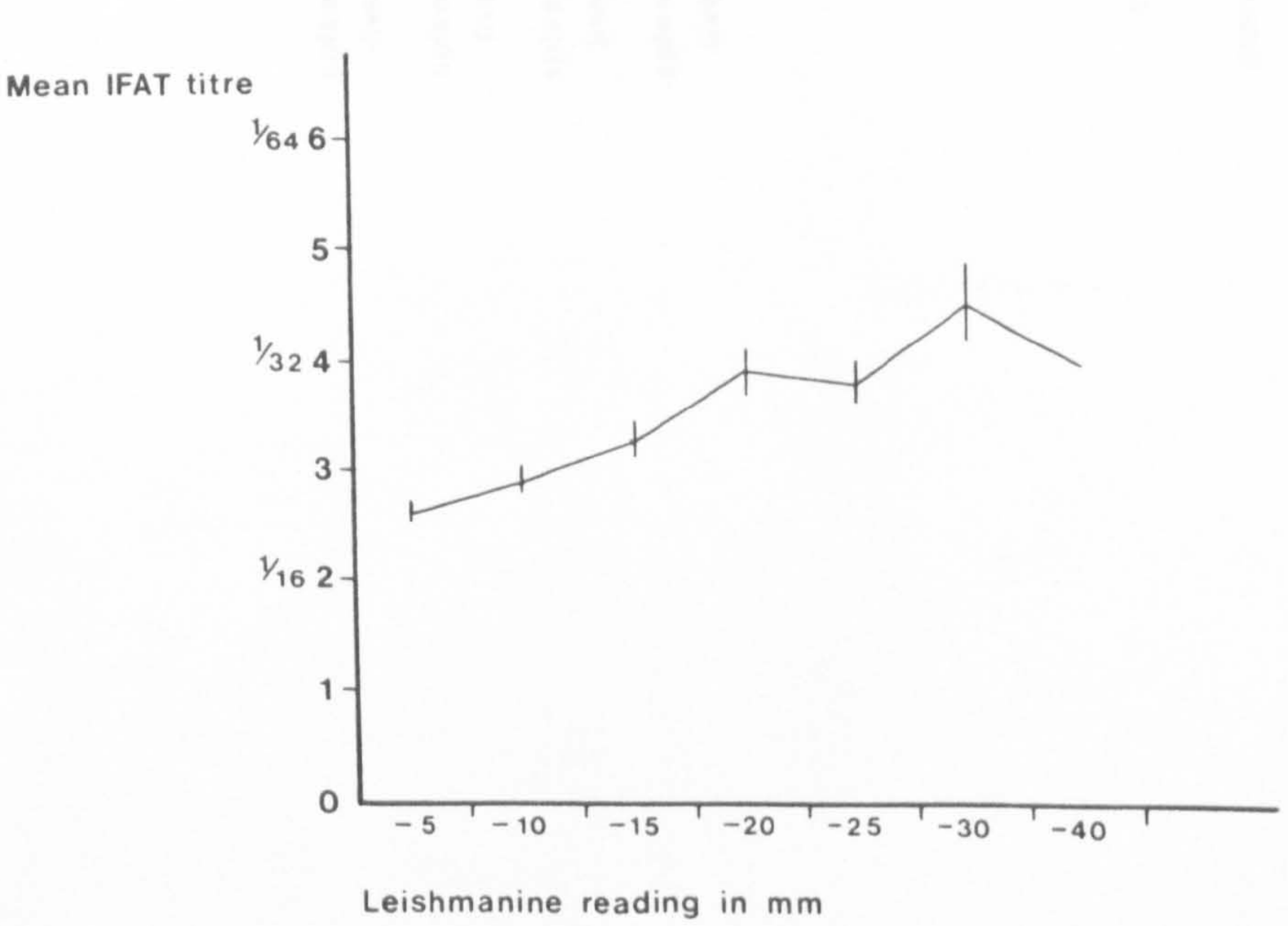


Figure 64 Abnormal findings among positives of the second survey.

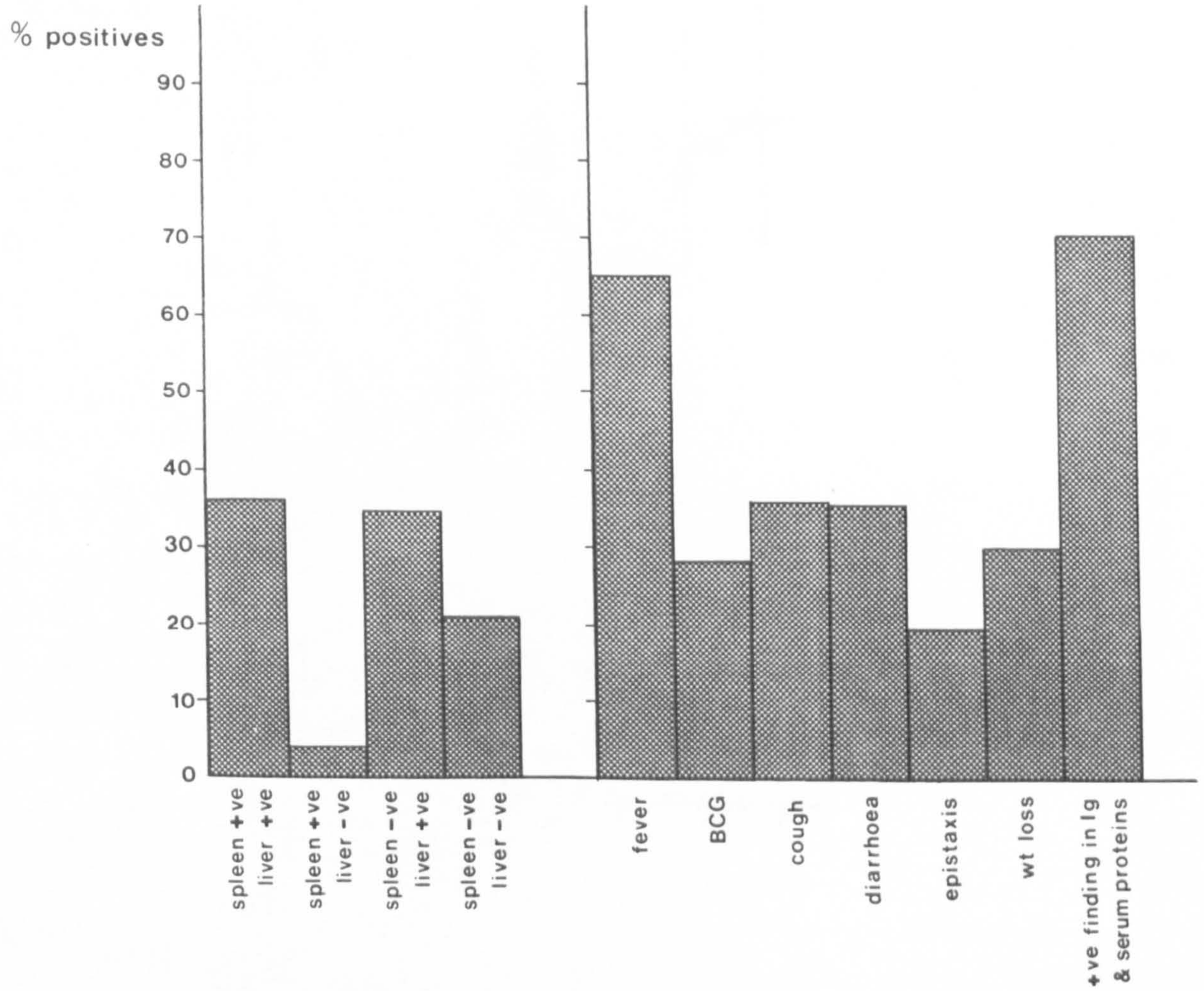


Figure 65 Showing the mean leishmanine reading for each age group among the IFAT seropositives in the second survey with ± 1 S.E.

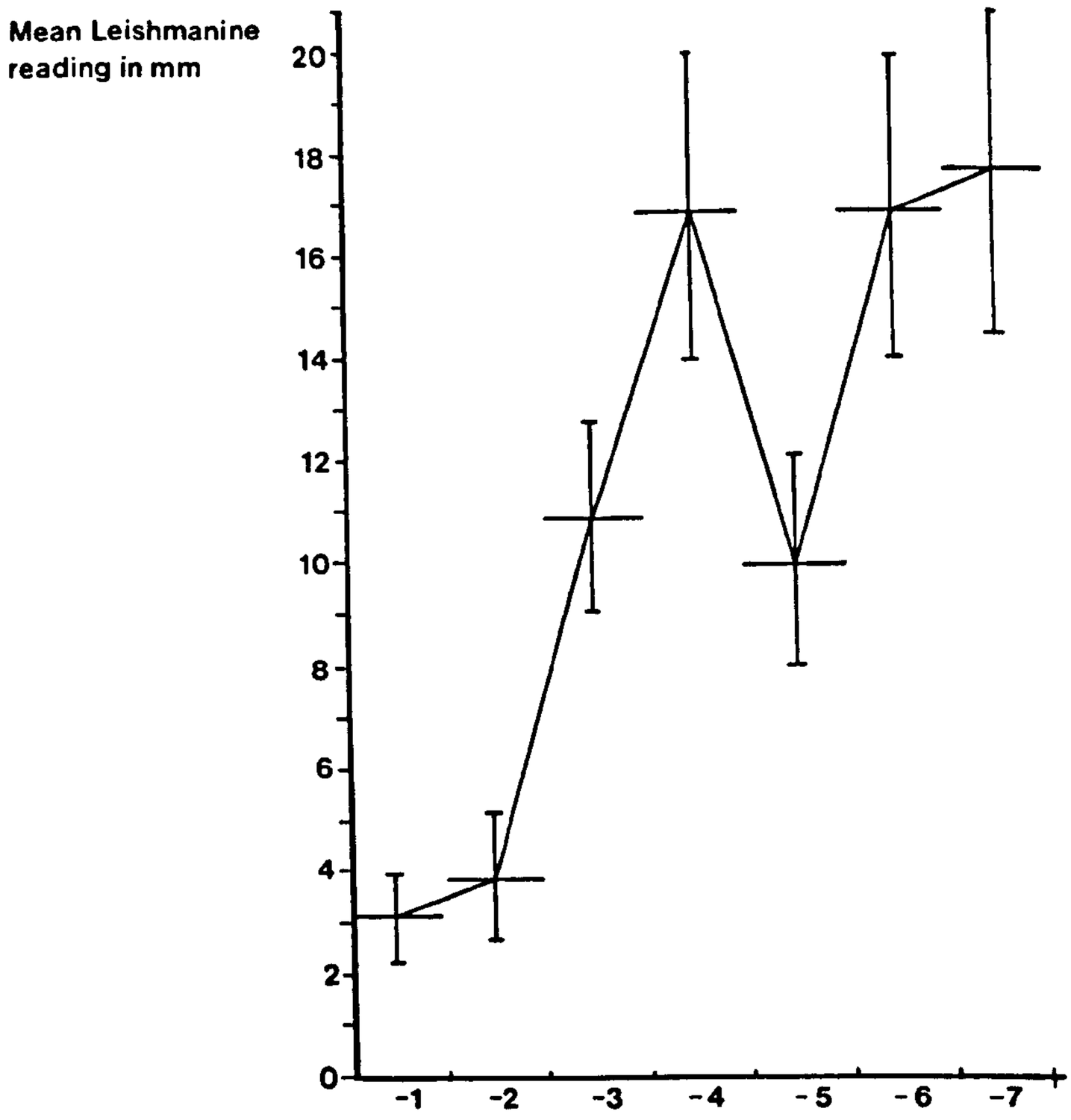


Figure 66 Showing the mean IFAT titre in each age group among the positives detected during the second survey with ± 1 S.E.

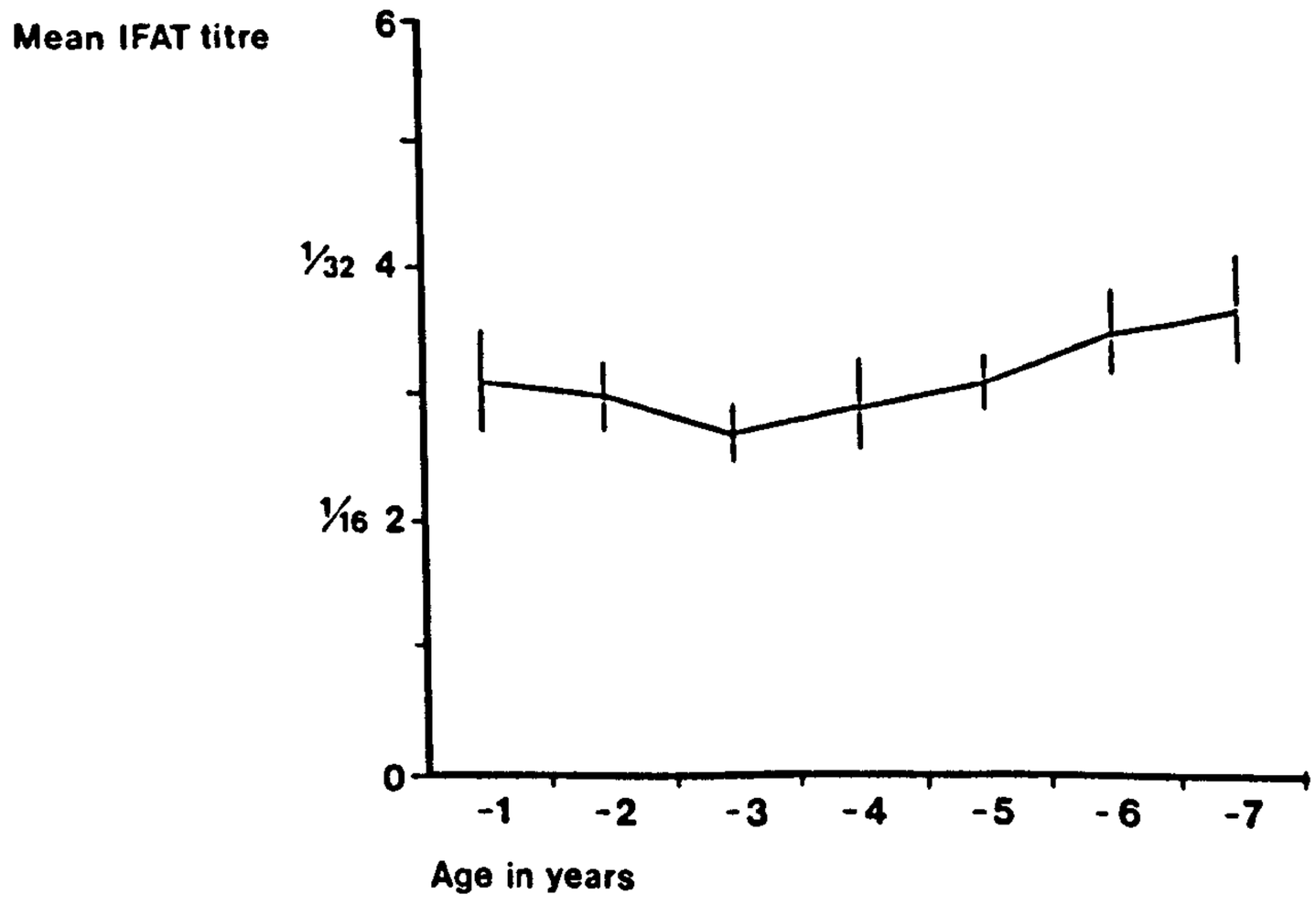


Table 35. Details of investigations done on the 98 IFAT sero-positive cases of the first survey. (IFAT positive is 1/16 and more).

	Number tested	% to total positives	Positives of those examined	% positives of those examined
Leishmanin	17	17.3%	14	82.4%
BCG	54	55.1%	27	50.0%
Serum proteins and immunoglobulins	10	10.2%	7	70.0%
Hepatomegaly and splenomegaly	89	90.8%	3.9	43.7%
Relative monocytosis	85	86.7%	40	47.1%
Relative lymphocytosis	85	86.7%	13	15.3%
Leucopaenia	9	9.2%	5	55.5%
Haemoglobin	61	62.2%	49	80.3%
History of cough	60	61.2%	12	20.0%
History of diarrhoea	32	32.77%	14	43.8%
History of epistaxis	21	21.4%	7	33.3%
History of weight loss	66	67.3%	24	36.4%
History of fever	92	93.9%	55	59.8%

Table 36. Details of investigations done on the 135 IFAT seropositive cases of the second survey. (IFAT positivity starts from a titre of 1/16)

	Number tested	% to total positives	Positives of tested	% positive tested to total tested
Leishmanin	89	65.9%	55	61.8%
BCG	115	85.2%	32	27.8%
Serum proteins and immunoglobulins	13	9.6%	9	69.2%
Hepatomegaly and splenomegaly	131	97.0%	97	74.0%
Monocytosis	121	79.6%	8	6.6%
Lymphocytosis	125	92.6%	31	24.8%
Leucopaenia	75	55.6%	43	57.3%
Haemoglobin	129	95.6%	51	39.5%
History of cough	116	85.9%	41	35.3%
History of diarrhoea	111	82.2%	39	35.1%
History of epistaxis	92	68.1%	18	19.6%
History of weight loss	111	82.2%	33	29.7%
Bone marrow	6	4.4%	4	3.0%
History of fever	134	99.3%	87	64.9%

diarrhoea. Some showed relative monocytosis as in Figure 61.

3. Both surveys.

The results of the work on the children of the study area for the whole year (both surveys first and second added together). The work began on 1st April 1979 and ended on 29th March 1980. 3403 under seven years old children were screened at least once during the two serological surveys and 5631 blood samples were collected from them. It was found that there were 98 positives in the first survey and 135 positives in the second survey, a total of 233 positives, but since there were 28 cases common to the two surveys the total positives actually will be 205, a positivity rate of 7.3% to the children covered, and 6% positivity rate of the total under seven years of age children in the study area. This is illustrated in Figure 69, 70 and 71.

Those 205 seropositive children live in 176 houses distributed as follows: 150 houses with a single case in each; 23 houses with double cases in each, and 3 houses with three cases in each. The location of houses with seropositive cases detected during both surveys could be seen in Figure 89, where the distribution is dispersed in space.

The 176 houses with positive cases constitute 15% of the houses in the study area, as shown in Figure 68. Houses found positive with these cases were either isolated or were located at the periphery of the village.

The following Table number 37 and Figure 67 show the incidence of the positive cases per one thousand of the general population according to the census.

Table 37. Per thousand positivity of the population in the study area as tested by IFAT.

Age	Population according to census	Positive in 1st survey	Age incidence/ 1000 of population	Positive in 2nd survey	Age incidence/ 1000 of population	Both surveys positive	Age incidence/1000 of population
-1	607	10	16.5	20	32.9	30	49.4
-2	537	14	26.1	21	39.1	35	65.2
-3	511	16	31.3	31	60.7	42	82.2
-4	447	18	40.3	14	31.3	27	60.4
-5	479	12	25.1	21	43.8	27	56.4
-6	407	15	36.9	15	36.9	24	58.9
-7	415	13	31.3	13	31.3	20	48.2
Total	3403	98	28.8	135	39.7	205	60.2

From Table 37 above the positivity seems to start early in life with the under one year of age, then the peak will be around three years of age, then the positivity declines with older children.

Figure 67 IFAT seropositivity rates per thousand of the population of the study area in the first, second and both surveys.

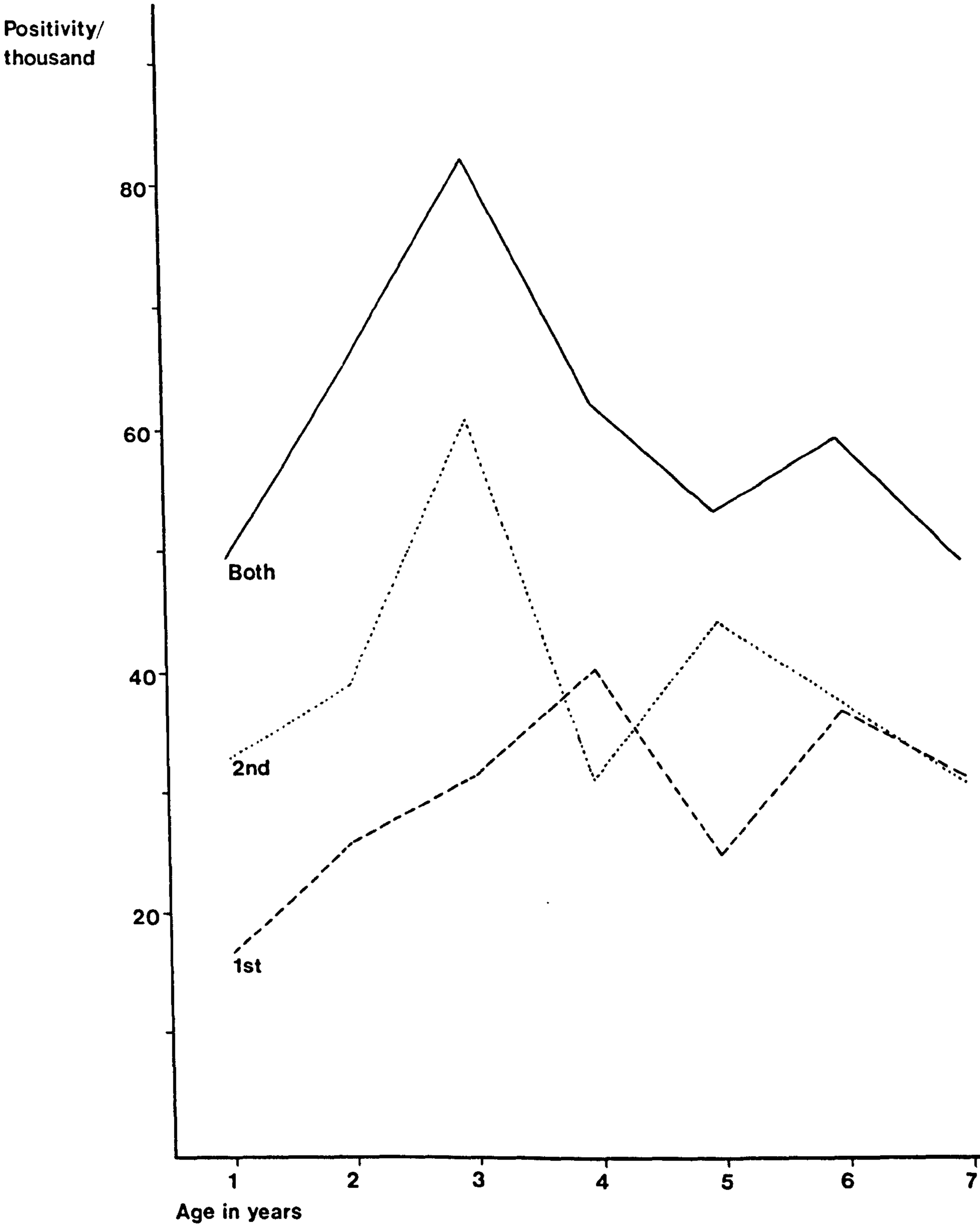


Figure 68 Showing percent of houses found with IFAT seropositive cases in the pilot, first survey, second and both surveys.

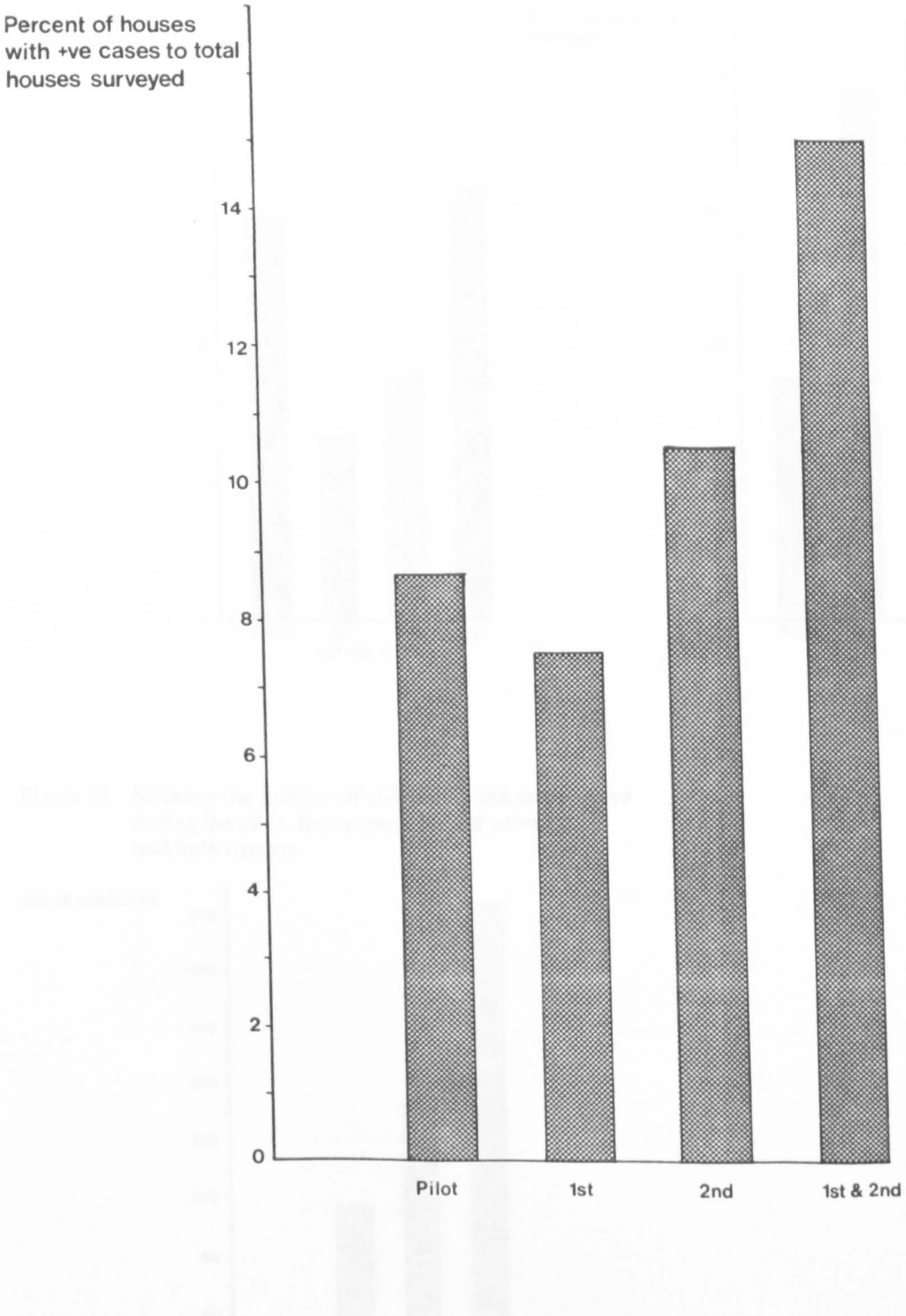


Figure 69 Seropositivity rate of children examined during the pilot, first, second and both surveys.

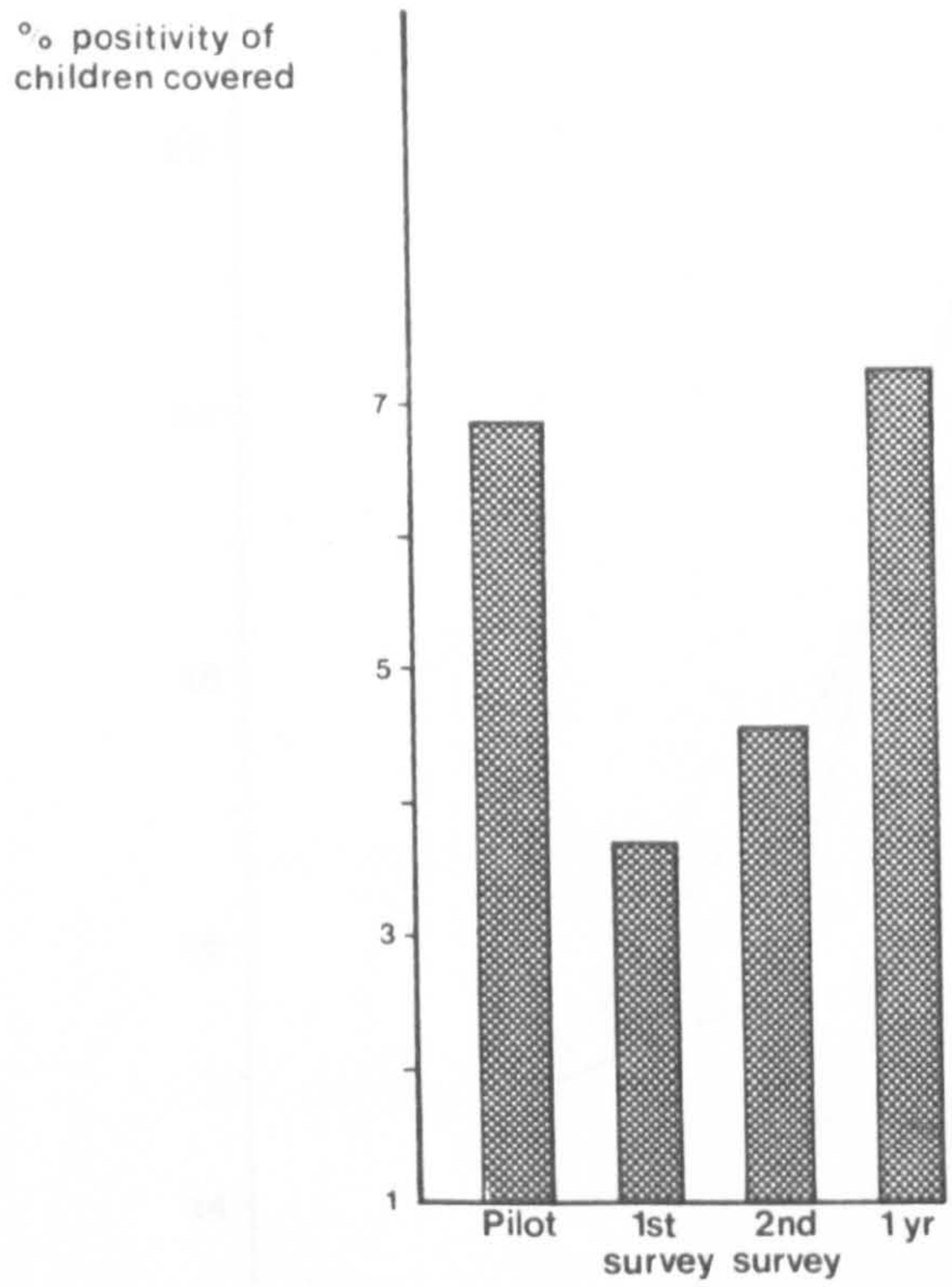


Figure 70 Showing the coverage of the target children by the pilot, first, second, and both surveys.

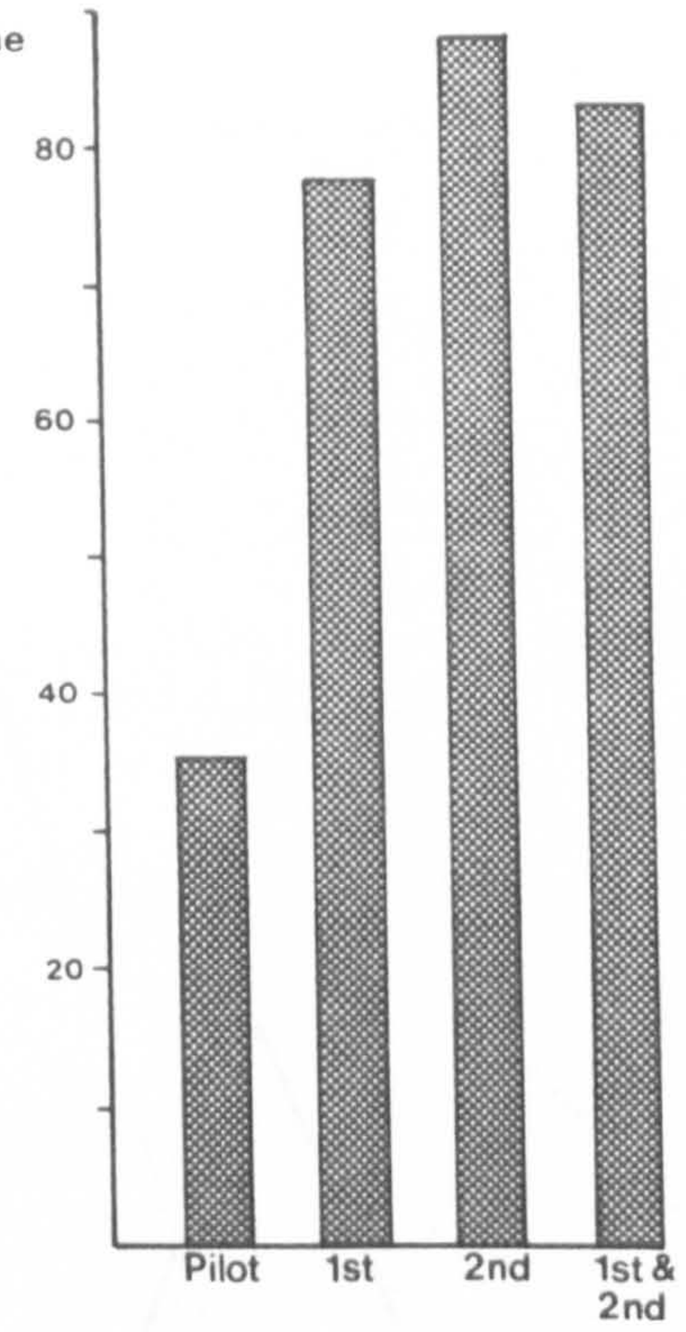


Figure 71 Showing the number of children found seropositive during the pilot, first survey, second survey, and both surveys.

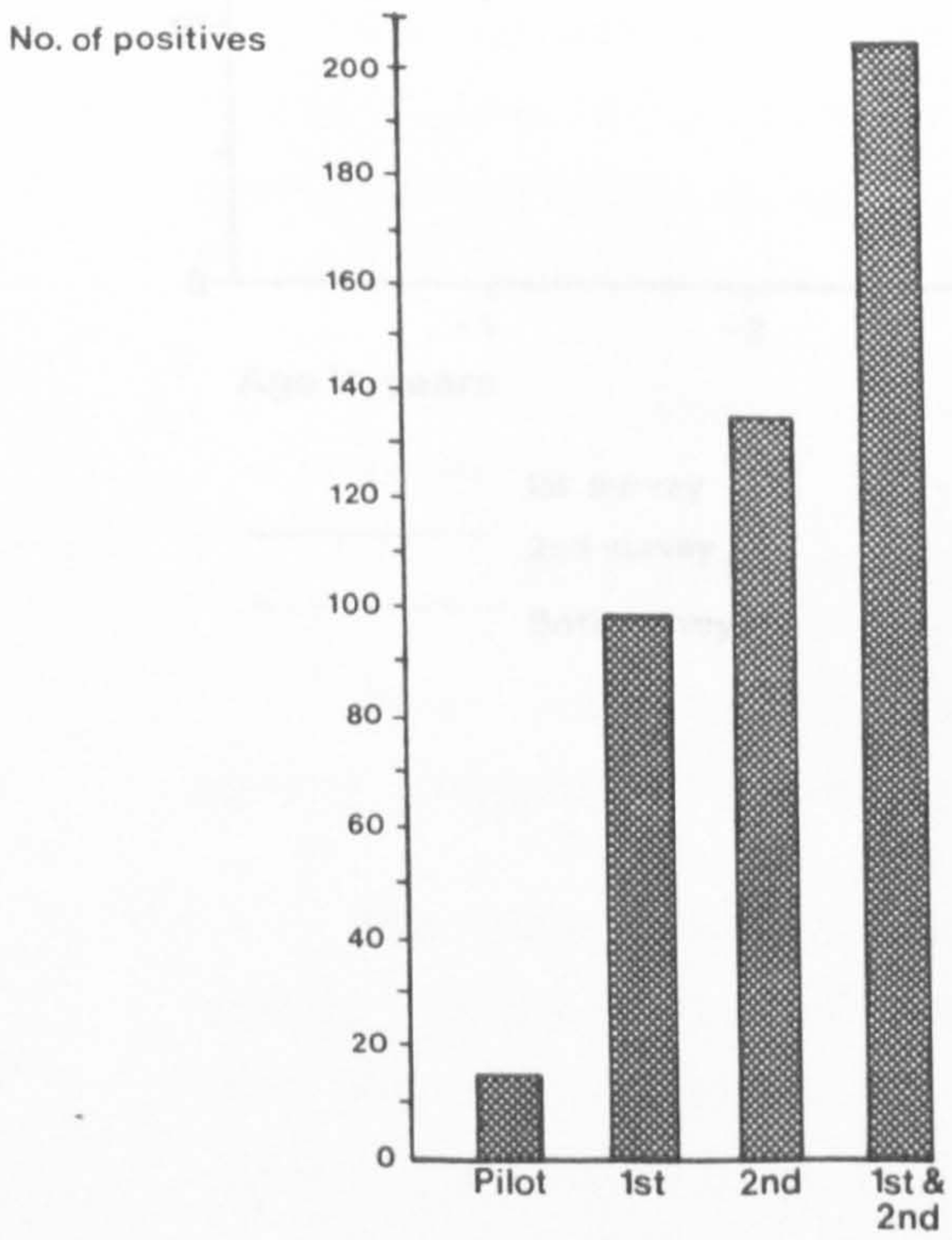


Figure 72 Age distribution of IFAT seropositives in the first, second, and both surveys.

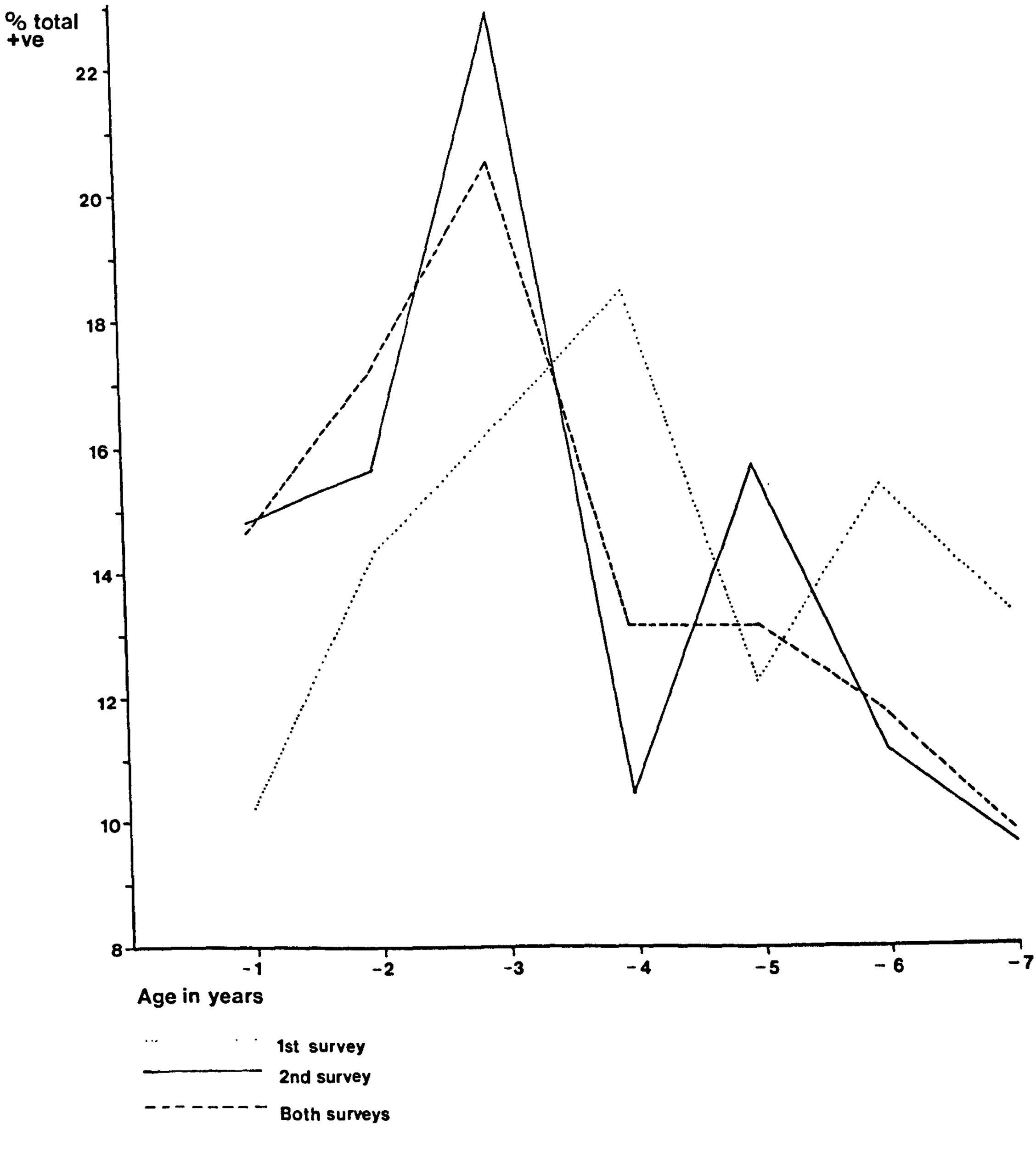


Table 38. The distribution of the IFAT seropositive cases in either survey according to age.

Age	Number	Percentage of total
-1	30	14.6%
-2	35	17.1%
-3	42	20.1%
-4	27	13.2%
-5	27	13.2%
-6	24	11.7%
-7	20	9.8%
Total	205	100.0%

Table 39. Sex distribution of IFAT seropositives in both surveys.

	1st survey		2nd survey		Common	Both	
	No.	%	No.	%	No.	No.	%
Males	48	48.9	74	54.8	15	107	52.2
Females	50	51.1	61	45.2	13	98	47.8
Total	98	100.0	135	100.0	28	205	100.0

The distribution of cases according to age, as shown in Table 38 and Figure 72, is similar to what has been shown about the distribution of parasitologically confirmed cases of visceral leishmaniasis discussed earlier:

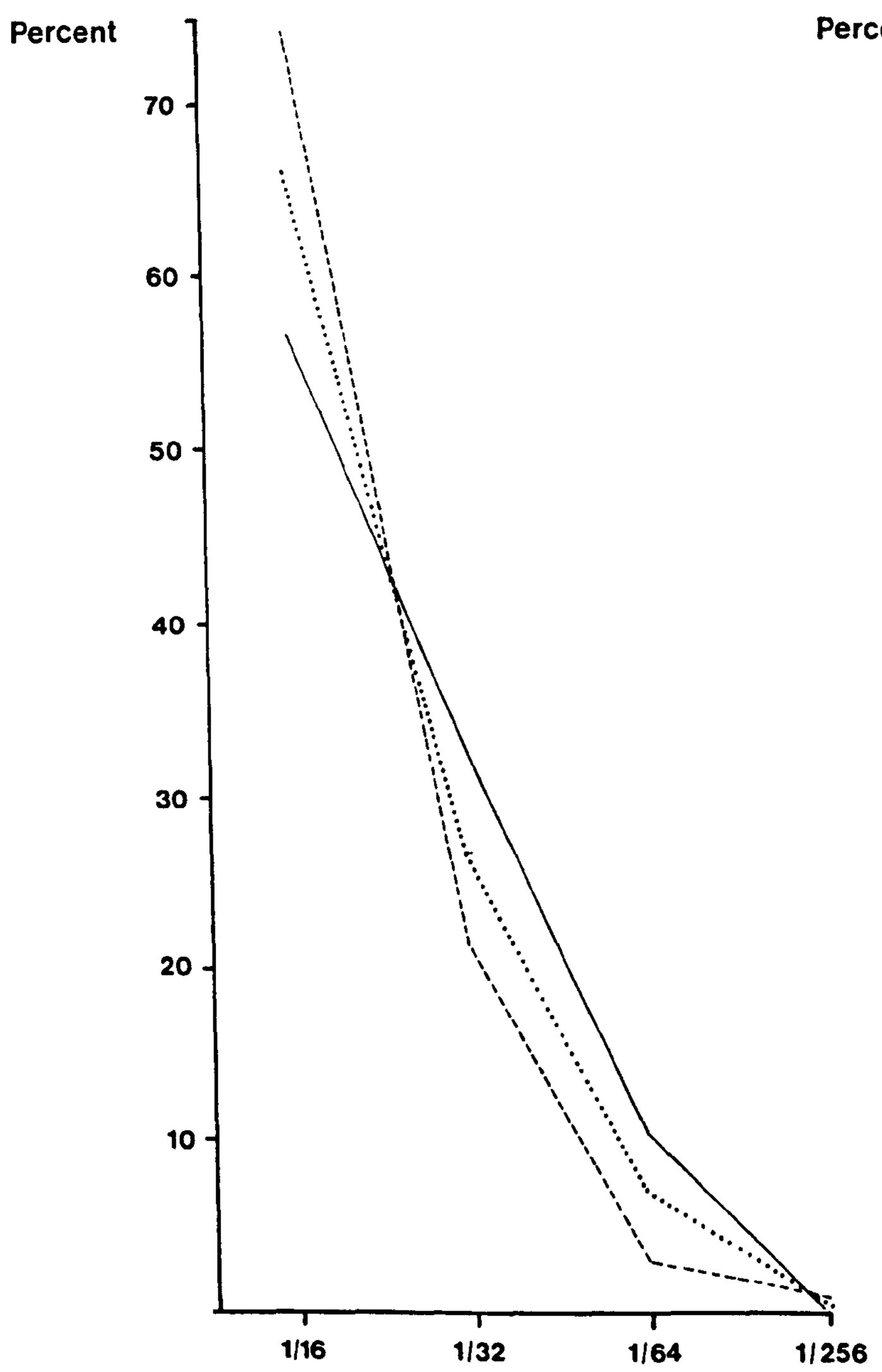
14.6% were under one year of age
13.7% were under two years of age
51.7% were under three years of age.

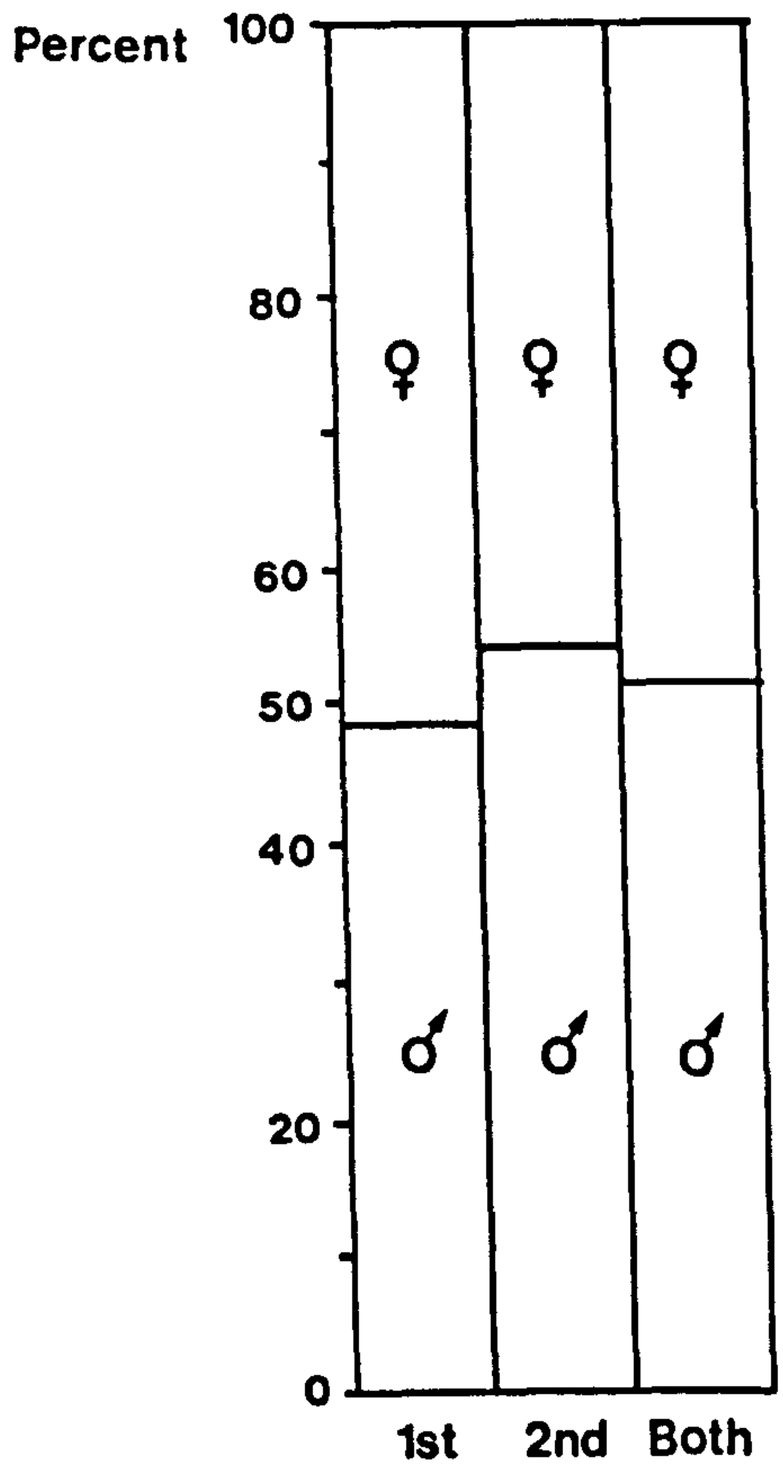
There was a shift of age peak towards younger ages in the second survey compared with the first.

The distribution of cases according to age showed an increase during the first year of life, reaching a peak at the third year and then the curve began to decline to a low level when reaching the seventh year of life.

So bearing in mind the lag time of the duration of positivity after infection, the only thing which favours more younger age groups in the confirmed cases of visceral leishmaniasis is the acuity of the

Figure 73 Showing percent distribution of IFAT titres among the positives in the study area in both surveys; it also shows their sex distribution.





disease and the severity of the symptoms.

107 (52%) of those positives were males and 98 (47.8%) were females. This is shown in Table 39 and Figure 73.

The distribution of the positive cases according to the IFAT titre of positivity, as illustrated in Table 40 and Figure 73, shows that

136 cases (66.3%) were having a 1/16 titre
54 cases (26.3%) with 1/32 titre
14 cases (6.8%) with titre, and
1 case (0.5%) with 1/128 titre.

Table 40. Showing the distribution of IFAT titre of the positives in the first, the second and in both surveys (IFAT positive is 1/16 and more).

IFAT titre	1st survey		2nd survey		Common	Both surveys	
	No.	%	No.	%	No.	No.	%
1/16	73	74.5	77	57.0	14	136	66.3
1/32	21	21.4	44	32.6	11	54	26.3
1/64	3	3.1	14	10.4	2	14	6.8
1/128	1	1.0	0	0.0	1	1	0.5
Total	98	100.0	135	100.0	28	208	100.0

Table 41. Mean IFAT titre against age in either surveys with the standard deviation, the standard error and the number in each age group.

Age	Mean IFAT titre	Standard Deviation	Standard error	Number
-1	2.7	1.53	0.28	30
-2	2.9	1.22	0.21	35
-3	2.6	1.17	0.18	42
-4	2.8	1.13	0.22	27
-5	2.9	1.01	0.19	27
-6	3.0	1.18	0.24	24
-7	3.8	1.75	0.39	20

It seems from this Table number 41 and from Figure 74 that mean IFAT titre has the tendency to increase with age, in the first seven years of life studied here.

The positivity rate of blood samples examined shows that it is low at the beginning of the survey and until April when it starts to increase, reaching a maximum positivity rate during December - February period, a picture which resembles the time incidence of visceral leishmaniasis in Iraq.

The following Table 42 and Figure 75 are illustrative of this.

Table 42. Positivity among sera collected according to the time of collection. (Positivity is an IFAT titre of 1/16 or more).

Months	Number of blood samples	No. of Positives	Percentage
April and May	2119	10	2.34
June, July and August	1602	60	4.70
September, October and November	3093	137	4.40
December, January and February	517	26	5.00
Total	5631	233	

The mean titre of IFAT of all the positives detected during the course of the study in one year whether a new case of a follow up of an already detected case distributed according to the month of collection shows the distribution presented in Table 43.

Table 43. Showing mean IFAT titre for each month of the work.

	Number of positives	Mean IFAT titre
April 1979	3	4.0
May	4	3.0
June	20	2.7
July	46	2.3
August	21	2.1
September	59	2.5
October	73	2.8
November	50	2.8
December	35	3.0
January 1980	17	2.8
February	33	3.0
March	4	4.0

Figure 74 Showing the mean IFAT titre in each age group among seropositives detected during the first and second surveys (± 1 S.E.)

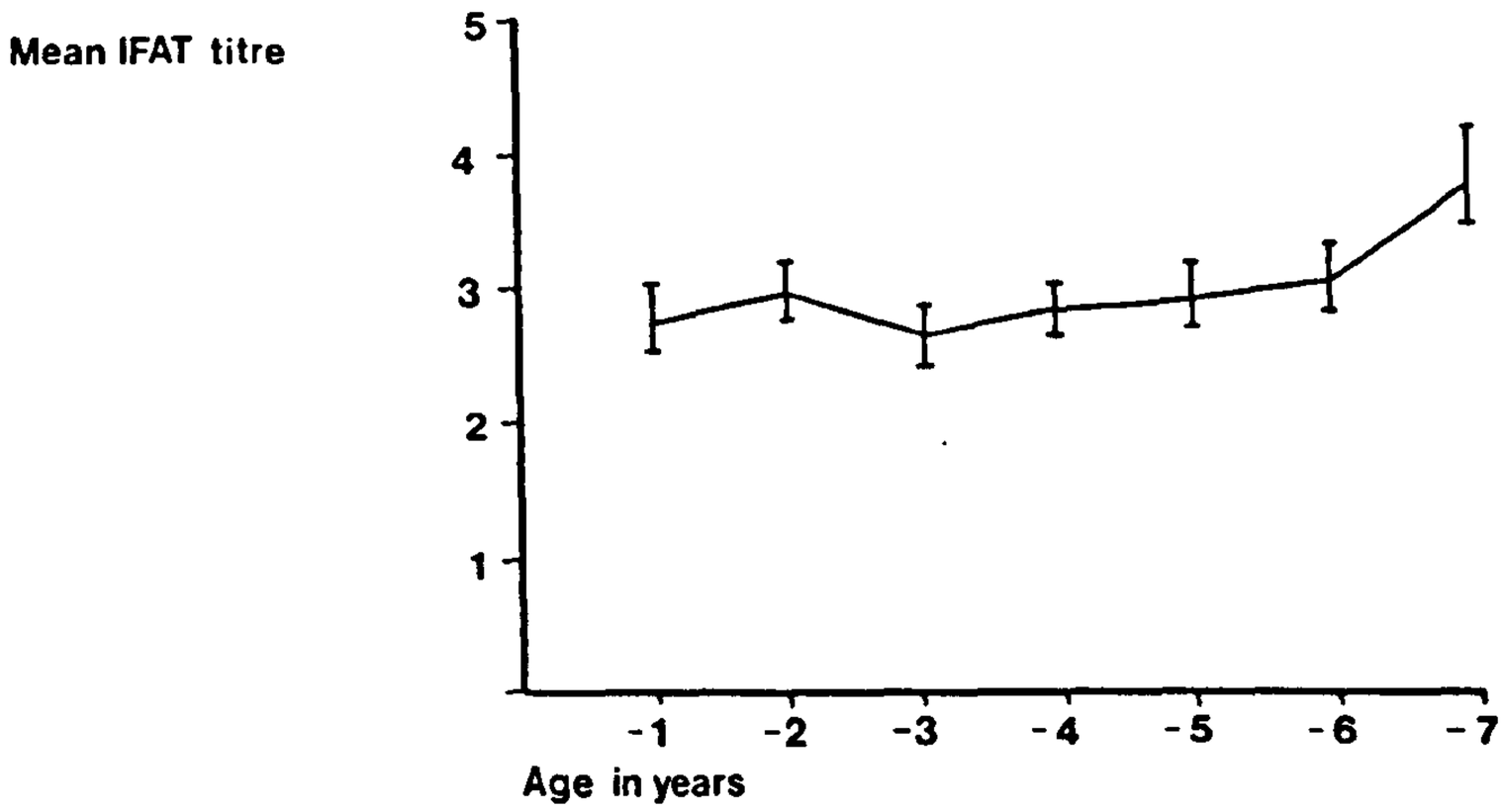


Figure 75 Showing the positivity rate of collected sera during the two surveys, distributed according to months.

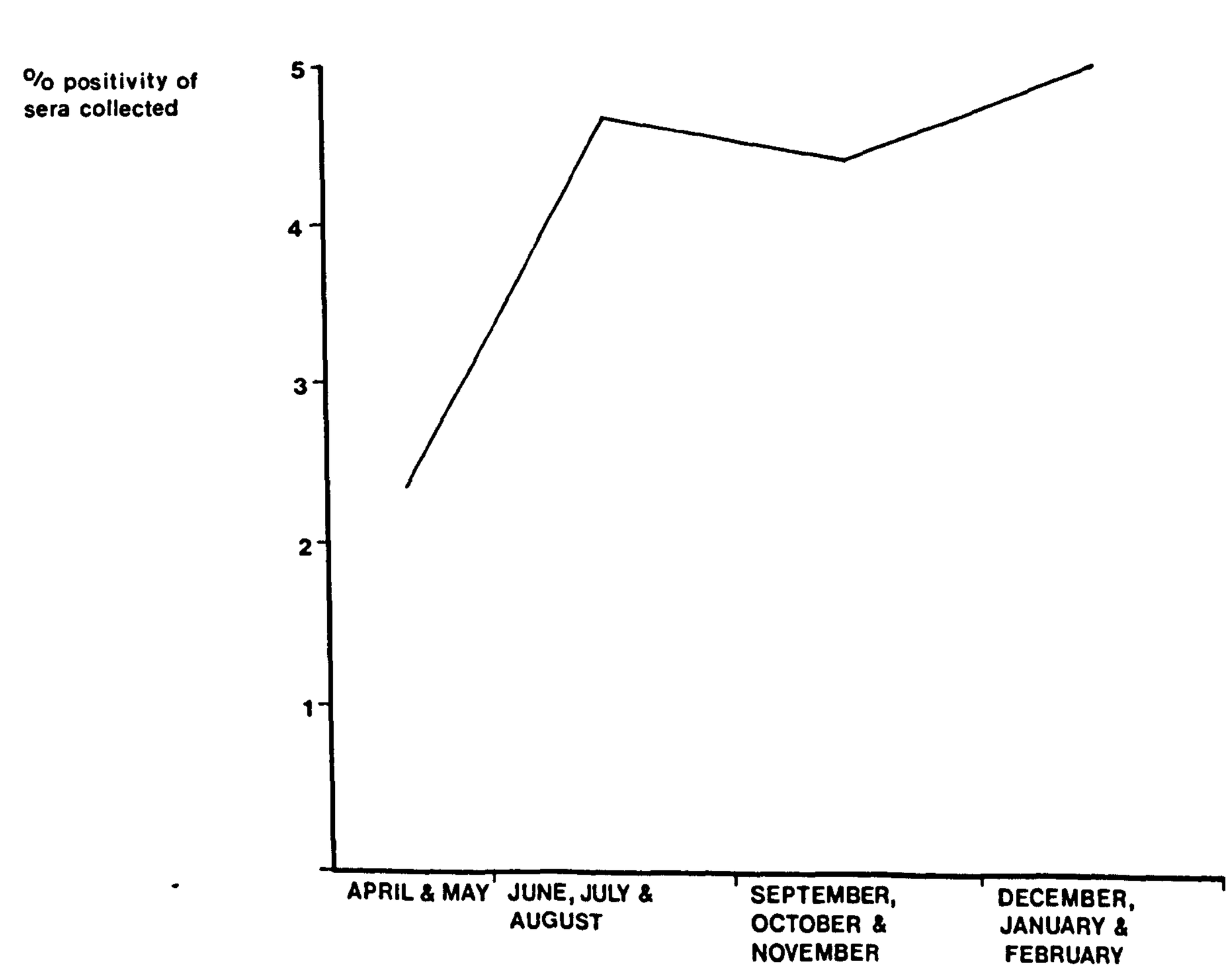
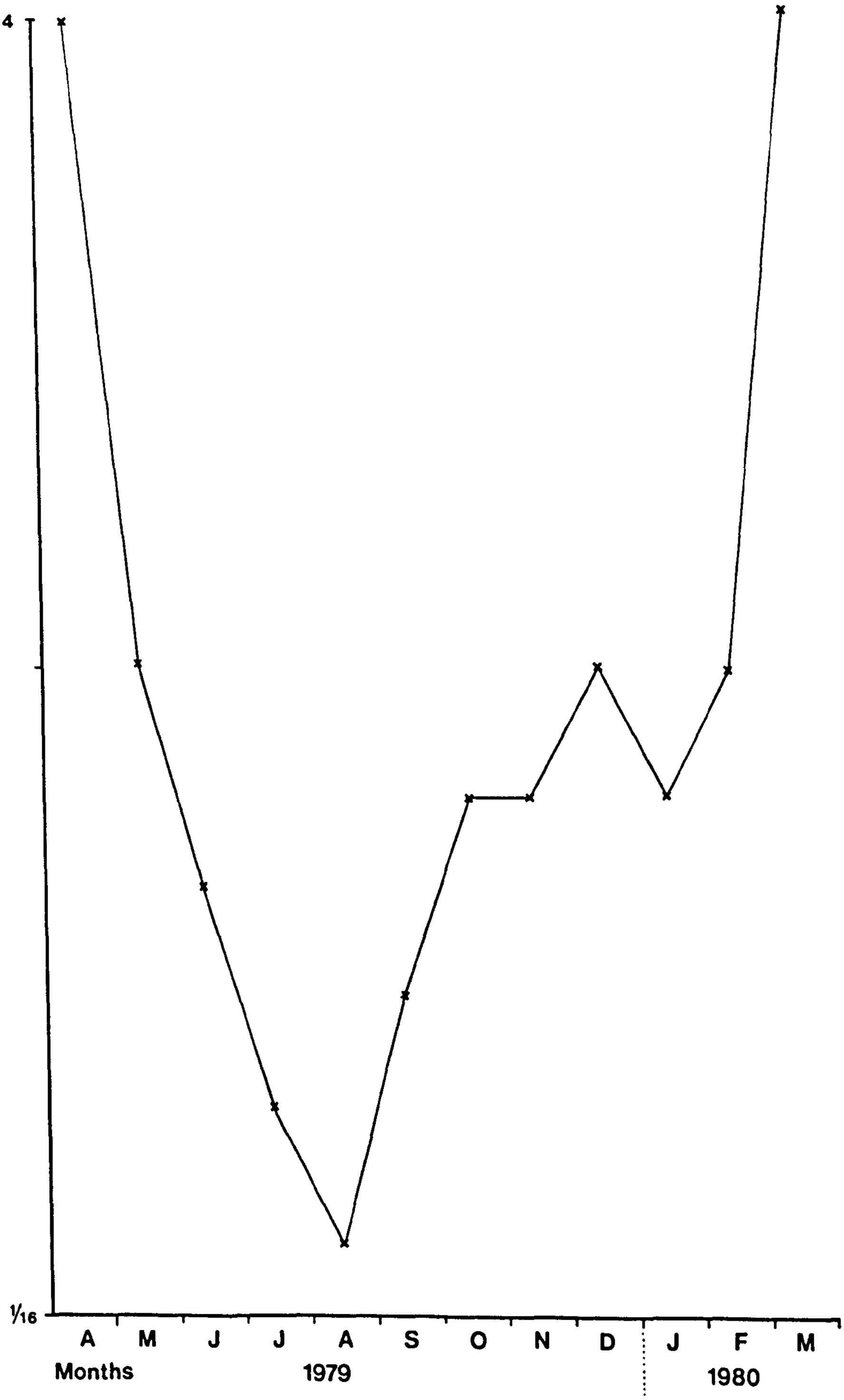


Figure 76 Showing the mean IFAT titre of positives detected during the two surveys.



Looking at Figure 76, which illustrates the same information, an obvious increase in mean titre readings coincides with the usual appearance of cases of visceral leishmaniasis in Iraq.

The following Figures 77 and 78 and Tables 44 and 45 show coverage in activity done during follow up of the positive cases; they also show the positivity of the activity.

The results show clearly that

65% of cases show enlargement of the spleen and liver
57% had leucopenia
2% of them were bone marrow positive
63% were leishmanin positive
66% show changes in serum proteins and immunoglobulin levels

Of 35 control seronegative children living in the area who were screened by physical examination only one showed positive spleen on deep inspiration (2.9%).

Table 44. Details of investigations done on 205 IFAT seropositive cases detected during one year. (IFAT positive is 1/16 and more)

	1st survey (98 positives)	% examined to total positives	2nd survey (135 positives)	% examined to total positives	Both 205 positives	% examined to total positives
Haemoglobin	61	62.2%	129	95.6%	168	82.0%
WBC	9	9.2%	75	55.6%	81	39.5%
Lymphocytes	85	86.7%	125	92.6%	189	92.2%
Monocytes	85		121	89.6%	185	90.0%
Liver and spleen	89	90.8%	131	97.0%	184	89.8%
Fever	92	93.9%	134	99.3%	199	97.1%
Cough	60	61.2%	116	85.9%	159	77.6%
Diarrhoea	32	32.7%	111	82.2%	132	64.4%
Epistaxis	21	21.4%	92	68.1%	104	50.7%
Weight loss	66	67.3%	111	82.2%	160	78.0%
BCG	54	55.1%	115	85.2%	150	73.2%
Leishmanin	17	17.3%	89	65.9%	97	47.3%
BM	0	0	6	4.4%	6	2.9%
BM +ve	0	0	4	3.0%	4	2.0%
IgG and serum proteins	10	10.2%	13	9.6%	15	7.3%

Table 45. Results of follow up investigations carried out on IFAT seropositive cases during the two surveys (IFAT positive is 1/16 and more).

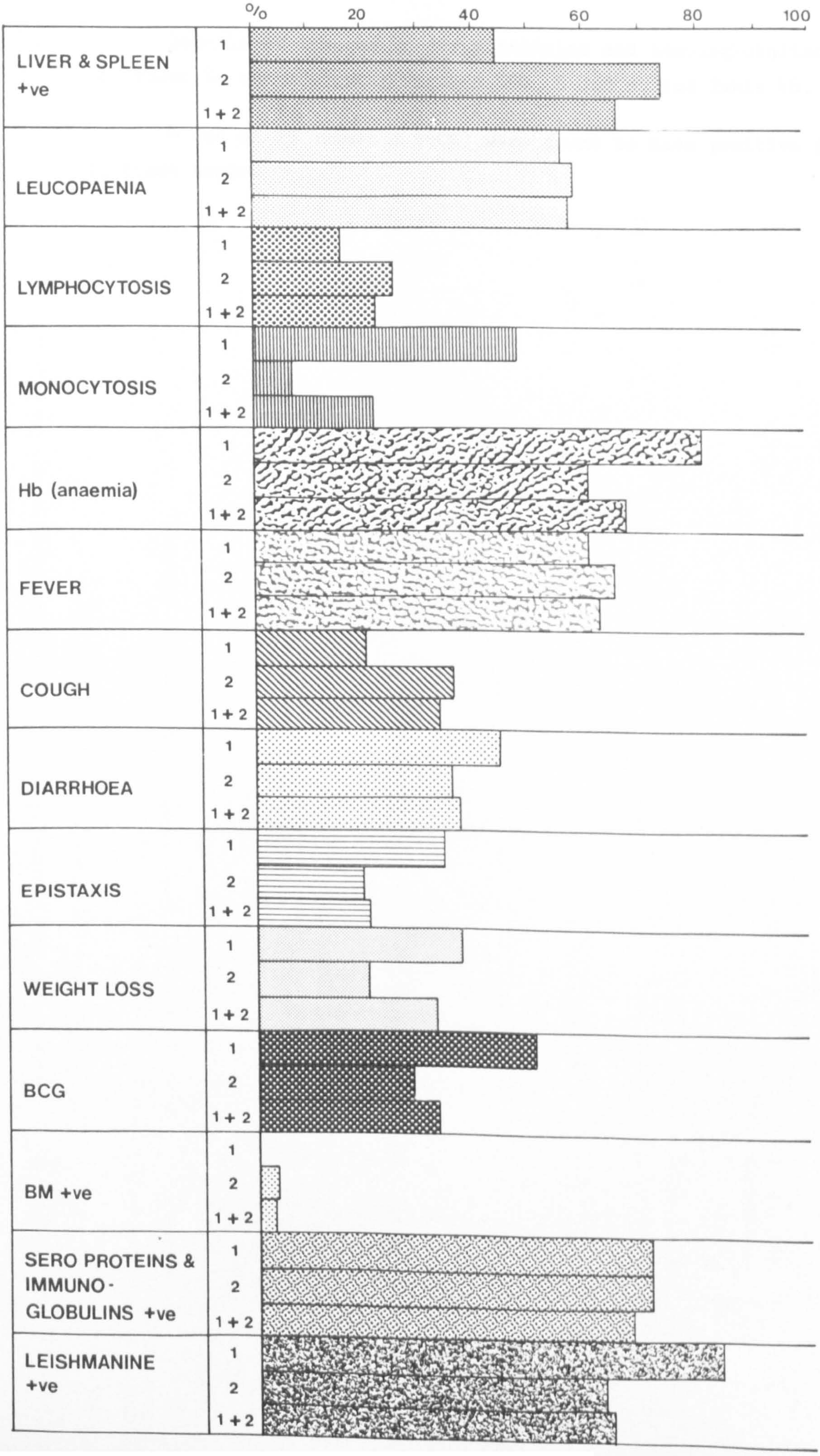
		1st survey No.examined	%	2nd survey No.examined	%	Common	Both surveys No.examined	%
	< 70	49	80.3	77	59.7	15	111	66.1
	70-81	12	19.7	51	39.5	7	556	33.3
Hb	> 80	0	0.0	1	0.7	0	1	0.6
	Total	61	100.0	129	100.0	22	168	100.0
	< 5000	5	55.55	43	57.3	2	46	56.8
Leuco-	5000-10000	4	44.44	32	42.7	1	35	43.2
cytes	> 10000	0	0.0	0	0.0	0	0	0.0
	Total	9	100.0	75	100.0	3	81	100.0
	< 25	20	23.5	12	0.6	5	27	14.3
Lympho-	25-33	52	61.2	82	65.6	12	122	64.6
cytes	> 33	13	15.3	31	24.8	4	40	21.2
	Total	85	100.0	125	100.0	21	189	100.0
	< 3	0	0.0	21	17.4	0	21	11.4
Mono-	3-7	45	52.9	92	76.0	12	125	67.6
cytes	> 7	40	47.1	8	6.6	9	39	21.1
	Total	85	100.0	121	100.0.	21	185	100.0
Liver	+ve	0	0.0	5	3.8	0	5	2.7
spleen	-ve	50	56.2	34	25.95	20	64	34.8
Liver spleen	+ve) -ve)	29	32.6	45	34.4	10	64	34.8
Liver spleen	-ve) +ve)	10	11.2	47	35.9	6	51	27.7
	Total	89	100.0	131	100.0	36	184	100.0
Fever	yes	55	59.8	87	64.9	19	123	62.8
	no	37	40.2	47	35.1	8	76	38.2
	Total	92	100.0	134	100.0	27	199	100.0
Cough	yes	12	20.0	41	35.3	1	52	32.7
	no	48	80.0	75	64.7	16	107	67.3
	Total	60	100.0	116	100.0	17	159	100.0
Diarrhoea	yes	14	43.75	39	35.1	5	48	36.4
	no	18	56.25	72	64.9	6	84	63.6
	Total	32	100.0	111	100.0	11	132	100.0

Table 45. (Continued)

		1st survey No. examined	%	2nd survey No. examined	%	Common	Both surveys No. examined	%
Epistaxis	yes	7	33.3	18	19.6	4	21	20.2
	no	14	66.6	74	80.4	5	83	79.8
	Total	21	100.0	92	100.0	9	104	100.0
Weight loss	yes	24	36.4	33	29.7	6	51	31.9
	no	42	63.6	78	70.3	11	109	68.1
	Total	66	100.0	111	100.0	17	160	100.0
BCG	yes	27	50.0	32	27.8	11	48	32.0
	no	27	50.0	83	72.2	8	102	68
	Total	54	100.0	115	100.0	19	150	100.0
BM	+ve	0	0.0	4	3.0	0	4	2.0
Serum proteins & immunoglobulins	+ve	7	70.0	9	69.2	6	10	66.7
Leishmanin	+ve	14	82.4	55	61.8	8	61	62.9

Figure 77 Coverage of IFAT seropositive children according to type of examination or test in the first survey, second survey and both surveys.

Figure 78 Results of IFAT seropositive findings in the first survey, second survey and in both surveys.



Details of changes in serum proteins and immunoglobulins are shown in the following Figures 79a, b and c, and Table 46.

9 out of 15 examined (60%) were found to have positive findings in these tests.

Table 46. Serum protein and immunoglobulin abnormalities found among IFAT seropositive cases detected during the two surveys (IFAT positive is 1/16 and more).

Name	1st survey	2nd survey	IgG	IgM	Hyperproteinaemia	Albumin	Globulin	α1	α2	β
F.S.	+	+							0.852	
M.M.	+	+	1760							
I.H.	+			200						
W.M.	+	+							0.905	
L.H.	+	+							0.936	
Kh.H.	+	+						0.526	1.147	1.004
F.J.	+									
H.K.	+	+								
H.A.	+	+			0.899				1.081	1.284
J.M.	+	+								
T.A.		+		196			3.916	0.502		
O.A.		+		215	10.15		4.780	0.450	1.110	1.110
K.I.		+		290					0.806	
H.H.										
Y.M.										
Percentage positivity			9.1%	36.4%	13.3%		13.3%	20%	46.7%	20%

Figure 79 Abnormal findings in serum proteins and immunoglobulins in the first survey (a), second survey (b), and in both surveys (c).

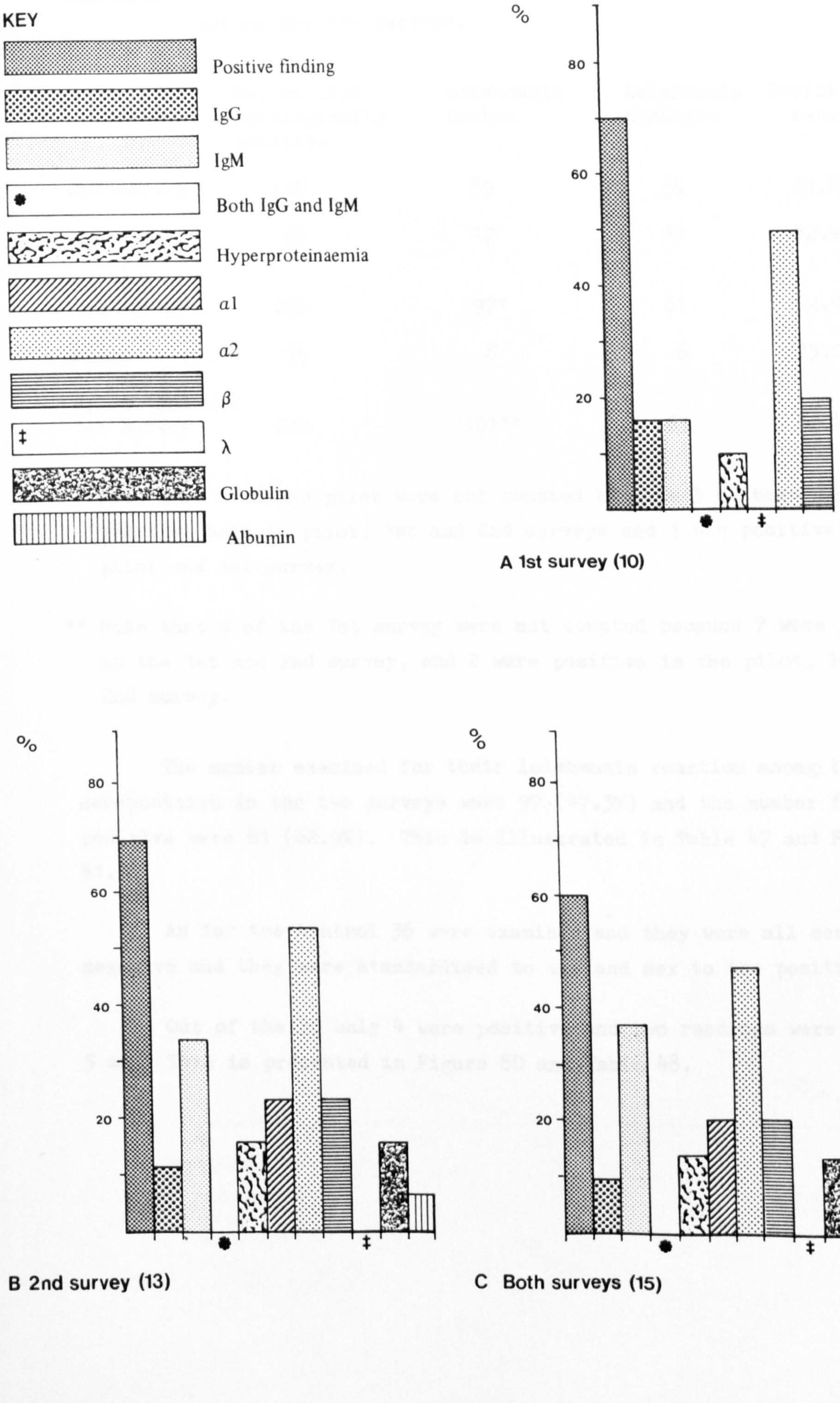


Table 47. Results of leishmanin tests on the IFAT seropositives detected during the two surveys.

	No. of IFAT serologically positive	Leishmanin tested	Leishmanin positive	Positivity rate
2nd survey	135	89	55	61.8%
1st survey	98	17	14	82.4%
2nd and 1st survey	205	97*	61	62.9%
Pilot	14	8	6	75.0%
Pilot, 2nd and 1st survey	213	101**	63	62.4%

* Note that 4 in the pilot were not counted because 3 of them were positive both in pilot, 1st and 2nd surveys and 1 was positive in the pilot and 1st survey.

** Note that 9 of the 1st survey were not counted because 7 were positive in the 1st and 2nd survey, and 2 were positive in the pilot, 1st and 2nd survey.

The number examined for their leishmanin reaction among the 205 seropositive in the two surveys were 97 (47.3%) and the number found positive were 61 (62.9%). This is illustrated in Table 47 and Figure 81.

As for the control 35 were examined and they were all serologically negative and they were standardised to age and sex to the positive group.

Out of the 35 only 4 were positive and two readings were below 5 mm. This is presented in Figure 80 and Table 48.

Figure 80 Percent distribution of leishmanine readings in the first, second, and in first and second surveys among the serologically positive and among serologically negative control children.

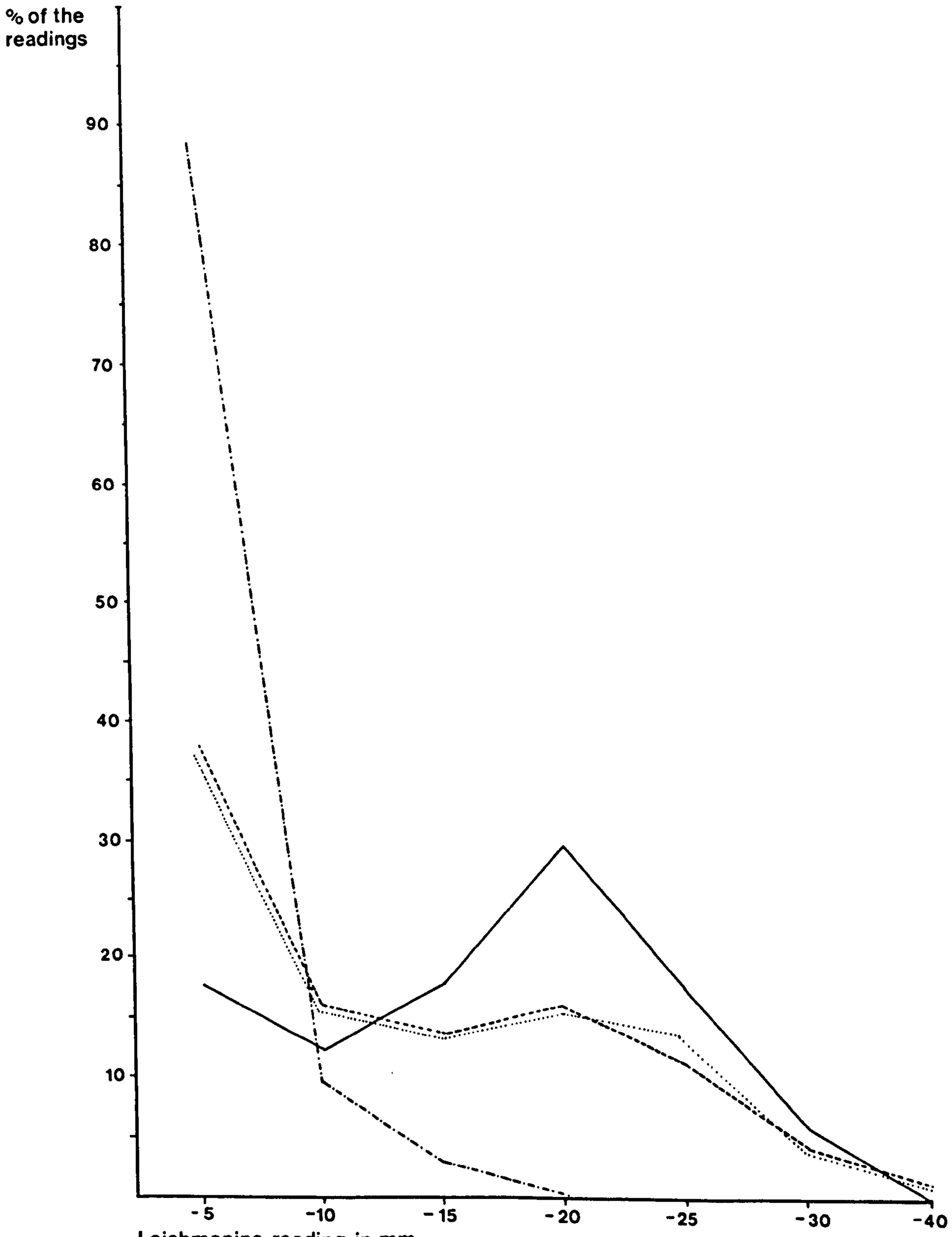


Figure 81 Leishmanine positivity.

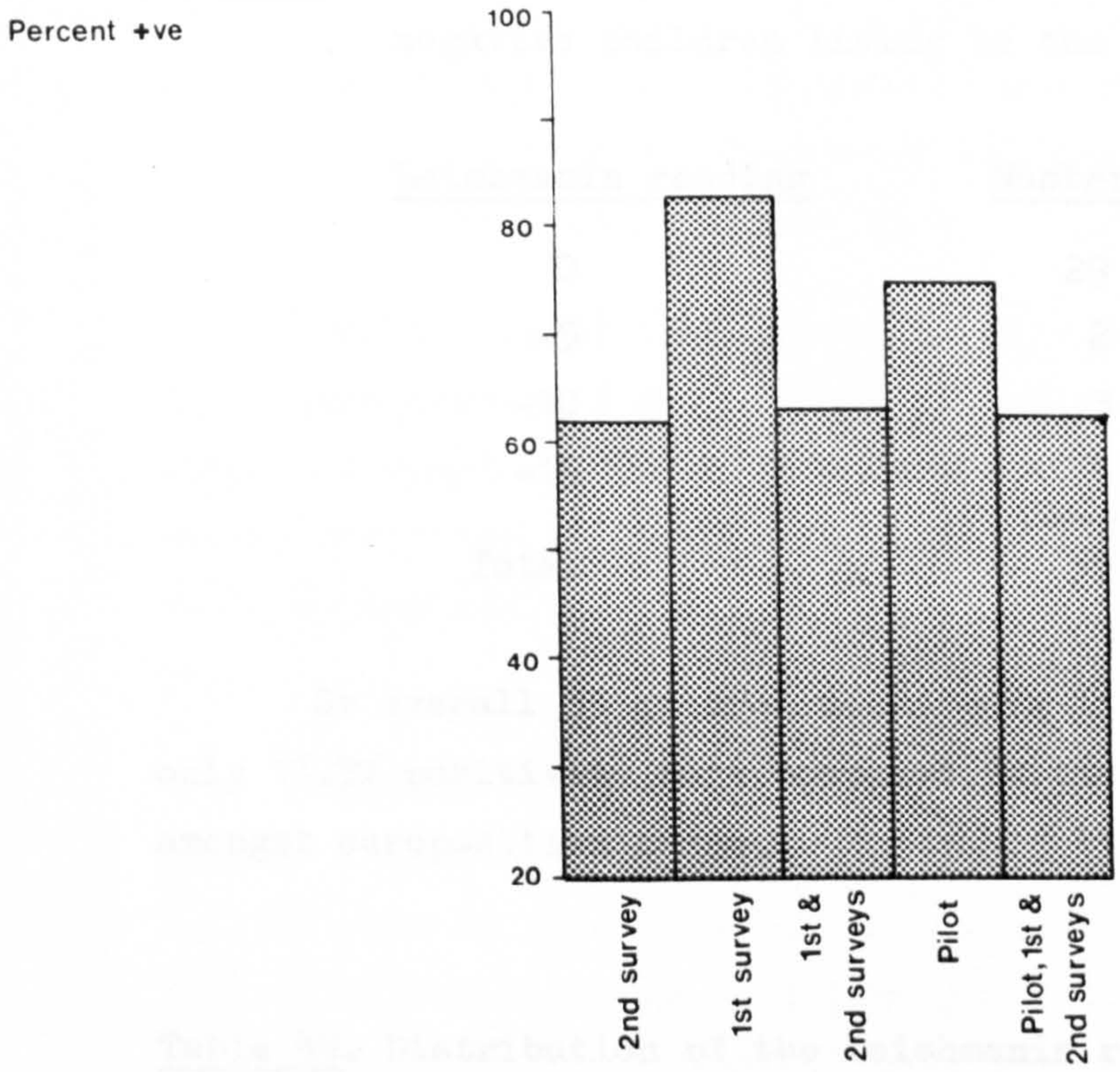


Figure 82 Showing the mean leishmanine reading for each age group among the positives in both first and second surveys.

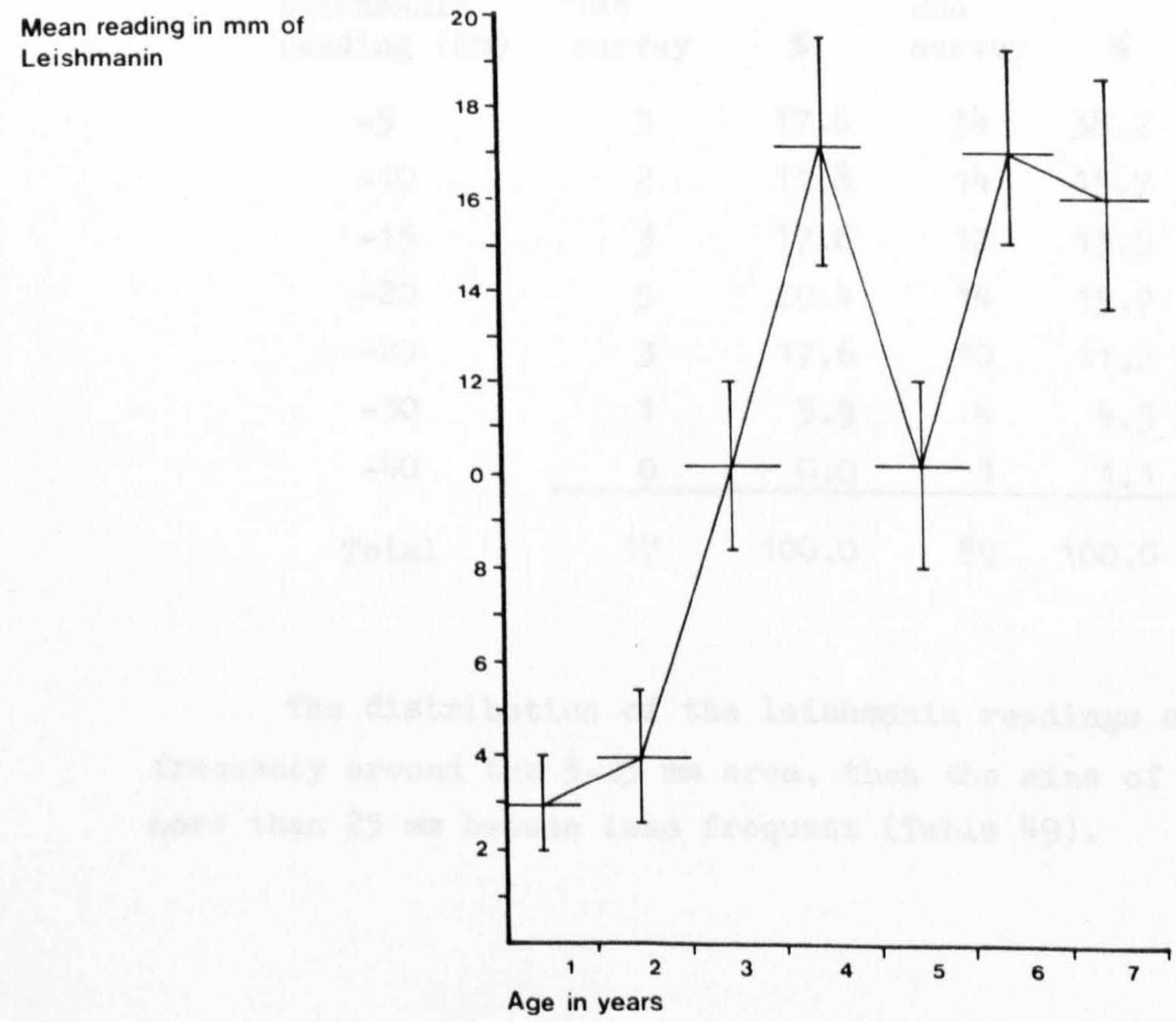


Table 48. Distribution of leishmanin readings amongst 35 sero-negative children living in the study area.

Leishmanin reading	Number	Percent	
0	29	82.8%)	
-5	2	5.7%)	88.5%
-10	3	8.6%	
-15	1	2.9%	
Total	35	100.0%	

So overall 35 control cases gave 88.5% negativity rate and only 11.5% positivity rate compared to the 62.9% positivity rate amongst seropositive cases.

Table 49. Distribution of the leishmanin readings among the IFAT positive cases in the first survey, in the second survey and in both surveys (IFAT positive is 1/16 and more).

Leishmanin reading (mm)	1st survey	%	2nd survey	%	Both surveys	%
-5	3	17.6	34	38.2	36	37.1
-10	2	11.8	14	15.7	15	15.5
-15	3	17.6	12	13.5	13	13.4
-20	5	29.4	14	15.7	15	15.5
-25	3	17.6	10	11.2	13	13.4
-30	1	5.9	4	4.5	4	4.1
-40	0	0.0	1	1.1	1	1.0
Total	17	100.0	89	100.0	97	100.0

The distribution of the leishmanin readings shows a high frequency around the 5-25 mm area, then the size of leishmanin reaction more than 25 mm become less frequent (Table 49).

In the positive cases detected during both surveys the mean leishmanin reading and rate of positivity of leishmanin seems to be increasing with age, as in Figures 82 and 84 and Tables 50 and 51, the under one year olds are almost all negative then readings start to increase and reach the peak at 4 - 6 years of age.

Positivity of those examined were higher in the first than in the second survey, a possibility of explanation is either that the sample examined in the first survey was small, or that in the second survey positives were found during the time when visceral leishmaniasis cases appear and delayed hypersensitivity did not have time to develop yet.

Another thing to be noticed is to compare the peak of age distribution of IFAT seropositives, as in Figure 72 with leishmanin reading against age, as in Figure 82, and in this respect it looks like the peak of higher readings of leishmanin follows the peak of age incidence of serapositives, the first have the peak around 4 - 6 years of age, the age incidence has the peak around three years of age. This difference is obvious and is due to the time lag between acquiring the infection and the time to develop the delayed hypersensitivity in the child.

Table 50 Leishmanin positivity by age among the seropositives detected in the first survey, second survey and in both surveys.

Age in years	First survey			Second survey			Both surveys		
	Number examined	Number positive	% positivity	Number examined	Number positive	% positivity	Number examined	Number positive	% positivity
-1	-	-	-	9	2	22%	9	2	22%
-2	3	1	33%	11	4	36%	14	5	36%
-3	1	1	100%	25	15	60%	25	15	60%
-4	4	4	100	8	7	88%	9	8	89%
-5	3	2	67%	16	10	63%	17	11	65
-6	2	2	100	12	11	92%	12	11	92%
-7	4	4	100	8	6	75	11	9	82%
Total	17	14	83%	89	35	82%	97	61	63

Table 51. Showing the mean leishmanin reading among the IFAT seropositives detected during the two surveys, according to age.

Age in years	Mean leishmanin reading for both surveys.	Standard deviation	Standard Error	Number
-1	3.1	3.22	1.07	9
-2	4.1	5.56	1.54	13
-3	10.8	10.17	2.03	25
-4	17.7	8.14	2.71	9
-5	10.5	9.02	2.19	17
-6	17	10.57	3.05	12
-7	16.6	9.3	2.8	11

Table 52. Leishmanin reading against mean titre of IFAT for both surveys.

Leishmanin reading	Mean IFAT titre	Standard deviation	Standard error	Number
-5	2.7	1.07	0.18	36
-10	3.2	1.28	0.33	15
-15	3.2	1.30	0.36	13
-20	3.5	1.67	0.43	15
-25	3.5	1.20	0.33	13
-30	4.5	1.91	0.96	4
-40	4.0			

It appears that mean IFAT titre seems to increase with positivity readings of the leishmanin, as seen in Table 52 and Figure 83.

Table 53. Leishmanin reading of IFAT seropositive cases detected during the two surveys distributed according to IFAT titre.

IFAT titre titre	Leishmanin reading in mm							
	-5	-10	-15	-20	-25	-30	-40	Total
1/16	26	7	6	6	4	1	-	50
1/32	10	6	6	5	8	1	1	37
1/64	1	1	1	4	1	2	-	10

Figure 83 Showing mean IFAT titre with ± 1 S.E. for each group of leishmanine readings in both the first and second surveys among the seropositives.

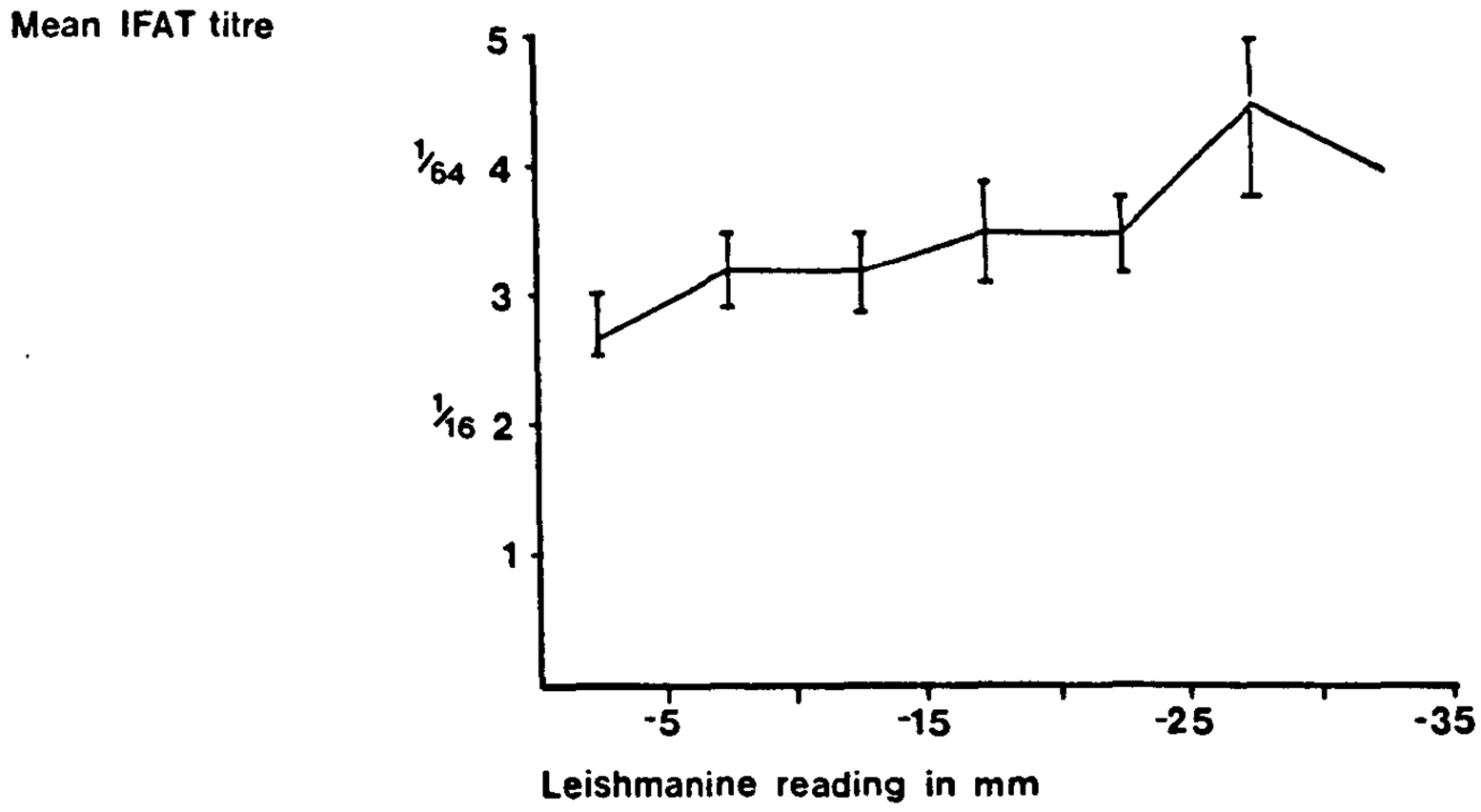


Figure 84 Showing positivity of leishmanine among IFAT seropositives in both surveys, distributed according to their ages.

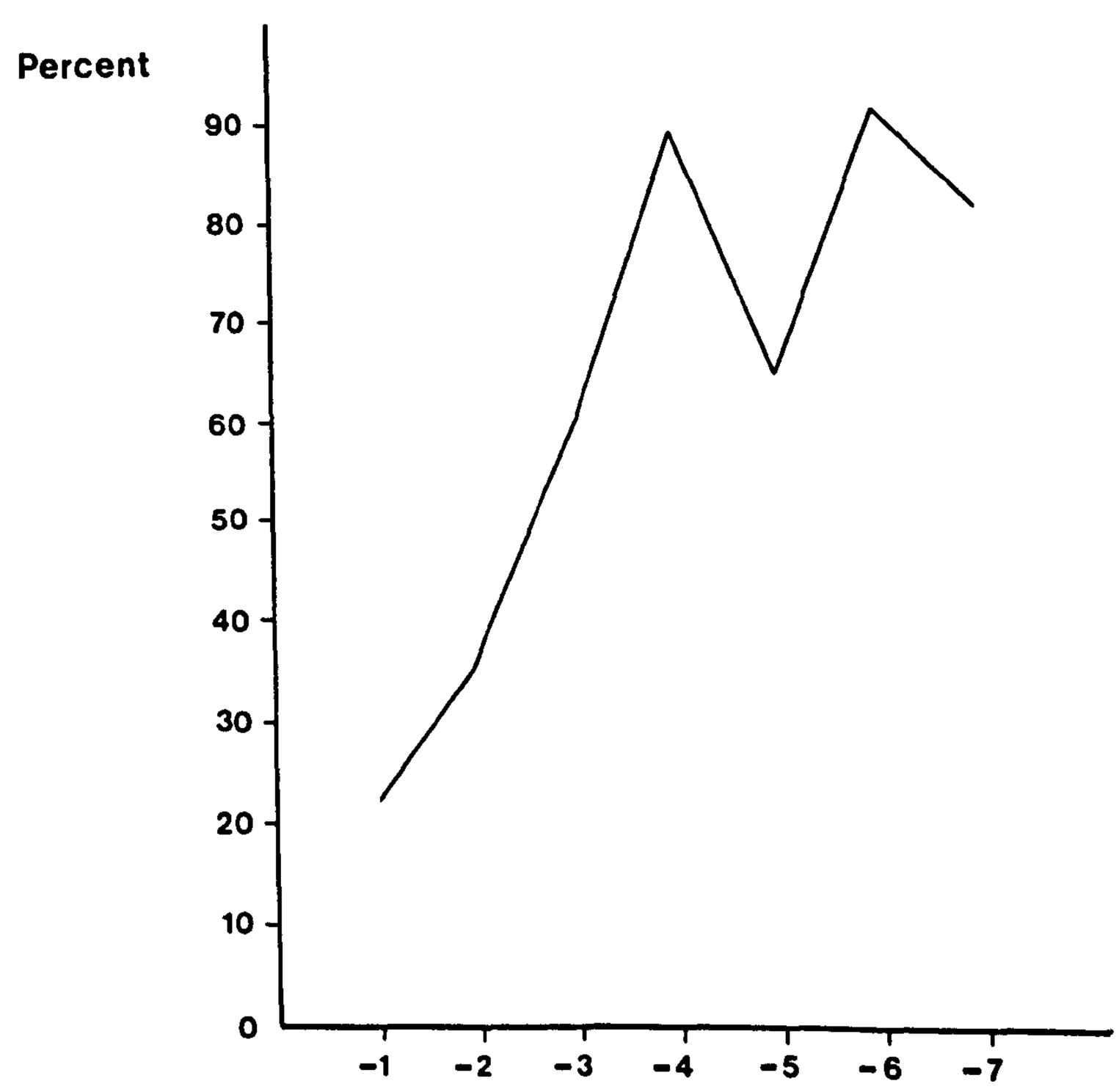


Figure 85 Showing mean ± 1 S.E. leishmanine reading for each titre of IFAT of the positives in the first and second surveys.

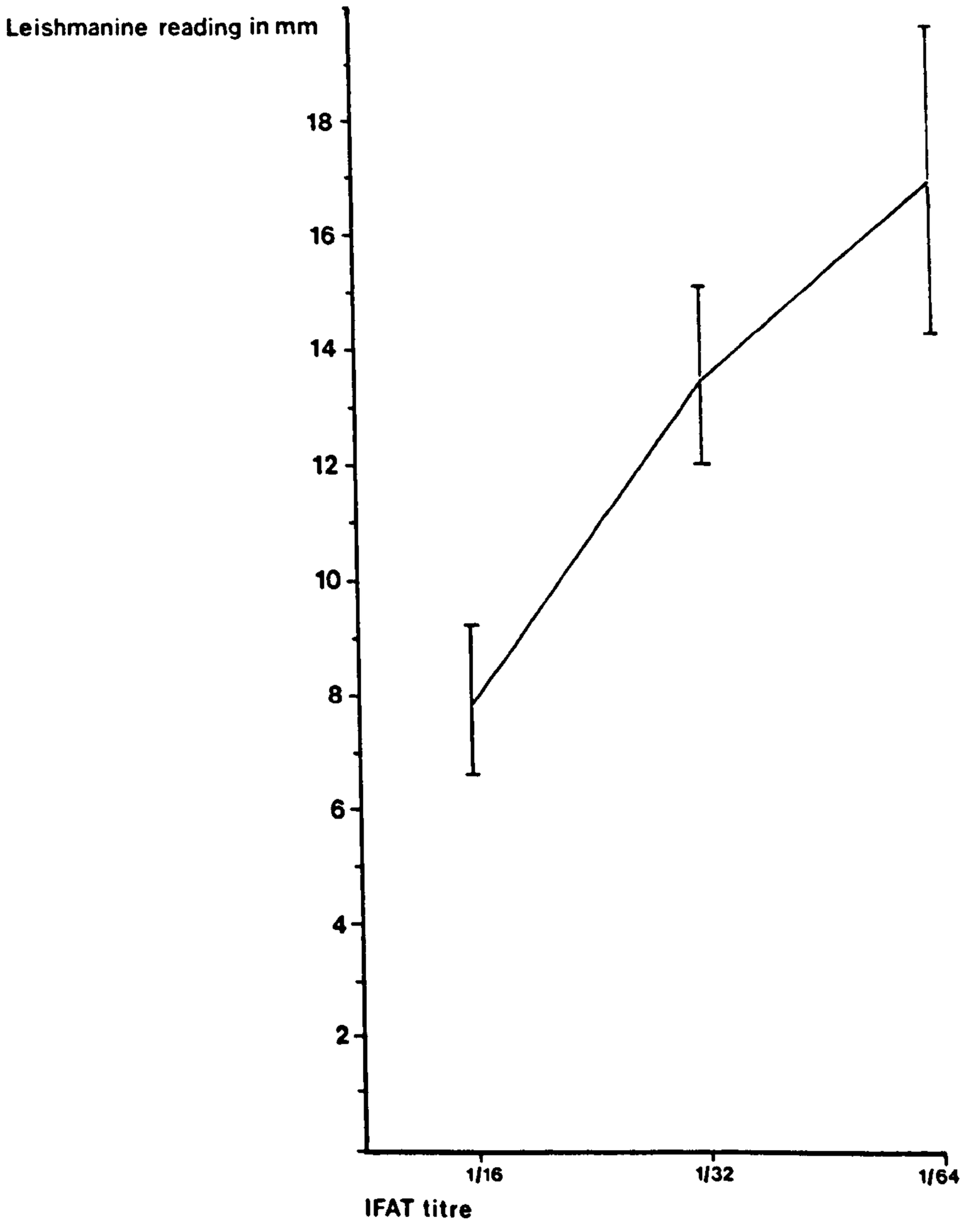


Figure 85 and Table 53, however, show that the low IFAT titre is associated with a low positive reading of leishmanin and as the titre rises the leishmanin reading increases, and it is more probable to get a positive leishmanin reading among high IFAT titres.

ELISA was done on sera of 132 of the IFAT seropositives (64% of the cases) and 60 of them (45.5%) were found to be positive also with this test. This could be seen clearly in Table 54 and Figure 86.

Reproducibility of the ELISA test used was done and sera were transferred to the Ross laboratories in London and it was found that there were agreements in the results when retesting the sera according to the following limits:

97% agreement with the negative sera tested by IFAT, 38% agreement with the results of positive sera having an IFAT titre of 1/16, 80% agreement with the results of positive sera having an IFAT of 1/32, and 100% agreement with the results of sera having an IFAT titre of 1/64 and 1/128.

Table 54. ELISA test of IFAT seropositives detected during the two surveys.

Total positives with IFAT		No.examined with ELISA	%	No. found positive with ELISA	% positivity
1st survey	98	69	70.4	31	44.9
2nd survey	135	87	64.4	44	50.6
Common	28	24	85.7	15	62.5
Both	205	132	64.4	60	45.5

Some of the positive cases were followed up until they were negative, as shown in Table 55 and Figures 87 and 88.

Figure 86 Showing the positivity detected by ELISA of the IFAT seropositives of the surveys.

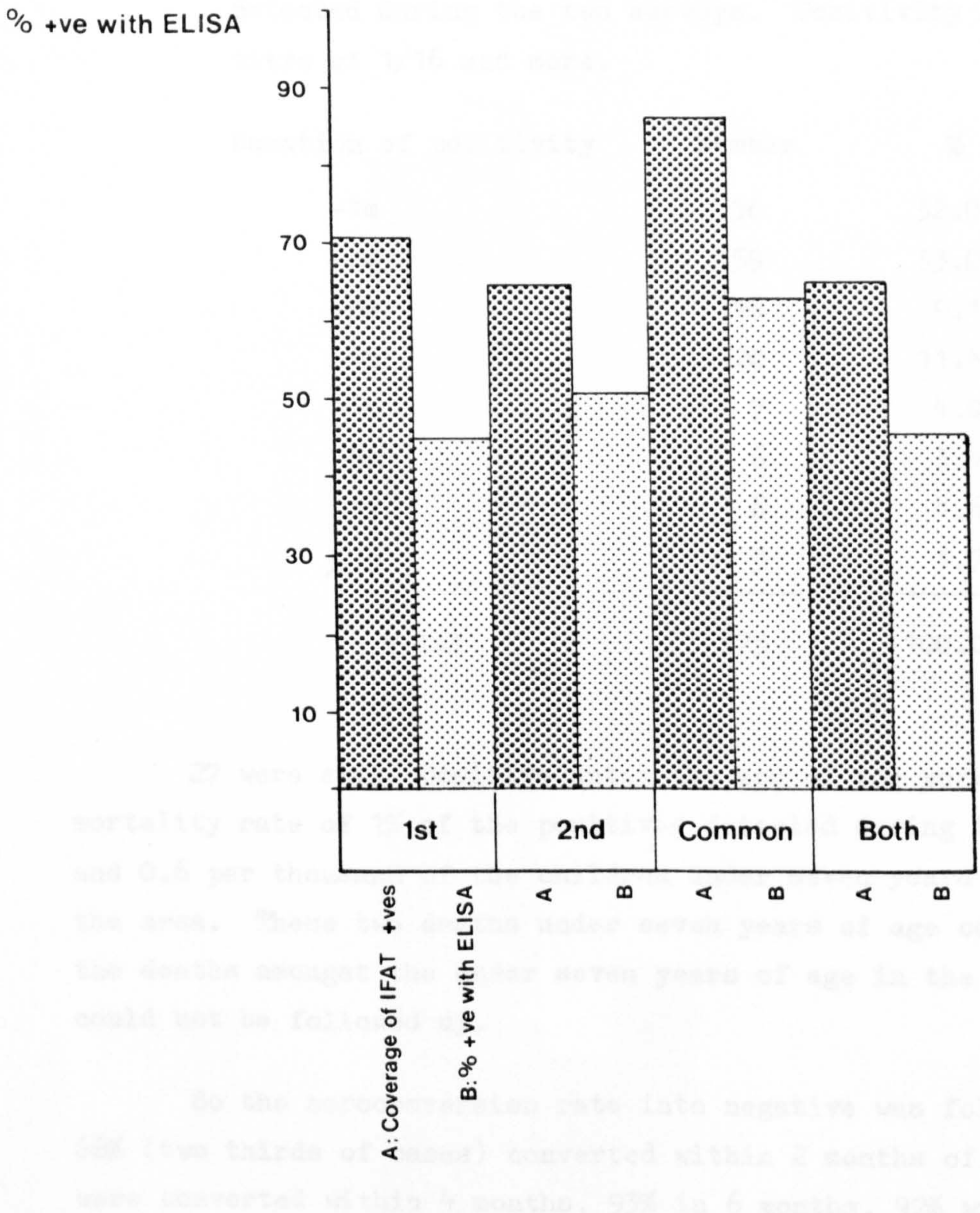


Table 55. Sero reversion to negativity among the IFAT seropositives detected during the two surveys. Positivity here is an IFAT titre of 1/16 and more.

Duration of positivity	Number	%
-1m	56	32.0
-2m	59	33.0
-3m	16	9.1
-4m	20	11.4
-5m	7	4.0
-5m	6	3.4
-1y	8	4.6
>1y	3	1.7
Total	175	100.0

27 were still positive until the end of the work, 2 died, a mortality rate of 1% of the positives detected during the two surveys and 0.6 per thousand of the children under seven years of age living in the area. These two deaths under seven years of age constitute 25% of the deaths amongst the under seven years of age in the area, And one could not be followed up.

So the seroconversion rate into negative was followed up and 66% (two thirds of cases) converted within 2 months of detection, 86% were converted within 4 months, 93% in 6 months, 97% within one year.

Figure 87 Showing the distribution of the period of positivity of those positive cases detected by both surveys and turned serologically negative after follow up.

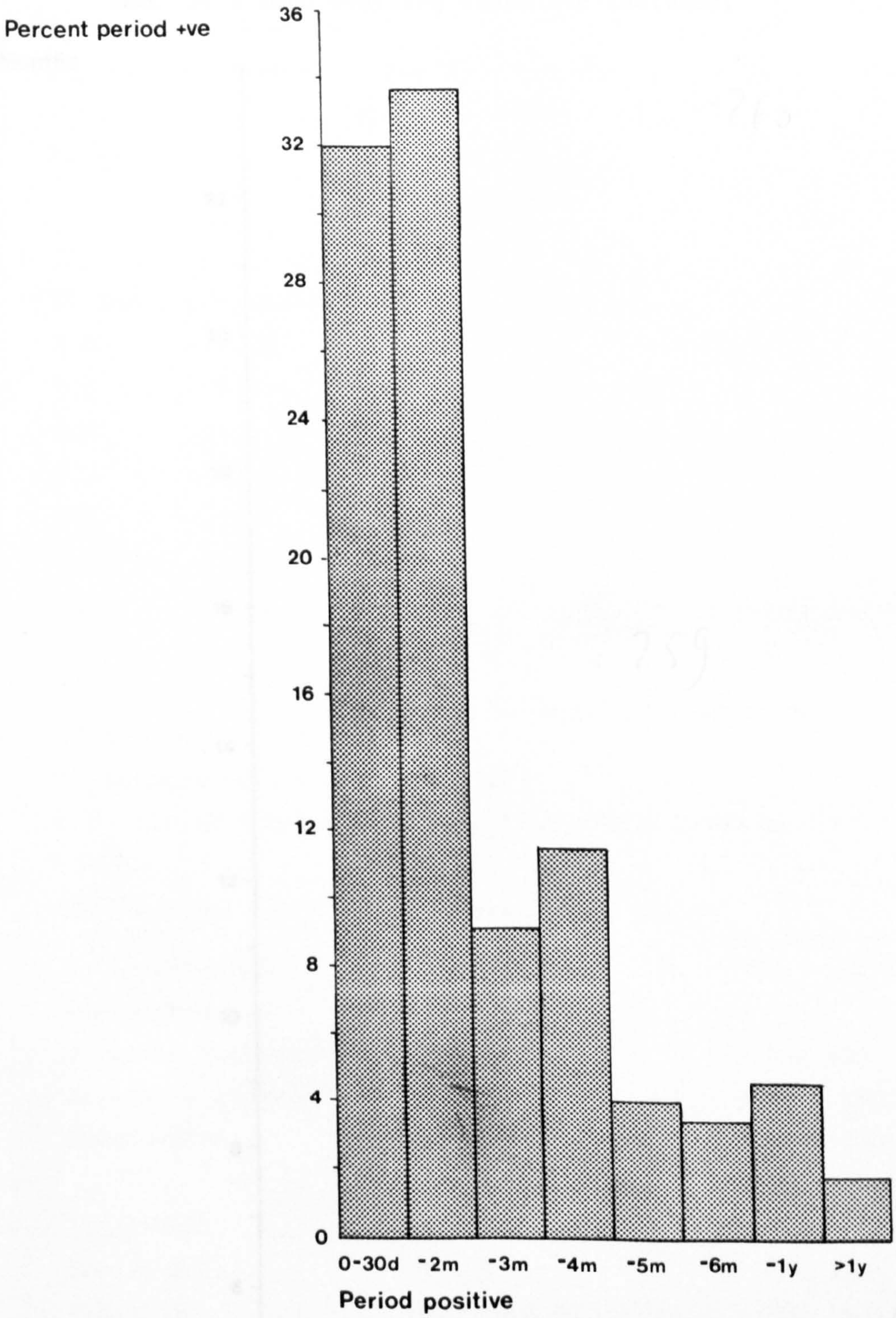
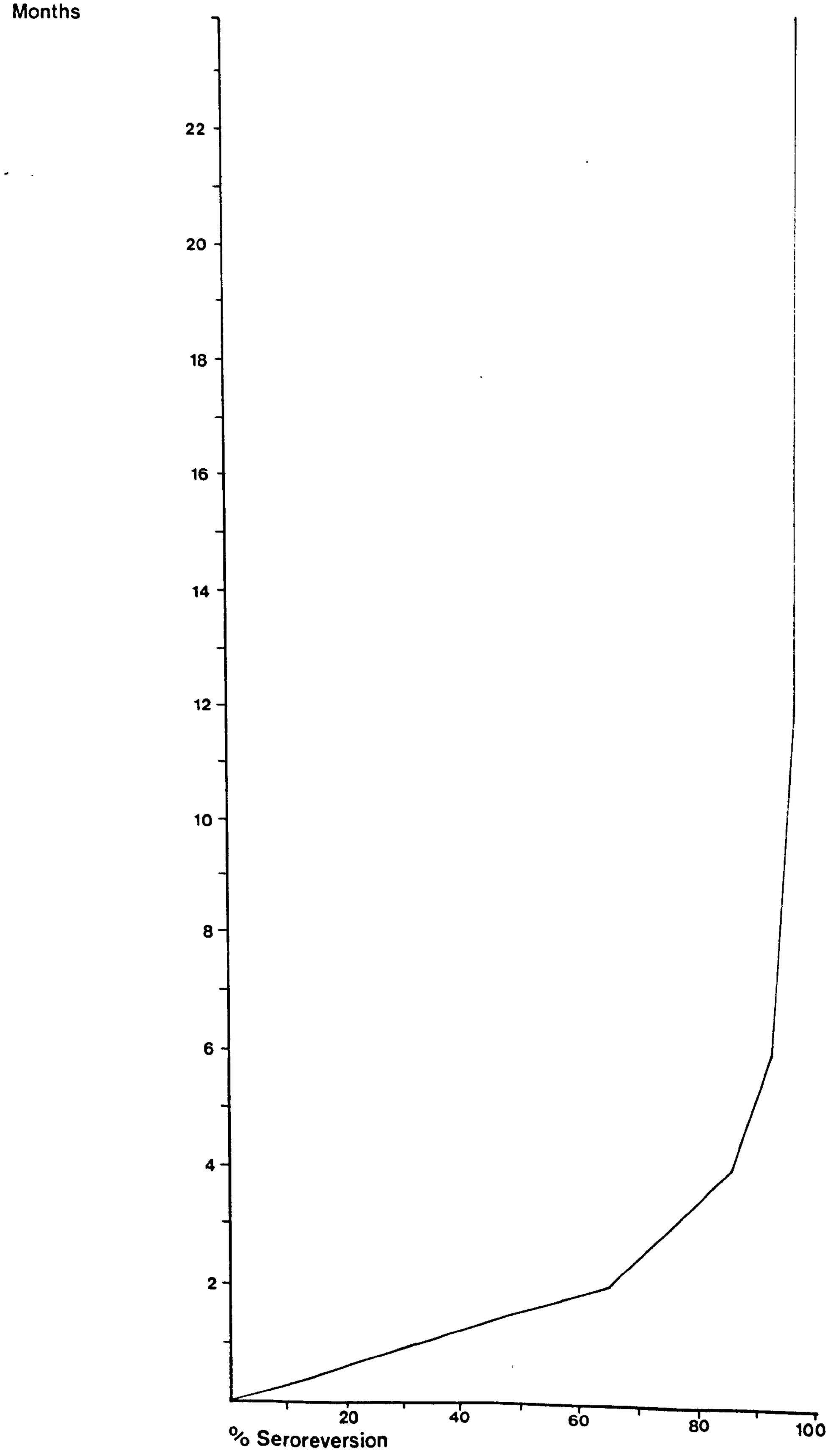


Figure 88 Serological follow-up of seropositive cases detected during the two surveys. Both surveys 175 cases. Only seroreverting cases are included.



Some normal values for tests used in this survey:

Actual anaemia below 70%	
Suggestive anaemia	Men 70 - 85%
	Women 70 - 80%
Normal	Men above 85%
	Women above 80%

Hb Scale (Tallquist)	Fergon
30%	= 4.7G
40%	= 6.3
60%	= 9.4
70%	= 10.9
80%	= 12.5
90%	= 14.1
100%	= 15.6

HB males 14 - 18 G/100ml
females 12 - 16 G/100 ml
newborn 16.5 - 19.5 G/100 ml
children (varies with age) 11.2 - 16.5 G/100 ml

Differential count of peripheral blood cells

		No./cmm
Total leucocytes	100%	5000 - 10,000
Myelocytes	0	0
Juvenile neutrophils	3-5	150 - 400
Segmented neutrophils	54 - 62	3000 - 5800
Lymphocytes	25 - 33	1500 - 3000
	%	
Monocytes	3 - 7	285 - 500
Eosiniphils	1 - 3	50 - 250
Basophils	0.0. - 0.75	15 - 50

Infants and children have relatively greater numbers of lymphocytes and monocytes, lymphocytes may amount to 45% in the first 3 years.

Platelets 150,000 - 350,000

Serum proteins:	G/100ml
Total proteins	6 - 8 G/100 ml
Albumin	3.5 - 5.5 G/100 ml
Globulin	
$\alpha 1$	0.1 - 0.4 G/100 ml
$\alpha 2$	0.4 - 0.8 G/100 ml
β	0.5 - 1 G/100 ml
λ	0.75 - 1.5 G/100 ml
IgG	564 - 1765 mg/100 ml.
IgM	53 - 180 mg/100 ml

To calculate the mean titre of IFAT different titres were given scores as follows:

Titre	Score
negative	0
1/16	2
1/32	4
1/64	6
1/128	8
1/256	10
1/512	12
1/1024	14

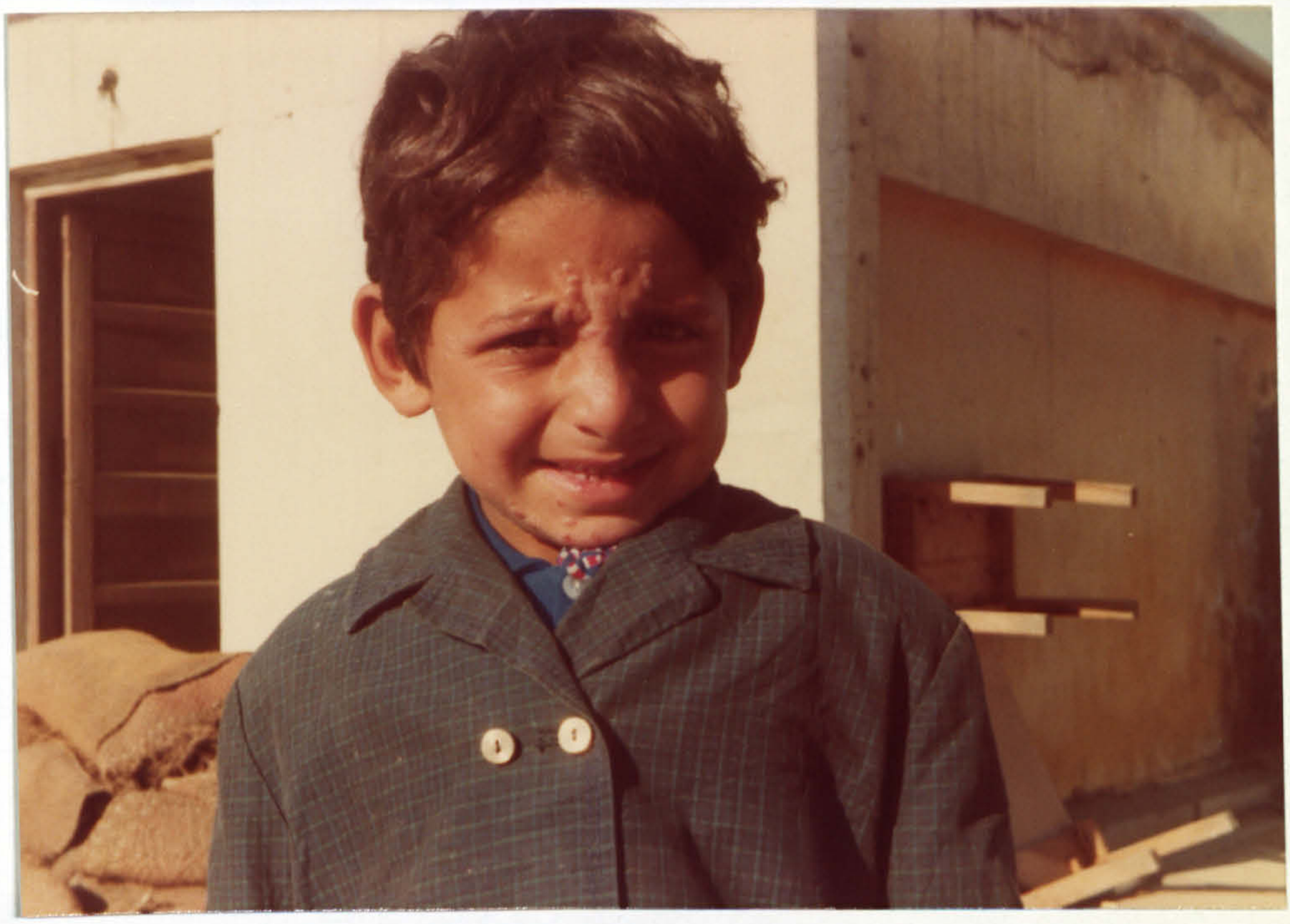
H. RESULTS OF SKIN WINDOWS

Negative human controls: the cover slips were removed after 24 hours and examined, they were found to contain clumped macrophages, they also contained polymorphs (neutrophils with some cosinophils).

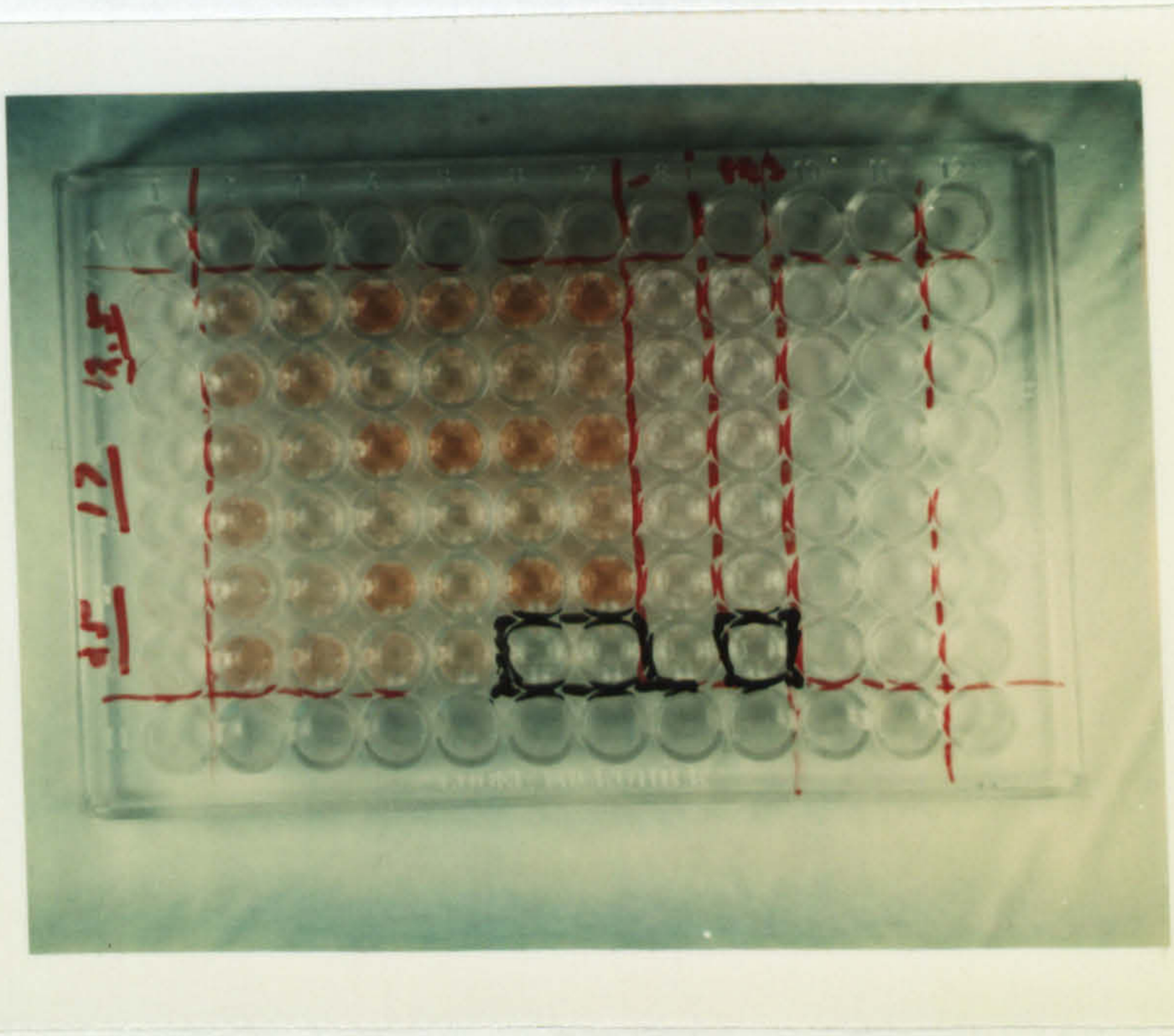
The same method was applied to two cases of visceral leishmaniasis, one before treatment and one after, both of them showed the same picture with no amastigotes to be seen, although the coverslips contained a lot of macrophages. So the skin window on human cases of visceral leishmaniasis gave negative results.

The results of the skin windows on the two control mice which were not infected showed a similar picture as was seen in man, clumping of macrophages with some polymorphs.

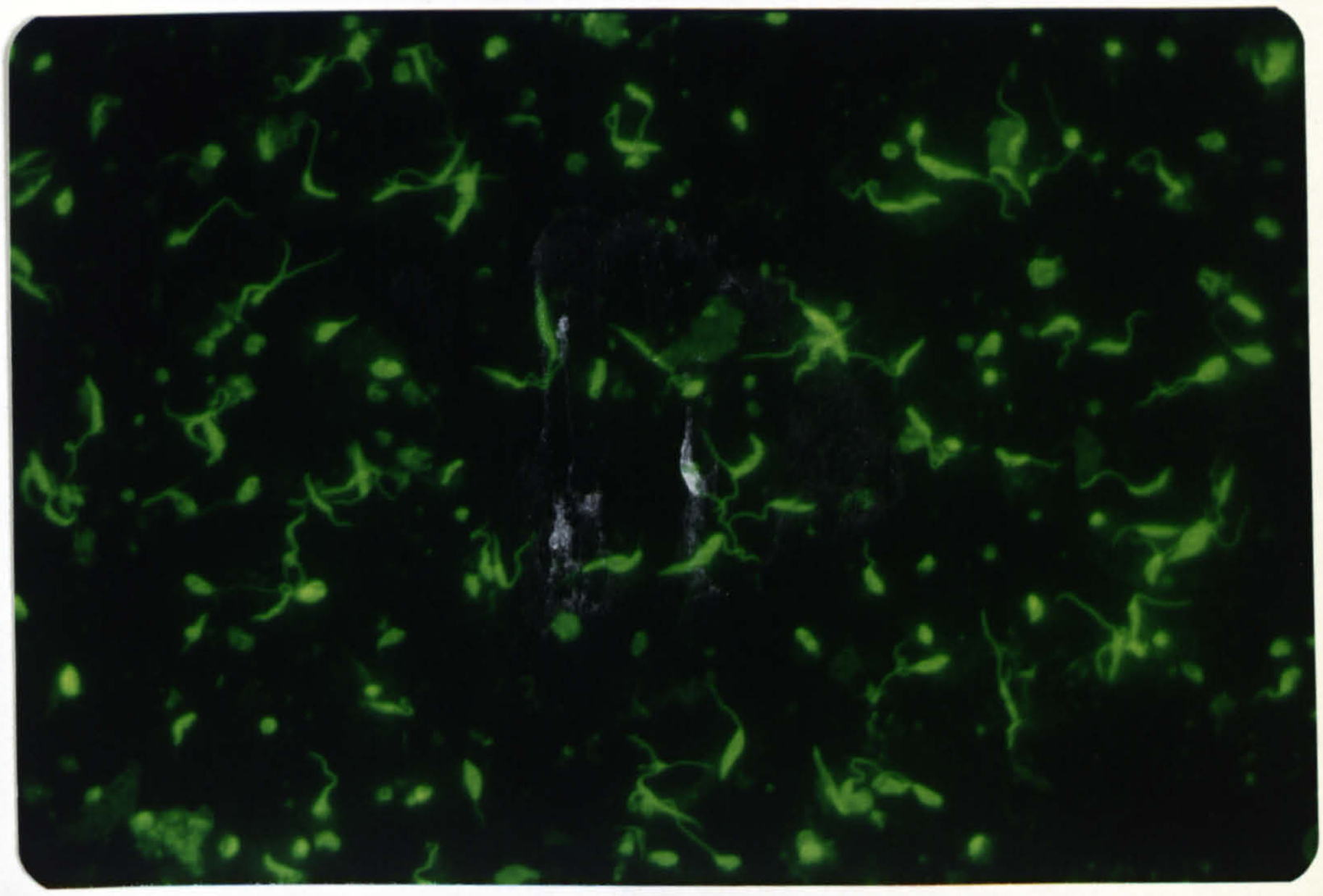
Skin windows on 12 infected inbred mice genetically susceptible towards infection with L. donovani, were examined at day 1 and 2 after embedding of the coverslips and also skin windows on heavily infected hamsters. All showed negative results and we could not get any amastigotes on the slide outside or inside macrophages although the latter were abundant on the coverslip.



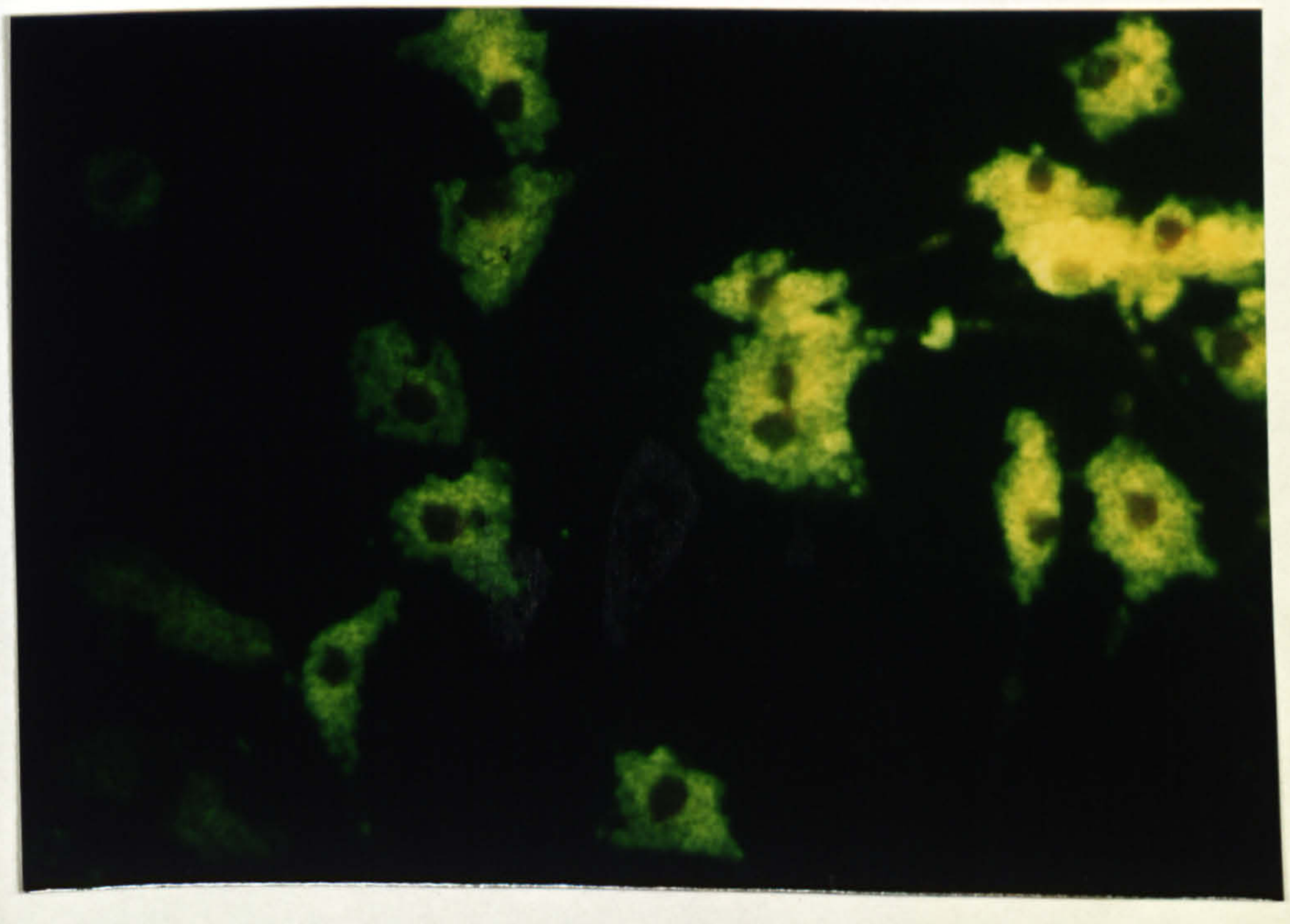
Photograph 1. Skin lesions developing in a case of visceral leishmaniasis



Photograph 2. Checker board of ELISA for the concentration of antigen used.

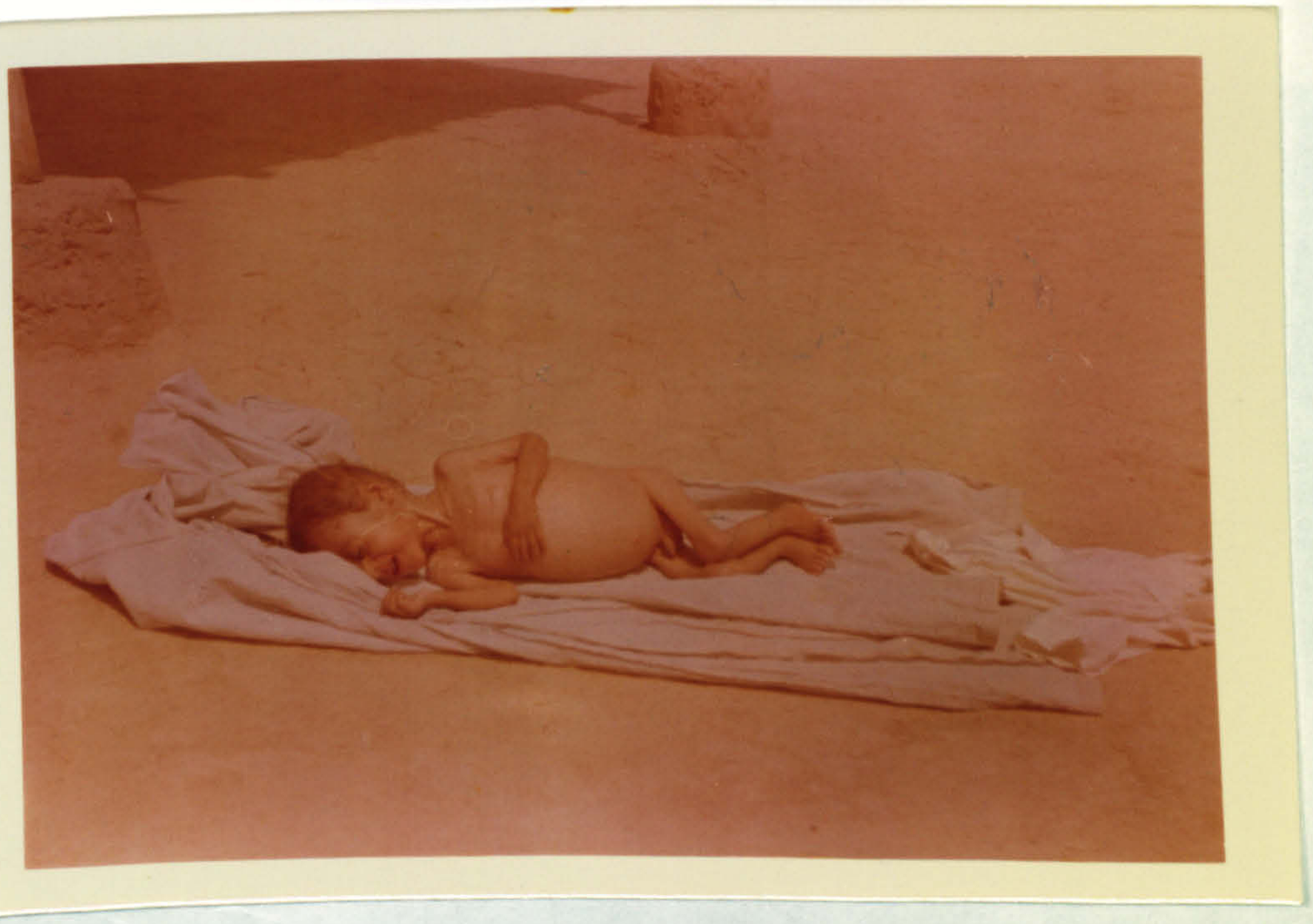


Photograph 3. Promastigotes used as an antigen in IFAT



Photograph 4 Intracellular amastigotes used as antigen in IFAT

Photograph 5. Team of blood collection



Photograph 6. Bone marrow positive case of visceral leishmaniasis.

Photograph 7. Houses in study area

Photograph 8. Roads in study area

Photograph 9. Channels in study area

Photograph 10. River Diala in the study area

Photograph 11. A dog from the study area

Photograph 12. A jackal shot dead and studied

Photograph 13. Animal burrows in the study area.

Photograph 14. A fox shot dead and studied.

I. RESULTS OF THE WORK ON THE POSSIBLE ANIMAL RESERVOIR

Studies on the possible animal reservoir of visceral leishmaniasis in Iraq included the work on

151 jackals (Canis aureus)

45 foxes (Vulpes vulpes)

1 wolf (Canis lupus)

and 65 dogs (Canis familiaris)

The work also included the study of

18 Mus musculus

and 68 Rattus rattus

The place of these studies was planned to be in the study area but for unforeseen administrative difficulties the whole work on the animal reservoir had to be transferred to other localities similarly important as foci of visceral leishmaniasis, these foci were around Baghdad and as shown on the map.

1. Jackals (Canis aureus, the Asiatic jackal)

151 jackals were shot during this work. They were studied by direct examination of their viscera, impression smears and cultures of the viscera were done in addition to culture of blood and other tissues, in an attempt to isolate the parasite. In addition to that, serum proteins were estimated by electrophoresis and blood was examined serologically by IFAT and ELISA, using the antidog of IgG commercially available conjugate.

Of those 151 jackals studied, 92 of them (61%) were males and 59 of them (39%) were females, as shown in Figure 90. Most of them were adult animals, except a very few of them which were young.

These jackals were collected from nine different well-known foci of visceral leishmaniasis, as illustrated in Table 56 and Figure 93. Six out of nine foci gave positive serological findings among the jackals examined, in some of the areas like Nomanyia a high positivity rate was detected among the jackals examined which reached 74%. One of those positive jackals had an IFAT titre of 1/128, another jackal with the same titre was found in Swaira focus which had a positivity rate of 60% among the jackals examined serologically.

Figure 90 Sex distribution of jackals studied.

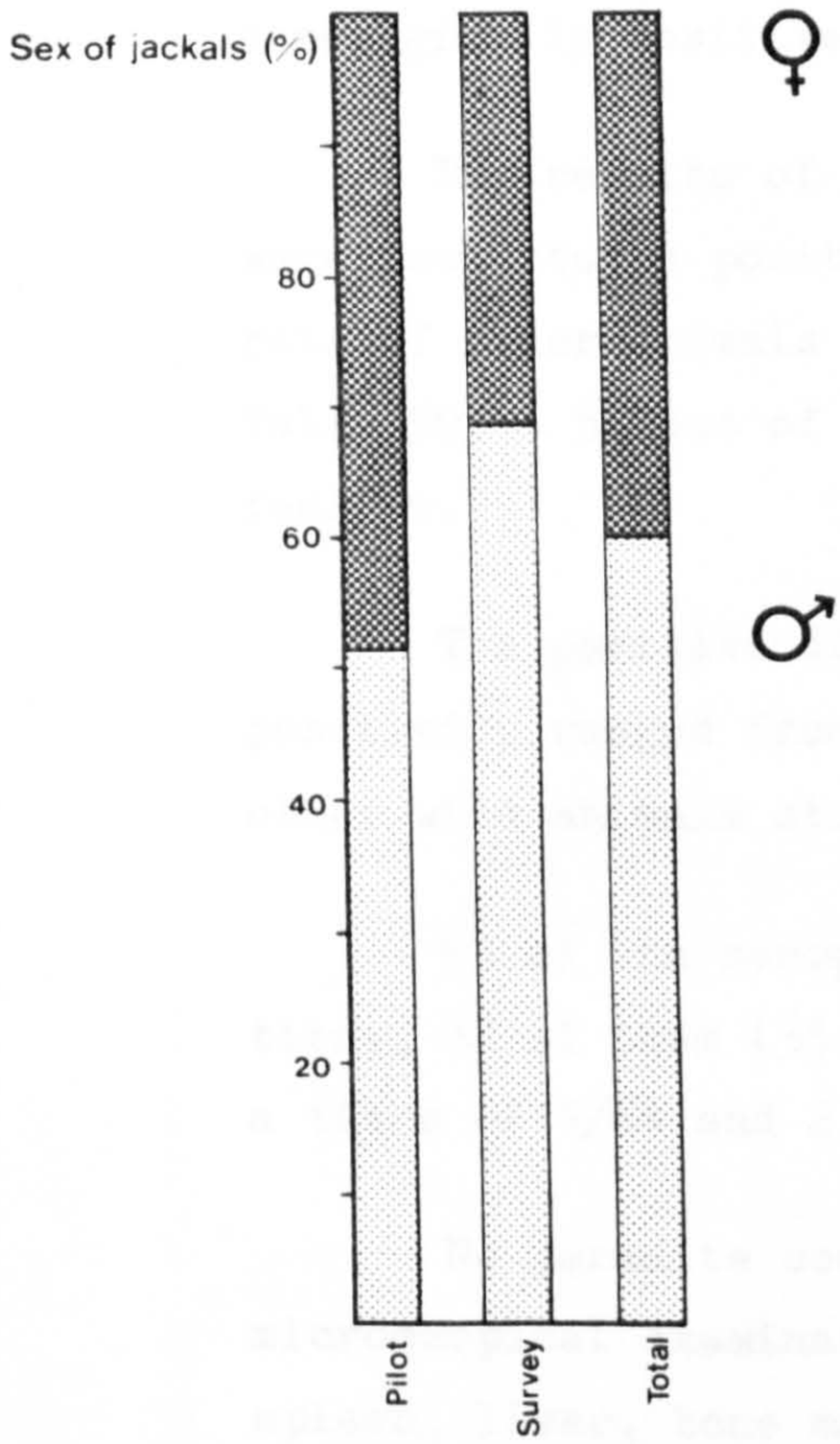


Figure 91 Positivity of animals examined.

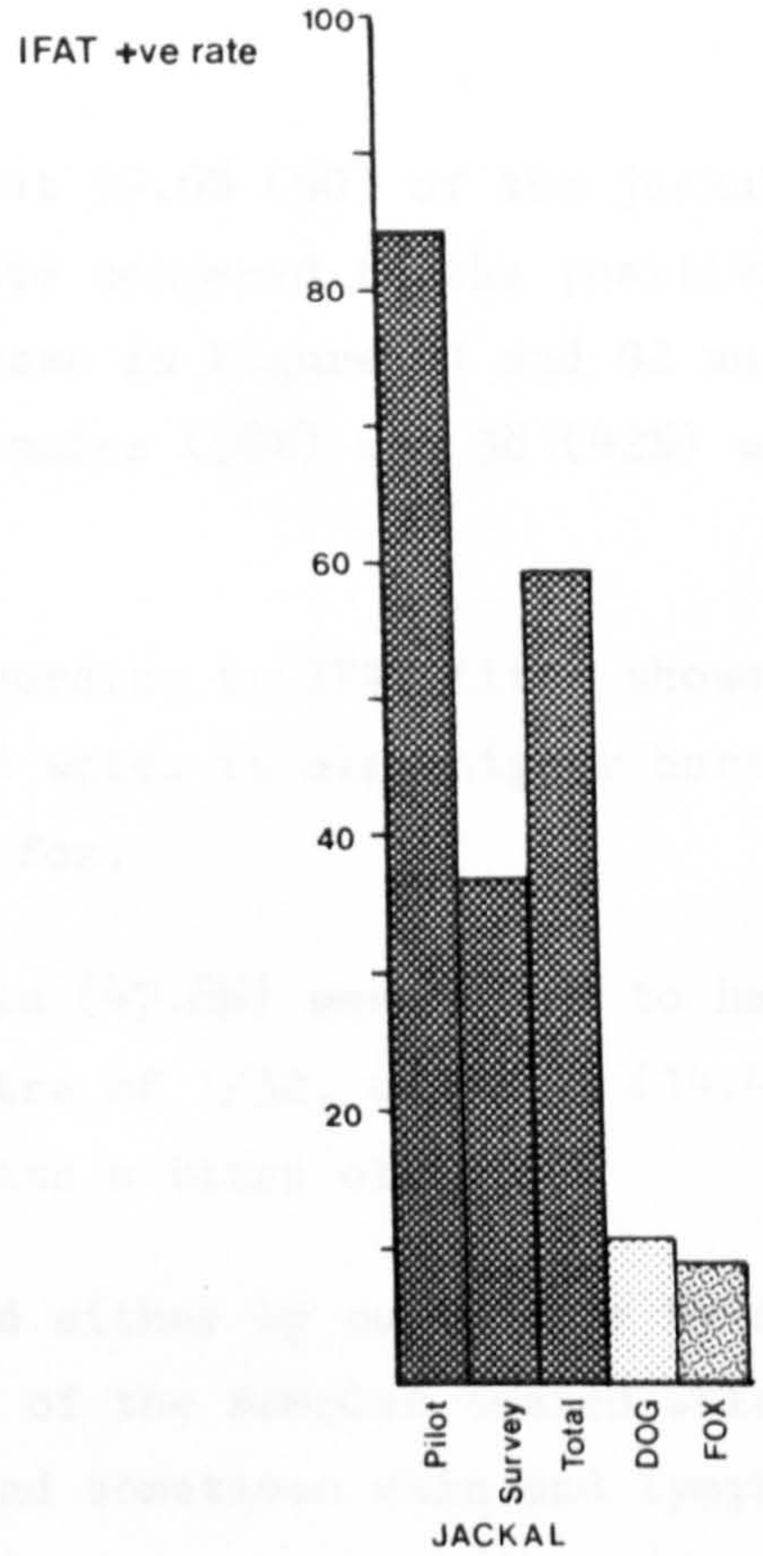
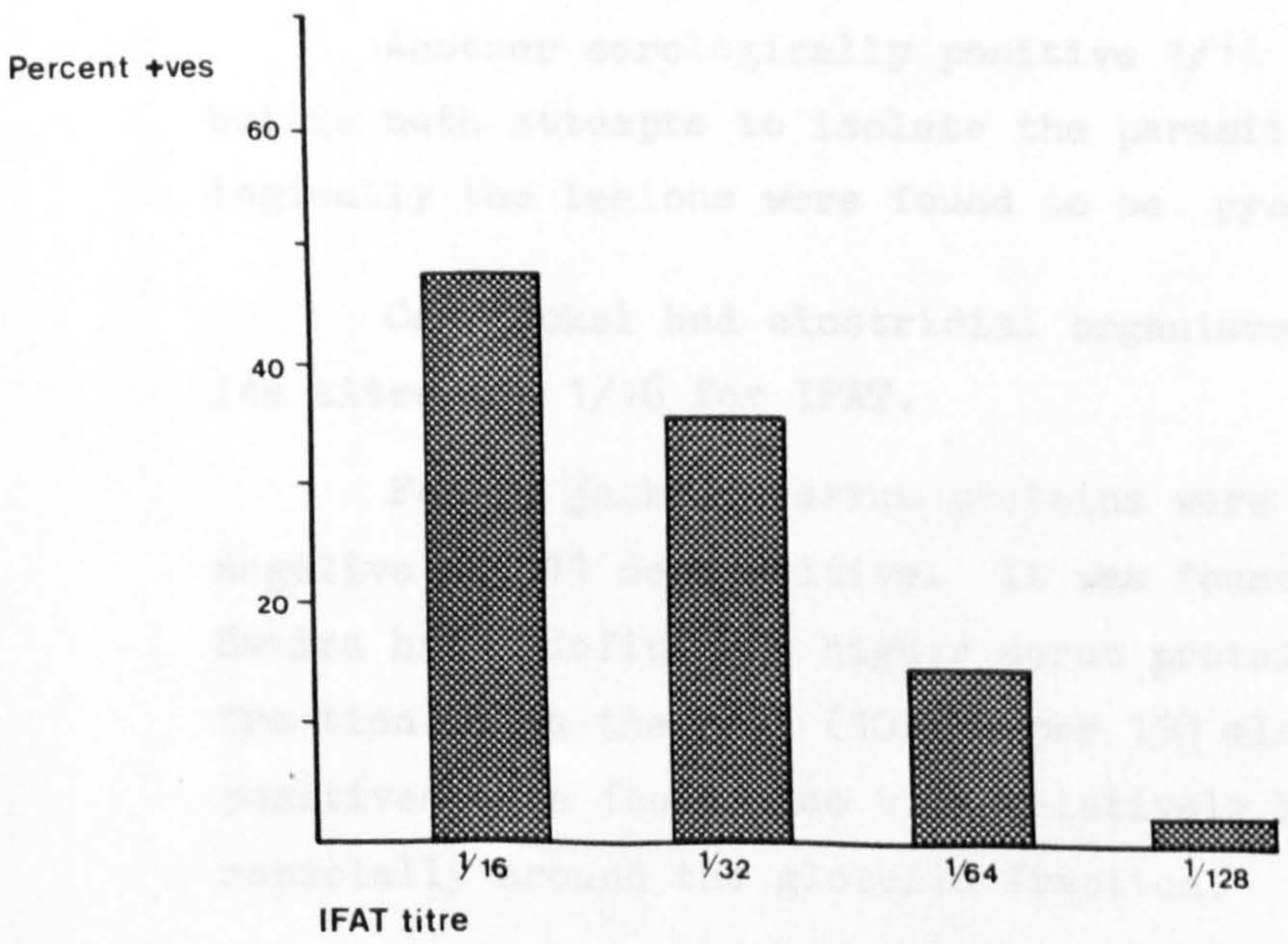


Figure 92 Distribution of IFAT titres of positive jackals.



Only one jackal was shot in the study area and this was found to be serologically positive (1/64).

The results of IFAT showed that 59.6% (90) of the jackals shot were found to be positive, a high rate compared to the positivity rate of other animals studied, as shown in Figures 91 and 92 and in Table 59. 52 out of those 90 were males (58%) and 38 (42%) were females.

The positive distribution according to IFAT titre shows that positivity ranged from 1/16 to 1/128 which is also higher here than with other wild animals studied like the fox.

43 of the seropositive jackals (47.8%) were found to have 1/16 titre, 32 of them (35.5%) with a titre of 1/32, other 13 (14.4%) with a titre of 1/64 and 2 more (2.2%) with a titre of 1/128.

No parasite could be isolated either by culture or by direct microscopical examinations from any of the samples tested which included spleen, liver, bone marrow, blood and sometimes skin and lymph nodes on different types of media.

One jackal from Khalis had a large spleen and also depilation with skin lesion, but serologically the jackal was negative.

Another serologically positive 1/16 jackal had a skin lesion but in both attempts to isolate the parasite failed and histopathologically the lesions were found to be pyogenic infection of the skin.

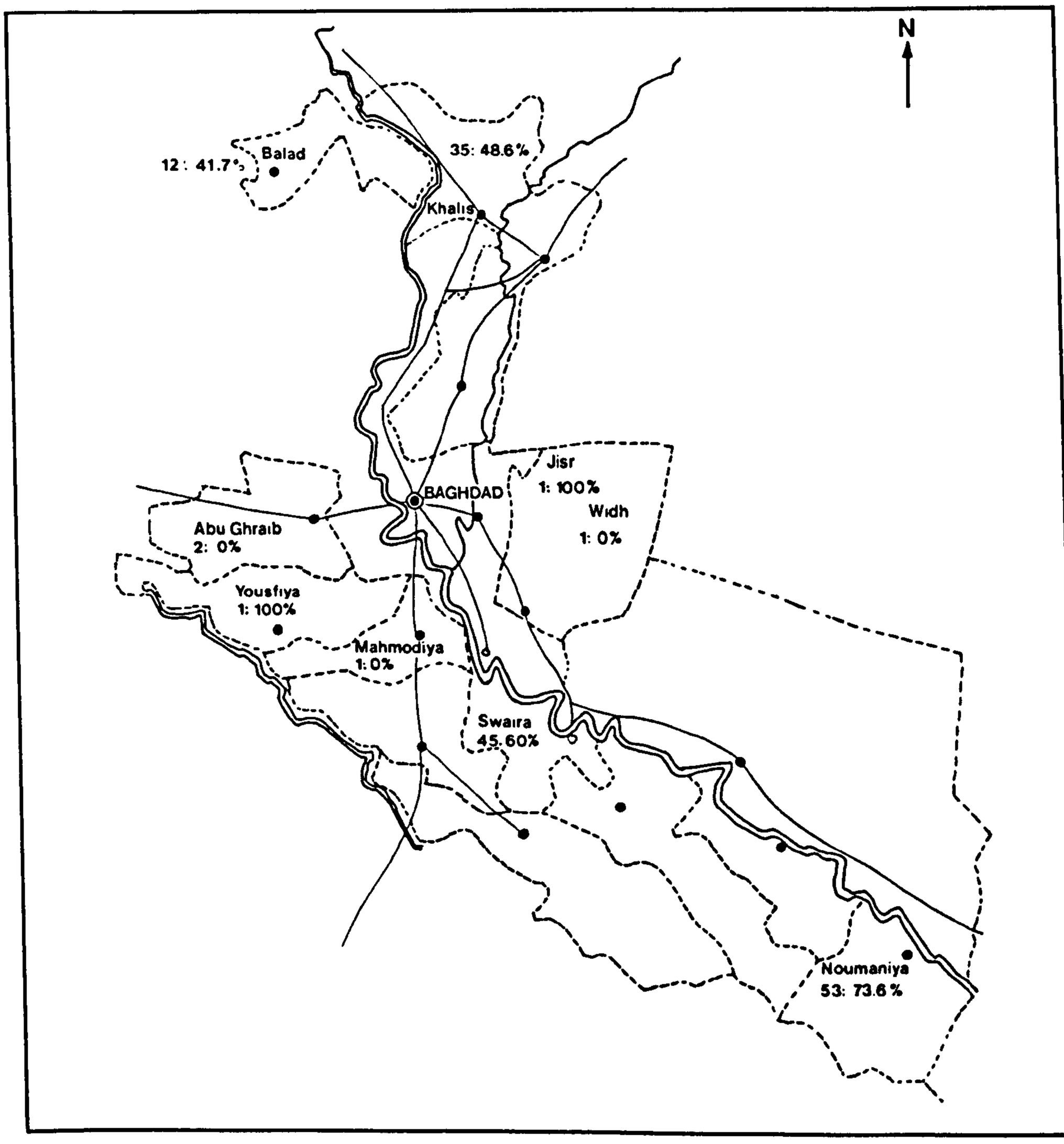
One jackal had clostridial organisms in the bone marrow smear, its titre was 1/16 for IFAT.

For 32 jackals, serum proteins were done, 21 of them were negative and 11 seropositive. It was found that the 1/128 jackal from Swaira had definitely higher serum proteins (especially the globulin fraction) than the rest (10.8 G per 100 mls). Some other seropositives were found also with relatively higher protein levels especially around the globulin fraction.

Table 56. IFAT seropositivity and positivity rates of jackals studied in the nine foci of visceral leishmaniasis.

	Jisr	Noumaniya	Swavia	Khalis	Yousfiya	Wihda	Mahmoudiya	Abu-Gharib	Bulad	Total
1/16		14	15	8	1				5	43
1/32		17	10	5						32
1/64	1	7	1	4						13
1/128		1	1							2
Total positives	1	39	27	17	1	0	0	0	5	90
Negatives	-	14	18	18	-	1	1	2	7	61
Grand total	1	53	45	35	1	1	1	2	12	151
Positivity rate according to area	100	73.6	60	48.6	100	0	0	0	41.7	59.6

Figure 93 Map showing areas where jackals have been studied, with their positivity.



2. Foxes (Vulpes vulpes, the red fox)

45 foxes were shot and studied from five foci of visceral leishmaniasis around Baghdad, as shown in Figure 94 and Table 57.

28 of them were shot in Swaira focus
8 in Wihda
7 in Mahmoudiya
1 in Khalis
1 in Salman Pak

27 of them were males (60%) and 18 (40%) were females
Four of them were positive with IFAT titre of 1/16, which constitutes only 8.9% positivity rate and they are distributed as follows:

1 in Swaira (3.57% positivity rate)
1 in Wihda (12.5% positivity rate)
2 in Mahmoudiya (28.57% positivity rate)

Two of the four were males and two females.

All cultures and direct examinations to reveal the parasite gave negative results.

One fox had microfilariae detected in its peripheral blood.

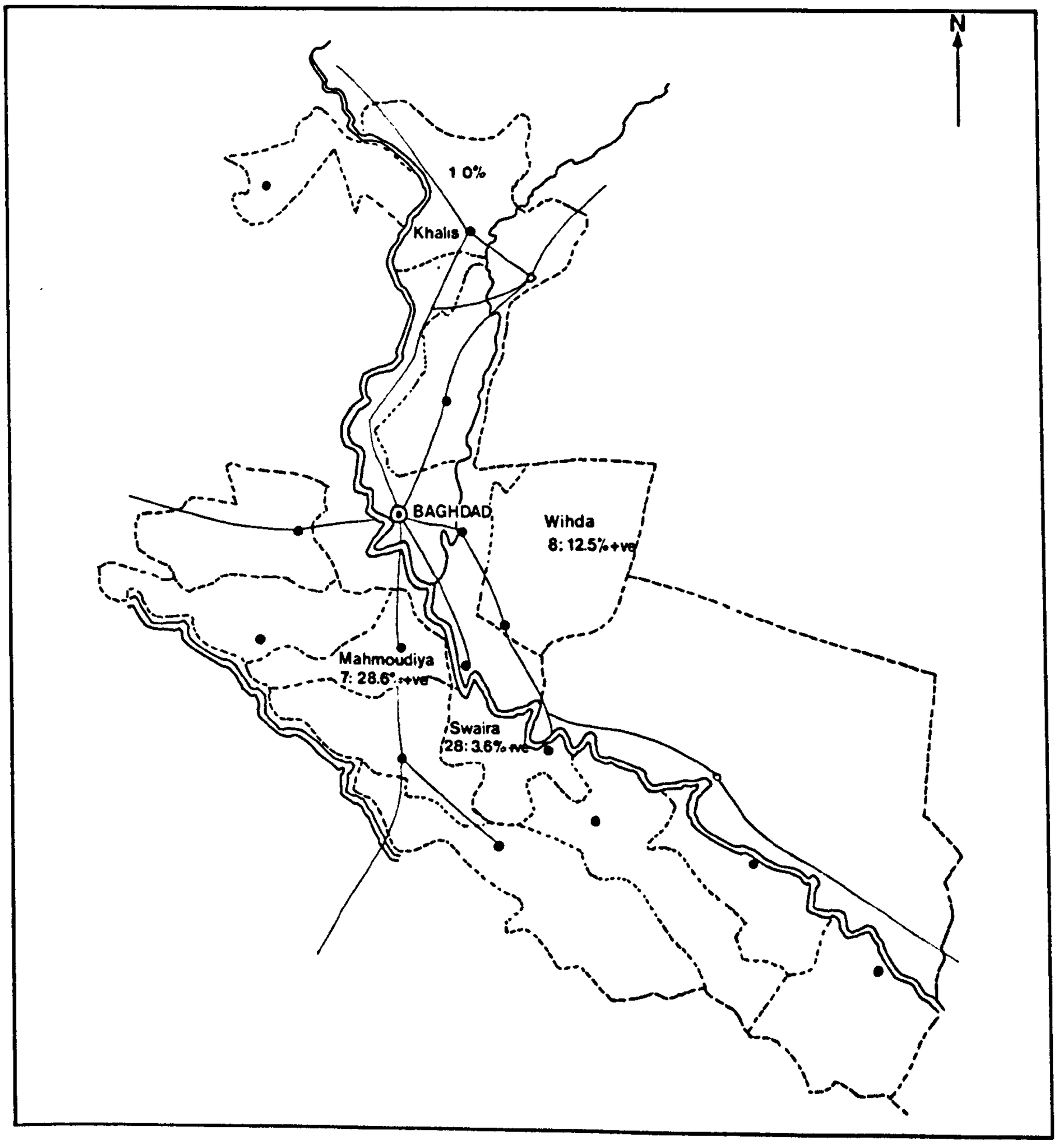
One had a skin lesion but histophathlogically it was not due to leishmaniasis and no parasite could be isolated from it by direct method or by culturing.

Serum protein estimation was done on 16 foxes (2 with 1/16 IFAT titres) and no notable difference was detected in the levels of serum proteins compared to the negatives.

Table 57. IFAT seropositivity and positivity rate of foxes studied in five foci of visceral leishmaniasis.

	Swaira	Wihda	Mahmoudiya	Khalis	Salman Pak	Total
1/16	1	1	2	-	-	4
-ve	27	7	5	1	1	41
Total	28	8	7	1	1	45
Positivity	3.57%	12.5%	28.57%	0%	0%	8.9%

Figure 94 Map showing area where foxes have been studied, with their positivity.



3. The dog (Canis familiaris)

65 dogs were shot in Baghdad city and neighbouring areas.

61 of them were stray dogs of Baghdad city and three from the study area and one from Balad from the house of a case of visceral leishmaniasis.

Most of these dogs were emaciated, the one from Balad looked ill, some of them were depilated.

Some investigations were done on them and the results were as follows:

No parasites could be isolated from the viscera or tissues of any of the dogs examined.

Seropositivity by IFAT was af follows: out of the 65 dogs examined only seven were seropositives (10.8%).

Positivity was distributed as follows, and as shown in Table 58.

IFAT titre of 1/16 - two only

IFAT titre of 1/32 - four, one from the house of a case of visceral leishmaniasis

IFAT titre of 1/128 - one only.

Three dogs from the study area proved to be negative although one of them had obvious depilation and was emaciated and looked ill. Serologically and by other methods all attempts to isolate the parasite were not successful.

Serum proteins were done on two serologically negative dogs from the study area only, no notable high levels of serum protein constituents were found.

Table 58. IFAT seropositivity and positivity rates of the dogs studied.

	Jisr	Balad	Baghdad city	Total
1/16	-	-	2	2
1/32	-	1	3	4
1/64	-	-	-	-
1/128	-	-	1	1
Total positive	-	1	6	7
negative	3	0	55	58
Total(grand)	3	1	61	65
Positivity rate	0	100	9.5	10.8

Table 59. Summary of the study done on canines as a possible reservoir of visceral leishmaniasis in different foci of the disease. Positivity here denotes a titre of 1/16 and more in IFAT.

		Jisr	Noumaniya	Swaira	Khalis	Yousfia	Wihda	Malmoudiya	Abu-Gharib	Balad	Salman Pak	Baghdad city	Total
Jackals	Total	1	53	45	35	1	1	1	2	12			151
	Positivity rate	100	73.6	60	48.6	100	0	0	0	41.7			59.6
Foxes	Total			28	0		12.5	28.57			1		45
	Positivity rate			0									9
Wolves	Total			1									1
	Positivity rate			0									0
Dogs	Total	3								1		63	65
	Positivity rate	0								100		9.5	10.8
Total	Total	4	53	74	36	1	9	8	2	13	1	63	262
Carnivores	Positivity	25	73.6	37.8	47	100	11	25	0	46	0	9.5	38.5

4. Wolf (Canis lupus)

One wolf only was shot and studied and this was in Swaira, all investigations and tests done were negative.

5. Mice

18 mice (Mus. musculus) were caught from different parts of Baghdad city and the usual search for the parasite and serological examinations were done. All proved negative with all these investigations.

6. Rats (R. rattus)

68 rats were caught in Baghdad city. Isolation of the parasite failed. Instead some Trypanosomes were detected in the blood of some of them. Serologically some were positive but this could be due to cross reaction with their Trypanosome infection.

27 were positive by IFAT, with 39.7% positivity rate.

J. RESULTS OF THE LIMITED SCALE ENTOMOLOGICAL STUDIES IN THE STUDY AREA

1. Results of the precipitin test.

The results of the precipitin test showed that 34% (11) sandflies had fed on Galliformes (chicken in this case) and the remaining 21 had fed on man.It is interesting to see that some sandflies caught inside people's bedrooms were fed on Galliformes, because in this area the chickens run freely in the houses.

2. Results of the first study which was done in June 1979.

The result on the human bait: It was shown that a human bait around the peak hour of activity of the sandflies was bitten 42 times in one hour.

The result of collection of sandflies from five indoor places of human dwellings by sucking by a tube on four consecutive days, as seen in Table 60, showed the total number of sandflies collected was 94, all of them were Phlebotomus papatasi females. 42 of them were blood fed (45%), 46 of them had laid eggs (49%), none of them was positive for promastigotes of Leishmania.

3. Results of the second study which was done in November 1979.

Results on human bait showed that around the peak hour of activity of sandflies a human bait was exposed to 25 bites of sandflies in one hour.

Result of collection of sandflies from 5 different indoor places of human dwellings by sucking by a tube on four consecutive days, as shown in Table 60: total number of sandflies collected was 88, 15 were not identified, 73 only were identified, 31 only were P.papatasi (43%) 37 were S. baghdadis (50.5%) and only 5 were S. squamipleuris (6.8%).

Of the 31 P. papatasi: 10 were fed (32%), and only had laid eggs (3.2%).

Of the 37 S. baghdadis: 19 were fed (51%), and only one had laid eggs (2.7%).

Of the 5 S. squamipleuris all of them were unfed and all did not lay eggs.

None of the sandflies dissected had promastigotes of Leishmania.

So from these studies it was found that more than 10 sandflies per room were found. Phlebotomus papatasi sandflies were most frequently found in the area in association with man and animals. It was found to be both anthropophilic and zoophilic, and man was exposed to 25 - 40 bites of sandflies in one hour outside the houses. But no parasites could be isolated from the sandflies dissected, relatively low percentage of sandflies were fed on the second study than the first.

Table 60. Results of work done on sandflies in the study area.

	1st Study (June 1979)						2nd Study (November 1979)					
	Total	% to total caught in study	Fed	% of fed to species	Eggs	% to species	Total	% to total caught in study	Fed	% of fed to species	Eggs	% to species
Phlebotomus papatasi	94	100	42	45	46	49	31	43	10	32	1	3.2
Sergentomyia baghdadis							37	50.5	19	51	1	2.7
S. squami-pleuris							5	6.8	0	0	0	0
Total identified	94						73					
Unidentified							15					
Total caught	94	100	42	45	46	49	88	100	29	33	2	2.3

K. RESULTS OF THE LONGITUDINAL STUDY OF INFECTION OF MICE WITH L.DONOVANI

Sera collected from the mice in this longitudinal study were examined by the two serological methods, the IFAT and ELISA, each serum was examined by those two methods at least twice, then the readings were grouped under the day after infection the blood was collected and according to the group of inbred mice tested, all these tests were done blindly.

The following are the Tables of the results. Tables 61, 62, 63 and 64 and Figures 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105 and 106 show the means of readings in different groups according to days of infection experimentwise.

Geometrical means of IFAT titre were used beginning with 1 as negative

2 as 1/16
2.5 as 1/32
3 as 1/64
3.5 as 1/128
4 as 1/256

The results of the work by IFAT and ELISA vary slightly between the two techniques and from experiment to experiment, but generally it was shown that in innately resistant mice there was very low reaction which could be considered as negative throughout the whole experiment.

Innately susceptible mice including the BALB/C show seropositivity by day 50 of the infection and then positivity increases until the end of the experiments at day 130 regardless of the parasitological course of infection, because it was seen by Bradley (1977) and Bradley and Kirkley (1977) that parasite loads in the liver will drop significantly in some susceptible mice. In this work there were high titres of antibody measured by IFAT and ELISA when parasitological loads fell to a low level as in the case of BIO mice. Sometimes antibody levels measured by the serological tests showed some significant difference between the innately susceptible cure BIO mice and the innately susceptible non cure mice BIO D2. This was seen in one experiment (542) by IFAT beginning for day 50 after infection and throughout the infection and it was shown by ELISA also at D126 in another experiment (554).

T-test was done in every possible way and only the following showed some significant results, Table 65.

Table 61. The results of ELISA, IFAT and parasite densities in the mice of experiment number 542.

		E L I S A				I F A T		PARASITES		
Day after infection	Mice	Mean	Standard Error	Number of Readings	Number of Animals	Geometrical Mean	Standard Error	Number of Animals	Mean of Log LDU	Number of Animals
50	B10	0.555	0.057	4	2	2.5	0	2	2.327	2
	$B10D_2$	0.167	0.022	4	2	1.5	0	2	2.950	2
85	B10	0.763	0.291	4	2	3.5	0	2	0.527	2
	$B10D_2$	0.290	0.008	4	2	2.7	0.039	2	3.674	2
129	B10	1.115	0.140	6	3	4.1	0.036	3	0.843	3
	$B10D_2$	0.473	0.111	4	2	2.7	0.039	2	3.521	2

Table 62. The results of ELISA, IFAT and Parasite densities of the mice in experiment number 552.

		ELISA			IFAT			PARASITES	
Day after infection	Mice	Mean	Standard Error	Number of readings	Number of animals	Geometrical mean	Standard error	Number of animals	Mean log of LDU	Number of animals
15	C57 L	-	-	-	-	1.08	0.035	4	0.385	2
	Balb C	0.107	0.012	6	3	1.14	0.029	8	2.763	3
	Balb B	0.089	0.002	6	3	1.09	0.026	8	2.829	3
	Balb K	0.097	0.009	5	3	1.34	0.043	8	2.842	3
30	C57 L	0.105	0.012	2	1	1	0	2	-	-
	Balb C	0.115	0.012	8	4	2.039	0.054	5	2.981	3
	Balb B	0.147	0.011	10	5	2.209	0.095	5	2.581	3
	Balb K	0.163	0.011	10	5	2.55	0.038	5	2.51	3
50	C57 L	0.109	0.012	8	2	1.58	0.199	2	0.455	2
	Balb C	0.274	0.035	20	8	3.98	0.018	8	3.12	3
	Balb B	0.257	0.019	20	8	4.22	0.019	8	2.592	3
	Balb K	0.3	0.026	16	8	4.37	0.008	8	2.593	3
85	Balb C	0.525	0.038	18	6	3.14	0.024	6	3.074	3
	Balb B	0.454	0.036	24	6	2.48	0.022	6	2.755	3
	Balb K	0.541	0.071	14	5	2.658	0.017	6	3.028	3
131	Balb C	0.513	0.03	9	3	5	0	3	3.24	3
	Balb B	0.587	0.062	8	3	4.66	0.015	3	2.71	3
	Balb K	0.512	0.051	10	3	4.1	0.036	3	2.314	3

Table 63. The results of ELISA, IFAT and Parasite densities in mice of experiment number 553.

		E L I S A				I F.A T		PARASITES		
Day after infection	Mice	Mean	Standard error	Number of readings	Number of animals	Geometrical mean	Standard error	Number of animals	Mean of log LDU	Number of animals
15	B10 D2	0.106	0.007	4	2	1	0	2	2.76	2
	B10	0.083	0.003	2	1	1	0	2	2.712	2
	C57 L	0.108	0.006	6	3	1	0	4	1.15	2
50	B10 D2	0.162	0.007	4	2	2.7	0.04	2	3.419	2
	B10	0.207	0.021	4	2	2.45	0.088	2	2.339	2
85	B10 D2	0.393	0.046	4	2	3.24	0.034	2	3.344	2
	B10	0.524	0.109	4	2	2.5	0	2	1.026	2
	C57 L.	0.151	0.020	4	2	1	0	2	0	2
133	B10 D2	0.436	0.062	8	4	3.75	0.017	4	3.328	4
	B10	0.837	0.217	4	2	4.246	0.026	2	0.468	2

Table 64. The results of ELISA, IFAT and Parasite densities in mice in experiment number 554.

		ELISA				IFAT			PARASITES	
Day after infection	Mice	Mean	Standard error	Number of readings	Number of animals	Geometrical mean	Standard error	Number of animals	Mean of log LDU	Number of animals
15	C57 L	0.119	0.014	4	2	1.5	0	2	1.053	2
	B10 D2	0.168	0.020	14	7	1.7	0.252	7	-	-
	B10	0.163	0.018	10	5	1.6	0.042	7	-	-
50	B10 D2	0.321	0.032	8	4	2.978	0.023	7	3.231	2
	B10	0.301	0.017	14	7	3.05	0.019	7	2.372	2
85	B10 D2	0.516	0.058	12	6	2.109	0.046	6	3.421	2
	B10	0.592	0.058	12	6	2.4	0.045	6	0.726	2
126	B10 D2	0.397	0.064	8	4	4.1	0.026	4	3.55	4
	B10	0.885	0.038	4	2	5	0	2	0.801	2

Table 65. t-test to show significant differences in immune response in different strains of mice as measured by IFAT and ELISA.

IFAT 553 D85

	B10	C57L	t value	DF	
SD	0	0			Result at 0.05
$\bar{x}$	1.5	0	3.16	2	significant
N	2	2			

IFAT 542 D50

	B10	B10D2	t value	DF	
SD	0	0	3.16	2	Result at 0.05
$\bar{x}$	1.5	0.5			is significant
N	2	1			

IFAT 542 D85

	B10	B10D2	t value	DF	
SD	0	0.4	7	2	Result at 0.01 is
$\bar{x}$	3.5	1.8			highly significant
N	2	2			

IFAT 542 D129

	B10	B10D2	t value	DF	
SD	0.6	0.4	3	3	Result at 0.05
$\bar{x}$	3	2			is significant

ELISA 554 D126

	B10	B10D2	t value	DF	
SD	0.064	0.188	3.402	4	Result at 0.05
$\bar{x}$	0.885	0.397			is significant
N	2	4			

Figure 95 Showing mean of ELISA readings in experiment number 542 with ± 1 S.E.

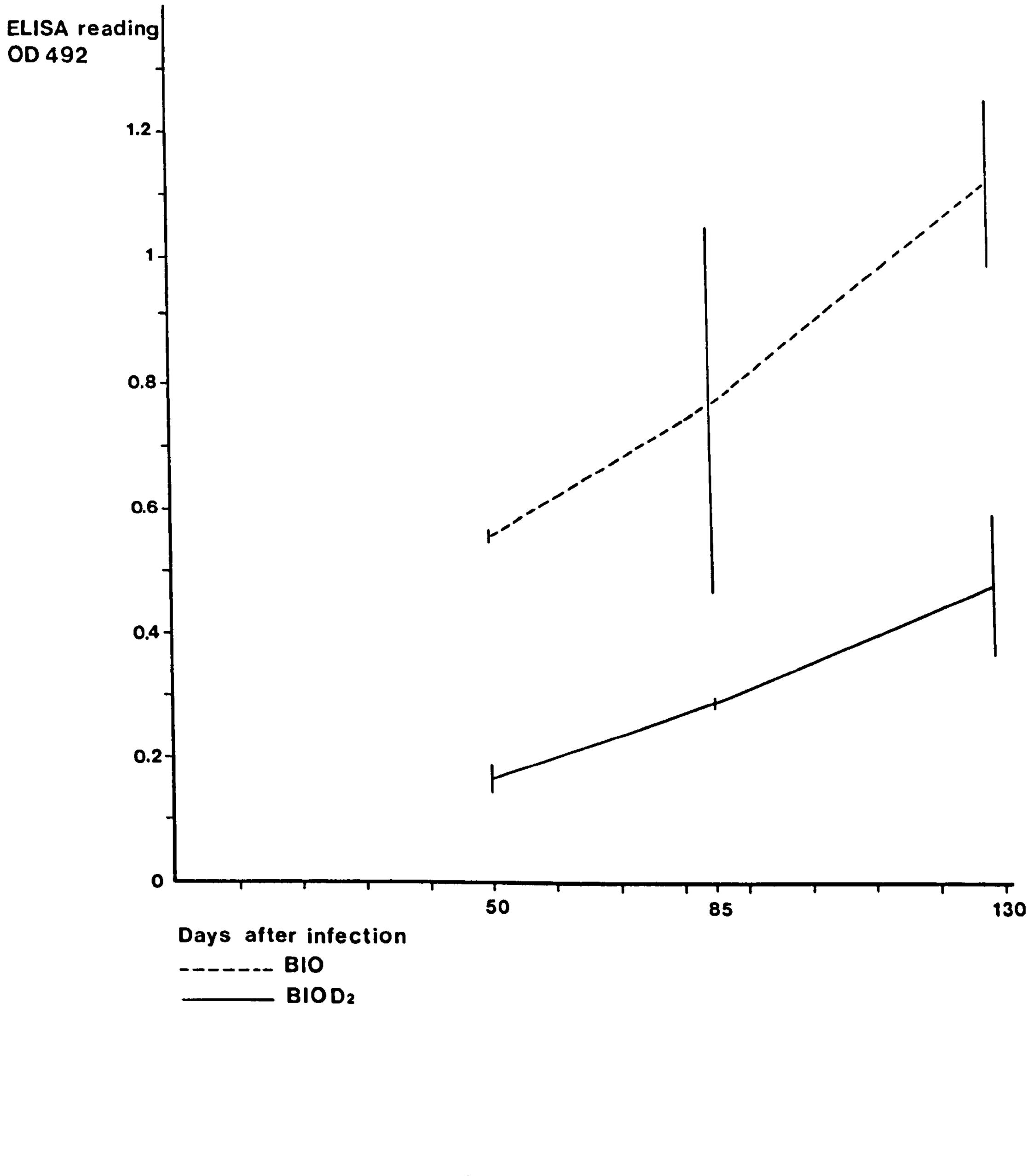


Figure 96 Showing geometrical means of IFAT in experiment 542 with ± 1 S.E.

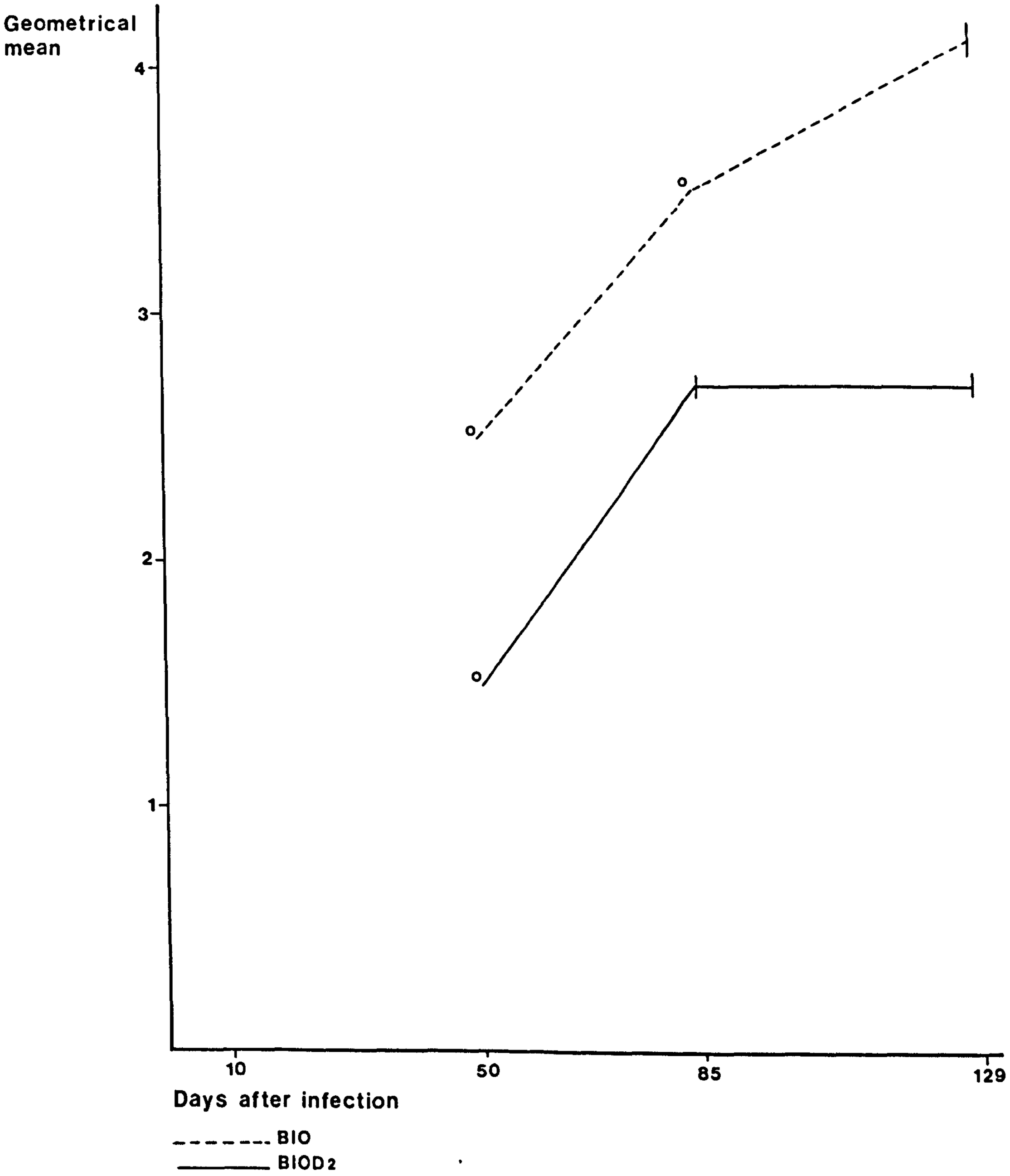


Figure 97 Showing LDU of liver of the mice infected of experiment 542.

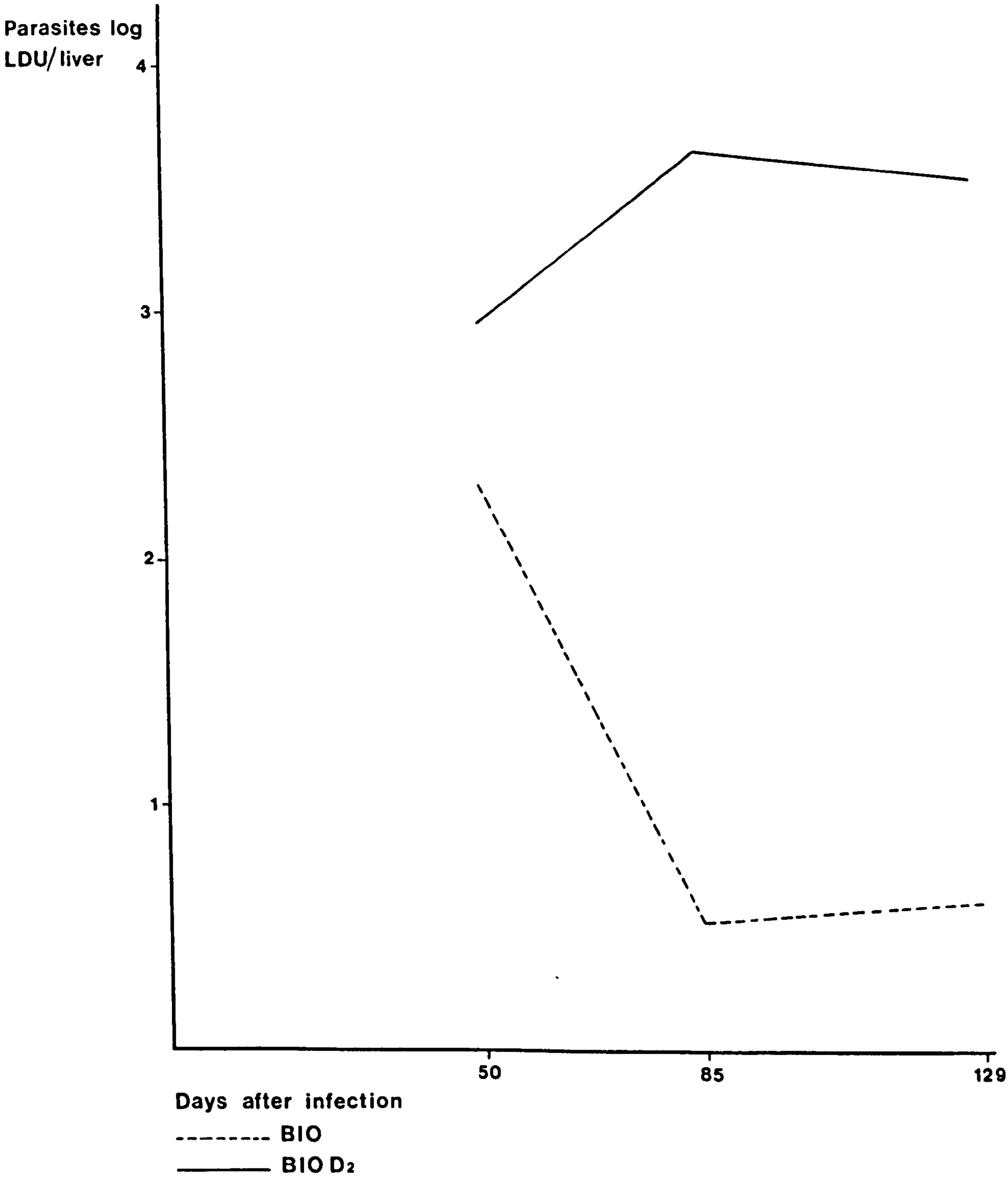


Figure 98 Showing mean ELISA reading in experiment number 552 with ± 1 S.E.

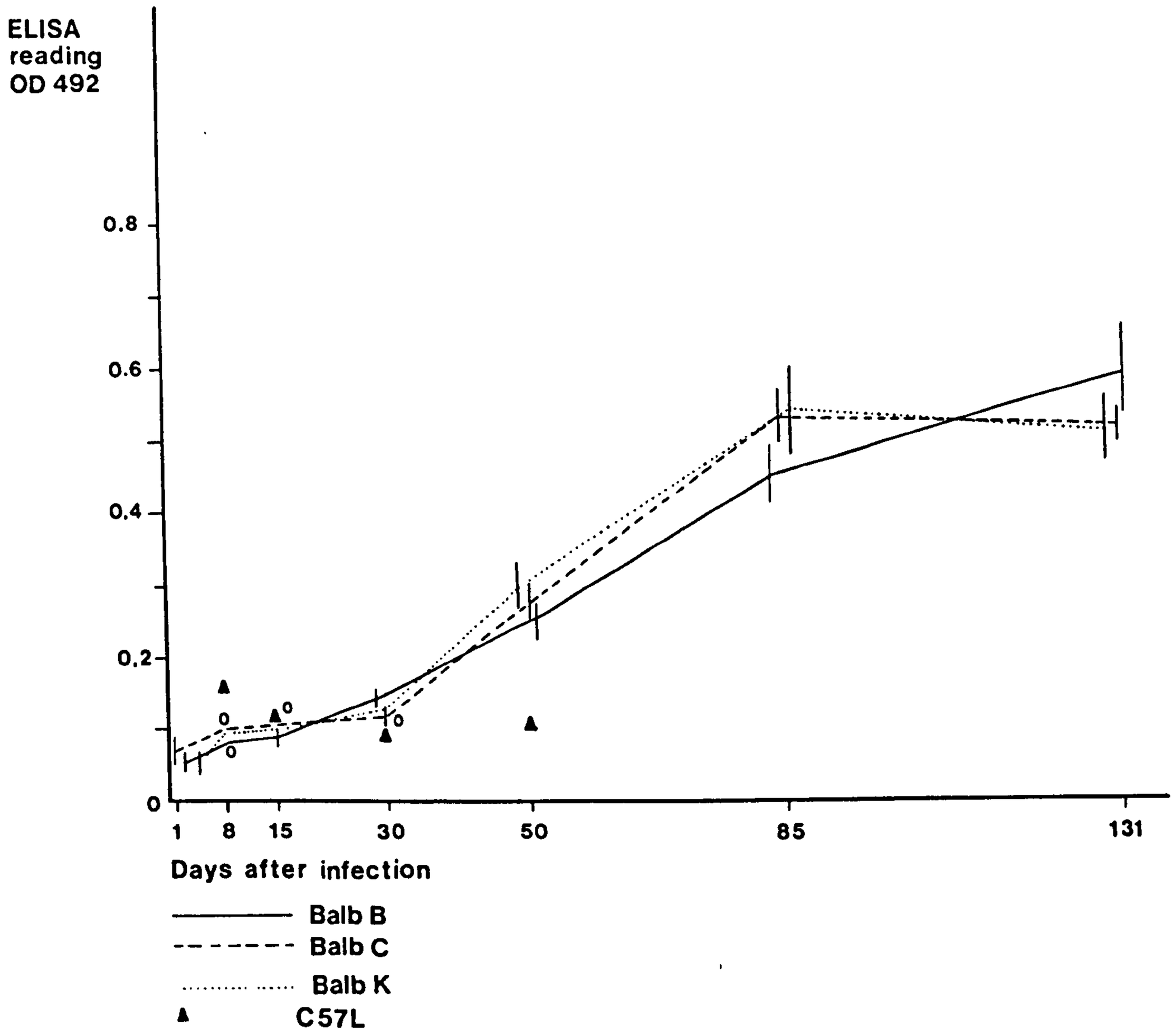


Figure 99 Showing geometrical mean of IFAT in experiment number 552 with ± 1 S.E.

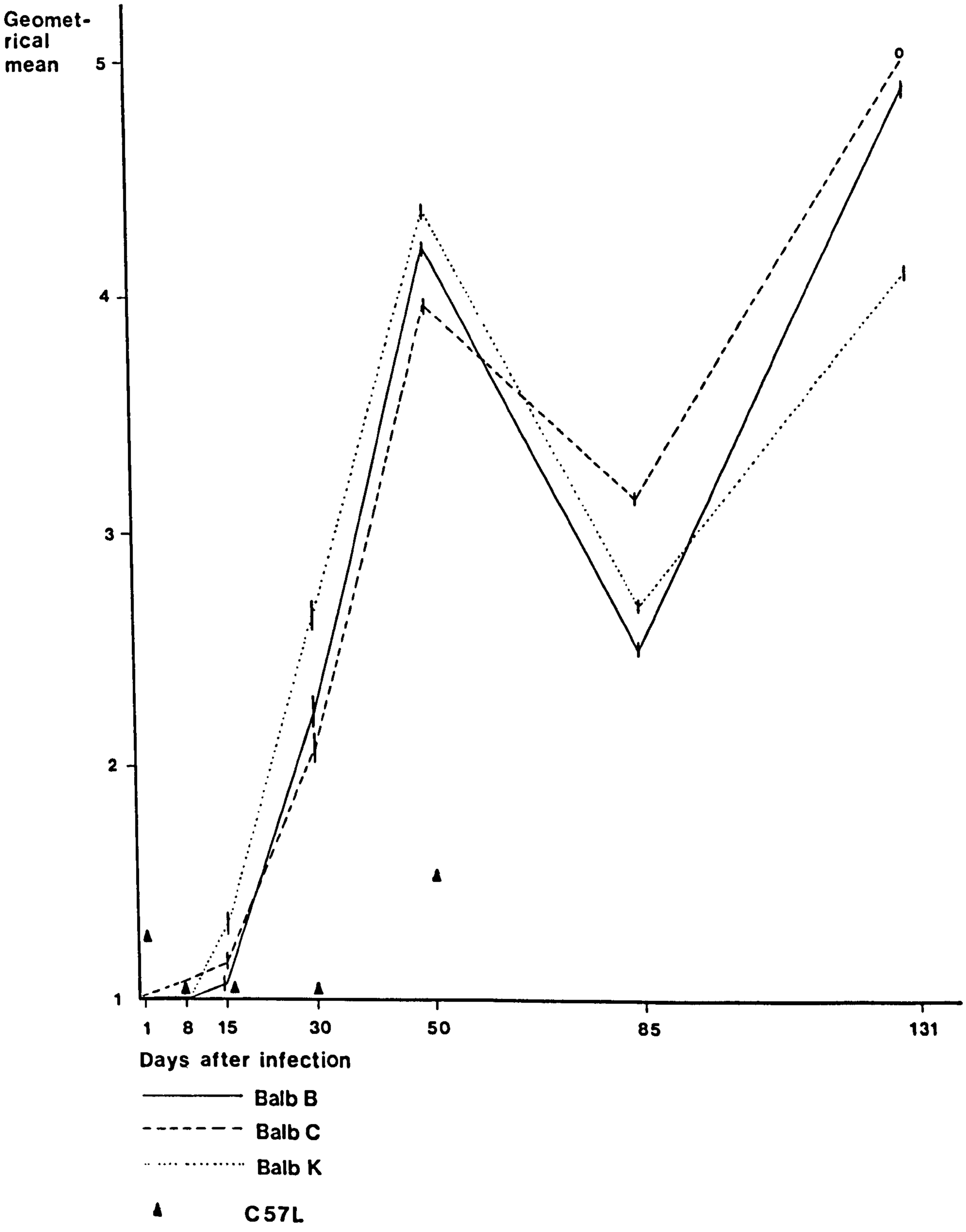


Figure 100 Showing mean LDU values of liver of mice studied in experiment number 552.

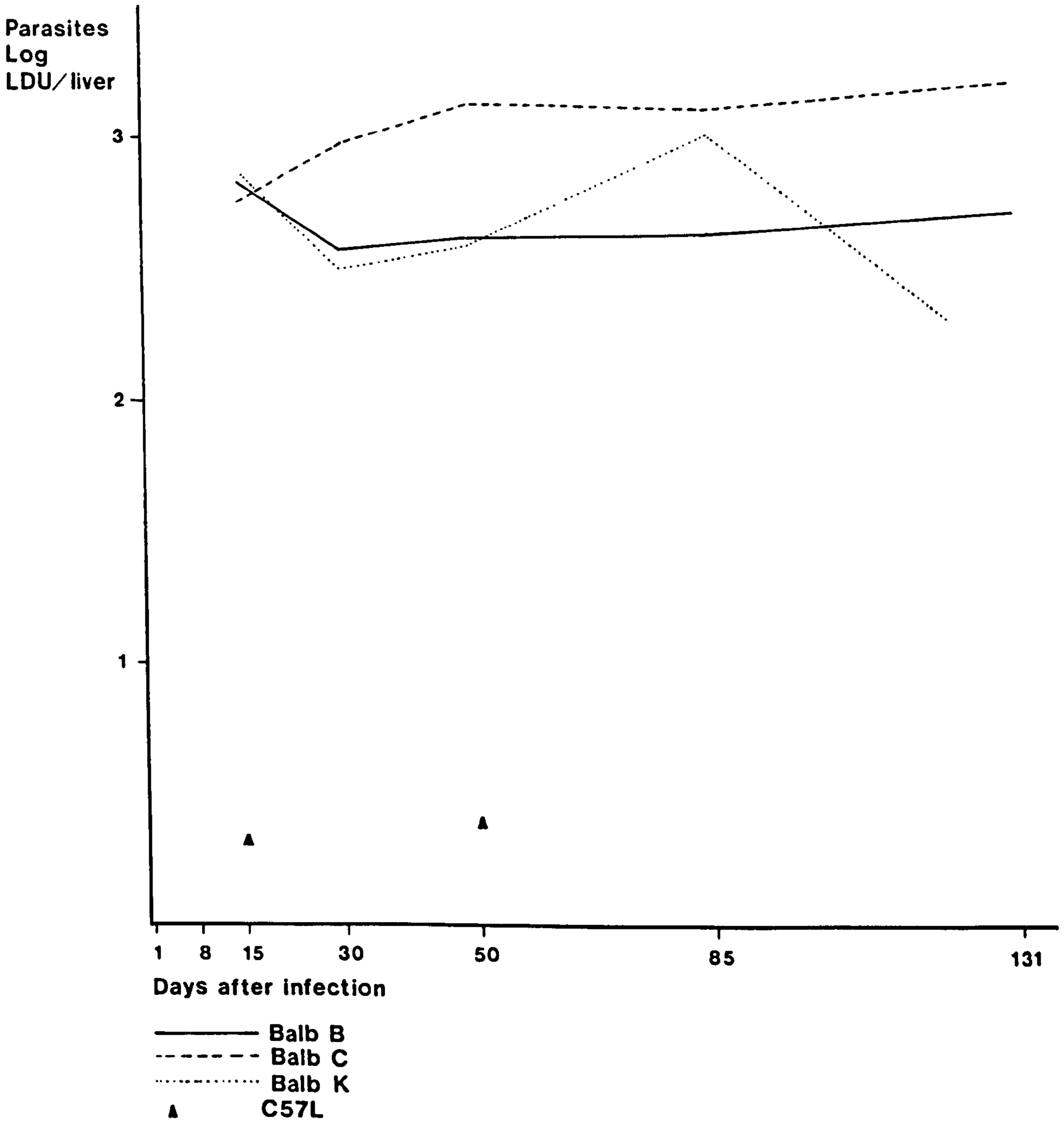


Figure 101 Showing mean ELISA reading in experiment number 553 with ± 1 S.E.

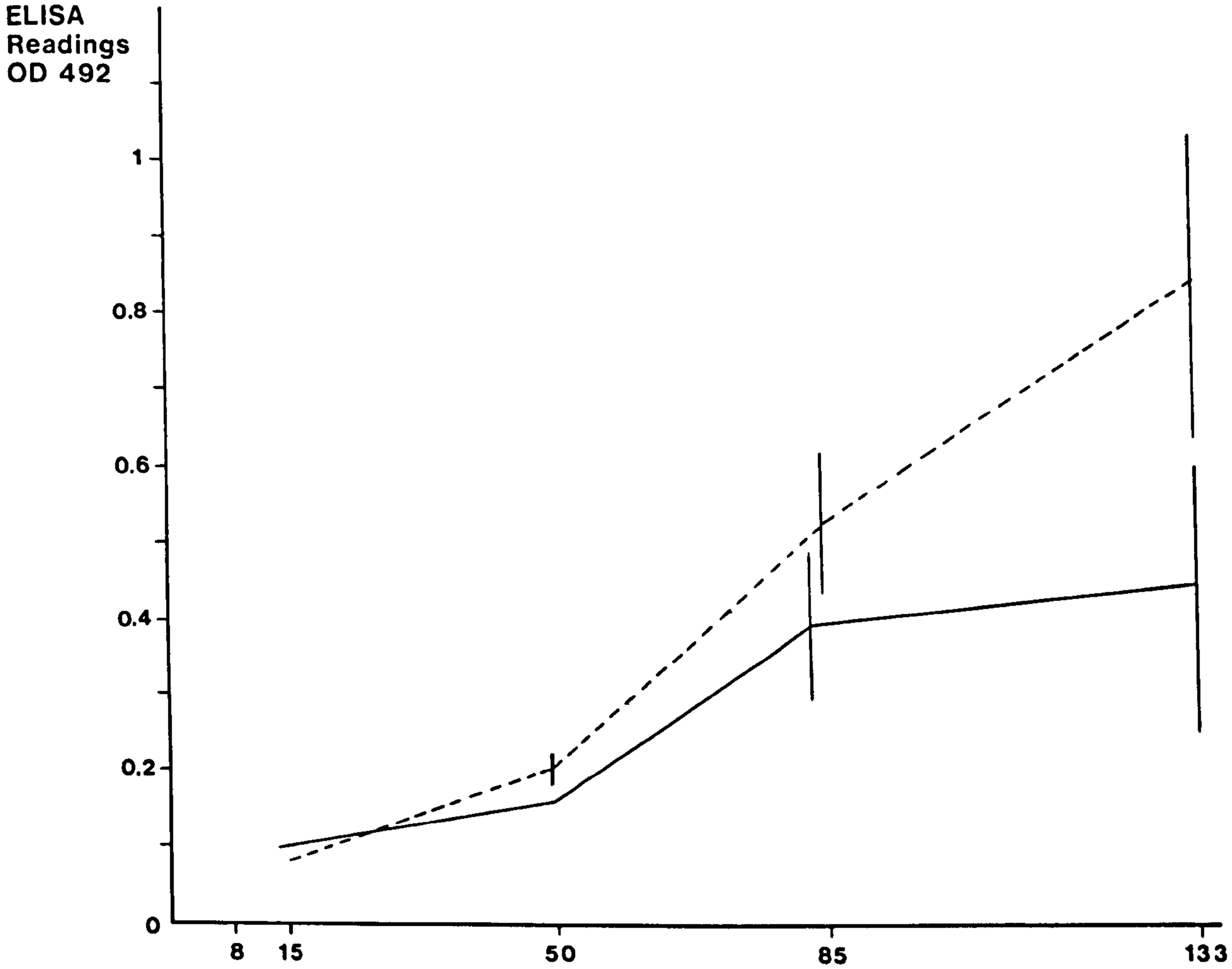


Figure 102 Showing geometrical mean of IFAT in experiment number 553, with ± 1 S.E.

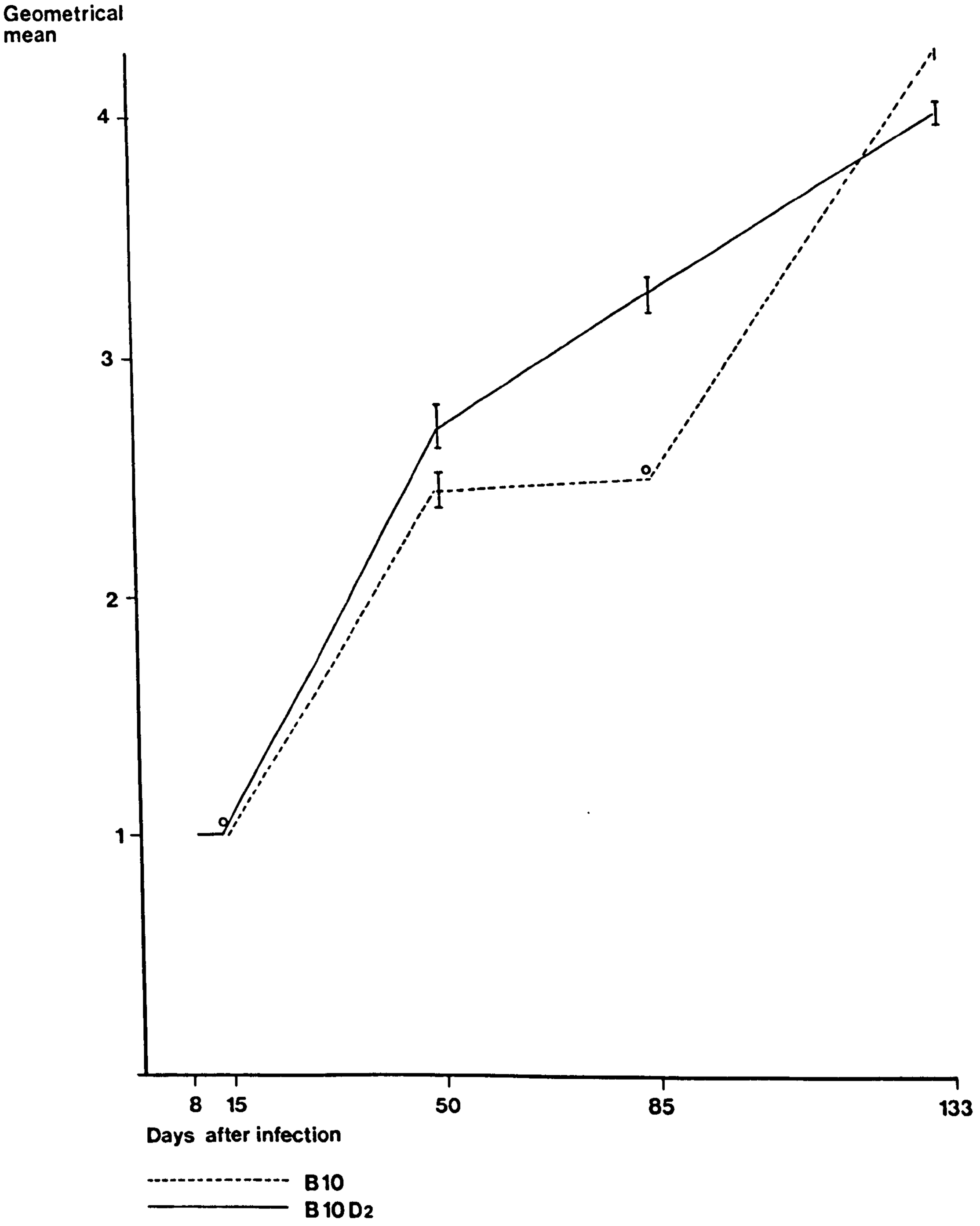


Figure 103 Showing LDU values of liver of mice studies in experiment number 553.

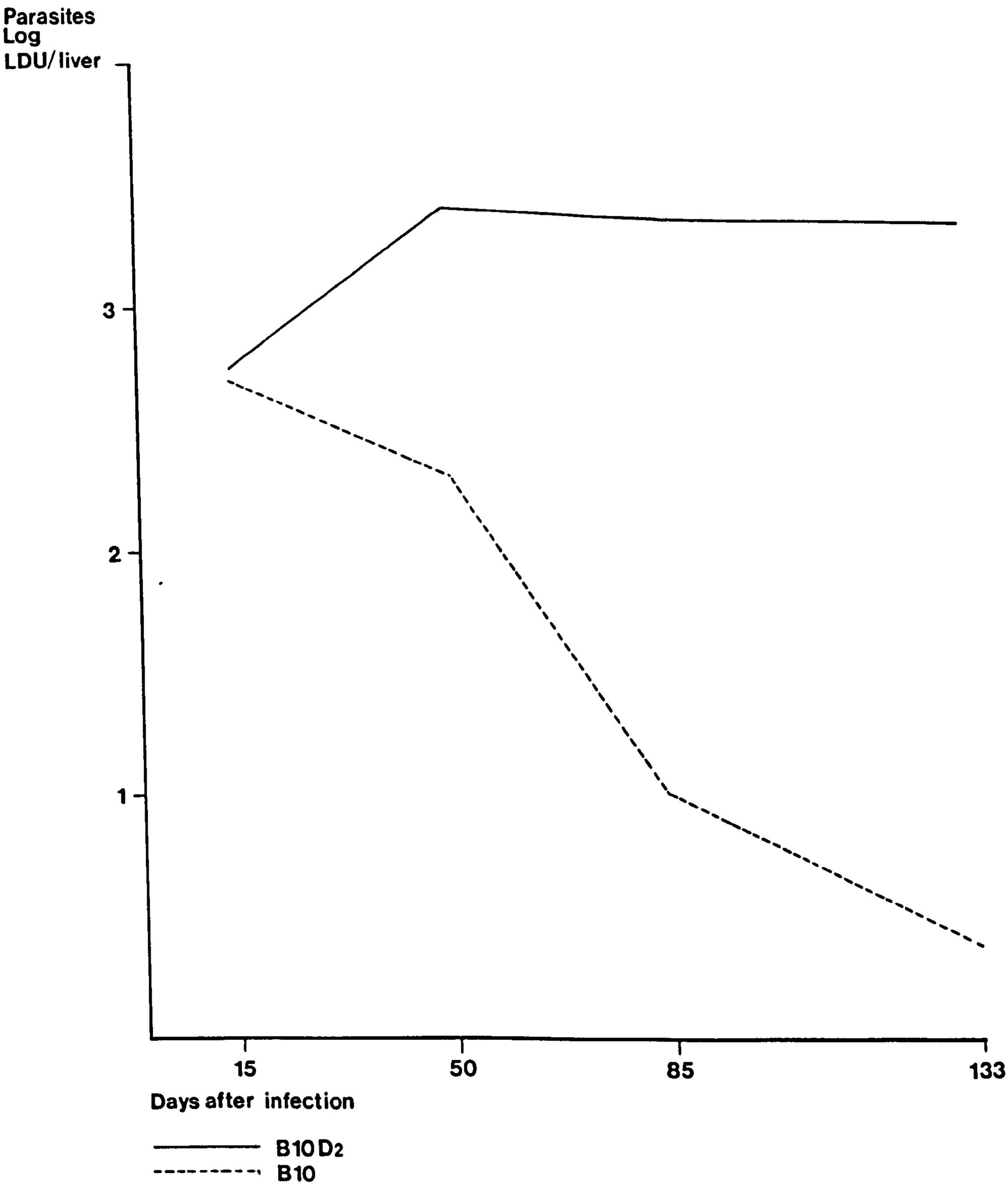


Figure 104 Showing mean ELISA reading in experiment number 554 with ± 1 S.E.

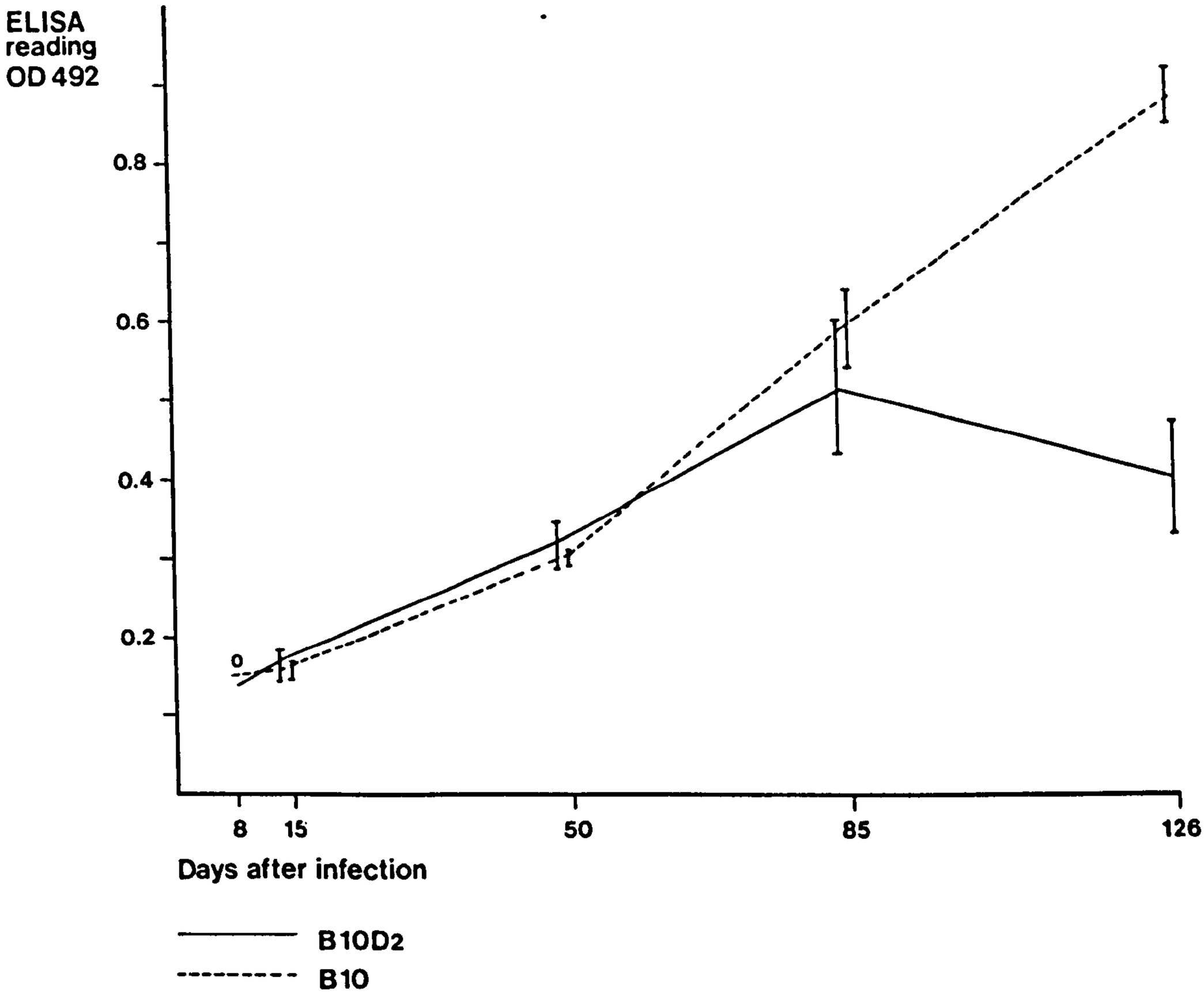


Figure 105 Showing geometrical mean of IFAT in experiment number 554, ± 1 S.E.

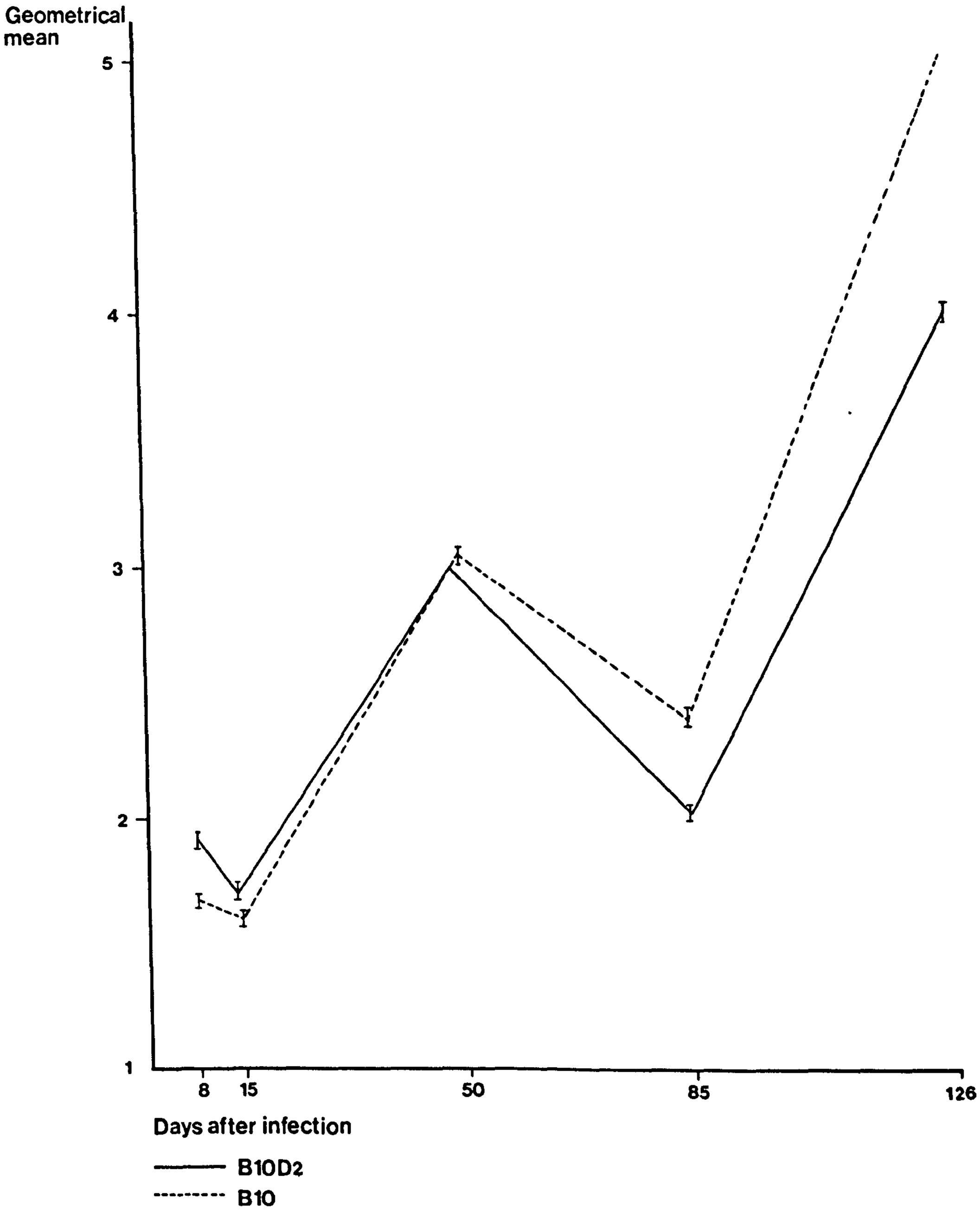
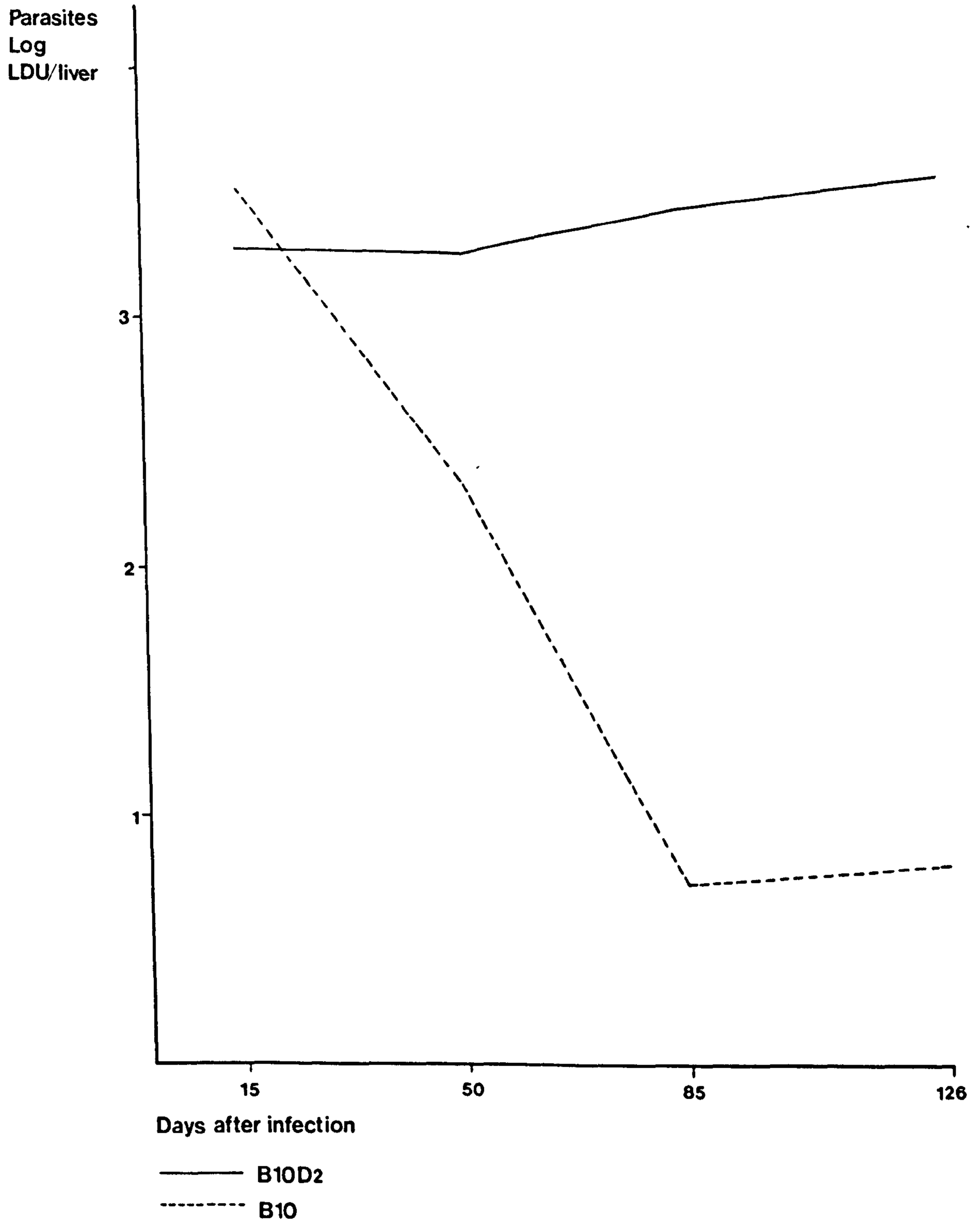


Figure 106 Showing LDU values of liver of mice studied in experiment number 554.



CHAPTER IV

GENERAL DISCUSSION AND SUMMARY

The use of serological methods to detect cases of visceral leishmaniasis was found to be a good alternative method for the conventional use of bone marrow puncture and detection of the parasite, this conclusion is reached after studying all the aspects of the serological tests used (IFAT and ELISA) in defined animal systems and in confirmed human cases, and it was found after doing checker boards for both that with positivity cut of IFAT 1/16 and of ELISA at 0.2 read spectrophotometrically at 492 nm a reliable diagnosis of the disease could be made excluding as much as possible cross reactivity with other diseases. IFAT was found to be more sensitive and ELISA was found to be more specific. These tests were easy to conduct and results were found reproducible.

The best antigen used for fluorescence from the point of ease of production was found to be the promastigote form of the parasite, although intracellular amastigotes were found more specific. The best antigen used for ELISA was found to be the sonicated promastigotes which later was ultracentrifuged at 100 000G. Checker boards for both tests which were done gave an optimal dilution of sera and conjugates and duration for the incubation.

For the purpose of studying specificity and sensitivity of the serological tests used 435 sera were collected from the hospitals, from different diseases, this included 66 bone marrow positive cases of visceral leishmaniasis. They were studied epidemiologically and otherwise and it was found that half the cases were within their first year of life, all were less than six years of age and distribution of cases according to sex showed no significant difference between the numbers of males and females affected. IFAT and ELISA titres increased with age, until it reached the peak at 3 years of age then began to decline again in older children. Comparing the peak of age incidence at first year of life with the peak of antibody production as measured by serology at three years of age, agrees with what was found during the follow-up of bone marrow positive cases of visceral leishmaniasis and their seroreversion, where it was found that about one third of the cases revert to negative titres, implying cure, within 9 months after their detection, 90% of them reverted to negative within two years after being detected and only 10% remained sero-positive for up to five years after being detected.

High mortality among the bone marrow positive cases of visceral leishmaniasis which were followed up was noticed (30.8%), death occurred from a few days to a few months after detection of the case, irrespective of treatment. Clinical and laboratory investigations showed that the classical abnormalities due to the disease were usually detected in these cases. It was found hepatomegaly could be detected in 100% of the cases and around 90% had splenomegaly, 80% had high IgG levels, 83% had high levels of globulins and in 71% of them leucopenia could be detected.

Such follow-up of positive cases was not done before and mortality rates given in literature were calculated from deaths occurring during the child's stay in the hospital, while in this study they were followed up to their places of residence and real mortality rates could be calculated, a mortality rate of 30% of those cases admitted to hospital within short periods after their detection may change our concept of the impact of the disease on cases detected and on the community in general, in spite of medical services being available.

Itwas also not known when seroreversion and real cure happens in a case of visceral leishmaniasis and the child's state of health was never assessed after leaving the hospital. It was found here that a child may leave the hospital after being treated and still have high antibody titres as measured by serological tests and high levels of immunoglobulins, and raised serum proteins specially the globulin fraction could be detected long after their discharge from the hospital. Splenomegaly and hepatomegaly could be detected months and probably in some cases years after the child had left the hospital. Sometimes it was found that a child may be admitted again in a different hospital receiving another course of treatment because of his condition.

Other points like the sex distribution and the insignificent differences in the distribution between males and females, along with the age distribution of the disease is shown in this study. Half of the cases registered were within their first year of life, their mean antibody titre as measured by serological tests used reached the peak at 3 years of life coinciding with the study of seroreversion.

Skin windows were not found to be successful in this study as they did not detect any positivity in the macrophages, and due to the negativity of the results in man and animals they were not pursued any further.

The true picture of visceral leishmaniasis in Iraq could not be well understood without studying defined population groups and following them up for their rates of infection among the risk age and other matters.

So a study of a defined population of 9889 people with 3403 children under seven years of age (34.4% of the total population) was conducted in an area around 300 sq. kilometres just south of Baghdad. This area was rural in nature, it included 1171 houses, 50% of the families living in these houses were of 6-10 people in size, houses were mostly dispersed and jackals and dogs were usually found.

Two surveys were conducted after a pilot study, the first survey before the usual time of recording of cases of visceral leishmaniasis, the other during the time. Children under seven years of age were screened for their levels of antibodies in their sera against the disease by the usual serological methods of IFAT and ELISA, and seropositives were studied more fully and followed up.

Matters that may affect the results of the work were accounted for like the problem of cross reactivity and serological tests used and the area was studied from the point of view of the disease pattern in it. It was found that no oriental sore could be detected during recent years, malaria cases had disappeared for a long time now, leprosy cases never occurred in the area, bilhazia cases were not registered and tuberculosis, as discussed before, leaves the under seven's mostly unaffected. Mapping and census of the area were done for the first time in the area by this work.

The major work included two surveys. Both surveys were conducted within one year and it was found that there were 205 seropositives, around 60 per 1000 of the under seven years of age children in the area. They were found in 176 houses (15% of the houses in the area), the houses with cases were mostly dispersed in space and if located in a village, they were found at the periphery of the village.

Positivity begins early in life, peak reaches around 3 years of age, then it begins to decline, more than 50% of those positives were under 3 years of age, this compared to age incidences of the disease among registered cases of the disease and may account for the difference in the severity of the disease in those hospitalised.

It was similarly found here that there is no statistically significant difference in sex distribution among those positives.

In general it was found that positivity rates among sera collected and examined increase in those months corresponding to the usual time of the appearance of cases of visceral leishmaniasis, similarly mean IFAT titres of those seropositives increase at that time.

Seropositivity was detected among the children aged under seven years during the study and after the child was negative in a previous serological examination; duration of conversion from seronegative to seropositive sometimes took one month. Those seropositives were studied and followed up and ten of them were hospitalised, bone marrow puncture was done on six of them, two showed negative results and four of them showed the parasites in their bone marrow, all ten received treatment, one of them died, he was one of the bone marrow negatives, another child died before it was possible to do bone marrow on him, so in total 2 died out of the 205 sero positives, a mortality rate of 1% of the positives and 0.6 per thousand of the children under seven years of age in the study area, and 25% of the deaths recorded among the under sevens during the study, and 20% of children hospitalised because of the disease.

Other investigations were done on those seropositives and it was found that 65% had their spleens and livers enlarged, 57% had leucopenia, 63% were leishmanin positive and 66% showed abnormality in serum proteins, all of which corresponds to what is usually seen in typical cases of visceral leishmaniasis, but with varying degrees of symptomatology which differ in terms of severity. Control sero negatives were found completely different, they showed about 90% negativity in leishmanin and they were negative in other respects also, like splenomegaly and clinical symptoms.

Leishmaanin testing of the seropositives showed that the size of the leishmanin reactions and the positivity rate of the test according to age tends to increase with age, beginning with negative readings in the first year of life, reaching a peak at 4 - 6 years of age, the lag time between the peak of age distribution at 3 years and peak of leishmanin reading at the age of 4 - 6 years is the time for the process of cure of those cases and the development of the delayed hypersensitivity. It was found also that seropositive cases detected during the second survey when tested with leishmanin gave less positivity than that of the first survey and that could be explained that the cases in the second survey were new

cases and thus did not have the chance to develop delayed hypersensitivity in comparison with those of the first survey. Other evidence supporting this is the higher rates of positivity among tne second survey and higher titres among the positives, and the shift of the age towards the young in the second survey.

It was found also that there was a greater chance of having a seropositive case to be leishmanin positive when he has a higher serological titre; this may be explained on the grounds that the disease has developed to a peak stage where the process of cure has begun and delayed hypersensitivity started to function.

When those two seropositives were followed up it was found that two thirds of the cases reverted to negative in two months time; and 93% in 6 months time, the quicker seroreversion here may be accounted for by the degree of symptomatology and hence the severity of the disease.

So in conclusion it seemed that the real picture of the disease may differ considerably from what is already known, because previously as the work is restricted to hospitals and hospital cases, the picture was not complete, and attack rates could not be worked out.

Now after this study had been done it is possible to work out attack rates of the disease in endemic areas, and to clarify more the epidemiological picture of the disease.

Suggestive evidence was found in this study that all seropositives detected were cases of visceral leishmaniasis because they have the abnormal findings usually found in typical cases of the disease, except that symptomatology and severity of the disease seem to extend along a spectrum beginning at one end with the typical case of the disease to the other end of the spectrum where signs and symptoms could be milder and conditions subclinical and child conditions may be passed unnoticed, compared with the control group, which were the seronegatives, which lacked these classical abnormalities. The age distribution compared to those which are hospitalised shows a peak around three years of age, their leishmanin positivity increased with age. The age distribution in general, and the clinical picture in particular are affected by many factors like the state of maturity of the immune system, the nutritional state of the child, the exposure rate of the child to the bites of infected sandflies and thus the size of the inoculum, the nearness of the location of the child to the normal habitat of the sandflies, and to other factors probably like

parasitic factors and genetic background.

Sex was found not to differ significantly.

Time of appearance of cases seems to be during the usual time of registering of the classical cases of visceral leishmaniasis in hospitals, and due to the length of the period of positivity they were detected along the whole year.

As to distribution in space it was found that cases usually occur in isolated dispersed houses where contact with infected sandflies from the reservoir of the disease is more probable. There were no obvious geographical clustering of seropositives.

The following figures and rates clarify the situation more: It was found that there were 205 seropositives, i.e. 60 per thousand of the under seven years of age, or 20 per thousand of the whole population were affected during one year, 10 of these cases were hospitalised and treated as cases of visceral leishmaniasis due to the severity of their state, i.e. around 3 per thousand of the under seven years of age in the study area, and one in a thousand of the whole population. Some of these showed parasites in their bone marrows.

2 children died in the area in one year because of the disease, that is around 0.6 per thousand of the children under seven years of age or 0.2 per thousand of the whole population die yearly because of the disease. It also means that around 5% of cases of visceral leishmaniasis in Iraq reach the hospitals and get registered and only around 1% of the real cases die yearly because of the disease, compared with the 30% mortality of acute hospitalised cases. 25% of the deaths in the under seven years of age were due to visceral leishmaniasis.

Work on the reservoir included the study of 151 jackals, 45 foxes, 1 wolf, 65 dogs, 18 mice and 68 rats. Work on canines was transferred to other endemic areas of the disease, due to administrative difficulties. In general no parasite could be isolated from any animal, but serological examination showed significantly high positivity rates (60%) among the jackals (Canis aureus) and high titres of positivity (up to 1/128 by IFAT) among them, compared to the low (10%) positivity in foxes and dogs - suggesting that the jackal is the animal to be suspected epidemiolically and serologically as the animal reservoir of visceral leishmaniasis in Iraq.

Small scale entomological investigations in the study area failed to reveal promastigotes in the sandflies dissected, but it showed that P. papatasi is more commonly found in association with man and animals, and that man is exposed to 25 - 42 bites per hour by sandflies in the study area. The sandfly density was high (10 per room).

Longitudinal follow-up of the serological reactions in inbred mice with different genetic backgrounds infected with L. donovani showed that there was very low reaction, nearly negative, in the innately resistant mice, while in the susceptibles, including the balbs significant levels of seropositivity were detected by day 50 of the infection, then positivity increased until the end of the experiment at day 130, regardless of the parasitological state of the animal. In some experiments significant differences in the antibody level as measured by the serological methods used could be detected between the cure and non-cure types of the innately susceptible mice, significantly higher levels were found in the susceptible cure mice, than with non-cure. Sometimes in the cure type high levels of serological positivity were encountered when the parasite density in the liver is low.

Overall, this study begins to use serological methods for elucidating the epidemiology of visceral leishmaniasis and suggests their potential for changing our understanding of the epidemiological processes involved.

CHAPTER V

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