

TITLE

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**EPIDEMIOLOGICAL SIGNIFICANCE OF IMMUNE  
STATUS OF COMMUNITIES IN KALA-AZAR  
ENDEMIC AREAS.**

**A CROSS SECTIONAL AND COHORT STUDY.**

**Dissertation submitted in partial fulfillment  
of the requirement of the Dr. MGR Medical  
university for the  
degree of M.Sc Epidemiology.  
MARCH 2001.**

## CERTIFICATE

This is to certify that the thesis entitled "EPIDEMIOLOGICAL SIGNIFICANCE OF IMMUNE STATUS OF COMMUNITIES IN KALA-AZAR ENDEMIC AREA" is a bonafide work by Dr.Rajan R.Patil in partial fulfillment of the rules and regulations for MSc. Epidemiology examination of The Tamilnadu Dr. MGR Medical University Chennai to be held in March 2001.

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## INTRODUCTION

Kala azar is a disease of public health problem predominantly in the third world countries. India Bangladesh and Nepal account for 90% of the disease burden of the world. It comes under the so called 'orphan diseases' owing to its neglect both at national and international level.

Kala-azar is an endemic disease in parts of India. It is a serious problem in the states of Bihar and West Bengal. Annual reported number of cases is 24665 with frequency of 36.8/100,000 in 1996 in Bihar. In West Bengal total reported cases for the same time period is 1987. UP occasionally is in news, with sporadic outbreaks. The case fatality rate of the disease in untreated cases is close to 90%, which with treatment reduces 15% and is 3.4% even in specialized hospitals<sup>1</sup>.

Planning for Kala azar is hindered by conflicting reports of official and actual figures. Just to give an instance, 54650 cases were reported from Bihar In 1990, but an expert team constituted by government of India in 1991 chaired by CP Thakur, put the actual numbers close to 250000. The latest annual figures were 15485 and 6750 cases in 1998(till May) of 1997<sup>2</sup>

Kala azar has re-emerged from near eradication all over the world. Its annual estimate for incidence and prevalence worldwide is 0.5 million and 2.5 million, respectively <sup>1</sup>.

India too showed the same trend, an epidemic of kala-azar in Bihar, (India) in 1950s died out within a decade, as a collateral effect to the DDT spraying which was part of the National Malaria Control Programme. Sand flies disappeared from the house sprayed with DDT to eliminate Anopheles mosquitoes. The disease became rare. When spraying was discontinued however, sand flies surviving in cattle shed returned to the houses and some became reinfected with Leishmania, possibly by feeding on the remaining Post kala-azar Dermal Leishmaniasis (PKDL) cases <sup>3</sup>

For prevention and control of the disease, epidemiological works at field level is a must to understand the burden of the disease. The diagnosis and treatment are important aspect of kala azar control programme. Reliable and valid diagnostic tools are required for the success of control programme.

WHO has laid emphasis on developing simple tests for diagnostic as well as screening programmes. The aldehyde and bonemarrow aspirate tests presently used are seen wanting in sensitivity and specificity. Moreover they can't be employed for the screening programmes. <sup>4</sup>

More studies are recommended to assess the utility of newer serological tests like Direct agglutination test for diagnostic as well as screening programmes<sup>5</sup>

Leishmanin skin test is valuable tool for epidemiological works to measure past and possibly future incidence in the world<sup>6</sup>

## JUSTIFICATION OF THE STUDY

- ◆ Information on the magnitude of kala-azar in endemic areas and among tribal communities is limited.
- ◆ Uncertainty regarding the usefulness of serological tests used in the screening as well as diagnosis of kala-azar.
- ◆ Need for developing indicators for assessing factors responsible for the disease transmission, which would be in turn help in assessing impact of intervention programmes.

## OBJECTIVES

1. To estimate the prevalence of Kala azar infection (present and past) in Pahadias and Santhal tribal communities of Jharkhand state.
2. To study the dynamics of the antibodies in cured cases of kala-azar for its implication on the validity serological test.

#### 4.1 HISTORY OF KALA-AZAR

Kala-azar has been occurring in India in epidemic and sporadic form over the past centuries. The earliest epidemics of Kala-azar were confused with those of malaria. However, the first recorded epidemic which could be attributed to the manifestations of the disease in India could be in 1824-25 in Jessore (Elliot). Burdwan fever of 1854-75 was also attributed to Kala-azar (roger). The first epidemic in Assam is supposed to have occurred in 1870 in Garo Hills. However, Carke first described the disease in Assam in 1882 in his Sanitary report of Assam based on the report on MC Naught , the then Civil Surgeon of Turf. Kaladukh in Purnea (Brown 1898) and the Kala-azar in Darjeeling (Ross 1899) are attributed to Kala-azar. The epidemic fever <sup>in</sup> dinaz pur and Rungpur Between 1871-76 and in Patna during 1856-59 could also be attributed to Kala-azar.<sup>7</sup>

Leishman in 1903 reported peculiar bodies in the spleen of a soldier who died of Dum Dum fever in Netley Hospital. Donovan in the same year reported detection of similar bodies in the spleen of the patients in Madras suffering from prolonged fever with splenomegaly. Therafter, conclusive evidence was obtained and the causative agent i.e., the parasite was named an after Leishman and Donovan.<sup>8</sup>

#### 4.2 KALA-AZAR DISTRIBUTION -PLACE AND PERSON.

Apart from several states in India, the disease is present in East and North Africa, Sudan, Bangladesh, parts of china and USSR, South Iran, Arabia, Mediterranean countries of Europe, Ethiopia, Kenya and South America, including Brazil and Venezuela. In India, the disease is most endemic in eastern half, but isolated pockets exist in almost all regions from where sporadic cases and even small outbreaks have been reported from time to time.

In Mediterranean region, infants and children are almost exclusively affected. In other endemic areas including India, though these are more frequent in children and the young, older age groups are also susceptible. Males are affected more than females. Kala-azar is more prevalent in rural settings where conditions exist for multiplication of the main vector sand fly *Phlebotomus argentipes*. Only females act as vectors.<sup>9</sup>

#### 4.3 RESERVOIRS OF INFECTION IN INDIA

No animal reservoir has been found in India and transmission of kala-azar occurs from man to man through the recognized vector *P. argentipes*. The cutaneous or dermal leishmaniasis is caused by *L. tropica* and this is restricted to Rajasthan where it is zoonotic.<sup>10</sup>

In some regions such as northeastern India, human appear to be their own reservoir and several factors serve to facilitate person-to-person

transmission. The vector *P. argentipes*, has a preference for human blood. The parasite is found in circulating monocytes in Indian cases of Kala-azar more frequently than usual. Dermal lesions that develop after the initial disease in Indian patients' may be an additional source of parasites for the vector.

#### 4.4 RESERVOIRS OUTSIDE INDIA

A common transmission cycle involving man and animal is seen between humans and dogs which act as canine reservoir. The domestic dog, as wild **canines** such as the fox, develop a chronic systemic disease very similar to that of humans when infected with *L. donovani*. But an additional unique feature of leishmanial in canines is the frequent presence of organisms in the skin including the nose and ears, which are favorite feeding sites of sand flies. An epidemiological cycle of parasite **involving wild foxes, domestic dogs, and human** via the vector *Lutzomyia longipapisi* has been documented in northeastern Brazil. The domestic dog has also been incriminated as an important reservoir host for visceral leishmaniasis of the Mediterranean region and in certain areas of China.

**Rodents** are the likely reservoir in the Sudan, and activity of the vector, *Phlebotomus orientalis*, is high in clumps of acacia woodland near villages. In Kenya, transmission of disease is associated with termite hills, which serve as resting places for the vector *P. martini*, and around

which village men gather in the evening. However, the animal reservoir in Kenya has not been identified.<sup>11</sup>

#### 4.5 VECTORS

Sinton originally suggested Sandfly as a possible vector in 1922.

There are about 600 species of the phlebotomine sandflies distributed through out the world and among these 70 are proven or suspected vectors of leishmaniasis. In India, the only proven vector of kala-azar is *phlebotomus argentipes*. The biology of this vector in West Bengal has been worked out in details. It breeds in cowsheds. They can attack man. The vector can invade the clean biotype from the nearby resting places, attacking man out doors, hence acquiring out door infection is also possible. It was revealed that they could take 5 blood meals in nature, especially in rainy season, indicating high vector potentiality. In this connection it may be mentioned that period of peak transmission usually corresponds to the time when vector population is relatively old (as in rainy season June to September).

*P. argentipes* may disperse 500 m from the place of release. The flies can invade the living rooms and both exit and entry activities concerning cowshed and human habitation.<sup>12</sup>

8. Age and sex have no influence on the epidemic pattern. There was a definite decline in cases of Kala-azar in India from 1961. In 1960 there was 3916 number of cases and in 1961 only 196 cases were recorded. In that time Kala-azar was not at all a public health problem. The factors responsible for the decline were associated to<sup>7</sup> :

- Effective treatment of cases,
- DDT spraying under NMEP during 1953-57 and under NMEP from 1958
- Increased immunity in the population

#### **4.9 INCUBATION PERIOD**

It has been difficult to estimate the incubation period of kala-azar. Usually it is between 2-6 months but could be as short as 10 days or as long as 9 years

The ratio of clinical to subclinical infection may range from 1:5<sup>4 12 15</sup>

#### **4.10 IMMUNITY IN KALA AZAR**

Kala-azar unlike other diseases (parasitic) is characterized by near absence of a second attack after successful cure of the first.

Leishmania parasites are able to persist very successfully in immunocompetent individual and they are also able to suppress the immune response suggesting that relationship of the parasite with man is very old.<sup>12</sup>

Visceral leishmaniasis has always been thought to be unique, in that infection of man with *L. donovani* resulted in kala-azar syndrome with little or no immune response, poor cellular tissue response, many parasites and no delayed hypersensitivity. However it was found that delayed hypersensitivity and a positive leishmanin skin developed after 6 months of treatment henceforth such cases were immune to reinfection.<sup>6</sup>

Resistance to *Leishmania* depends upon the development of specific cell mediated immunity. The capacity of the individual to mount such response varies, and consequently Leishmaniasis presents a spectrum of disease in the same way that leprosy does. At one end of the spectrum lie visceral leishmaniasis and diffuse cutaneous Leishmaniasis, characterized by abundance of parasites, absence of lymphocytes in lesions, insensitivity to leishmanin, and poor prognosis. At the other end lie the self-healing sore, with relatively scanty parasites, marked lymphocytic infiltration, and leishmanin sensitivity. Accompanying cell mediated immunity is delayed hypersensitivity to numerous parasite antigens, which causes the destructive pathology of leishmanial ulcers, especially in chronic conditions of espundia and Leishmaniasis recidiva in which the normal balance between immunity and hypersensitivity has been lost. The mechanism underlying these abnormal immune response are not understood.<sup>16</sup>

The major biological properties of viscerotropic leishmaniasis is its invasive character which permits escape from the cellular defenses of the host at the portal entry. The rapidly multiplying parasites within the cells of mononuclear phagocytic system disseminate widely in the tissues of the host particularly in the haemopoietic and lymphopoietic system. .

The initial response to Kala-azar seems to stimulate both specific and non specific increase of immunoglobulins. The specific response to leishmania antigens is not perhaps protective. The non-specific increase of immunoglobulins in Kala-azar may be the result of deviation to systemic lymphoreticular system of antigens, which are normally mopped up by the kupffer cells of liver.

Spontaneous cure as well as 'premunition' or 'sterile' resistance due to sub clinical infection in endemic zones is more frequently encountered in Indian Kala-azar. It appears that in Kala-azar, there is exaggerated stimulation of the production of immunoglobulins some specific and other non-specific. On the other hand, it fails to stimulate requisite CMI necessary for spontaneous cure and sub clinical infection, suggests that the human host can mount up sufficient immunological reactions both humoral and cell mediated, to protect the individual. CMI probably plays the crucial role but the T cells are suppressed during active phase of the disease. <sup>17</sup>

The parasite stimulate several antibodies producing B-cell including group and genus-specific (polyclonal) as well as species specific (monoclonal) cells. Since the antibodies elicited are more of a reactionary than protective in nature.

The seropositivity in kala-azar is a long lasting (few years), during any serological assessment a positive test, is an indication of recent or past infection and not active disease.

There are several hypotheses on why the antibodies linger very long after cure :<sup>16</sup>

1. Agglutination antibody have longer life span
2. a clinical infection could have persisted, eliciting circulating antibodies, treatment having not been sufficient to result in parasitological cure
3. cured patients had been re-exposed to Leishmania in endemic area.<sup>18</sup>

These circulating antibodies of humoral immunity offers very little help in protecting against re-infections. The Cell mediated immunity (CMI) which is suppressed during the active disease, develops well following treatment or self resolution. The leishmanin skin test is, always negative during the active phase of the disease and becomes positive after six months of initiation of treatment.

With development CMI, patients recover from infections and achieve lifelong immunity. No record of second attack of visceral leishmaniasis is there. Positive leishmanin test, acquired naturally from inapparent infections also protects from infection.<sup>12</sup>

In the absence of recovery in Leishmaniasis, there is bonemarrow suppression, impairing the immune response to host of infections like tuberculosis, pneumococcal pneumonia etc. Leishmaniasis has been recognized as one of the infection that may complicate the course of AIDS.<sup>15</sup>

#### **4.11 CLINICAL MANIFESTATIONS OF LEISHMANIA DONAVANI INFECTION**

##### **UNAPPERENT INFECTION.**

At this stage the infection by the parasite although is unable to produce disease, results in stimulation of immune system.

##### **OLIGOSYMPOMATIC/SUBCLINICAL INFECTION**

In this condition there might be transient clinical expression in the form of fever and hepatosplenomegaly. Many of these individuals might cure themselves spontaneously without specific treatment. A small proportion of these cases might progress to develop frank kala-azar and /or dermal leishmaniasis within a period ranging from 4 weeks to few months.

## LYMPHATIC LEISHMANIASIS

This is condition of generalised lymph node involvement by the parasite leishmania donavani, without clinical signs of enlargement of liver or spleen.

## KALA-AZAR.

Kala-azar is the severest form of clinical expression <sup>of</sup> infection <sup>with</sup> leishmania donavani in man and is a result of uninhibited multiplication of the parasites because of cell mediated immunosuppression. If not treated, majority of the cases end up fatally.<sup>7</sup>

Clinically it presents with fever of varying duration and types often for years. The onset is usually insidious, especially in indigenous peoples who may feel well and have good appetite despite daily bouts of fever. In over 80 percent of cases, however, the fever eventually develops a characteristic pattern with twice daily elevations and may undulate as in brucellosis. Splenic enlargement is not necessarily rapidly progressive, nor does the size of the spleen correlate with duration of the disease. In many instances, however, it reaches the right iliac fossa. The liver enlarges more slowly and becomes palpable in 20% of the cases and is likewise firm and not tender.<sup>19</sup>

Pigmentation of skin. - around malar bones and temples and around the mouth (hence labeled black sickness- kala-azar) Hair becomes dry, thin and brittle.

Haemorrhagic manifestations- epistaxis seen often, haemorrhages of skin, mucous, membranes and retina are rare.

In the most acute cases fever and toxemia may be the only signs

Tongue is always nearly clean.<sup>20</sup>

### **POST KALAZAR DERMAL LEISHMANIASIS (PKDL)**

PKDL is perhaps second to no other disease in regard to its enigmatic etiopathogenesis. Although the same parasite causing kala-azar is responsible, very little is known about the mechanism of development of PKDL. This is a condition, which primarily affects skin and in late cases may involve mucus membranes of eye, respiratory tract. GI tract and genitalia, In majority of cases there would be a past history of Kala-azar however a sizeable proportion do not provide such past histories, although there are evidences of prior visceralisation.

Some Kala-azar cases may develop post Kala-azar dermal leishmaniasis (PKDL) this apparent change in viscerotropic properties of *L. donovani* to dermatotropism is believed to be induced by immunoregulatory mechanism in cured Kala-azar patients.

**PKDL shows important differences from Kala-azar, which are important in terms of epidemiology.**

- ◆ About 20% of Indian patients develop a rash one to two years after treatment or spontaneous recovery. The lesions develop slowly and

may last for several, 20 years. In Africa the rash develops in 2 percent of cases, usually during treatment.<sup>17</sup>

- ◆ Maximum number of cases have been described from India and Bangladesh, In other endemic regions PKDL is rare or absent.<sup>8</sup>
- ◆ Unlike kala-azar, PKDL is never fatal and can remain active and a potential source of sand fly infection in untreated patient for at least 35 years. Unless free treatment and free transport to clinics are offered poor patients, they seldom report for treatment of disease with relatively mild symptoms.<sup>3</sup>
- ◆ Possibility of PKDL cases, as a potential source of reservoirs of infection in a community, except for theoretical basis has never been verified.<sup>12</sup>

#### **4.12 DIAGNOSIS OF KALA-AZAR**

In Kala-azar endemic areas, all the fever cases of more than two weeks duration, which do not respond to anti-malarials, should be suspected for Kala-azar

Laboratory confirmation diagnosis is by demonstrating *Leishmania donavani* in Stained smears from bone marrow, spleen, liver, lymph glands or blood and by recovery of the parasites by culture of these materials on NNN media

1. Examination of peripheral blood smear, leukopenia with relative neutropenia is seen
2. There is marked production globulin by the plasma cells in Kala-azar. This increase in globulin is detected by aldehyde test. It becomes positive only after 2-3 months of infection. Napier's aldehyde test: one ml of clear serum from the patient and a drop of formaline kept at room temperature. A positive reaction is gellification and opocification of serum within 3-30minutes.
3. Serological tests such as by indirect immunofluorescence (IFT ) & Enzyme linked immunosorbent Assay (Elisa)

#### **4.13 DIAGNOSTIC TESTS**

##### **DIRECT EVIDENCES.**

This method involves demonstration of the parasite in the smear (for amastigotes) culture (to demonstrate promastigote)

##### **BONE MARROW EXAMINATION**

Bone marrow aspirations is a painful technique and also needs experienced medical persons. However the greatest disadvantage of the technique is its poor sensitivity, which ranges from 55%-70% depending upon the duration and severity of the disease. Because of its pain, the people have not accepted this method very well.<sup>7</sup>

## **SPLENIC ASPIRATION**

Although splenic aspiration gives a positivity rate of about 85%-90%, this method is totally unsuitable for many rural situations because of the danger of intraperitoneal hemorrhage leading to death due to abnormality in blood coagulation during Kala-azar. The other disadvantage of the method is that it cannot be done in smaller sized spleen (which should be more than 4-5 cms. Below the left costal margin) thereby failing to diagnose early cases when the spleen size would be very small.<sup>7</sup>

## **ALDEHYDE TEST**

One drop of 30% formaldehyde or commercial formalin is added to 1 ml. Of serum of patient's blood. The tube is well shaken and placed at room, temperature. Jellification with opacity like the white of parboiled egg occurring within 60 minutes is a positive reaction. This test becomes positive within 1-2 months of development of Kala-azar.

## **4.14 SEROLOGICAL TEST.**

Serological tests derive their main advantage from the fact that the antibodies appear much before the parasites are demonstrable in the clinical materials at a significantly early stage, so much so that by using these methods one can identify prospective kala-azar cases <sup>21</sup>

### **Advantages <sup>7</sup>**

1. diagnosis of active cases of all forms of leishmaniasis
2. diagnosis of infection, before clinical presentation
3. screening a population for endemicity of the disease
4. screening a population for mass diagnosis
5. as a surveillance tool to monitor intervention activities.

In patients with kala-azar antibody production is vigorous and rapid. Various techniques of serodiagnosis of kala azar are based on polyclonal stimulation of B-cell (non -specific) or clonal stimulation of B-cell (specific tests) <sup>22</sup>

#### **4.15 NON-SPECIFIC TESTS**

Infection by *L.donavani* stimulates production of immunoglobulins by B-cells. In contrast, production of albumin is hampered leading to reversed albumin-globulin ratio. This increased production of immunoglobulins is used quite frequently as diagnosis of Kala-azar at less- equipped, peripheral laboratories. Some of these tests are Napier's aldehyde test and Chopra's antimony test. These tests are easy to perform but have high false-positive rate due to over production of immunoglobulins in many other diseases. Since these tests fail to detect cases of early leishmaniasis, the sensitivity of these tests is around 85% only.

#### 4.16 SPECIFIC TESTS.

Specific serological techniques are based on demonstration of antibodies produced against the circulating parasitic antigens. The specificity of various tests depends on the antigen or its epitome used in the test, as the parasite will stimulate several antibodies producing B-cell including group and genus- specific (polyclonal); as well as species specific (monoclonal) cells. **Therefore, the sensitivity may depend on the test and its methodology by the specificity will depend on the antigen rather than method used.**

**Some serological tests are:**

**DAT - Direct** agglutination test.

This test has been found to have a sensitivity of 96.5-100% and specificity 91-95%. Its strength is its high sensitivity and specificity in early diagnosis of kala-azar patients. The draw back is that it remains positive for more than 5 years after complete cure.

**ELISA -** The sensitivity of ELISA is nearly 100%, but specificity is not very high as cross-reaction with were from patients with TB and toxoplasmosis has been recorded.

**Dot-ELISA** - is another method that has been developed in which interpretation can easily be made by visual inspection of reaction end points, obviating the need for ELISA reader. 19

#### 4.17 STUDIES ON VALIDITY OF DAT TESTS.

- ◆ A sensitivity of 100% and specificity of 99.3% and 100% were reported by HARITH et al (1986).<sup>23</sup>

In one study on direct agglutination test for visceral leishmaniasis, IFAT and ELISA were applied to sera of patients with visceral leishmaniasis, African, American trypanosomiasis other parasitic infection and healthy controls. **The sensitivities of 3 tests were comparable (96.3% to 100%):** excluding patients with African and American trypanosmiasis, **the specificities of DAT and IFAT were 100% and ELISA 87.3%** <sup>5</sup>

#### *Following studies recommend different cut off values :*

Study done in India showed, serum dilution of 1:800 differentiates a case of visceral leishmaniasis from healthy controls. Therefore any serum specimen with titre of  $\geq 1:800$  was considered to contain anti-leishmanial agglutinating antibodies, Fifty-six of 58 sera (**96.5%**) from confirmed cases of visceral leishmaniasis had anti leishmanial antibodies, while none of the clinically suspected cases, apparently healthy control subjects, tuberculosis or malarial cases had significant titres i., e  $\geq 1:800$ . The mean titre of agglutinating antibodies in visceral leishmaniasis cases was significantly higher than that of the control subjects. Although the mean titres for patients with malaria and tuberculosis were higher than those of both the endemic and non-

endemic control subjects how ever the highest titres in these groups were below the cut-off value 1:800. <sup>24</sup>

Relatively high levels of agglutinating antibodies in apparently healthy subjects from an endemic area, compared to these in subjects living in non-endemic areas might be due to previous exposure of the former to *L. Donavani*, through the bite of an infected vector, resulting in some degree of humoral response. The relatively high titres in sera from cases of tuberculosis may be due to the presence of antigenic determinants of *L.donavani* promastigotes cross react with mycobacterium tuberculosis<sup>24</sup>.

- ♦ In patients with recent kala-azar, titres of 1:5200 or higher were found. Cured Kala-azar patients treated 4-14 months before testing, showed the titres in the range of 1:3200 to >1:5200. Healthy and diseased controls had titres below 1:1600 with the exception of African trypanosomiasis patients who showed titres 1:200 to 1:12800, overlapping with the titres of cured Kala-azar patients. Where trypanosomiasis is not a consideration, a titre of 1:1600 could considered indicative of visceral leishmaniasis, **the sensitivity and specificity were then 100%**. <sup>23</sup>

A titre of 1:3200 or greater in an area considered to be free of African Trypanosomiasis is highly predictive of visceral leishmaniasis (recent or Past).. In areas where trypanosomiasis occurs, further serum dilution

The DAT appears to be a useful tool for screening, with a high sensitivity and a high predictive value of negative test. However, the DAT cannot differentiate between past kala-azar, sub-clinical infection, and active disease. <sup>25</sup>

Anti-leishmanial antibodies have been demonstrated in clinical cases of visceral leishmaniasis by variety of immuno diagnostic tests but these tests may give false positive results with case of typhoid, malaria and tuberculosis. Thus attempts have been made to develop a simple but specific diagnostic serological tests for the early identification of cases of Visceral leishmaniasis. The DAT has been shown to be not only highly sensitive but also a specific and simple test for confirming the diagnosis of visceral leishmaniasis. However, further investigation is required to assess the specificity and sensitivity of DAT in different geographical areas. <sup>24</sup>

Since DAT has a high negative predictive value the absence of antibodies detectable by DAT could tend to rule out active kala-azar in clinically suspected cases. Furthermore assessing the titres of agglutinating antibody could monitor effectiveness of therapy. However, investigation on larger number of cases is required before a final conclusion can be drawn.

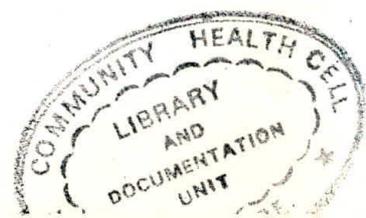
#### 4.19 LEISHMANIN AS SCREENING TEST.

Leishmanin is a suspension of 10 to the power of 6 promastigotes in 1 ml of .5 percent phenol saline. The test is an index of delayed hypersensitivity. It has been also used as important epidemiological tool to assess the immune status of population against leishmanial infection and the spread of disease within and from an epidemic focus. It is not species specific. It is used to map out the extent of past infection in community and may be of help in diagnosis in individual patients.<sup>16</sup>

Interpretation of the results of a leishmanin test survey requires consideration of factors such as the time during the epidemic when the survey was conducted, past experience of the community of such epidemics, the age of the community at that particular geographical location and the frequency of population exchange.<sup>26</sup>

True second attack of Kala-azar after successful recovery from the first is almost unknown although there are some isolated reports needing verification.<sup>27</sup> The sensitivity of leishmanin test is reported to be 93%<sup>28</sup>

The leishmanin test is specific for leishmaniasis but is not species specific. Leishmanin positivity increases with age in endemic areas of cutaneous leishmaniasis and in endemic kala-azar areas leishmanin positivity is acquired without any clinical evidence of infection and varies inversely with incidence of kala-azar indicating population immunity. Leishmanin positivity has also been shown to develop without clinical signs of infection in Northern Italy during an outbreak of kala-azar.<sup>6</sup>



Leishmanin positivity denotes an immune state that prevents further infection provided no immunosuppressive factors intervene and this immunity has been demonstrated. In old endemic foci where there are frequent contacts with infected sand flies only new generation of children are likely to contract the infection since the older generation are already immune. This phenomenon is aided by the fact that foci often remain constant, strictly limited to the same district or to the same group of houses or even the same house for years. It could be used as indirect diagnostic reaction. If someone is suspected of having kala-azar and the leishmanin test is positive in one or more members of household, this observation along with negative reaction in the patient can suggest the diagnosis.

These observations suggest that the leishmanin skin test is valuable tool to measure the past present possibly future incidence of kala-azar in all areas of the world.<sup>6</sup>

Interpretation should be done carefully inter observer variation is significant.<sup>29</sup>

## 5 MATERIALS AND METHODOLOGY

### 5.1 STUDY AREA

#### **Litipara block (Jharkhand)**

Litipara block is the most predominant tribal block in Pakur district of Jharkhand state with 25% Pahadias and 50% Santhal tribes.<sup>30</sup> It is one among the six blocks of Pakur district of newly created Jharkhand State. Jharkhand literally means 'Jhar' (cluster of thick forest) and 'khand' (tract of road.<sup>31</sup>). Tribal population in this area is mainly dependent on the forest produce, which they either sell in the market or exchange for the food grains.

Out of four villages chosen for the study, one is inhabited by Santhal tribe and rest three villages are inhabited by the Pahadia tribe

Pahadias have a dravidian background, speak dravidian language who migrated from south India.<sup>32</sup> The total number of this tribe is less than a lakh, there is concern that they might become extinct. Government has banned family planning programmes in this community.

#### **5.2 NORTH 24 PRAGANAS DIST. (West. Bengal)**

Residents of north 24 Parganas district of West Bengal district. Predominantly rural area. The paddy and fisheries form the economic lifeline of the region. Muslims are in significant numbers in the population

### **5.3 TESTS USED**

**DAT** - After cleaning the thumb, the skin was punctured with a sterile lancet, a large drop of blood was allowed to fill the circles on the DAT filter paper. It was allowed to air dry .<sup>25</sup>

**LEISHMANIN SKIN TEST** - After antiseptic measures, Injecting .1 ml of antigen intradermally into the volar surface of the forearm . A palpable nodule, 5 mm or more in diameter after 48-72 hours, is considered positive.<sup>16</sup>

## **METHODOLOGY**

### **5.4 For Community based - cross sectional study**

1. Census of entire population of the four tribal study villages was conducted for sampling purpose. Every individual in each family according to hierarchy in the family was enumerated.
2. Population in the census record formed sampling frame. Through Systematic Random sampling technique, every fourth individual was enlisted as the study sample.
3. The sampled individuals were visited at their place of residence, and purpose of the study was explained
4. Voluntary informed consent was obtained before taking necessary clinical information and subjecting them to the screening tests.

### **5.5 Hospital based -cohort study.**

1. Data of treated Kala-azar cases from Government Hospital in 24 paraganas district of West Bengal, was obtained for the period of 1999 & 2000.
2. cases were selected on the basis of completion of treatment of 0,3,6,9 &12 (treatment cohorts) months from the reference month August 2000 ( $\pm 10$  days) was short-listed.

3. The cases were traced and explained the purpose of the study. After obtaining the voluntary informed consent, they were subjected to DAT.
4. The same treatment cohorts were repeat tested again after three months (Nov.2000) by applying DAT test.
5. The titres of phase one and phase two tests were analysed for dynamics of the antibodies for the period.

	- Govt. school
	- well (গুটি)
	- tube well working
	- tube well not working
	- Animal's house
	- Broken house
	- Puja place (পূজা ঘর)
	- Home way
	- P.W.D. Road (S.H. 24)
	- Shop

\* 2 of S.M. Siblings

SONADHANI PAHARIA

WAY to SATIA

KISBHITA VILLAGE

T. 5. 2

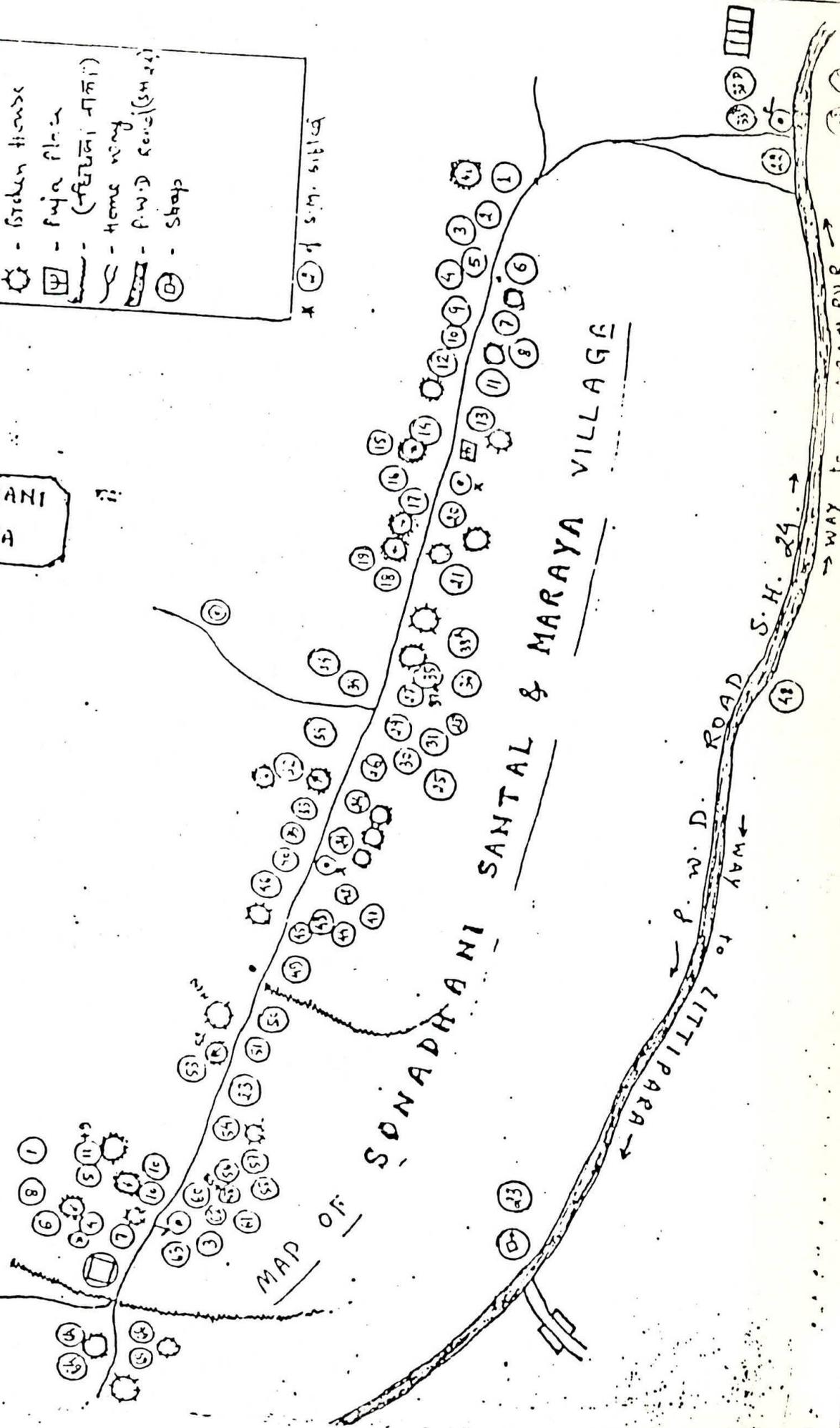
MAP OF SONADHANI

SANTAL & MARAYA VILLAGE

P. W. D. ROAD S. H. 24

WAY to LITIPARA

WAY to AFAMPUR



## RESULTS

### 6.1 AGE AND SEX DISTRIBUTION OF STUDY POPULATION\*

Table -1.

AGE GROUP	MALE	FEMALE	TOTAL
0-9	26 (41.3%)	37 (58.7%)	63
10-19	12 (48%)	13 (52%)	25
20-29	20 (56.6%)	16 (44.4%)	36
30-39	12 (48%)	13 (52%)	25
40-49	8 (88.9%)	1 (11.1%)	9
Above 50 year	8 (80%)	2 (20%)	10

168

\* about 22 people could not be included in this table since their age was not available.

## **6.2 PREVALENCE OF LEISHMANIN SKIN TEST POSITIVITY**

In Paharias and Santhals community

**Prevalence - 44.4%** (95%CI = 33%-55%)

## **6.3 PREVALENCE OF DAT SEROPOSITIVITY $\geq 1:800$**

In Paharias and Santhals community

**Prevalence - 44%** (95%CI=35%-53%)

## 6.4 ANNUAL RATE OF INFECTION IN AGE GROUPS

Table No-2.

AGE GROUPS	CUMULATIVE INCIDENCE	ANNUAL RATE OF INFECTION
1-10 yrs	19.4%	4%
11-25 yrs	61.1%	5%
26-40 yrs	66.7%	3%
≥ 40 yrs	36.4%	.8%

### CALCULATION OF ANNUAL RATE OF INFECTION

$$e^{-It} = 1 - CI$$

$$I = \frac{-\ln(1-CI)}{t}$$

where,

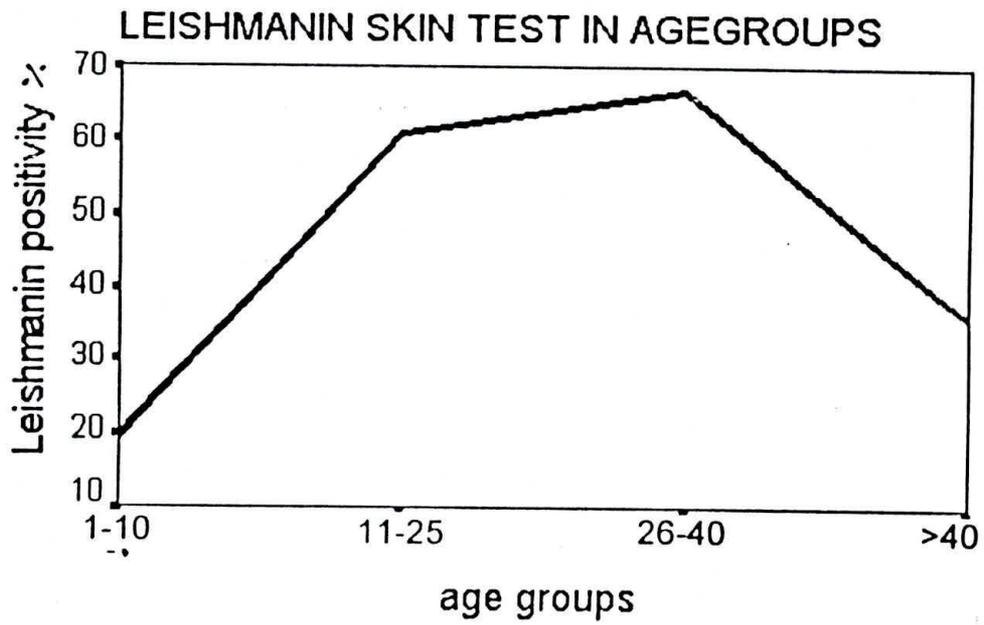
CI= Cumulative Incidence.

I = Incidence.

t = Time (median age)

Graph No. 1

LEISHMANIN TEST POSITIVITY RATES AMONG AGE GROUPS



## 6.5 EFFECT OF SEX ON LEISHMANIN SKIN TEST

Table -3.

MALE	(48)	45.8%
FEMALE	(51)	43.1%

no. in parenthesis is no. of people examined  
% is percentage positivity

$$X^2 = .67 ( P > .05 ) \quad OR = 1.12 \quad [ 95\% CI .47 \text{ to } 2.66 ]$$

## 6.6 EFFECT OF AGE ON LEISHMANIN SKIN TEST

Table - 4.

1-10 yrs	(36)	19.4%
11-25 yrs	(18)	61.1%
26-40 yrs	(33)	66.7%
≥ 40 yrs	(11)	36.4%

no. in parenthesis is no. of people examined  
% is percentage positivity

$$X^2 \text{ for linear trend} = 5.287 ( P < .05 )$$

## 6.7 EFFECT OF DAT TEST ON LEISHMANIN SKIN TEST

**Table -5.**

DAT $\geq$ 800	(35)	48.6%
DAT < 800	(43)	44.2%

no. in parenthesis is no. of people examined  
% is percentage positivity

$$X^2 = .15 \quad (P > .05) \quad OR = 1.19 \quad [ 95\% CI = .44 \text{ to } 3.22 ]$$

## 6.8 EFFECT OF FAMILY SIZE ON LEISHMANIN SKIN TEST

**Table -6.**

FAMILY $\geq$ 6	(30)	73.3%
FAMILY < 6	(59)	37.3%

no. in parenthesis is no. of people examined  
% is percentage positivity

$$X^2 = 3.03 \quad (P = .08) \quad OR = 2.06 \quad [ 95\% CI = .84 \text{ to } 5.06 ]$$

## 6.9 EFFECT OF SPLEEN SIZE ON LEISHMANIN SKIN TEST

Table -7.

PALPABLE	(43)	32.5%
NON PALPABLE	(30)	53.6%

no. in parenthesis is no. of people examined  
% is percentage positivity

$$X^2 = 4.35 \quad (P > .05) \quad OR = .42 \quad [95\% CI = .17 \text{ to } 1.03]$$

## 6.10 EFFECT OF FEVER ON LEISHMANIN SKIN TEST

Table -8.

FEBRILE	(20)	35%
AFEBRILE	(79)	46.8%

no. in parenthesis is no. of people examined  
% is percentage positivity

$$X^2 = .91 \quad (P > .05) \quad OR = .61 \quad [95\% CI = .19 \text{ to } 1.87]$$

## 6.11 EFFECT OF SEX ON DAT POSITIVITY

**Table -9.**

MALE	(77)	46.75%
FEMALE	(69)	40.5%

no. in parenthesis is no. of people examined  
% is percentage positivity

$$X^2 = .56 \quad (P > .05) \quad OR = .78 \quad [ 95\% CI = .38 \text{ to } 1.58 ]$$

## 6.12 EFFECT OF AGE ON DAT POSITIVITY

**Table -10**

1-10 yrs	(43)	39.5%
11-25 yrs	(29)	48.3%
26-40 yrs	(40)	50%
≥ 40 yrs	(17)	41.2%

no. in parenthesis is no. of people examined  
% is percentage positivity

$$X^2 \text{ for linear trend} = .197 \quad (P > .05)$$

**6.20 EFFECT OF CUT-OFF TITRE  $\geq$  1: 6400 ON VALIDITY OF  
DIRECT AGGLUTINATION TEST**

**Table-18.**

	D+	D-
DAT $\geq$ 800	38	2
DAT <800	44	116
	82	118

Sensitivity- 46%

Specificity - 98%

Likelihood ratio- 23

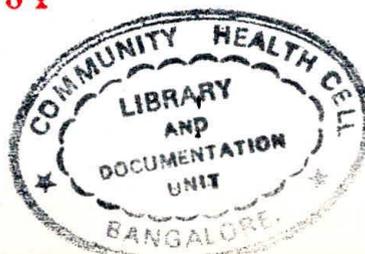
**6.21 MEAN TITRE LEVELS OF ALL TREATMENT COHORTS IN  
PHASE-1**

**Table-19.**

<b>TREATMENT COHORTS</b>	<b>LOG MEAN TITRE</b>	<b>GEOMETRIC MEAN TITRE.</b>
UNDER TREATMENT(0)MONTH	4.05	11220
3 MONTHS	3.51	3235
6 MONTHS	3.60	3981
9 MONTHS	3.46	2884
12 MONTHS	3.60	3981

**Note :** That even after 12 months of treatment the titre remain very high above the seropositive cut off of >1:800 within the treatable range.

DIS-317  
06754 001



**6.22 MEAN TITRE LEVELS OF ALL TREATMENT COHORTS IN  
PHASE-2**

**Table-20.**

<b>TEATMENT COHORTS</b>	<b>LOG MEAN TITRE</b>	<b>GEOMETRIC MEAN TITRE.</b>
3 Months	3.97	9332
6 Months	3.26	1819
9 Months	3.17	1479
12 Months	2.72	524
15 Months	2.96	912

note that The Nine month group has become sero negative < 800 in the 12th month in 2nd phase.

**6.23 COMAPARISON OF MEAN TITRES OF ALL THE TREATMENT  
COHORTS.**

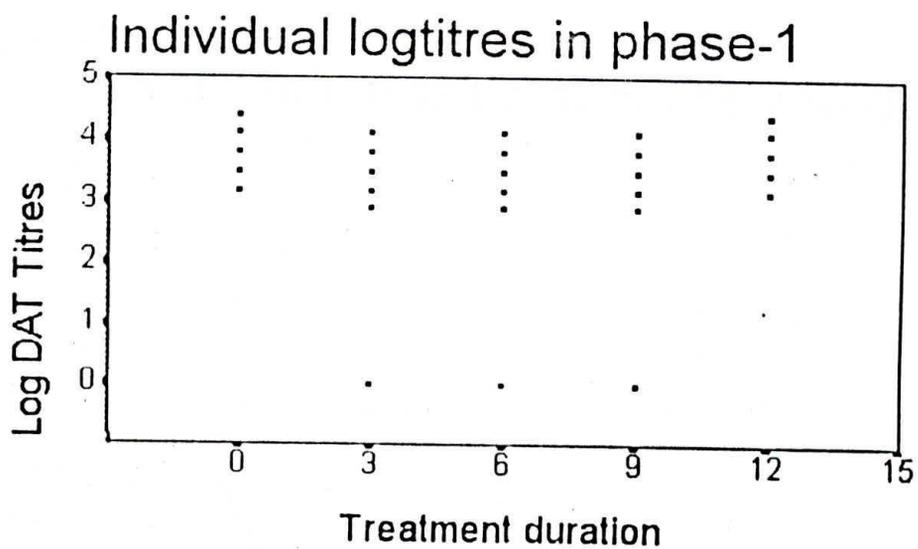
**Table-21.**

<b>TREATMENT COHORTS</b>	<b>GEOMETRIC MEAN TITRE. IN PHASE-1</b>	<b>GEOMETRIC MEAN TITRE IN PHASE-2.</b>
0 Month	11220	9332
3 Month	3235	1819
6 Month	3981	1479
9 Month	2884	524
12 month	3981	912

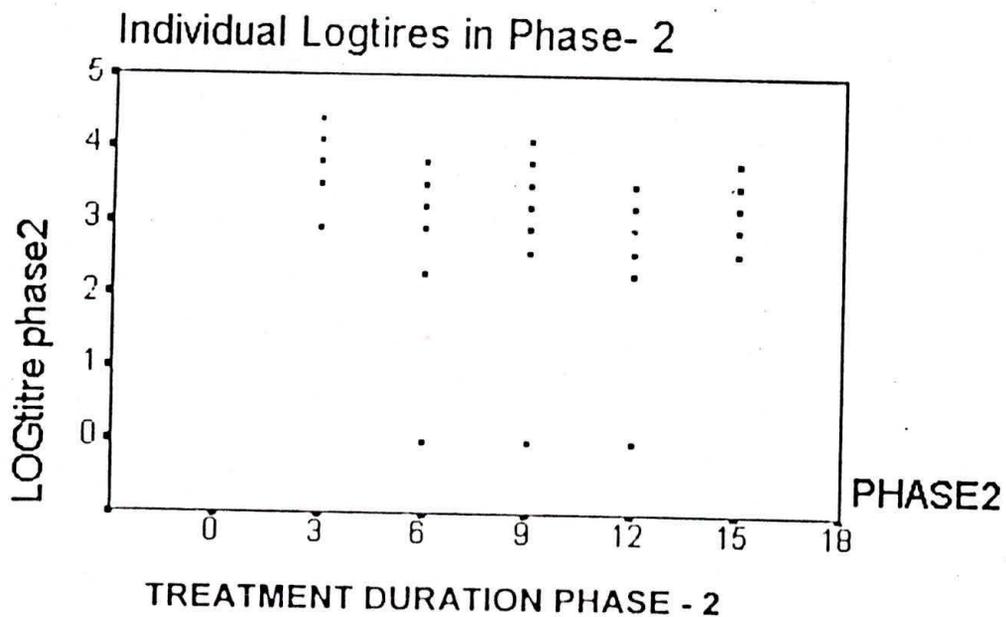
note the differences increasing widely with increasing time .

Graph-2

INDIVIDUAL LOG TITRES OF TREATMENT COHORTS IN PHASE ONE AND PHASE TWO



Graph-3.



interesting finding, especially so when compared annual rates of infection for disease like TB which is about 1% in India.

While Leishmanin test is a good indicator of the past infection in the community shown by hypersensitivity to the leishmanin antigen by cell mediated response, it does not give any information about recent infection which mainly elicit humoral response in the body. Since our objective was to study the prevalence of leishmania infection in the community irrespective of whether it was past or present, we had to employ another serological test called Direct Agglutination test, which reacts to the circulating antibodies. Leishmanin skin test in recent infection or active disease will be negative due to suppression of cell mediated immunity there by not picking up active infections.

With Leishmanin picking up the past infection and DAT picking up the recent infection and active disease we could have a better assessment disease burden in the community.

In our study area prevalence of sero positivity ( titre  $\geq 1:800$ ) to leishmania infection was 44% ( 95%CI=35%-53%). By employing DAT test we could capture extra 18 (23.1%) cases in the community in whom the leishmanin skin test was negative.

Having both serological and leishmanin test result to our disposal, our interest was to look for any correlation between between the two results

and especially to see whether leishmanin skin test positivity was higher in seronegative as compared to the seropositive. No negative correlation was seen.

Earlier studies have shown male preponderance in kala-azar <sup>6, 34</sup> a retrospective study conducted on the Bihar epidemics in 70s by CP Thakur <sup>34</sup> showed the male : female ratio was 5.5 :1. In our community such phenomenon was not observed. In our hospital study there was male preponderance, however this raises two key issues whether the probability of disease given infection is lower for females or is it due to health seeking behavior difference between sex.

The young and Middle age groups showed higher rates of leishmanin skin test positivity 61% and 66.7 % respectively compared to the extremes in the age spectrum 19.4% in children and 36.4% in above 40 years groups. This is attributed to the high transmission of infection in kala-azar endemic areas, where the population get infected early in childhood, consequently by middle age, half of the population would have experienced leishmanial infection and hence be immune to disease. Middle aged group showing higher infection is also reported by CP Thakur<sup>34</sup>. One possible reason for the low leishmanin skin test positivity in old age groups could be due to fading immunity. Nandy et al <sup>26</sup> reported 19.2% in 1-10 years group 25.6% after 40 year. Studies from Mediterranean region<sup>6</sup> showed age group 50-70 year with maximum leishmanin positivity. Badaro et al reported predominantly higher higher

infection in children under 15 in Brazil, where adults get rarely infected.<sup>34</sup>

In sharp contrast to leishmanin skin test, there was little difference in DAT sero positivity in different age groups.

The absence of association between Family size and kala-azar can be explained on the fact that, over crowding has no bearing on the transmission of leishmania infection, unlike in diseases like tuberculosis or measles.

We looked for any association between DAT sero positivity with fever and splenomegaly as surrogate variables for kala-azar, since the probability of the kala-azar is higher in cases with these signs in endemic area, but no association could be found. Reasons for absence of correlation between seropositivity with surrogate variables like fever and splenomegaly could not be explained since DAT is a test for recent infections.

High titre of antibodies can be elicited in subclinical and asymptomatic cases. Although the mean level in such cases will always be lower than clinical cases.<sup>35</sup>

Incidentally DAT posed more questions in our study than it could solve, 1. what is the status of the individuals who are apparently healthy, being negative to the leishmanin skin test but seropositive to DAT.

2. Why there was no difference in seropositivity rates between different age groups as in leishmanin skin test.

We attempted to find the answers for the above questions by conducting a hospital based study with the objective of studying dynamics of the sero titre in kala-azar cases after the cure. We began studying the dynamics of the antibody level in cured kala-azar cases, by measuring the antibody levels in two phases. In the first phase blood samples were collected 82 kala azar cases based on their completion of treatment like 0( just completed),3,6,9,12 months prior to the reference month (June) when our community study was conducted, 15 cases were recruited in each treatment cohort. Same cases were repeat tested after 3months.

The results of the hospital based study showed declining pattern in the antibody levels in 82 cases successfully treated for kala-azar, but did not show any definite trend in relation to time. The titre seem to decline with time but the consistency was absent. The mean titre level during the treatment was 11220, which went down to 3235 at 3rd month then increased to 3981 at 6 months, dipping to 2884 in 9 months again. The upsurge to 3981 at 12 months could not be explained, which in effect meant the antibody titres returning back to 6 month level. The fluctuations especially the upsurge towards the later months could not be accounted for. Since Only one cohort (9 month ) showed its mean titre dipping from 2884 to 529 we could not tell with conviction, how long do the kala azar cases take to convert into sero-negative status

which needs an elaborate cohort study which is out of scope for our present study.

We could show the means of differences between the logtitre values of phase one and phase two was significant F statistics=7.41 ( $p<.05$ ) The differences increased with the time after completion of the treatment. This implied that the fall in antibodies is slower in the beginning and gradually accelerate with the time.

The variation and inconsistency in the antibody dynamics lowers validity as diagnostic test, which explains misclassification of many normal people in the community as diseased through seropositivity with higher titres, seriously undermining the validity of DAT as diagnostic test.

Since the validity of any given test is dependent on prevalence of the disease, we were prompted to look into the validity of DAT with different cut-off titre. For this reason we recruited (n=82) treated kala-azar cases from referral hospital of West Bengal into case series and study subjects from community without fever (n=118) were included into control series. We looked at the effect on the validity of DAT with different cut off titres. The cut off titre of 1:1600 gave better validity (sensitivity=.84 and specificity=.82) than currently used cut off titre of 1:3200 (sensitivity=.63 and specificity=.83)

## 8. SUMMARY AND CONCLUSION

Kala-azar is a major public health problem In our country. We have to tackle it by giving due priority. Our study showed 44.4% leishmanin skin test positivity and 44% DAT seropositivity , indicating high prevalence rates of leishmania infection in the tribal community . The middle age groups showed higher rates of positivity. Leishmanin skin test showed significant association with age. The male to female ratio for disease was similar, we did not find the male preponderance. Leishmanin skin test had clear cut results for population as to past infected or not.

DAT was found to be good epidemiological tool for community survey but its validity as diagnostic test was not very high. There was no association between DAT seropositivity and other variables like age,sex or surrogate variables for kala azar like splenomegaly and fever. DAT results showed ambiguity in classifying the population into recent and past infection.

Our hospital based study showed declining trend in the antibody levels with no definite trend in relation with time. The variation in declining antibody level was high. The means of differences of antibody levels in the phase-1 and phase- 2 was statistically significant. The effect on the DAT Validity was measured by adopting different cut-off titres of antibody levels. The titre of 1:1600 was found to have the best diagnostic

validity for DAT in Santhal parganas area with sensitivity of 84% and specificity of 86%. The likelihood ratio for 1:1600 cut-off titre was 4.6. This was much better than the 1:3200 cut-off titre presently used in the area which had specificity of 83% and a low sensitivity of 63%.

Our study gives the baseline information of the magnitude of the disease and infection in the tribal communities in Santhal parganas area. Clear Idea of the magnitude is necessary in any area for taking on intervention programmes. The impact of the intervention programme should be measurable in any evaluation.

For disease control and prevention, test/s used for community surveys and screening programs should conform to criteria like Validity, repeatability, acceptability, simplicity, rapidity, safety, ease of administration and low cost<sup>36</sup>. Both Aldehyde and Bone marrow tests which are the most widely used test for diagnosing kala azar, are not feasible for screening purposes.

Sternal bone marrow puncture and aspirate the definitive test for Kala-azar diagnosis is very painful procedure, intimidating the patients. Moreover it involves technical expertise and therefore is not feasible in field conditions.

Aldehyde test is a very simple but has low sensitivity and specificity. It continues to be mainstay for lab diagnosis of Kala-azar infection in rural

and semiurban areas where the disease burden of kala-azar is maximum. Aldehyde test is not positive until a few months after disease onset thus further missing the disease in undetected cases of Kala-azar

DAT test is a very simple test to carryout, where in collection of specimen is very simple, involving collection few drops blood sample from finger prick on the filter paper, which could then be sent to concerned laboratory, even by post. Thus, ideally suited for Sero-epidemiological work

The DAT and Leishmanin skin tests are most widely used epidemiological tool all over the world for conducting field studies to assess the magnitude of the disease in the community

## 9. LIMITATION

Bone marrow test could not be carried out for definitive diagnosis of kala-azar .

The Hospital cases could not be followed for longer time due to time constraints.

Many of the cases of kala-azar in referral hospital were diagnosed on the basis of DAT

## 10. RECOMMENDATION

Use of multiple test in series or parallel to increase sensitivity and specificity, for diagnosis, in the absence of Bone marrow test.

An elaborate Cohort study to understand the long term dynamics of antibodies.

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## 11. APPENDIX I

### PROFORMA FOR COMMUNITY BASED STUDY

ID NO.	
NAME	
GUARDIAN NAME	
AGE	
SEX	
FAMILY SIZE	
TRIBE	
VILLAGE	
H/o KALAAZAR	
C/O OF FEVER	
SPLEEN SIZE	
DAT TITRE	
LEISHMANIN SKIN TEST	

**11.2 APPENDIX II**

**PROFORMA FOR THE HOSPITAL  
BASED STUDY**

ID NO	
NAME	
AGE	
SEX	
VILLAGE	
TREATMENT MONTH	
DAT	