

# VISCERAL LEISHMANIASIS: A SEROEPIDEMIOLOGICAL STUDY OF 289 CHILDREN FROM ENDEMIC FOCI IN AZAD JAMMU AND KASHMIR BY INDIRECT FLUORESCENT ANTIBODY TECHNIQUE

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## Abstract

Between January'85 and August'87, 22 cases of VL were seen at National Institute of Health, Islamabad. Three (14.6%) came from the previously known endemic region of Gilgit, 15 (68.1%) from different localities in Azad Jammu and Kashmir (AJ&K), and 4(17.3%) from neighbouring foci in NWFP and Punjab. Mean age of the patients was 4.2 years, (Range, 10 months to 57 years) median 2.5 years and mode 2 years. High levels of Leishmania antibodies were detected by Indirect Immunofluorescent Antibody Technique (IFAT) in all cases. Leishmania were isolated from bone marrow aspirates of 2 patients and isoenzyme characterization performed in one of these, the organism was typed as *Leishmania infantum sensu stricto*. Sera from 289 children residing in 5 endemic localities in AJ&K was tested for Leishmania specific antibodies by IFAT and low levels of these antibodies were detected in 15.4% of the cases (JPMA 39: 225, 1989).

## INTRODUCTION

Human visceral leishmaniasis was first reported in Pakistan from the northern areas in the late fifties and early sixties<sup>1,2</sup>, the disease was thought to be restricted to foci in Gilgit and Skardu. Since the advent of this decade, sporadic cases began to occur not only in different foci of AJ&K but also in some extreme north eastern areas of Punjab as well as nearby localities of NWFP<sup>3</sup>. The disease is acquired through the bite of infected phlebotomine sand flies. Amastigotes multiply within mononuclear phagocytes throughout the reticuloendothelial system including spleen liver, lymphnodes and bone marrow. Clinically, the disease is marked by intermittent fever, anaemia, lymphadenopathy and hepatosplenomegaly. Progressive weight loss, cachexia and malaise, followed by bacterial superinfections and can sometimes result in death. In general once hepatosplenomegaly occurs host defence mechanisms are not able to prevent further parasitic multiplication<sup>4</sup>. Patients with VL during the acute stage have high levels of globulins and produce Leishmania-specific antibodies. Following successful treatment, these antibody levels decline and a gradual, cell mediated, delayed type hypersensitivity response to leishmanial antigen is elicited which manifests over several months, and resistance to infection usually develops<sup>5</sup>. Bone marrow aspiration has been used extensively for the diagnosis of VL. This is an invasive procedure and may lead to serious complications if performed by individuals not fully trained in this technique. IFAT, a relatively more sensitive procedure, is now widely used for the diagnosis and seroepidemiological studies of VL<sup>6</sup>. This pilot study was undertaken to detect leishmania antibody levels in patients and determine baseline antibody levels in endemic populations in AJ&K using IFAT.

## **MATERIALS AND METHODS**

### **a) Selection of Localities:**

Five villages in AJ&K (Total population, 2600) were selected for seroepidemiological survey. From these villages atleast 7 active cases had been reported in the past two year period. These villages are located in the vicinity of 3 major towns in AJ&K i.e. Rawalakot, Dirkot and Chikar. The houses in these villages are generally scattered along the hills in this region.

### **b) Collection of Sera for IFAT:**

Sera was obtained from 22 patients admitted in different hospitals of Rawalpindi and Islamabad. .Sera samples were also collected from 289 children (under 12 years) residing in 5 localitjes mentioned above. Of these 289, venous blood was drawn from 90. From the remaining 189 children, 100 microliter blood samples were drawn by finger pricks and dried on No. 4 Whatman filter paper. These were appropriately numbered and stored at -20C Serum was eluted in 1 ml Phosphate buffered saline (PBS, pH 7.4) to give a starting dilution of 1 in 20.

### **c) Preparation of Antigen:**

Leishmania was isolated from a case of VL and an invitro culture was maintainS in NNN medium. Promastigotes were harvested from this culture and after three washings in PBS, stored overnight at 4°C in 1% formol PBS. This was washed three times again with PBS and diluted to contain 30 to 40 parasites per high power field. One drop of this antigen was placed on each spot of a 12 well multispot slide (flow Laboratories). The antigen slides were air dried at room temperature, properly wrapped in aluminum foil and placed in self sealed polytbene bags, with a few crystals of silica gel. The slides were stored at -70°C till use.

### **d) Conjugate:**

Commercially available antihuman IgG fluorescence isothiocyanate (FITC) conjugate obtained from Sigma, was used at a working dilution of 1:20 in PBS. At this dilution no fluorescence was observed in the negative control.

### **e) Test Procedure:**

The antigen slides were brought to room temperature before use. 25 micro liter of test serum was applied to each antigen spot and allowed to react for 30 minutes at room temperature inamoist chamber. The slides were washed 3 times in PBS through serial changes of 10 minutes each. Antihuman IgG FITC conjugate was then applied and incubated for 30 minutes at room temperature in moist chamber. Washing procedure was repeated. The slides were mounted in a preparation of 90% glycerol in 0.3M Carbonate/Bicarbonate buffer (pH 9.0) and covered with coverslips. Both positive and negative control sera were used each time the test was performed.

### **f) Fluorescence Microscopy**

Slides were examined with an Olympus fluorescent BH2 microscope using a dark field condenser and 400 X magnification. Light source was 12 volts, 50-60HZ halogen lamp.

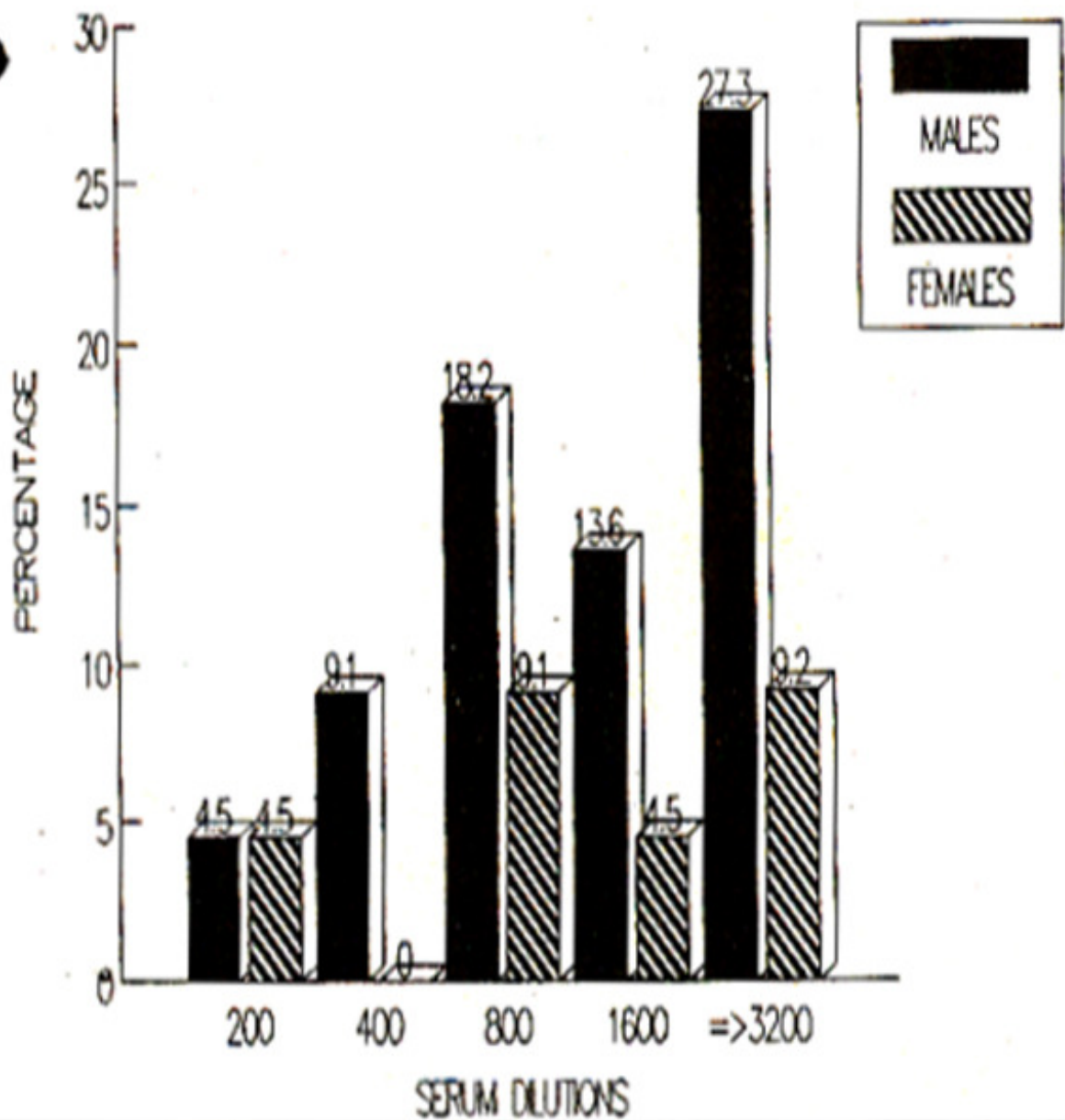
### **g) Isoenzyme Characterization:**

Culture on NNN medium was obtained from bone marrow biopsy of a two year old male patient admitted in a local hospital. Isoenzyme characterization on this sample was performed at aWHO recognized center, Instituto Superiore Di Santa in Rome, Italy.

## **RESULTS**

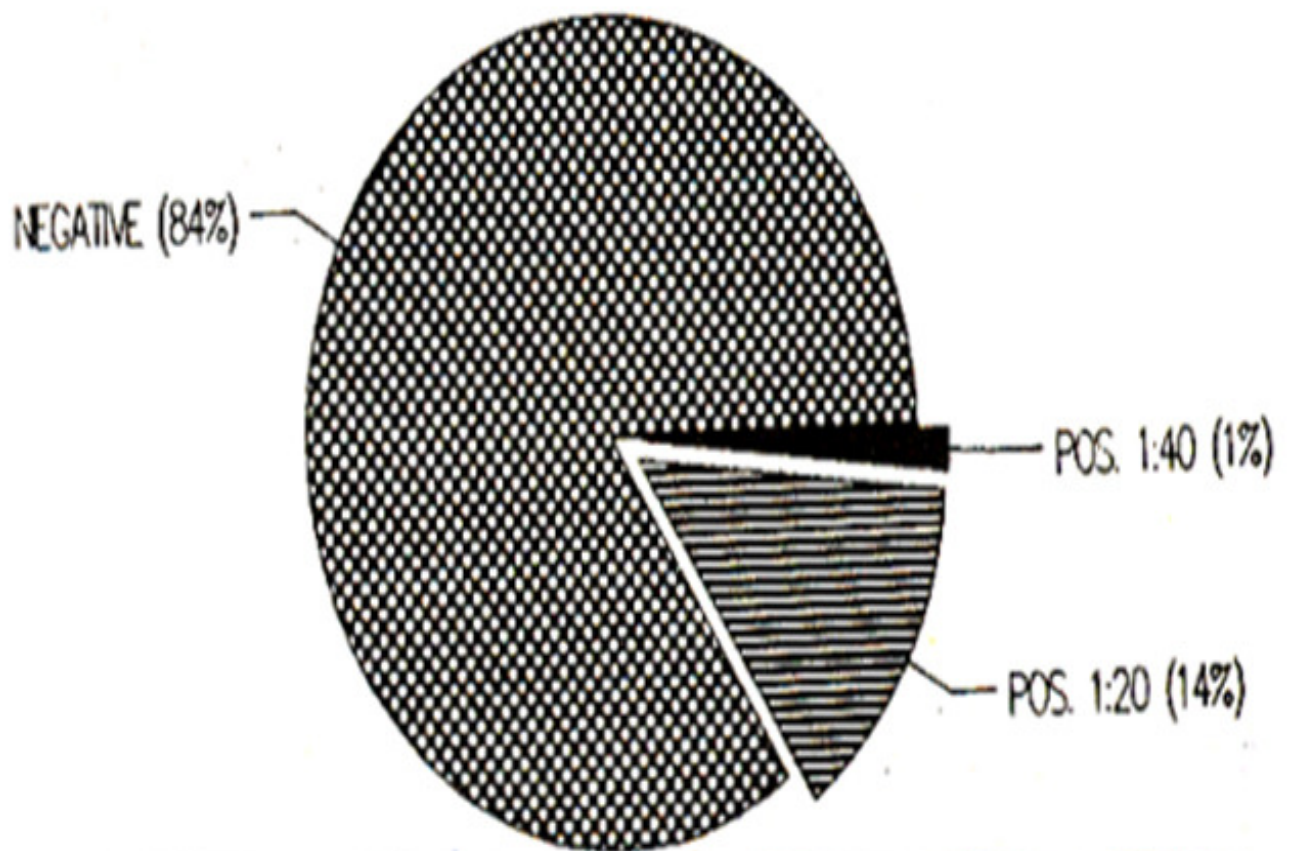
### **Serology of Patients**

All confirmed cases of VL demonstrated very high titers of Leishmania antibodies (Figure 1).



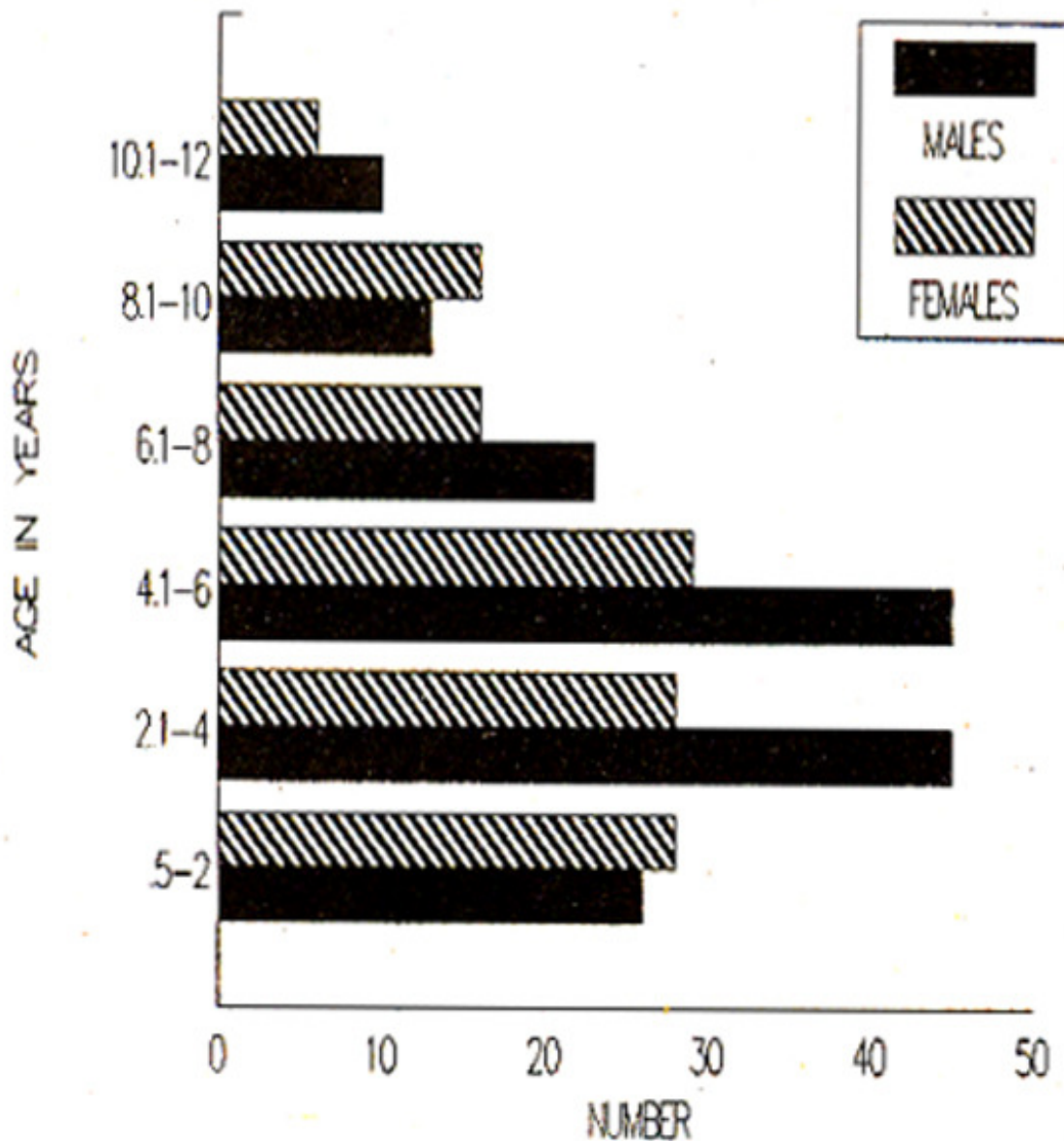
**Figure 1. IFAT results in 22 children with visceral leishmaniasis.**

9.1% were positive at titers of 1:400, 27.2% at 1:800, 18.2% at 1:1600 and 36.4% had titers of 1:3200 or more. Serology of Children in Endemic Areas Of the 289 children examined (Figure 2),



**Figure 2. Serology of 289 children in five endemic foci of VL in AJ&K. IFAT results.**

none showed clinical evidence of disease. IFAT results did not reveal detectable antibodies to Leishmania in 84.6%. 14.1% were positive at serum dilutions of 20, and 13% were positive at dilutions of 40 (Figure 3).



**Figure 3. Seroepidemiology of 289 children from endemic areas. Age and Sex distribution.**

Sera from 40 control children living in nonendemic regions were all negative for leishmania antibodies.

#### **Isoenzyme Characterization**

Strain characterization on one isolate, identified the parasite strain as *Leishmania infantum* sensu stricto. Reference stocks used for identification included *L. infantum* s. st., *L. infantum* NH variant *L. infantum* NH MDH ASAT variant and *L. donovani*. Enzyme patterns were examined for enzymes such as PGM, PGJ, GOT, G6PD, P6GD, ME, MDH, LDH, MPI and NH.

#### **DISCUSSION**

IFAT has been used to study leishmania antibodies in patients suffering from YL. All patients exhibited high levels of such antibodies, except two children, in whom the levels were rather low (Positive at

dilution of 200). Both these patients had received a course of Pentavalent Antimony before their serum sample was tested. Successful treatment of VL with pentavalent antimonials results in a rapid decline in leishmania specific antibodies and this is used as an indicator to monitor and assess the response to treatment in patients<sup>7,8</sup>. Titers over 1:256 are considered to be diagnostic for VL<sup>9</sup>, and in our study, 91% of the patients presenting with clinical disease had antibody levels higher than 1:400 (Figure 1). Of the 22 patients in the study, the disease was diagnosed both by a positive bone marrow aspiration and high levels of leishmania antibodies detected by tEAT in 18. IFAT alone was used successfully for diagnosis in the remaining four, and two of these patients had IFAT antibody levels of 1:800 while the other two had titers in excess of 1:3200. Clinical studies in this region show evidence that the disease is of the Mediterranean type, affecting most commonly young children. The disease is manifested by prolonged fever, anaemia, hepatosplenomegaly, generalised lymphadenopathy, weakness, wasting and emaciation. Laboratory investigations in patients revealed pancytopenia and markedly increased levels of gammaglobulins. Leishmania isolate obtained from bone marrow of a two year old male child admitted in a local hospital was typed as *Leishmania infantum* ss on isoenzyme characterization. Seven cases occurred in five villages of AJ&K. A randomly selected sample of 289 children under 12 years of age, from these villages revealed low levels of Leishmania antibodies. Of these, 14.1% had antibody titers of 1:20 and only 13% had levels of 1:40, while 84.6% of the children were negative for leishmania specific antibodies. Sera obtained from 40 control children from non endemic area were also negative. In one house hold where a six year old female patient had recently expired of VL, none of her family members including 3 sisters (age range 2-10 years) showed any clinical evidence nor had history suggestive of past disease. They were also negative on serology. In another village a 5 year old girl who had suffered from the disease two years before, and had since fully recovered demonstrated an antibody level of 1:20 in her serum. Careful followup of the children with low levels is therefore essential. Asymptomatic and subclinical forms of VL are known to occur.<sup>10</sup> In a prospective study in Brazil<sup>11</sup>, of the 86 children with leishmania antibodies 23.3% remained asymptomatic, 17.4% developed classical VL within weeks of index serology. The remaining 59.3% children showed subclinical disease manifested by mild constitutional symptoms malaise, diarrhoea, poor work-play tolerance and intermittent hepatomegaly. Of those showing subclinical disease 25% progressed to classical VL, and the remaining resolved their illness after a prolonged period. As Geimsa stained smears of bone marrow aspirates are usually negative for leishmania in the absence of classic Kala-azar, infection rates in populations can be determined by sensitive serological techniques such as IFAT, EUSA, RIA<sup>12</sup> and by delayed hypersensitivity skin testing<sup>13,14</sup>. Identification of vector in this region has not yet been achieved. Although a large variety of phlebotomine sandflies are found in Pakistan<sup>15</sup>, the ones that have been identified in the northern areas include *Ph.chinensis*, *Ph. sergenti*, *Ph.kendeliki burnei* and *Ph.major*. Promastigotes have not been found in any of the species so far. Dogs and other wild carnivores such as foxes, jackals and wolves are primarily believed to be the reservoir of disease in most endemic areas<sup>16</sup>. The prevalence of human infection cannot be directly correlated with that in dogs. A number of domestic dogs were examined for evidence of clinical disease such as cutaneous lesions, onychogryphosis, keratoconjunctivitis, rigidity of posterior limbs etc. None were found to be affected. Sera from 4 dogs tested for leishmania antibodies by hemagglutination, yielded negative results. Recently however, sensitive and more reliable techniques such as IFAT<sup>17</sup> have been successfully employed in the detection of antibodies in the reservoir host. In conclusion, therefore it is observed that sporadic cases of VL are occurring in the northern parts of the country, most commonly affecting young children. The disease was first reported from the northern areas about three decades ago and was thought to be restricted to foci in that region. However, since the advent of this decade, cases of visceral leishmaniasis began to be reported from different foci in Azad Jammu and Kashmir. Even more alarming is the fact that the disease has also been reported from regions of the North West

Frontier Province as well as Punjab that neighbour AJ&K. There is thus conclusive evidence that the disease is gradually spreading southwards in the country. In order to limit this disease the exact epidemiological pattern needs to be defined and knowledge regarding both vector as well as the reservoir host is essential before comprehensive control efforts can be undertaken.

## ACKNOWLEDGEMENTS

The authors are indebted to Drs. Gramiccia, M. and Gradoni, L. of the Instituto Superiore Di Santa, in Rome, Italy, for isoenzyme characterization of a *Leishmania* isolate. Support of Drs K Abbas and Z. Kundi from Polyclinic and Holy Family hospitals at Islamabad/Rawalpindi is also gratefully acknowledged. Thanks are also due to Mr. Manzar All, Technician, Immunology Department, NIH, for helping us in field studies. This study was sponsored by the WHO/TDR Special Program for Research and Training in Tropical Diseases.

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