A rising trend in kala-azar in Varanasi district, Uttar Pradesh, India: A recent survey using direct agglutination test

Suman Gupta, Suman Tiwari, Ashim Kumar Bagchi and J. C. Katiyar

Division of Parasitology, Central Drug Research Institute, Chattar Manzil Palace, Lucknow 226 001, India

Sporadic cases of kala-azar reported from certain districts of eastern UP prompted us to study the seroepidemiological profile of the disease in Varanasi district. A total of 2182 blood samples were collected and screened by the direct agglutination test (DAT). Five hundred and ninety cases comprising of various categories of disease status responded positively to DAT, with the antibody titre ranging between 1 : 3200 and 1 : > 102400. Ten asymptomatic subjects positive by DAT later developed full-blown disease, indicating the usefulness of the test in early diagnosis of kala-azar.

THE routine diagnosis of visceral leishmaniasis is based on one or more of the following methods: (i) the microscopical detection of parasites in smears of lymph node, bone-marrow (BM) or splenic aspiration, (ii) culturing of the parasite from patient material, or (iii) serological tests for the detection of anti-leishmanial antibodies. A relatively new and very sensitive technique for detection of *Leishmania* parasites in blood, BM, lymph node, or spleen material is polymerase chain reaction (PCR)[1] but the application of this technique in the diagnosis of leishmaniasis is still in its infancy. Further, the test is not suitable for community-based diagnosis.

BM biopsy has a rather low sensitivity of 55–70%, while the procedure involving spleen puncture although 90% sensitive is risky, painful and needs skilled investigators[2,3]. Compared with the first two categories mentioned above, serological tests have the advantage that blood sampling is relatively easy, with little inconvenience for the patient, and that many samples may be processed simultaneously.

Several serological tests have been reported for the diagnosis of visceral leishmaniasis. Most promising among these are the tests based on enzyme-linked immunosorbent assay (ELISA)[4–6], dot-ELISA[7,8], indirect fluorescent antibody test (IFAT)[9] and direct agglutination test (DAT)[10,11].

Results of a large-scale comparative evaluation of dot-ELISA and DAT carried out in kala-azar-endemic villages of Muzaffarpur (Bihar, India) have recently been reported[12].

Since the last few years, sporadic cases of kala-azar have been reported from some districts of eastern UP (Varanasi, Gonda, Basti, Gorakhpur)[13,14], and no systematic survey on the epidemiological profile of kala-azar has been made in these areas, and Varanasi being the most infested district for kala-azar (unpublished reports), we ventured to undertake seroepidemiological survey of the disease in Varanasi using DAT. (The test standardized by us is now being marketed by Span Diagnostic Ltd., Surat, with the trade name of 'LEISHMA TEST'.)

For survey, the stratified sampling method was used. In the beginning, the whole district was classified into seven strata. On the basis of scattered reports of kala-azar available at that time, we selected the sample size proportional to the population size from each strata (Dariyapur, 357; Ramnagar Malahia, 1100, Ramna Malahia, 281; Sujabad 66; Golaghat, 30; Rastapur, 218 and Ashram Samadhi, 130). Thus a total of 2182 blood samples were collected in three rounds by finger prick on Whatman No. 1 filter paper[13]. All the samples after elution were screened by DAT at 1 : 3200 test dilution. A detailed case history of each subject regarding name, age, sex, present symptoms, treatment history (present and past, if any) was recorded.

The antigen for the test was prepared from trypsin-treated Coomassie brilliant blue stained promastigotes of *Leishmania donovani* (Man/IN/80/Dd8) as described by Harith *et al.*[11] except that the organisms were grown in L-15 plus 10% foetal calf serum (FCS) medium[15] in place of RPMI-1640 plus 15% FCS.

The test was carried out essentially as described by Harith *et al.*[10] using Laxbro V-shaped well microtitre plates. Briefly, serum samples were diluted in a diluent containing 0.9% (w/v) NaCl, 0.2% (w/v) gelatin (Difco Laboratories, Detroit, Mich) and 0.78% (v/v) 2-mercaptoethanol. Serum samples were serially diluted double fold (1 : 200 to 1 : 409600). The first well of plate was used as antigen control containing only the diluent and no serum. Equal volume (50 µl) of antigen suspension (70 million promastigotes/ml) was added to each well, including the controls. We routinely employed 3–4 h incubation period at 18°C to 20°C before reading of the DAT results.

The titre is defined as the highest dilution at which agglutination is still visible. In comparison to the blue dot present in the negative control wells, this agglutination shows as blue mats. Chi-square test was applied to test the significance among the percentage of seropositive cases in different wards. Significance of positivity rate between male and female subjects was tested by *Z*-test for proportion. Mann–Whitney U-test was applied to evaluate significance between different age groups.

Of the 2182 subjects from seven wards surveyed, 590 were found positive for kala-azar (27% positivity) by DAT (Figure 1). The seropositives comprised of cases with fever and allied symptoms and frank *Leishmania-*

related symptoms, post-treated cases and asymptomatic subjects (Table 1).

Figure 2 shows that percentage of seropositive cases differs significantly ($P < 0.01$) among the wards surveyed. Rastapur, Golaghat and Ramna Malahiya were highly endemic whereas Ramnagar Malahiya, Sujabad and Ashram-Samadhi had very low positive cases.

All the ten cases having clinical symptoms of kala-azar (fever, weight-loss, hepatosplenomegaly) showed positivity by DAT. The antibody titre ranged from 1 : 3200 to 1 : 102400 (Figure 1, Table 1). Seven of the cases were also positive by BM biopsy. All the ten cases irrespective of parasite positivity responded well to the stibanate therapy.

Out of 197 cases with history of fever, cough and anorexia, 131 (66.5%) were positive to DAT.

Amongst the 27 cases who were undergoing treatment for kala-azar or who had completed treatment within 6 months, 26 cases (96.2%) showed positive response by DAT. After 4–5 months, 11 cases could be followed-up, two became negative in DAT, one converted into PKDL and the rest 8 showed declining trend in antibody titre (Figure 1, Table 1).

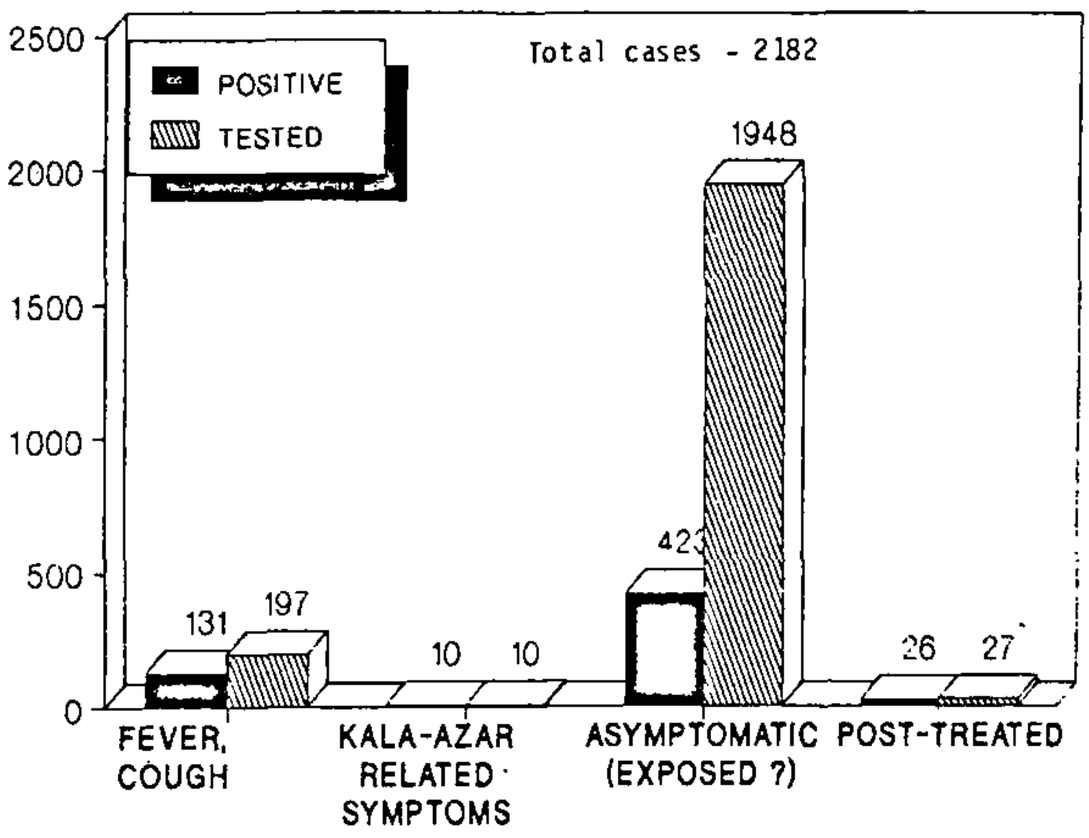

Figure 1. Results of kala-azar survey in Varanasi district by direct agglutination test (DAT).

Four hundred twenty-three (423) subjects out of 1948 with no symptoms of kala-azar were also found positive in DAT. These presumably were exposed cases. The antibody titre ranged between 1 : 3200 and 1 : 12800.

Ten asymptomatic subjects found positive in the first visit when retested on follow-up 4–5 months later were found to have full-blown disease, indicating the usefulness of DAT in the early diagnosis of leishmaniasis. The findings point towards future kala-azar epidemic (Figure 1, Table 1) in Varanasi.

Among the 130 cases from Ashram-Samadhi, 33 cases were leprosy patients. All these gave negative response by DAT conforming with earlier findings[10,12] of no cross reaction with leprosy.

Of the 2182 subjects, 1306 (59.9%) were males and 876 were females (40.1%), and DAT positivity was 25.3% and 29.6% respectively (Figure 3). Although the percentage positivity in females was higher only by 4.3%, it was statistically significant ($P < 0.05$).

Although age-wise there is no perceptible difference in the seropositivity (NS), the percentage of female sero-positives was little higher (39%) than males (34%) in the 25–34 years age group (Figure 4).

Sporadic cases of kala-azar have been reported from a few districts of eastern UP[13,14]. A large number of visceral leishmaniasis cases were reported from Bhadohi district adjacent to Varanasi[13]. It was believed that the disease had been transmitted to the local population by infected labourers coming from Darbhanga district (Bihar) about a few years ago and engaged in the carpet industry in Bhadohi.

Unconfirmed visceral leishmaniasis is a diagnostic dilemma for clinicians in kala-azar-endemic centres. The clinicians have to choose between the risk of delaying or withholding treatment for visceral leishmaniasis patients and prescribing anti-leishmanial drugs for others free of *Leishmania* infection.

Table 1. Results of kala-azar survey in Varanasi district by direct agglutination test

Total cases surveyed: 2182

Category	No. of cases studied	No. of cases positive by DAT	Per cent positivity
Cases with fever and cough	197	131	66.5
Cases with kala-azar and related symptoms	10	10	100.0
Asymptomatic (exposed?) subjects	1948	423*	21.7
Patients undergoing treatment or post-treated cases	27	26	96.3

Test dilution, 1 : 3200.
*Ten subjects on follow-up developed full-blown disease.

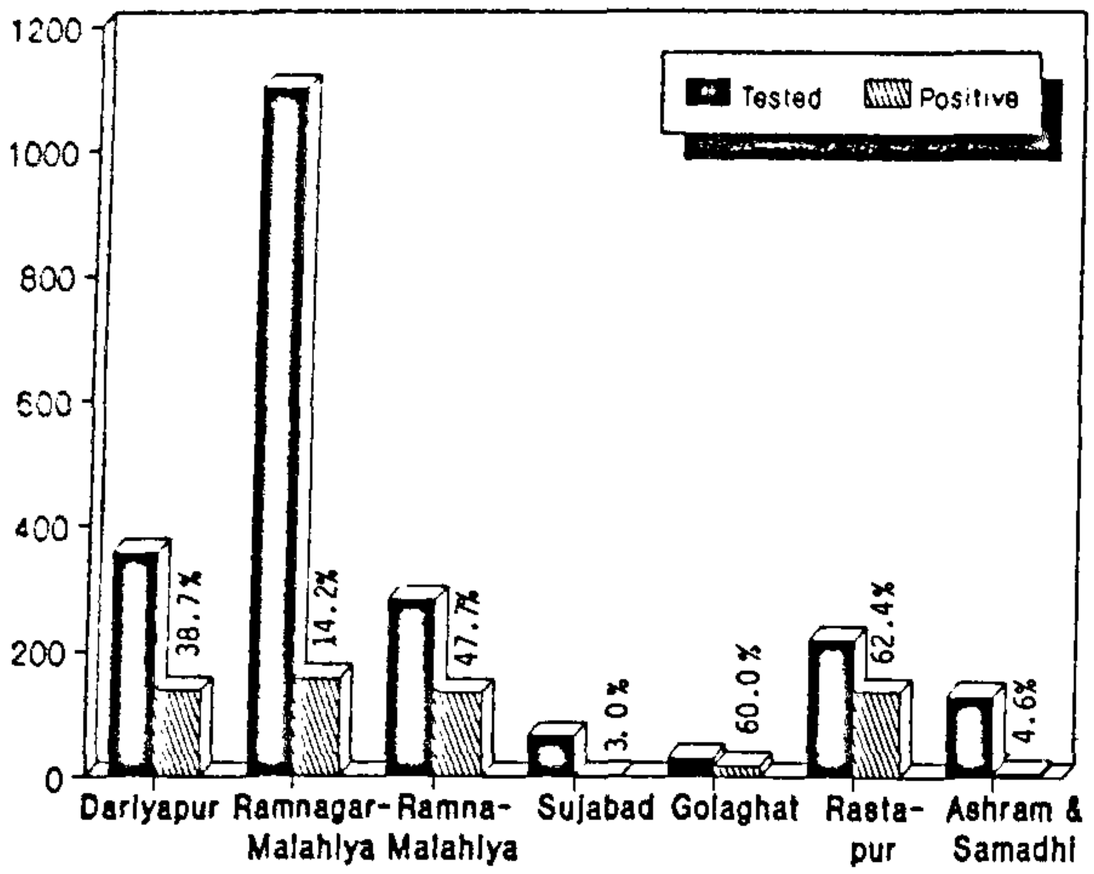

Figure 2. Prevalence of seropositives in different wards of Varanasi surveyed.

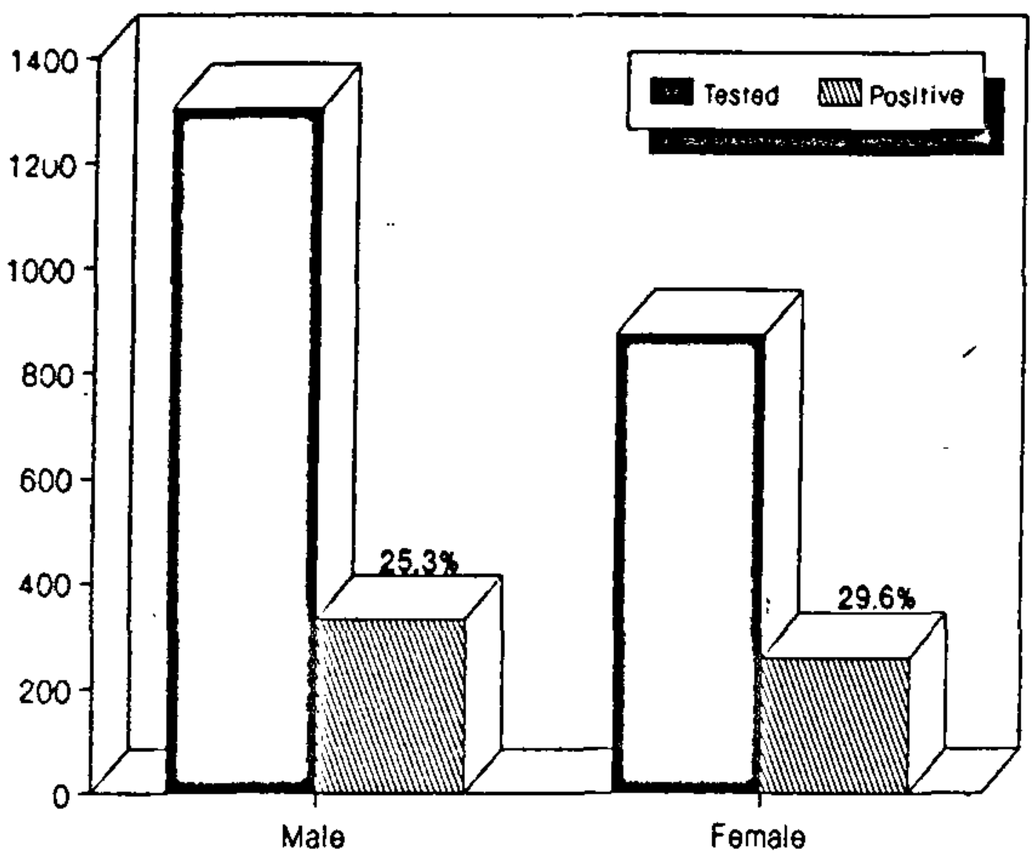

Figure 3. Prevalence of seropositives by sex.

DAT originally developed by Harith *et al.*[10] has shown high order of sensitivity and specificity in different countries[11,16–18]. The ease of conducting the test and cost effectivity make the test very useful for community-based diagnosis. Hence, this test was employed in the present study.

We estimated the seroprevalence rate using DAT in seven wards of the Varanasi district covering 2182 native population. Among these, three wards have shown significant seropositivity. The overall prevalence rate was found to be 27% and these comprised of cases with different disease status.

Ten cases possessed positive symptoms of kala-azar and of these, seven were also positive by BM biopsy.

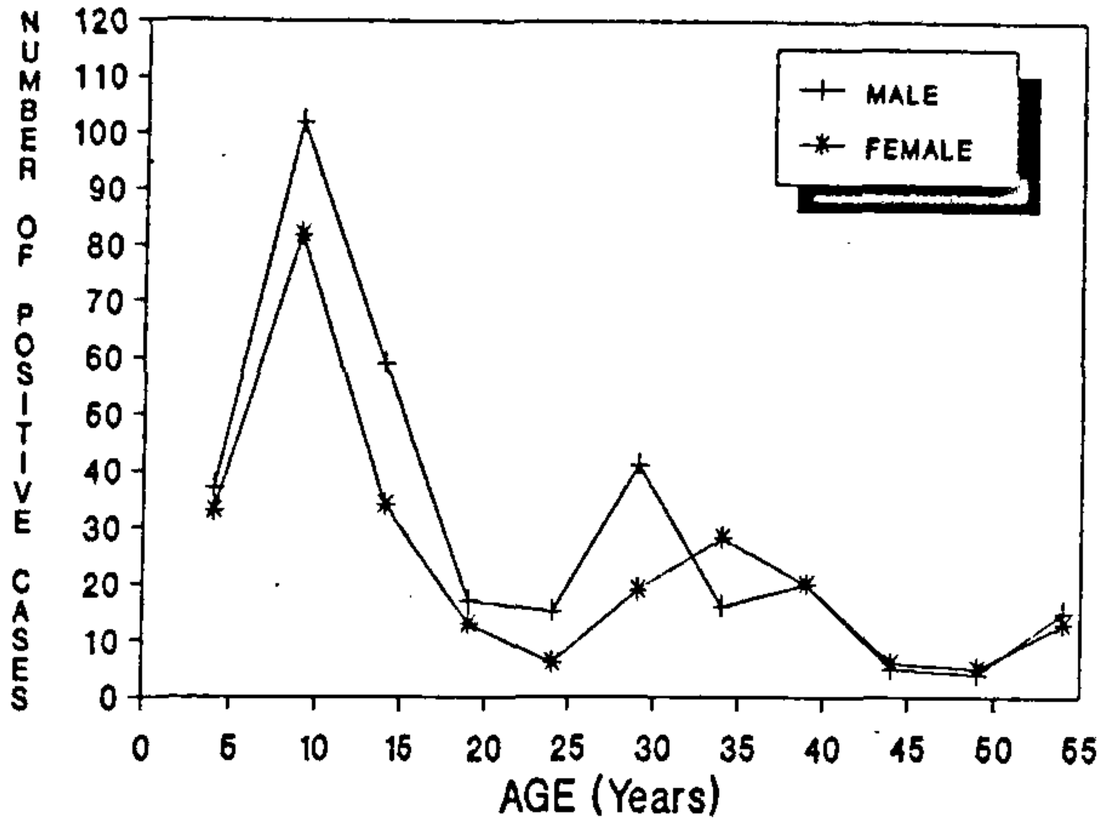

Figure 4. Age-wise direct agglutination test response in male and female seropositives.

In the rest three, the parasitological confirmation could not be achieved. All the ten DAT positives responded well to the stibanate therapy. A large number (423) of asymptomatics found positive to kala-azar is an alarming signal.

In leprosy-ashram, 33 cases suffering from leprosy and found negative in DAT further endorsed our earlier findings[12] on the specificity of the test. As reported earlier[19], we also found females to be more susceptible to kala-azar infection than males.

Certain reports[20,21] indicate that children are more prone to kala-azar than the adults, but our study did not show any pattern in favour of any age-group. The reason could be the limited population size studied and the extension of survey in other wards may reveal clear picture.

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Active transposable elements in Indian maize germplasm and assorted genetic testers

B. M. Prasanna and K. R. Sarkar

Division of Genetics, Indian Agricultural Research Institute, New Delhi 110 012, India

The distribution of genetically active maize transposable elements, *Uq*, *Mrh*, *En* (*Spm*), *Cy* and *Bg*, was surveyed in diverse maize germplasm acclimatized or indigenous to India. Active *Uq* elements were uncovered in several entries, including TEW Drought Tolerant Synthetic-2, NEH collections – K-2, M-5, M-6 and M-10, CM111, *ACR*-marked haploidy tester line 6187, besides *g r-g* and *c1* anthocyanin gene testers. Active *En* elements were detected in M-6 and M-10, while a weakly active *En* element was found in the Pool 16 population. *Mrh* and *Uq* elements with very low mutability were also found in TEW Drought Tolerant Synthetic-2 and EEY-DMR, respectively. Active *En* and *Bg* elements were detected in some *C-I*-marked lines such as 6191A and 6193F, respectively. The study revealed the widespread occurrence of *Uq* in Indian maize germplasm. The pervasiveness of active transposons such as *Uq* in maize germplasm and genetic testers without conscious selection for their presence is interesting in view of the proposed role of mobile elements in generation of genetic variability.

A transposable element system consists of a receptor and its respective regulator (a receptor is usually a deficient derivative of a regulator). The interaction between a receptor and a regulator of an element system is usually specific so that a receptor for an element like *Ubiquitous* (*Uq*) will reveal the presence of Uq but will not give any information on the presence or absence of other regulators. This specificity is the basis for distinguishing controlling element systems from each other. Several transposable element systems of maize have been well-characterized at the genetic level and detailed molecular analysis of some elements and the mutable alleles generated have already been made in the last decade[1–3].

Mobile Element Receptor Line (MERL) contains a receptor element (non-autonomous) for a specific element system in a homozygous state. These lines usually have colourless (non-variegated) kernel phenotype unless an active element of that system is present in the genome in which case the kernels will have coloured spots (excision events) on a colourless background. Probing the maize material to detect active mobile elements using MERL is a rapid, simple and accurate way. Molecular probing is not ideally suited to reveal the actual content of genetically active transposable elements, since (i) multiple copies of sequences homologous to the elements such as *En* and *Ac* are found in maize, which do not necessarily represent the number of active elements present; 30–50 homologous sequences may be found for a specific element in a plant containing only one genetically active element for which probing is carried out; and (ii) some elements such as *Uq*, *Mrh* and *Cy* have not been cloned.

The genetic strategy using MERL has been successfully used by Peter A. Peterson and associates[4–6]. The pervasiveness of genetically active mobile elements, such as *Uq* and *Mrh*, in the maize breeding material as well as lines native to Mexico, South America and USA, and the significance of such activity in the maize breeding material has been emphasized earlier[7–8]. The consequent effects of imprecise excision of the plant transposable elements on base pair sequences of the target genes in which they are inserted and the possibility of generation of genic and protein diversity has been well-established[9–11]. Detection of the widespread occurrence