

# Use of Immunoglobulin G Avidity To Determine the Course of Disease in Visceral and Post-Kala-Azar Dermal Leishmaniasis Patients

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**In the present study, anti-*Leishmania* immunoglobulin G (IgG) avidity was used to estimate the approximate time of disease manifestation. Significant differences ( $P < 0.0001$ ) were found between the levels of anti-rKE-16 IgG avidity in leishmaniasis patients with recent and chronic diseases. More than 76% of patients with an illness duration of less than 6 months had avidity of less than 70%, 94% of patients had less than 80% avidity, and all (100%) patients with illness of more than 6 months had avidity values higher than 70%. The study showed that avidity could successfully be used to pinpoint the duration of leishmaniasis.**

Visceral leishmaniasis (VL) is a chronic parasitic disease and is considered 1 of the 10 main tropical illnesses by the World Health Organization (8). It is usually a fatal disease if not treated and is characterized by irregular fever, malaise, loss of weight, hepatosplenomegaly, and anemia with or without lymphadenopathy. Human immunodeficiency virus (HIV) coinfection in leishmaniasis worsens the situation (7, 8).

Laboratory diagnosis of VL or kala-azar can be made by various methods, such as detection of parasite or its antigen in the relevant tissues or blood or nucleic acid amplification techniques. However, the most widely used method is detection of specific antileishmanial antibodies in the serum or plasma (8). Even though there are several test systems for antibody detection, no serological techniques are able to differentiate between recently acquired and chronic stages of disease. Determination of the precise time of infection may help in the treatment and control programs of leishmaniasis, as the incubation period of this infection may vary from 3 to 18 months depending upon endemicity, exposure to repeated infection, and host immune status, etc. (10).

In response to visceral *Leishmania* infection, a strong immune response takes place, and polyclonal hypergammaglobulinemia, consisting of high levels of all immunoglobulin classes, i.e., immunoglobulin A (IgA), IgM, and IgG, is the rule (10, 13). The IgG antibodies produced at the early stage of infection will have low affinity (or avidity), and this affinity force will increase with the passage of time. This phenomenon has been exploited to pinpoint the time of infection in various infectious diseases, e.g., rubella, cytomegalovirus, and toxoplasmosis, etc. (1–6, 12). However, avidity testing of antileishmanial IgG antibodies has not been used, to the best of our knowledge, for determining the time period of visceral leishmaniasis as yet. Therefore, the present study was undertaken to determine the avidity of IgG against a recombinant antigen of *Leishmania*

*donovani* and to evaluate its value in pinpointing the duration of illness.

Serum samples were obtained from 50 parasitologically confirmed patients with leishmaniasis. Parasitological diagnosis was made by demonstration of Leishman-Donovan bodies in bone marrow or splenic aspirates of suspected cases of visceral leishmaniasis or in skin biopsy specimens from suspected cases of post-kala-azar dermal leishmaniasis (PKDL) at the All India Institute of Medical Sciences, New Delhi, India. A detailed history of illness and its duration was noted. Accordingly, the patients included in this study comprised 17 cases (34%) of active kala-azar disease manifested in the last 6 months (group A), 20 cases (40.00%) of untreated kala-azar disease manifestation for more than 6 months (group B), and 5 cases (10.00%) of drug-resistant kala-azar with a disease duration of more than 4 months (group C). Eight cases (16.00%) of PKDL (group D) were also included in the study. In all of these patients, who had previously been completely cured of visceral leishmaniasis, PKDL had developed within the 6 months preceding (Table 1). Fifty serum samples from healthy controls (voluntary blood donors) and 150 samples from patients with other diseases (symptomatic HIV seropositive [50 samples], pulmonary tuberculosis [50 samples], hepatitis B surface antigen positive [30 samples], toxoplasmosis [10 samples], and malaria [10 samples]) were also included.

The recombinant antigen Ld-rKE-16 is a 39-amino-acid protein, cloned and expressed in *Escherichia coli*, from the C terminus of the kinesin protein of *Leishmania donovani* in our laboratory (14). A commercial firm, SPAN Diagnostics Ltd., India, has commercialized this antigen after preparing a rapid spot/dot test device (Signal-KA), based on flowthrough technology.

In a parasitologically confirmed case of VL and PKDL, an IgG avidity enzyme-linked immunosorbent assay (ELISA) was performed by using the patented Ld-rKE-16 antigen (14) and following the protocol of Beghetto et al. (2) with minor modifications. Briefly, the 96-well ELISA plates (Dynatech) were coated with Ld-rKE-16 at a concentration of 50 ng/well overnight in bicarbonate buffer (pH 9.2) at 37°C and washed three

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TABLE 1. Details of leishmaniasis patient samples taken for IgG avidity study

Patient group	No. of patients	Duration of illness (mean $\pm$ SD) (days)
Visceral leishmaniasis		
Recent infection (<6 mo)	17	66.47 $\pm$ 46.52
Chronic (>6 mo)	20	284.01 $\pm$ 138.91
Drug resistant	5	241.01 $\pm$ 112.93
PKDL	8	315.01 $\pm$ 111.19
Total	50	

times with phosphate-buffered saline (PBS) with Tween 20, pH 7.2, washing buffer. After blocking the plates with 1% bovine serum albumin in PBS (pH 7.2) for 1 h at room temperature (20°C), the plates were washed again three times with the same buffer and stored at +4°C for up to 2 weeks. All samples were tested in duplicate in the same plate, in row A as control wells (PBS) and in row B as urea wells. The patient sera were diluted 100-fold in round-bottom dilution vials in PBS (pH 7.2), and 200  $\mu$ l of the prediluted sample was added to both microwells (A and B) and incubated at 37°C for 1 h. After incubation, the wells were washed three times with PBS with Tween 20 washing buffer. In wells in row B, 200  $\mu$ l of dissociation buffer (6 M urea [Sigma Chemical, Co.] with 0.05% Tween 20 in PBS) per well was added, while in wells in row A, instead of 6 M urea, 200  $\mu$ l of washing buffer was added. The plate was incubated at 37°C for 10 min, followed by washings. After the washings, 100  $\mu$ l of anti-human IgG-horseradish peroxidase conjugate (Adaltris Italia S.p.A., Italy) was added to each well. The plate was again incubated at 37°C for 45 min, followed by the addition of 50  $\mu$ l of TMB chromogenic substrate (Adaltris Italia S.p.A., Italy) for 30 min at room temperature. The reaction was stopped by the addition of 1 N H<sub>2</sub>SO<sub>4</sub> solution, and absorbance was read at 450 nm in an ELISA reader (Anthos, Austria). For each sample, the IgG avidity was calculated as the ratio between the optical density at 450 nm (OD<sub>450</sub>) obtained for well B and that obtained for well A. In other words, if the OD value of well B was reduced to 40% or more of the OD value of well A for the same sample, it was considered low avidity. The test was performed with appropriate negative and positive serum controls. Statistical analysis of the results was done with the Sigma plot (version 9.0) program for evaluation of Student's *t* test (paired and unpaired) and with Microsoft Excel (version 7.0) for general statistical calculations, such as arithmetic mean and standard deviation. *P* values of <0.05 were considered significant.

Of the 50 parasitologically confirmed patients, 42 patients had kala-azar (groups A, B, and C), and 8 patients had PKDL (group D). Of the total, 20 (40%) were children (below 15 years) and 30 (60%) were adults, with the youngest being a 7-month-old male child who acquired leishmaniasis through blood transfusion and the oldest being a 65-year-old female. The overall mean age of patients with visceral leishmaniasis was 21.73  $\pm$  17.61 years, while the mean age of PKDL patients was 31.5  $\pm$  18.57 years. These findings are in concordance with previous observations (9). There was a male-to-female pre-

dominance in both VL (2.5:1) and PKDL (5:1) cases, which is along expected lines, as males have more exposure to sandfly bites than females (9). Most of the patients (90%) were from Bihar, while two (4%) were from Uttaranchal, a sub-Himalayan state of north India, one was from Assam (eastern India), one was from Madhya Pradesh (central India), and one was from Kashmir. Six patients were HIV positive. All HIV-positive patients were males and in their most productive age group, with a mean age of 27.65  $\pm$  7.5 years.

All of the 50 parasitologically proven cases were correctly diagnosed by rKE-16 ELISA and rapid Immunodot test (Signal KA). There was no false-positive result in healthy and other disease controls. Thus, the rKE-16 antigen had 100% sensitivity and specificity for kala-azar diagnosis in both test formats, as reported earlier (14) as well as in this study. This antigen also works efficiently on HIV-positive kala-azar patients (7).

The IgG avidity results indicated that, like other infections, the avidity assays could be used successfully for estimating the time and duration of leishmania infection. In patients who developed kala-azar disease within the last 6 months, the mean anti-rKE16 IgG avidity was found to be low (54.94%  $\pm$  19.23%) compared to the mean avidity of 94.70%  $\pm$  5.01% in patients who had leishmaniasis for more than 6 months (group B) and correlated well with the duration of illness as shown in Fig. 1. The IgG avidity ranged from 15.09% to 76.00% in this group; the duration of illness also ranged widely. If the 80% avidity was taken as the cutoff value, the duration of illness in 16 of 17 (94%) patients with an illness duration of less than 24 weeks (group A) could correctly be diagnosed. Even after lowering the cutoff values to 70%, three-fourths of the patients (13/17 [76.4%]) could be diagnosed correctly in this group. The most important finding of this study was that all patients (100%) who had the illness for more than 24 weeks had an avidity of more than 70%. This difference was statistically highly significant (*P* < 0.0001). It is possible that the history of illness of less than 24 weeks given by four patients with an avidity of more than 70% was not reliable, as all of these patients were illiterate and ignored mild symptoms.

Chronically sick patients unresponsive to treatment (group C) also had high avidity (93.59%  $\pm$  3.77%). As expected, the difference between groups A and C was also significant (*P* <

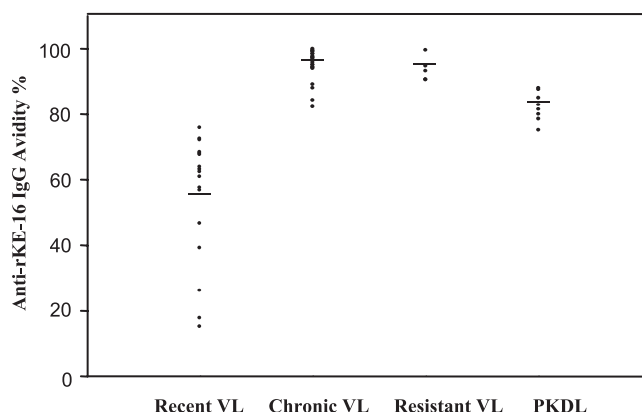


FIG. 1. Scatter diagram to show titers of anti-rKE-16 IgG avidity in patients with leishmaniasis at different clinical stages.

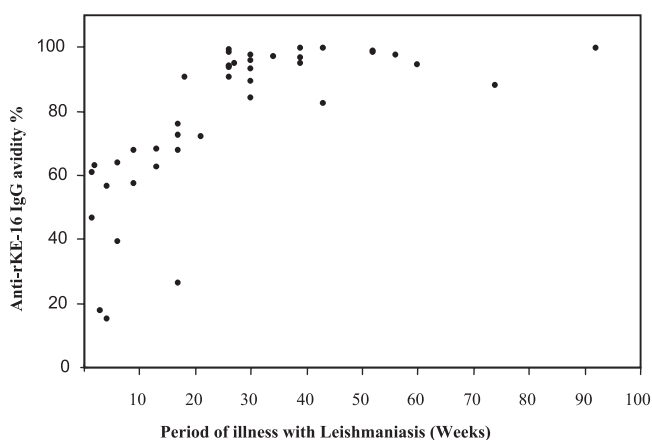


FIG. 2. Values of anti-rKE-16 IgG avidity in patients with visceral leishmaniasis and its correlation with the duration of illness.

0.01), but it was statistically insignificant ( $P < 0.37$ ) between groups B and C. The PKDL patients (group D) had moderately high avidity ( $82.16\% \pm 4.41\%$ ), which was significantly higher ( $P < 0.01$ ) than that of group A, but the difference was insignificant in comparison to groups B and C (Fig. 2).

Singh et al. (13) reported that diagnosis of recent infection of leishmaniasis using antileishmanial IgM and/or IgA detection methods can be used but the method is not highly rewarding. Even some cases of PKDL, supposed to be the most chronic form of leishmania infection, can demonstrate IgA and IgM antibodies, while some VL patients with acute illness may not show detectable levels of IgA and IgM classes of antibodies (13). PKDL is considered a sequel to visceral leishmaniasis and develops 6 months to several years after apparently successful treatment of VL (8). In India, patients with PKDL are also considered reservoirs for kala-azar outbreaks. However, we had previously postulated that PKDL manifestation may be acute upon chronic infection by another genotype (11). The finding of moderately high ( $82.16\% \pm 4.41\%$ ) avidity that is significantly ( $P < 0.001$ ) lower than that of the chronic kala-azar cases ( $94.70 \pm 5.01\%$ ) despite the mean illness period of  $315.01 \pm 111.19$  days strengthens our postulation. In this direction, we have already started the genetic analysis of VL and PKDL strains and found significant genetic heterogeneity between the two isolates (unpublished data).

Therefore, we can conclude that the anti-rKE-16 IgG avidity estimation could be used as a precise method of calculating the accurate date of leishmanial clinical disease, as for other infectious diseases. The patients with chronic illness who are unresponsive to treatment will have an avidity of more than

70%, while those who are newly infected and showed clinical manifestations within the last 6 months will have avidity values of less than 70%. These findings can be used routinely for diagnosis, prognosis, and field epidemiological purposes. Avidity can also be used for monitoring parasitological clearance in response to treatment.

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