Diagnosis of visceral leishmaniasis: comparative potential of amastigote antigen, recombinant antigen and PCR

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ABSTRACT

Development of simple, economical and non-invasive tests for the early diagnosis of visceral leishmaniasis (VL) or kala-azar (KA) remains a challenge, and serological studies based on antigen prepared from the amastigote stage of *Leishmania donovani*, the stage that causes infection, are lacking. In the present study, circulating antibodies to total antigen isolated from the promastigote and amastigote stages of the parasite, as well as to recombinant K39 (rK39) antigen, are measured by enzyme-linked immunosorbent assay (ELISA) and the results compared with a polymerase chain reaction (PCR) test for KA diagnosis. In 116 samples of KA examined, the amastigote antigen gave significantly higher mean absorbance values in ELISA than did the promastigote antigen. The sensitivity for KA detection was significantly higher using the amastigote antigen (94%) than the promastigote antigen (90.5%). Analysis in 91 controls showed that specificity was higher with amastigote antigen (92.3%) than with promastigote antigen (86.8-89.0%). Reliability of ELISA diagnosis with amastigote antigen was only marginally lower than that with rK39 ELISA or with the PCR test. Easy availability and low cost of indigenous amastigote antigen, together with the simplicity of ELISA compared with PCR, make ELISA based on amastigote antigen a promising choice for the diagnosis of KA.

KEY WORDS: Enzyme-linked immunosorbent assay. Kala-azar. Leishmania donovani. Polymerase chain reaction.

Introduction

Kala-azar (KA), or visceral leishmaniasis (VL), is a major public health problem with annual incidence and prevalence of 0.5 and 2.5 million, respectively, of which 90% cases occur in the Indian subcontinent and Sudan.[1] In India, KA is endemic in the eastern part of the country and often becomes epidemic, claiming the lives of thousands and causing severe morbidity in hundreds of thousands.

The causal organism *Leishmania donovani* is a dimorphic parasite, the flagellated promastigote form of which is found in the insect vector and the non-motile intracellular amastigote stage resides within macrophages of the human host. KA is characterised clinically by fever, weight loss, hepatosplenomegaly and pancytopenia, and has a high mortality rate in untreated cases.

Routine laboratory diagnosis of KA is by microscopic detection of Leishmania amastigotes in Giemsa-stained smears of spleen, bone marrow or lymph node aspirates. These processes are invasive methods, require considerable skill and are associated with the risk of fatal hemorrhage. Moreover, diagnosis by microscopy has limited sensitivity and specificity because morphologically similar organisms such as trypanosomes and amoebae may be a source of confusion.

Over the years, a number of indirect immunological methods such as direct agglutination tests,[2,3] indirect immunofluorescent antibody tests,[4] enzyme-linked immunosorbent assay (ELISA)[5-8] and a recombinant K39 (rK39) strip test[9] have been developed. Recently, the polymerase chain reaction (PCR) has proved to be a rapid, sensitive and specific method for detection of parasite in clinical samples.[10,11]

Serodiagnosis with various crude antigens using whole promastigote or soluble promastigote antigen has been used widely but is compromised by limited specificity and sensitivity. The recombinant antigens dp72 and rK39[12,13] have proved to be good diagnostic agents but the availability of recombinant antigens is often a limiting factor in developing nations in which the disease is endemic.

In comparison, total antigen prepared from amastigote stage of *L. donovani* is expected to be more useful for correct diagnosis as the amastigote stage is the replicating form that leads to active disease and induction of a specific immune response in KA patients. However, studies on the use of amastigote antigen for KA diagnosis are very limited. One of the initial studies to recommend the use of amastigote antigen for field diagnosis of KA used amastigotes isolated from splenic tissue of infected hamsters.[14]

Amastigotes are generally difficult to isolate in pure form and in large quantity. However, with the advent of methods for axenic amastigote culture, such difficulties have been

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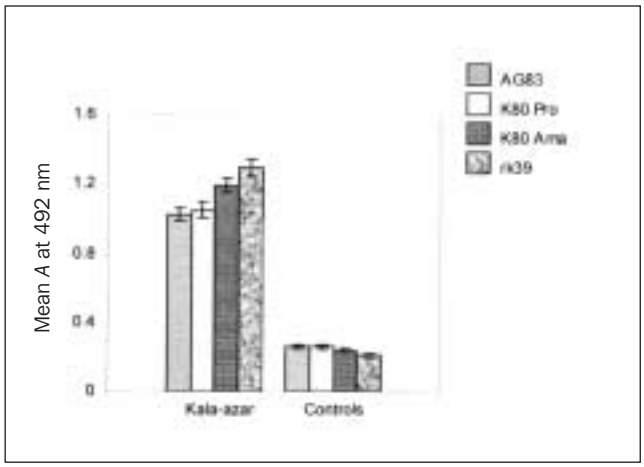

Fig. 1. Antibody levels determined by ELISA with antigen derived from AG83 promastigotes, indigenous promastigotes and axenic amastigotes of L. donovani and recombinant K39. Mean A values at 492 nm determined by ELISA for patient and control sera were plotted.

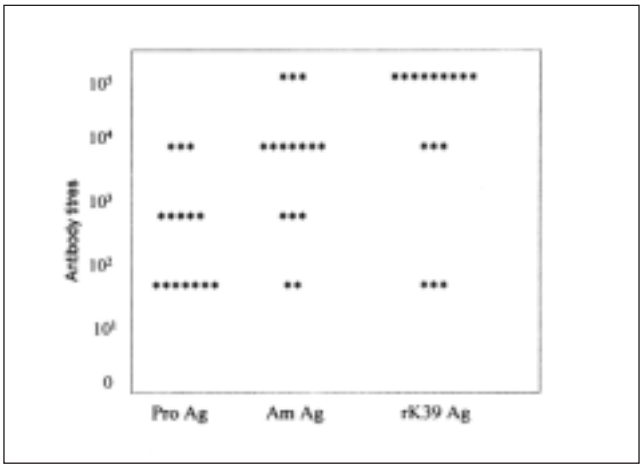

Fig. 2. Titres of antibody against antigens derived from indigenous promastigotes, axenic amastigotes and rK39 antigen in sera from patients with kala-azar.

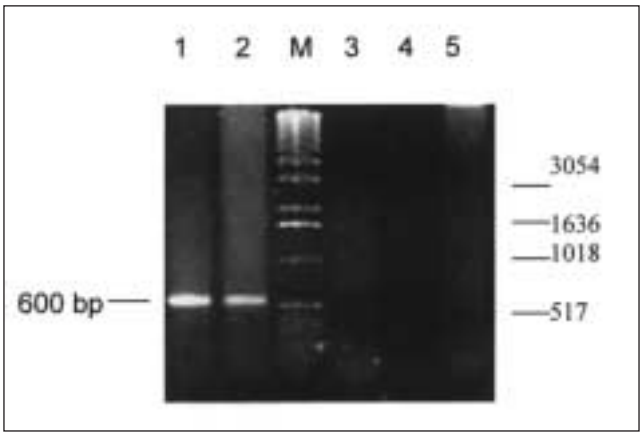

Fig. 3. PCR assay of clinical samples. Lane 1: KA blood; lane 2, KA bone marrow; lane 3: malaria; lane 4: tuberculosis; lane 5: control from the area of endemicity.

overcome successfully.[15,16] A recent study convincingly demonstrated the serodiagnostic utility of an amastigote antigen prepared from cultured axenic amastigotes.[17].

Here, we report a comprehensive study to evaluate the diagnostic potential of total amastigote antigen in 207 clinical samples, using axenic amastigote cultures propagated from indigenous *L. donovani* isolates.

Material and methods

Clinical samples

Serum samples were collected from 116 KA patients from Bihar reporting to Safdarjung Hospital, New Delhi, and informed consent was obtained from each. The patients presented with clinical symptoms of KA such as fever, hepatosplenomegaly, anaemia and leucopenia. The patients were evaluated clinically and confirmed by demonstration of amastigotes in bone marrow aspirates (BMA). All serum samples were taken at pretreatment stage.

Control groups comprised individuals with confirmed malaria (n=18, peripheral blood smear positive), pulmonary tuberculosis (n=19), healthy individuals from endemic regions (n=21) and normal healthy persons (n= 33) from Delhi, a non-endemic area.

Promastigote culture

Promastigotes of *L. donovani* strain AG83 (MHOM/IN/AG/83) originally isolated from a KA patient were propagated in M199 medium with 25 mmol/L HEPES (pH 7.4) supplemented with 10% fetal calf serum (FCS) as described previously.[18] Bone marrow aspirates from KA patients were used to set up cultures in M199 with 25 mmol/L HEPES and 20% FCS. These indigenous promastigotes were propagated for 3-4 passages in M199 medium with 10% FCS before antigen preparation.

Axenic amastigotes culture

Axenic amastigotes from an indigenous isolate of *L donovani* were grown following a procedure described previously[16] but with slight modification. Bone marrow aspirate samples from KA patients were collected in RPMI medium (pH 5.5) supplemented with 20% FCS and incubated at 35°C in 6% CO_2. The cultures were passaged every fourth day (5-6 cycles) before raising the temperature to 37°C. Axenically grown amastigotes were maintained by cycling between promastigotes and amastigotes every fourth day.

Antigen

Promastigotes and amastigotes from *L. donovani* isolated from bone marrow aspirates of KA patients and AG83 promastigotes were harvested during the late log phase. After extensive washing with cold phosphate-buffered saline (PBS) the cells were lysed by boiling for 3-5 min in buffer containing 50 mmol/L Tris-HCl (pH 7.0), 3% sodium dodecyl sulphate (SDS) and 1 mmol/L phenyl methyl sulphonyl fluoride. Protein concentration in the lysate was determined using the DC protein assay kit (Bio-Rad) as described previously.[19]

Enzyme-linked immunosorbent assay

Detection of anti-leishmanial antibody in serum samples was carried out using a standard ELISA method.[20] Polystyrene microtitre plate (Corning) wells were coated

Table 1. Comparison of the results of ELISA for the four serological assays based on antigens from AG83 promastigotes, indigenous promastigotes and axenic amastigotes, and rK39 antigen. A value of 0.45 was used as the cut-off value in all cases

	ELISA result with different antigens			
	AG83 Pro Ag	Pro Ag	Am Ag	rK39 Ag
Kala-azar	105/116(90.5%)	105 /116 (90.5%)	109 /116(94%)	111 /116(95.7%)
Tuberculosis	3/19(15.7%)	1/19(5.2%)	1/19(5.2%)	0/19(0%)
Malaria	2/18 (11.11%)	3/18(16.6%)	2/18 (11.11%)	1/18(5.5%)
Endemic control	3/21(14.5%)	4/21 (19%)	3/21(14.5%)	3/21(14.5%)
Healthy control	2/33(6%)	2/33 (6%)	1/33(3%)	0/33(0%)

Values indicate positive samples/total samples (percentage positive)

Table 2. Comparison of the results of the species-specific PCR assay and ELISA for the four serological assays based on antigens derived from AG83 promastigotes, indigenous promastigotes and amastigotes and rK39 antigen. A value of 0.45 was used as the cut-off value in all the cases.

		ELISA result with different antigens			
	PCR	AG83 Pro Ag	Pro Ag	Am Ag	rK39 Ag
Kala-azar	53/55	50/55	50/55	52/55	52/55
Controls	0/70	7/70	6/70	4/70	1/70
Endemic Controls	2/21	3/21	4/21	3/21	3/21

with 10 ng rK39 protein or 200 ng promastigote or amastigote crude antigen. The wells were blocked with 5% skimmed milk for 2 h at 37°C and washed (x3) with PBS containing 0.1% Tween 20 (PBST). The plates were incubated for 2 h with patients' sera at various dilutions as specified.

Wells were washed (x3) with PBST and incubated with anti- human IgG conjugated with horseradish peroxidase for 2 h at 37°C. After washing with PBST, ortho phenylene diamine substrate (Sigma) was added together with hydrogen peroxide to produce a colour reaction. The reaction was stopped by addition of 1N H_2SO_4 and absorbance (*A*) was measured at 492 nm using an ELISA reader (Titertek Multiscan Plus). Each sample was assayed in triplicate, along with appropriate controls.

The ELISA reader was set to subtract the reading of a blank control from the test samples. The cut-off value was derived from the mean *A* obtained with normal human sera from an area of endemicity with the three antigens. The cut-off value (0.45) was twice that of the mean *A* obtained.

DNA isolation and PCR analysis

Depending on the availability of clinical samples, DNA was isolated from peripheral blood obtained from KA patients (n=55) and controls (malaria [n=18], tuberculosis [n=19], healthy controls from endemic [n=21] and non-endemic areas [n=33]) using a method described previously.[10] Briefly, blood (0.2 - 1.0 mL) was treated with RBC lysis buffer and the resultant buffy coat subjected to lysis overnight in NET buffer with 100 µg/mL proteinase K and 1% SDS. DNA was extracted by phenol/chloroform extraction and ethanol precipitation. 100 ng DNA from both patients and controls was amplified using Ldi primers designed from kinetoplast DNA of parasite as described previously.

The primer sequences used were 5′-AAATCGGCTCCGAGGCGGGAAAC-3′ and 5′-GTACACTCTATCAGTAGCAC-3′. The reaction mixture (50 µL) contained 200 mmol/L of each dNTP, 50 ng of each primer, 1.5 mmol/L $MgCl_2$ and 1.25 units *Thermus aquaticus (Taq)* DNA polymerase in PCR buffer (Gibco BRL). Each reaction mix was overlaid with mineral oil and amplified for 40 cycles at 94°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 2 min, preceded by an initial denaturation of 2 min at 94°C. Final extension was for 3 min at 72°C. Amplification products were analysed by electrophoresis in 1% agarose gel in TAE buffer (40 mmol/L Tris acetate, 1 mmol/L EDTA) and samples producing 600 bp bands were recorded as positive.

Data analysis

Statistical analysis was done using unpaired Student's *t*-test to compare the mean *A* of ELISA using different antigens. $P<0.05$ was considered significant.

Results

Mean *A* for KA samples was highest with rK39 antigen even though the serum dilution was double (1 in 200) that used for promastigote and amastigote antigens (1 in 100) (Figure 1). Under the experimental conditions used, mean *A* values for the amastigote antigen ELISA were comparable with mean *A* for the rK39 ELISA ($P>0.05$). The amastigote antigen

gave significantly higher mean *A* ($P<0.05$) compared to promastigote antigen derived from either BMA of KA patients or the AG83 strain. Moreover, the amastigote antigen and rK39 antigens gave lower mean *A* for control samples compared with the promastigote antigens.

ELISA sensitivity to detect KA cases was 90.5% (105/116), 90.5% (105/116), 94% (109/116) and 95.7% (111/116) for AG83 promastigotes, indigenous promastigotes, amastigotes and rK39 antigen, respectively (Table 1). Control samples were diagnosed correctly in 95.6% (87/91) and 92.3% (84/91) using rK39 and amastigote antigen, respectively. The indigenous promastigote antigen and AG83 produced the correct diagnosis in 89% (81/91) and 86.8% (79/91), respectively, in control groups. The estimated sensitivity of the ELISA test at 95% confidence interval ranged from 94.3% to 99.3% for rK39 and 93.8% to 99% and 87% to 95.3% for amastigote and promastigote antigens, respectively.

Antibody titre in 15 randomly chosen samples was determined using indigenous promastigote antigen, axenic amastigote antigen and rK39 antigen. rK39 antigen produced the highest titre with majority of samples (Figure 2). With amastigote antigen the majority of samples (10/15) gave a titre above 10^{-4}, while with promastigote the titre was less than 10^{-3} in the majority (12/15) of cases.

55 KA cases were taken for comparative evaluation by PCR and ELISA. The PCR test used in 55 KA cases was a species-specific assay based on kinetoplast DNA primers, as described previously.[10] Positive samples gave 600 bp band after amplification (Figure 3). The PCR test proved positive in 53/55 cases, while ELISA test with promastigote, amastigote and rK39 antigens proved positive in 50/55, 52/55 and 52/55 cases, respectively.

Discussion

Here, we report the screening of four different antigens to find a suitable indigenous antigen to detect antileishmanial antibody in KA patients. A total of 207 clinical samples were screened with antigen prepared from the two stages (promastigote and amastigote) of the parasite, and results were compared with the recombinant k39 antigen. Higher sensitivities were achieved with amastigote and rK39 antigen (94% and 95.7%, respectively) compared to those achieved with the promastigote antigen (90.5%).

A number of studies have attempted to detect antileishmanial antibodies using antigen of promastigote origin in ELISA;[5,9,14] however, there are few reports of the use of amastigote antigen to detect antibodies in KA.[17] Recently, we evaluated the potential of crude promastigote and amastigote antigens in the diagnosis of post kala-azar dermal leishmaniasis and showed that ELISA sensitivity and specificity were higher with the amastigote antigen.[21] In addition, purified 200 kDa amastigote antigen and soluble amastigote antigen have both been shown to be useful in the diagnosis of KA.[14,17]

In the present study, the potential of amastigote antigen in the diagnosis of KA is clearly demonstrated, not only because of the higher sensitivity and specificity achieved but also by observation of high antibody titres. This is to be expected because parasites are present in the amastigote form inside mammalian hosts and this is the form that induces specific immune response in human host.

The recombinant protein rK39 that is expressed abundantly in the amastigote form has been reported to be a very sensitive antigen in the diagnosis and prognosis of KA.[13,22,23] In the present study, although rK39 produced the highest sensitivity and specificity of all the antigens evaluated, corresponding results with amastigote antigen were comparable and a significant improvement over those achieved with the promastigote antigen. Thus, the indigenous amastigote antigen provides a useful tool for diagnosis of KA, particularly when the recombinant antigen is not available or affordable as is the case in many regions of the world where the disease is endemic.

Earlier studies demonstrated the superiority of PCR assay to direct microscopy and ELISA.[24] In studies of VL in immunocompromised patients, PCR was found to be much more reliable than culture, microscopy and serological tests.[25,26]

In the present study 55 cases of KA were subjected to comparative evaluation by both ELISA and PCR. The PCR assay employed was a species-specific test developed recently.[10] Although PCR sensitivity for *L. donovani* infection was better than that achieved with ELISA, the sensitivity and specificity of the latter with the amastigote antigen were only marginally lower. AS KA is endemic in areas with poor socioeconomic conditions, ELISA using the indigenous amastigote antigen would provide a more economical and practical assay for diagnosis, as PCR is expensive and requires sophisticated facilities. □

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