

## Original Article

# Diagnosis of Kala-azar by rK39 based ELISA and KAtex

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### Abstract

*This cross sectional study was carried out in SSMC for a period of one year from July 2007 to June 2008 to evaluate rK39 based ELISA and KAtex tests for diagnosis of Kala-azar. Total 150 patients were selected clinically. For this study rK39 ICT was regarded as confirmatory test according to national guideline. Among 150, total 100 cases were confirmed to be positive for Kala-azar by rK39 ICT test. Age and sex matched 25 healthy persons each from endemic and non-endemic area were taken as control. Among the 100 sero confirmed cases KAtex was positive in 87 (sensitivity of 87%) while ELISA was positive in 98 cases (sensitivity 98%). Among the 50 controls none were positive by KAtex test while 1 (one) ELISA test was positive resulting in specificity of these tests 100% and 98% respectively. The result of the study clearly showed superiority of rK39 antigen based ELISA in respect of sensitivity and KAtex in respect of specificity.*

### Introduction

Visceral leishmaniasis (VL) is prevalent in more than 80 countries in Asia, Africa southern Europe and South America. *L. donovani* is the main causative parasite for VL<sup>1</sup>. There are an estimated 500, 000 new cases of VL and more than 50,000 deaths from the disease each year<sup>2</sup>, a death toll that is surpassed among the parasitic diseases only by malaria<sup>3</sup>. The majority ( $\geq 90\%$ ) of cases occur in just six countries: Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil. Among them India, Nepal and Bangladesh harbour an estimated 67% of the global VL disease burden<sup>4</sup>. Visceral Leishmaniasis (VL) is a re-emerging serious public health problem in the Indian sub-continent. The disease has been reported from 109 districts of Bangladesh, India and Nepal including 45 districts from Bangladesh<sup>5</sup>. Laboratory diagnosis of

leishmaniasis can be made by (1) demonstration of parasite in tissue of relevance by light microscopic examination of the stained specimen, in vitro culture, or animal inoculation; (2) detection of parasite DNA in tissue samples (3) immunodiagnosis by detecting parasite antigens and specific or nonspecific antileishmanial antibodies (immunoglobulin) from urine and blood samples or by assay for leishmania-specific cell-mediated immunity. The diagnostic gold standard for VL is to confirm the presence of *Leishmania* spp. either through cultures or microscopic visualization of parasites from aspirate of bone marrow, spleen, lymph node or liver. Though the sensitivity of splenic smears could be as high as  $>95\%$ ,<sup>6</sup> it carries the risk of severe/fatal haemorrhage; On the otherhand bone marrow aspiration is painful, cumbersome and has a

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low sensitivity (60-85%)<sup>6</sup>. Culture cannot be used for routine clinical diagnosis, as it requires expensive equipment and expertise. On the other hand serological methods are highly sensitive and relatively non-invasive and are comparatively more suited for diagnosing VL in endemic regions. They are either based on detection of antibodies or antigens. Tests based on the detection of antibodies, such as the direct agglutination test (DAT) in sera and a recombinant 39 amino acid antigen (rK39)-based rapid strip test, have become available in recent years<sup>7</sup>. Direct agglutination test (DAT), based on agglutination of the trypsinized whole promastigotes has been found to be useful in several endemic countries<sup>6</sup>. The major disadvantage of DAT is the relatively long incubation time of 18 hours and the need for serial dilutions of blood or serum. ELISA has been used as a serodiagnostic tool in leishmaniasis. The technique is highly sensitive, but its specificity depends upon antigen used. rK 39 is a 39-amino acid repeat that is part of a kinesin-related protein in *Leishmania chagasi* and which is conserved within the *L. donovani* complex. An rK39-based ELISA showed excellent sensitivity (93-100%) and specificity (97-98%) in many VL-endemic countries<sup>7</sup>. A simple, rapid, easy to perform dipstick test using rK39 is available in the form of antigen impregnated nitrocellulose paper strips adapted for use under field conditions. A meta-analysis that included 13 validation studies of the rK39 ICT showed sensitivity and specificity estimates of 93.9%(95% CI, 87.7-97.1) and 95.3% (95% CI, 88.8-98.1) respectively. Recently, the excellent diagnostic performance of rK39 ICT was confirmed in India and Nepal<sup>8</sup>. According to national guideline on Kala-azar elimination an endemic patient with clinically suspected Kala-azar is designated as confirmed Kala-azar if rK 39 test is found positive.

Antigen detection is more specific than antibody-based immunodiagnostic tests. This method is also useful in the diagnosis of disease in cases where there is deficient antibody production (as in AIDS patients). A new latex agglutination test (KATEX) for detecting leishmania antigen in urine of patients with VL has showed sensitivity between 68 and 100% and a specificity of 100% in preliminary trials<sup>9</sup>. The antigen is detected quite early during the infection and results of animal experiments suggest that the amount of detectable antigen tends to decline rapidly following chemotherapy<sup>10</sup>. So it can be said that serum and urine as a specimen for diagnosis of Visceral leishmaniasis is a better choice than bone marrow, lymph node or splenic aspirate. Among the serological methods ICT using

rK39 antigen is easy to perform and field applicable. But ELISA using rK39 antigen and KAtex are also promising in respect of sample collection and diagnostic superiority found in many countries. Due to the re-emergence of Kala-azar in Bangladesh different diagnostic methods has to be analyzed and compared to find out a simple, field applicable rapid and reliable diagnostic method which is the mainstay to prevent the spread of the disease. So the purpose of the study is to find out the superiority between two easily sample collectable methods.

## Materials And Methods

This cross sectional study was performed in the Department of Microbiology, Sir Salimullah Medical College, Dhaka from July 2007 to June 2008. A total number of 150 clinically suspected patients of Kala-azar of different age groups who were admitted in indoor of Sir Salimullah Medical College (SSMC) and Mitford Hospital, Mymensingh Medical College Hospital (MMCH) and Bhaluka Health complex, Mymensingh were included in the study. Laboratory work was performed in the Department of Microbiology, Sir Salimullah Medical College, Dhaka and partly in Department of Microbiology MMC Bhaluka health complex. Patients were selected clinically according to Bern et al<sup>11</sup>, 2006 guideline/criterian. Total 50 age and sex matched healthy people from endemic and non-endemic area were taken as control. Age and sex matched healthy individuals having no past history of prolong fever, splenomegaly or other clinical features suggestive of VL or no history of receiving antimonial drug were taken as controls. There were Twenty five healthy people from the village Dhalia of Bhaluka thana in Mymensingh district as endemic control and Twenty five healthy people from Mirpur of Dhaka city as non-endemic control. Patients who have already received SAG (Sodium antimony gluconate) for treatment, or have a previous history of kala-azar were excluded from the study.

**Legal procedures:** Before collection of all the samples verbal as well as written consent was taken from the patients and/or their attendants. Ethical clearance was taken from ethical review committee. Statistical analysis was done following a standardized formula.

### Laboratory procedure:

**Blood collection:** With all aseptic precaution three ml of venous blood was collected from each patient and control. Collected blood was kept in a sterile screw capped test tube for separation of serum. Sera from 150

patients were taken for antibody detection by ICT against the specific antigen rK39. ICT positive patients were undergone for ELISA and KAtex tests.

Urine collection: Approximately 5 ml of urine sample was collected from all the patients as well as the controls into a clean sterile container.

### Specimen storage

Serum and urine samples were stored in refrigerator (2-8°C) for up to 7 days. For long-term preservation samples were transferred in -20°C refrigerator.

#### Tests:

ICT (Immunochromatographic test): Principle:

The Kala-azar Deect™ Test for VL is a qualitative, membrane based ICT for the detection of antibodies to Visceral Leishmaniasis in human serum. The membrane is pre-coated with novel recombinant VL antigen on the test line region and chicken anti-protein A on the control line region. During testing, the serum sample reacts with the dye conjugate protein A-colloidal gold conjugate which has been pre-coated in the test device. The mixture then migrates upward on the membrane chromatographically by capillary action to react with recombinant VL antigen on the membrane and generates a red line. Presence of this red line indicates a positive result, while its absence indicates a negative result. Regardless of the presence of antibody to VL antigen, as the mixture continues to migrate across the membrane to the immobilized chicken anti-protein A region, a red line at the control line region will always appear. The presence of this red line serves as verification for sufficient sample volume and proper flow and as a control for the reagents. The test was done according to the manufacturer's instruction and the result was read in 10 minutes.

Urinary antigen detection by latex agglutination test (Katex): (Kalon Bio. UK. 2005) KAtex was done on the urine samples. Urine sample, taken from suspected VL patient was pretreated (boiling for 5 minutes) to inactivate heat-labile materials (protein antigens) which may cause a false-positive reaction. Test latex sensitized with antibodies raised against *L. donovani* antigen was then mixed with the sample on a glass slide. If Leishmania antigen present in the urine sample, it will cause cross-linking (agglutination) of the sensitized latex. The reaction was read after mixing for two minutes. Agglutination of sensitized latex indicates presence of Leishmania antigen in urine and thereby visceral leishmaniasis. The degree of agglutination were noted and interpreted according to the manufacturer's instruction.

### ELISA for antibody detection:

The kalazar Detect™ ELISA is an enzymatically amplified "two-step" indirect immunoassay to detect antibody against rk39 antigen of Leishmania. In this assay, standards, controls and unknown serum samples are incubated in microtitration wells which have been coated with specific recombinant antigens. After incubation and washing, the wells are treated with polyclonal goat anti-human IgG labeled with the enzyme horseradish peroxidase (HRP). After a second incubation and washing step, the wells are incubated with the tetramethylbenzidine (TMB) substrate. An acidic stopping solution is then added and the degree of absorbance measurement at 450nm. The absorbance measured is directly proportional to the concentration of antibodies to recombinants present.

**Calculation of Cut-off:** The cut-off was calculated based on the average OD values from 199 normal sera.

$$\text{Cut-off} = \text{Average OD} + 3\text{SD} = 0.101 + 0.289 = 0.390$$

**Interpretation of results:** OD values > cut-off was considered positive for the presence of specific antibodies to recombinant antigen. The test procedure was done according to the manufacturer's guideline and >0.390 OD was regarded as positive, between 0.390 and 0.250 were regarded as equivocal and < 0.250 OD was regarded as negative sample according to the manufacturer's guideline.

### Result

Clinically suspected 150 patients were undergone rK39 ICT test and among them 100 were positive. Serum samples of these patients were tested for ELISA and urine samples for KAtex. Serum and urine of 50 controls were also tested for ELISA and KAtex. ELISA was positive in one and none were positive for KAtex. Table I shows the result of KAtex and ELISA in ICT positive cases. Table II shows the sensitivity, specificity, Predictive value positive (PVP) and Predictive value negative (PVN) of KAtex and ELISA.

**Table I:** Comparison of Ab and Ag detection tests from blood and urine (n=100)

KAtex	ELISA	ICT	Total
+ve	+ve	+ve	87
-ve	+ve	+ve	11
-ve	-ve	+ve	2

**Table II:** Cumulative sensitivity and specificity PVP and NVP of KAtex, and ELISA

	KAtex	ELISA
Sensitivity	87%	98%
Specificity	100%	98%
Predictive value positive (PVP)	100%	99%
Predictive value negative (NVP)	79%	96%

## Discussion

Kala-azar is nearly always fatal if untreated<sup>12</sup>. The clinical presentation of VL is not sufficiently specific to guide any treatment. Highly accurate (both sensitive and specific), cheap and simple rapid diagnostic tests (RDTs) are therefore crucial for case-management of VL. Early case detection followed by adequate treatment is also important to control VL because, as yet, no vaccine is available and the long-term impact of vector control is unclear. Since the 1980s, the main objective of VL diagnostics development has been to replace the direct demonstration of parasites in tissue smears, by a 'field test' that is more appropriate for use in a VL-endemic context. The rK39 ICT is surely promising to meet this goal. The test is easy to perform, rapid, cheap and give reproducible results. And in a suspected case in endemic area its positivity gives confirmation of Kala-azar according to the National guidelines for Kala-azar elimination in Bangladesh.

This cross sectional study was carried out in SSMC for a period of one year from July 2007 to June 2008 to evaluate KAtex and rK 39 ELISA tests for diagnosis of Kala-azar. Among 150 clinically suspected patients 100 patients were positive for rK39 ICT Controls were 50 in number consisting of 25 healthy persons from endemic area and 25 healthy persons from non-endemic area. ELISA has been used as a potential sero diagnostic tool for almost all infectious diseases, including leishmaniasis. The technique is highly sensitive, but its specificity depends upon the antigen used. In this study using rK39 antigen, sensitivity and specificity of ELISA was 98% (Table-II). Other studies also supports that rK39 based ELISA has excellent sensitivity (93-100%) and specificity (97-98%) in many VL-endemic countries<sup>13</sup>. All ICT positive cases were also positive by rK39 ELISA in a study conducted in Bangladesh<sup>14</sup>. In this study, sensitivity and specificity of KAtex was found to be 87% and 100% (Table-II). This test showed

100% specificity and 68% to 100% sensitivity<sup>15</sup>. Gavgani et al.<sup>16</sup> (2008) found sensitivity of KAtex to be 77.77% and specificity 98.24% in their study.

However, the sensitivity of this test was disappointingly low in clinically suspected patients in a VL-endemic area in Nepal<sup>17</sup> where low sensitivity (47.4%) against excellent specificity (98.7%) was observed. They found a significant increase in the KAtex sensitivity with the duration of fever, spleen size and the parasite intensity in the tissue aspirate; the first two probably reflects the duration of the illness. Parasite density is probably well-correlated with antigen load in urine<sup>17</sup>. This study showed high sensitivity and specificity in KAtex test. Higher sensitivity may be explained by the increased parasite load of the patient as most of the patients in our country seek medical attention at later stage. Mean duration of illness before treatment is 4 months<sup>18</sup>. In this study KAtex test was done within 7 days of urine collection mostly that may also contribute to the higher sensitivity.

## Conclusion

In this study ELISA with rK 39 antigen was found to be highly sensitive. KAtex test although highly specific it lacks in sensitivity. KAtex test may be used to confirm active cases as it detects antigen and shows high specificity. Combination of KAtex with any other serological test may be regarded as an alternative to invasive methods of Kala-azar diagnosis where facilities are inadequate.

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