Original Article

Detection of urinary leishmanial antigen by latex agglutination test (*KA*tex) in kala-azar patients

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Abstract

Background: A new unique latex agglutination test (*KA*tex) that detects a stable, non-protein, disease specific parasite antigen in the freshly voided urine of patients suffering from active kala-azar has been introduced by Kalon Biological Ltd. UK. This is absolutely non-invasive method of diagnosis for visceral leishmaniasis and suitable for implementation as a rapid diagnostic tool at the point of care. ***Objective:*** Diagnostic potential of *KA*tex was evaluated among clinically suspected kala-azar patients in an endemic zone of Bangladesh. ***Methodology:*** *KA*tex was done using freshly voided urine according to the manufacturer's instructions for sixty (60) clinically suspected patients of kala-azar admitted in Rajshahi Medical College Hospital (RMCH), Bangladesh and forty (40) healthy controls during December 2005 to June 2006. Leishmania nested Polymerase Chain Reaction (Ln-PCR) using peripheral blood buffy coat was performed for all study population (100) and Ln-PCR positive cases were considered as confirmed cases of kala-azar. ***Results:*** Out of 60 clinically suspected kala-azar patients, 56 were Ln-PCR positive and 53 of 56 Ln-PCR positive cases were *KA*tex positive (sensitivity, 94.64%; Mantel-Haenszel Chi sq. 79.66, p= 0.0000, confidence interval [CI], >95 to 100%). None of the healthy controls was found positive by Ln-PCR but 2 of 40 were *KA*tex positive (specificity, 95%; confidence interval [CI], >95 to 100%). The positive and negative predictive values of *KA*tex were noted as 98.10% and 92.85% respectively. **Conclusion:** This limited prospective study suggests that *KA*tex is an absolutely non-invasive urine-based antigen detection test with high sensitivity and specificity and may be useful for screening active kala-azar patients, particularly suitable for field use.

Key words: Visceral leishmaniasis, Kala-azar, *KA*tex, Ln-PCR, sensitivity, specificity.

Introduction

Visceral leishmaniasis (VL) or Indian kala-azar is a vector borne parasitic disease caused by an obligate intracellular haemoflagellate of the genus Leishmania[1]. Infected human acts as reservoir and the *L. donovani* is considered to be the agent for Indian kala-azar. The VL has recently been shown to be far more prevalent and of greater public health importance than was previously recognized. The disease is geographically widespread threaten 350 million people worldwide with 500,000 cases of visceral leishmaniasis every year. Surprisingly, 90% of world's VL burden occurs in Bangladesh, Brazil, India, Nepal and Sudan with an estimated 75,000 deaths annually[2]. The current prevalence is estimated to be 45,000 cases with more than 40.6 million populations are at risk of developing the disease in Bangladesh. Out of 64 districts, kala-azar has been reported from 45 districts[3].

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Isolation of the parasite in culture or demonstration in relevant tissues like spleen, bone-marrow, buffy coat or lymph node by light microscopic examination of the stained specimen remain “gold standard” and naturally, leads to the definitive diagnosis of leishmaniasis[4]. However, these diagnostic methods are cumbersome, time consuming and involve painful invasive procedure rendering their limited scope in routine clinical practice. The development of Polymerase Chain Reaction (PCR) in mid 1980s has provided a powerful approach to the application of molecular biology techniques to the diagnosis of leishmaniasis[5]. PCR assays with kinetoplast DNA specific primers have been shown to have excellent sensitivities and specificities[6,7]. Further, PCR with buffy coat preparations to detect *Leishmania* has been found to be 10 times more sensitive than that with whole-blood preparation[8].

Immunodiagnosis is a valid and attractive choice in case of kala-azar like many other infectious diseases and detection of antigen is considered to be more specific than antibody-based tests. Further, antigen detection is more useful to differentiate between present and past infections and in situation where there is deficient antibody production such as in HIV leishmania co-infection. A new unique latex agglutination test (*KA*tex) for detecting leishmanial antigen in urine of patients with active VL has been introduced recently[9]. It detects a stable, non-protein, disease specific parasite antigen in the freshly voided urine of patients with an active infection[10]. The test has shown its sensitivity between 68 and 100% and specificity around 100% in preliminary trials[11-14]. The urinary antigen is detectable in both the promastigote and amastigote stages of the parasite. Monoclonal antibodies against *Leishmania* glycoconjugates strongly react with this molecule. This result suggests that the detected antigen is highly specific and diagnostic for VL. Moreover, *KA*tex becomes positive within one week of infection in case of active disease and performed better than any of the serological tests when compared to microscopy[15].

The aim of this prospective study was to evaluate the diagnostic accuracy of the newly available absolutely non-invasive urine-based antigen detection test for clinically suspicious patients of kala-azar admitted into RMCH, which is a tertiary care hospital in the Northern area of Bangladesh and handles kala-azar patients round the year.

Methodology

Study Population: The study included 60 clinically suspected patients of visceral leishmaniasis of different age and sex those who were admitted at different Medical and Paediatric wards of Rajshahi Medical College Hospital (RMCH), Bangladesh and 40 healthy controls of comparable age and sex - 20 from non-endemic and 20 from endemic areas of kala-azar. Controls were free from having past history of prolong fever, splenomegaly or other clinical features suggestive of visceral leishmaniasis and declared healthy after being physically examined by the physician.

Blood collection: Taking all aseptic precaution, 5.0 ml of blood was collected into an EDTA containing clean test tube from all patients and controls.

Collection of Buffy coat: After 30 minutes of collection of blood it was centrifuged @ 4000 rpm for 10 minutes. Using tip of the micropipette, 500µl of buffy coat was aspirated from the middle layer of the tube containing concentrated leukocytes and was kept into a 1.5ml microcentrifuge tube to be preserved at -20^{0}C for PCR amplification.

DNA extraction: DNA from the buffy coat was extracted for PCR by following procedure of QIAgen DNA Blood Mini Kit (Quagen, Hilden, Germany, Cat No. 51106) according to manufacturer’s instructions. The DNA was eluted in 0.2 mL of AE buffer (supplied with the

Quagen kit). The purity of the DNA was satisfactory since a ratio of OD at A_{260}/A_{280} was within 1.7-1.9 for all DNA samples. We used molecular grade water instead of blood as an extraction control to check for carry-over contamination in every run of DNA extraction and PCR amplification.

<u>*PCR protocol:*</u> We used a previously reported *Leishmania* specific nested PCR (Ln-PCR) with primers targeting the parasite's SSU-rRNA region[16]. An advantage of this Ln-PCR is its high sensitivity and specificity due to the use of second set of *Leishmania* specific primers designed to an internal sequence of the first PCR products (R223 5'-TCCCATCGCAACCTCGGTT-3′ and R333 5′-AAAGCGGGCGCGGTGCTG-3′). For the first PCR run, we used Kinetoplastida specific primers (R221 5′-GGTTCCTTTCCTGATTTACG-3′, and R332 5′-GGCCGGTAAAGGCCGAATAG-3′).

In the first PCR, 2μL of extracted DNA were amplified in a final volume of 25 μL containing 12.5 μL of Biorad iQ Supermix (catalog number 170-8862 that contains 100 mM KCL, 40 mM Tris-HCl pH 8.4, 1.6 mM deoxynucleoside triphosphates, 50 unit/mL iTaq DNA polymerase, 6.0 mM $MgCl_2$). 0.3 μmol/L of each Kinetoplastida-specific primers R221 and R332 and additional 3.0 mM $MgCl_2$ were also added. Amplification was performed on a BioRad's MyCycler. The PCR programme was run for 40 cycles that consisted of denaturation at 94^0C for 30s, annealing at 64^0C for 30s and extension at 72^0C for 30s.

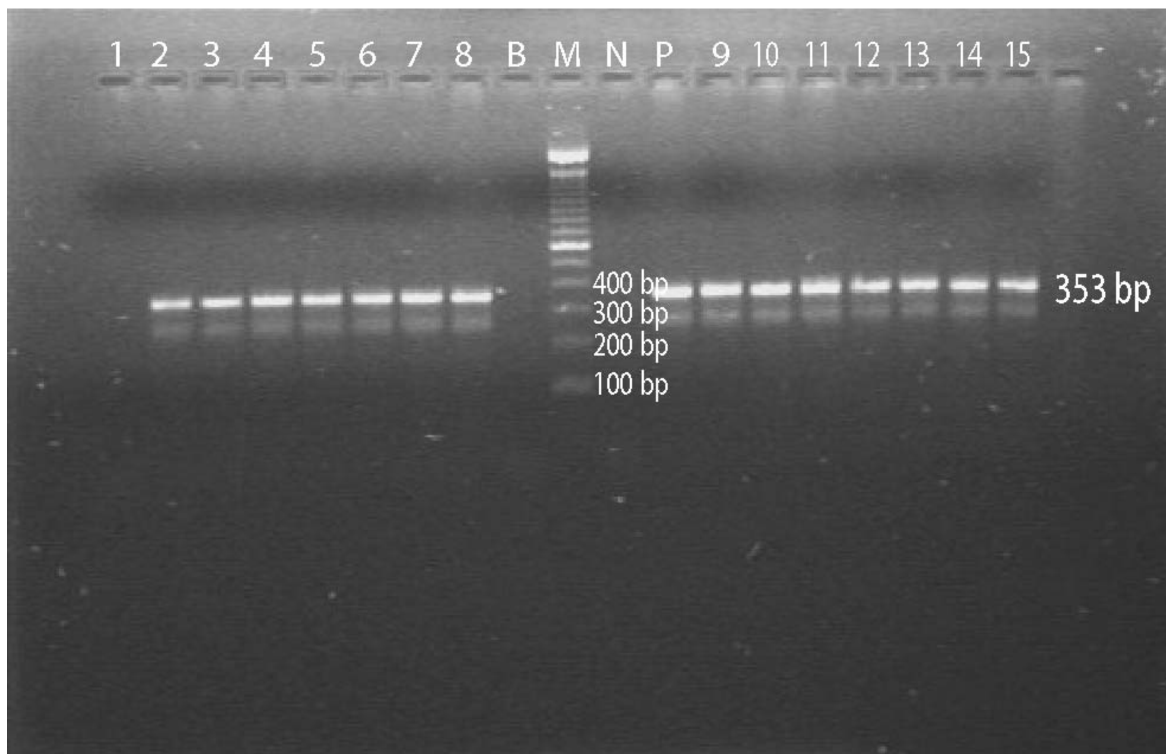

Figure 1: Ln-PCR amplified DNA in gel

Lane 1, no DNA, lane 2-8, positive amplified bands of 353 bp size from VL patients, lane N, negative control (healthy human blood), lane M, molecular size marker, lane B, blank (molecular grade water), lane P, positive control (cultured promastigote DNA), lane 9-15, positive amplified bands from VL patients.

Prior to the second amplification or nested PCR, the amplified products from the first run were diluted at 1:50 with molecular grade water and 1 μL was added to a 25 μL reaction volume, as described above, containing 0.15 μmol/L of the *Leishmania*

specific primers R223 and R333. For the second round of amplification, 35 cycles were used consisting by denaturation at 94^{0}C for 30s, annealing at 65^{0}C for 30s and extension at 72^{0}C for 30s. In both amplifications, Taq DNA polymerase activation was performed at 95^{0}C for 3 min, and a final extension at 72^{0}C for 5 min was included. Amplification products were separated by electrophoresis on 2% agarose gel with 100 bp DNA 'ladder' (Invitrogen, USA, Cat. No. 15628-019) as molecular-size marker, and stained with ethidium bromide (0.1 mg/mL). Stained gels were visualized and photographed under UV light emission with a UV transilluminator (BioRad, Milan, Italy, S.N. 75S/03589). Amplification products were visualized and positive samples yielded a PCR product of 353 bp (Fig. 1). In every run, molecular grade water and healthy human DNA were used as negative controls, and DNA from cultured promastigotes served as positive control.

*Kala-azar latex agglutination test (KA*tex*):* Approximately 5.0 ml of urine was collected into a sterile test tube from each patient and control for latex agglutination test (*KA*tex) to be performed on the same day of collection. Urine sample was pre-treated to inactivate heat-labile material capable of causing a false-positive reaction. Test latex, sensitized with antibodies raised against *L. donovani* antigen was next mixed with the sample on the glass slide. The reaction was read after mixing for two minutes. Antigen present in the sample made cross-linking (agglutination) of the sensitized latex and was indicative of VL. For every assay, the negative control was done in a reaction zone next to the test sample to distinguish between a weak positive and negative result[9]. The results were expressed as positive or negative.

Case definition

Leishmania nested Polymerase Chain Reaction (Ln-PCR) positive cases were considered as confirmed cases of kala-azar.

Statistical analysis: The sensitivity and specificity were calculated by using the following formulae: *Sensitivity* = [number of samples with true-positive results / (number of samples with true-positive results + number of samples with false-negative results)] x 100; *Specificity* = [number of samples with true-negative results / (number of samples with true-negative results + number of samples with false-positive results)] x 100. The positive and negative predictive values were calculated as the number of samples with true-positive results/ (number of samples with true-positive results + number of samples with false-positive results) and as the number of samples with true-negative results/ (number of samples with true-negative results + number of samples with false-negative results), respectively.

Ethical consideration: The protocol of this study was approved by the *'Institutional Review Committee'* of Rajshahi Medical College, Bangladesh for ethical issues related to this research. Informed written consent was obtained from each patient or legal guardian and control before venipuncture to collect blood and for urine.

Results

Characteristics of clinically suspected kala-azar patients: Clinically suspected patients of kala-azar included forty four male and 16 female. Median age of the patients was 25 years with quartile 14-35. All patients had splenomegaly and chronic fever with a median duration of four months. Other common symptoms such as history of weight loss and darkening of skin were found in 98.33% and 95% of the patients respectively (Table-I).

Laboratory results of patients and controls: Figure-1 shows the photograph of amplified DNA in gel. Results of *KA*tex among Ln-PCR positive cases and controls are shown in Table-II. Out of 56 Ln-PCR positive patients, *KA*tex was found positive in 53 cases, indicating its sensitivity as 94.64%. All non-endemic controls (20)

were negative but 02 of 20 (10%) endemic controls were *KA*tex positive, indicating its specificity to be 95%.

Table I: Characteristics of clinically suspected kala-azar patients

Characteristics	*Number*	*Percentage*
Male	44	73.33
Female	16	26.67
Fever	60	100.00
Duration of fever in months (Median, quartiles)	4.74 (2.5-6)	-
Anemia	59	98.33
Anorexia	52	86.67
Weight loss	59	98.33
Darkening of skin	57	95.00
Splenomegaly	60	100.00
Hepatomegaly	55	90.16
Lympadenopathy	00	00
Past history of kala-azar	05	08.33
History of kala-azar in the family	16	26.67

Table II: Laboratory results of patients and controls

Groups	*KA*tex	
	Positive	*Negative*
Ln-PCR-positive cases (n = 56)	53 (94.64)	03 (05.36)
Endemic controls (n = 20)	02 (10.00)	18 (90.00)
Non-endemic controls(n = 20)	00 (00.00)	20 (100.00)

Figures within parenthesis indicate percentage

Diagnostic indices of KAtex: Table-III shows sensitivity, specificity, positive predictive value and negative predictive value of *KA*tex as 94.64%, 95%, 98.10% and 92.85% respectively.

Discussion

Improved as well as non-invasive diagnostic tool is of crucial importance for any attempt to control visceral leishmaniasis in the endemic areas. Present day serological tests like direct agglutination test (DAT), rK39 ELISA or Immunochromatographic dipstick tests which detect anti-leishmanial antibodies though not standardized have high sensitivities and variable specificities shown in different studies. However, the presence of antibodies in healthy controls from areas of endemicity is a major drawback to their use as diagnostic tool[17,18]. On the contrary, detection of antigen is considered to be more specific than antibody-based tests and it broadly correlates with active disease.

In the present series, we have compared the positivity of *KA*tex that detects urinary leishmanial antigen with Ln-PCR carried out in DNA extracted from buffy coat among kala-azar patients and healthy controls of different age groups. Considering the very high diagnostic sensitivity (99%) and specificity (100%) of PCR yielded in cases of Indian Kala-azar,[19] we were encouraged to utilize PCR method as confirmatory test for kala-azar. The observed sensitivity (94.64%), specificity (95%), positive predictive value (98.10%) and negative predictive value (92.85%) of *KA*tex in the present study are in accordance with most of the studies carried out both in home and abroad. In a recent study carried out in India,

Table III: Diagnostic indices of KAtex

Test	Sensitivity	Specificity	Positive predictive value	Negative predictive value
*KA*tex	94.64%	95%	98.10%	92.85%

*KA*tex was found to be 87% sensitive and 99% specific[13] and De Colmenares et al (1995) from Spain reported its sensitivity and specificity as 96% and 100% respectively[20], which are more or less consistent with our findings. Sensitivity and specificity of *KA*tex were noted as 100% and 91.4% respectively against parasitologically confirmed cases of kala-azar in one Bangladeshi study,[21] which is also in accordance with our findings. In a study conducted in Nepal, the sensitivity and specificity of *KA*tex were found as 47.7% and 98.7% respectively[22]. The reason for 03 false *KA*tex-negative cases in the present study could be limitation of this test to detect low level of urinary leishmanial antigen that may be present in some patients. On the other hand, 02 false *KA*tex-positive cases noted among endemic controls, suggests its further limitation to predict negative value of this test. It is reasonably assumed that the variable sensitivity and specificity of *KA*tex could be due to geographical location, endemicity of the disease and off course host's factors.

The present study has several limitations. First, we could not investigate the actual reasons for *KA*tex negative cases among kala-azar patients. Secondly, the number of negative controls is less than what has been suggested by the experts concerning the comparison of VL diagnostic tools, thus leading to an overestimated specificity of *KA*tex method used herein[23].

Early correct diagnosis of kala-azar is important as the treatment is of long duration and can be complicated with many side effects. The availability of accurate laboratory tests is therefore, essential. Although the choice of diagnostic tests depends upon many factors and varies greatly in different situations but for any particular test to be introduced, it must be acceptable in terms of its sensitivity, specificity and reproducibility. *KA*tex has been found to be reliable and absolutely non-invasive diagnostic test for kala-azar and our findings have reinforced further that this test could be a good alternative to conventional parasitological or more sophisticated molecular PCR-based diagnosis of kala-azar. But only a limited number of studies have so far been done which is not adequate to refer it as a versatile test for field use. It is recommended that more studies with larger population are necessary for its further diagnostic evaluation.

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References

1. Leishman WB. On the possibility of occurrence of trypanosomiasis in India. *Brit Med J* 1903; 1: 1252-1254.

2. Murray CJL, Lopez AD. Global Health Statistics: A Compendium of Incidence, Prevalence and Mortality Estimates for Over 200 Conditions. Murray CJL, Lopez AD, eds. Global Burden of Disease and Injury Series. Volume II. Boston: Harvard University Press, 1996.

3. Moazzem H. National Guidelines and Training Module for Kala-azar Elimination in Bangladesh, Published by Directorate General of Health Services, Dhaka, Bangladesh, 2008.

4. Sundar S and Rai M. Laboratory diagnosis of visceral leishmaniasis. *Clin Diag Lab Immunol* 2002; 9(5): 951-958.

5. Boelaert M, El-Safi S, Mousa H, Githure J, Mabati P, Gurubacharya V, Shrestha J, Jacquet D, De Muynck A, Le Ray D and Vander Stuyft P. Multicenter evaluation of repeatability of the

direct agglutination test for visceral leishmaniasis. *Trop Med Int Health* 1999; 4: 31-37.

6. Adhya S, Chatterjee M, Hassan MQ, Mukherjee S, Sen S. Detection of Leishmania in the blood of early kala-azar patients with the aid of the polymerase chain reaction. *Trans R Soc Trop Med Hyg* 1995; 89: 622-624.

7. Fisa R, Riera C, Ribera E, Gallego M, Portus M. A nested polymerase chain reaction for diagnosis and follow-up of human visceral leishmaniasis patients using blood samples. *Trans R. Soc Trop Med Hyg* 2002; 96(Suppl. 1): S191-S194.

8. Lachaud L, Chabbert E, Dubessay P, Reynes J, Lamothe J and Bastein P. Comparison of various sample preparation methods of PCR diagnosis of visceral leishmaniasis using peripheral blood. *J Clin Microbiol* 2001; 38: 613-617.

9. Kalon Biological Ltd. Aldershot, Hants, United Kingdom, 2005.

10. Sarkari B, Chance M, and Hommel M. Antigenuria in visceral leishmaniasis: detection and partial characterization of a carbohydrate antigen. *Acta Tropica* 2002; 82: 339-348.

11. Attar ZA, Chance ML, El-Safi S, Carney J, Azazy A, El-Hadi M, Dourado C, Hommel M. Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. *Acta Tropica* 2001; 78: 11-16.

12. Nahar K. Diagnosis of visceral leishmaniasis by a latex agglutination test (KAtex), by detecting leishmanial antigen from urine samples. M.Phil. (Microbiology) thesis. 2005.

13. Sundar S, Agrawal S, Pai K, Chance M, Hommel M. Detection of leishmanial antigen in the urine of patients with visceral leishmaniasis by latex agglutination test (KATEX). *Am J Trop Med Hyg* 2005; 73(2): 269-271.

14. Vilaplana C, Blanco S, Dominguez J, Gimenez M and Ausina V. Noninvasive Method for Diagnosis of Visceral Leishmaniasis by a Latex Agglutination Test for detection of Antigen in Urine samples. *J Clin Microbiol* 2004; 42(4): 1853-1854.

15. Bahadur S, Chance M, Hommel M. Antigenuria in visceral leishmaniasis: detection and partial characterization of a carbohydrate antigen. *Acta Tropica* 2002; 82: 339-348.

16. Cruz I, Canavate C, Rubio JM, Morales MA, Chicharro C, Laguna F, Jimenez-Mejias M, Sierra G, Videla S, Alvar J. A nested polymerase chain reaction (Ln-PCR) for diagnosing and monitoring *Leishmania infantum* infection in patients co-infected with human immunodeficiency virus. *Trans R Soc TropMed Hyg* 2002; 96: 185- 189.

17. Singh S and Sivakumar R. Recent advances in the diagnosis of leishmaniasis. *J Post Grad Med* 2003; 49: 55-60.

18. Sundar S, Pai K, Sahu M, Kumar V and Murray HW. Immunochromatographic strip test detection of anti k39 antibody in Indian visceral leishmaniasis. *Ann Trop Med Parasitol* 2002; 96: 19-23.

19. Maurya M, Singh RK, Kumar B, Salotra P, Rai M, and Sundar S. Evaluation of PCR for Diagnosis of Indian Kala-Azar and Assessment of Cure. *J Clin Microbiol* 2005; 43(7): 3038-3041.

20. De Colmenares M, Portus M, Riera C, Gallego M, Aisa MJ, Torras S, Munoz C. Detection of 72-75 kD and 123 kD fractions of leishmanial antigen in urine of patients with visceral leishmaniasis. *Am J Trop Med Hyg* 1995; 52: 427-428.

21. Alam M, Shamsuzzaman AK, Musa AK, Khan AH, Mahmud MC, Hossain MA, Ahmed AA. Antigen Detection from Urine of Kala-Azar Cases by Latex Agglutination Test. *Mymensingh Med J* 2008; 17(1): 22-27.

22. Rijal S, Boelaert M, Regmi S, Karki BMS, Jaquet D, Singh R, Chance ML, Chappuis F, Hommel M, Desjeux P, Van der Stuyft P, Le Ray D and Koirala S. Evaluation of a urinary antigen-based latex agglutination test in the diagnosis of kala-azar in eastern Nepal. *Trop Med Int Health* 2004; 9(6): 724.

23. Boelaert M, Bhattacharya S, Chappuis F, El-Safi SH, Hailu A, Mondal D, Rijal S, Sundar S, Wasunna M, Peeling RW. Evaluation of rapid diagnostic tests: visceral leishmaniasis. *Nat Rev Microbiol* 2007; S30-S39.