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Evaluation of a urinary antigen-based latex agglutination test in the diagnosis of kala-azar in eastern Nepal

S. Rijal[1], M. Boelaert[2], S. Regmi[1], B. M. S. Karki[1], D. Jacquet[2], R. Singh[1], M. L. Chance[3], F. Chappuis[4], M. Hommel[3], P. Desjeux[5], P. Van der Stuyft[2], D. Le Ray[2] and S. Koirala[1]

1 *B.P. Koirala Institute of Health Sciences, Dharan, Nepal*
2 *Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium*
3 *Liverpool School of Tropical Medicine, Liverpool, UK*
4 *Geneva University Hospital, Geneva, Switzerland*
5 *Disease Control, Prevention and Eradication, World Health Organization, Geneva, Switzerland*

Summary

BACKGROUND We evaluated the diagnostic accuracy as well as the reproducibility of the urine latex agglutination test 'KAtex' in the diagnosis of kala-azar in patients recruited at a tertiary care centre in Dharan, Nepal, between November 2000 and January 2002.

METHODS All patients presenting with fever of 2 weeks or more and splenomegaly were consecutively enrolled. Bone marrow and – if negative – spleen aspirates were examined for *Leishmania donovani*. Serum and urine samples were taken in duplicate for the Direct Agglutination Test (DAT) and KAtex. The reference laboratory determined sensitivity and specificity of KAtex. Reproducibility between both laboratories was assessed.

RESULTS KAtex was performed on urine from 155 parasitologically confirmed kala-azar and 77 non-kala-azar cases (parasitology and DAT-negative). KAtex showed a sensitivity of 47.7% (74/155, 95% CI: 39.7–55.9) and a specificity of 98.7% (76/77, 95% CI: 93.0–100.0). Reproducibility of KAtex showed a kappa of 0.684 ($P < 0.001$, $n = 232$).

CONCLUSION KAtex evaluation showed high specificity, low sensitivity and moderate reproducibility. A urine test for kala-azar could become a real breakthrough in kala-azar management if its reproducibility and sensitivity could be further improved.

keywords visceral leishmaniasis, sensitivity and specificity, diagnostic accuracy, urine antigen detection test, Nepal

Introduction

Visceral leishmaniasis (VL) or kala-azar is considered a major public health problem in Nepal where it is endemic in the south-eastern Terai region, with an estimated 5.5 million people at risk of acquiring the disease (HMGN 2001/2002). The recommended method for diagnosis of VL is the microscopic demonstration of amastigotes of *Leishmania donovani* (LD bodies) from spleen or bone marrow aspiration, although the sensitivity of the latter technique has been shown to be only 70–86% (WHO 1984; Zijlstra *et al.* 1992). Moreover, these techniques require expertise both from the physician and the laboratory technician and in Nepal LD microscopy is limited to reference hospitals. As the disease affects the poorest of the poor in remote rural regions, most patients lack access to these reference centres. The diagnosis of VL in those areas is thus usually based on non-specific clinical features (cachexia, anaemia, prolonged fever and hepato-splenomegaly) along with a positive formol-gel test.

Serological tests for the detection of antibodies have been developed in the pursuit of an alternative to parasitology. Their main advantage is non-invasiveness but they do not discriminate between clinical, subclinical or past infection, and cross-reaction with other pathogens is possible. The Direct Agglutination Test (DAT) developed by El Harith *et al.* (1986, 1988) has excellent diagnostic accuracy (Boelaert *et al.* 2004), but its use in Nepal has so far also been limited due to the expertise required for its execution. The indirect immunofluorescence antibody test (IFAT) requires an immunofluorescence microscope and is thus neither appropriate nor affordable for decentralized diagnosis. A test based on 39-amino acid repeat recombinant leishmanial antigen from *L. chagasi* (rK39) has been introduced in an enzyme-linked immunosorbent assay (ELISA) (Badaro *et al.* 1996; Zijlstra *et al.* 1998) and,

later, in a lateral flow dipstick format (Sundar *et al.* 1998). The latter is very easy to use in the field and the initial study showed 100% sensitivity and 98% specificity (Sundar *et al.* 1998). However, an evaluation in Sudan showed only 67% sensitivity (Zijlstra *et al.* 2001). Moreover, this particular format of the dipstick is no longer available. Another version tested in India proved to be a good diagnostic guide in kala-azar suspect cases (Sundar *et al.* 2002). Sarker *et al.* (2003) found in Bangladesh high sensitivity in confirmed kala-azar patients and high specificity in healthy endemic controls and patients suffering from other conditions. In Nepal, an early version of this dipstick showed a specificity of only 71% in controls who presented as clinical suspect cases of kala-azar (Chappuis *et al.* 2003); however, more encouraging results were obtained with later generations of this dipstick (Bern *et al.* 2000; Boelaert *et al.* 2004).

Recently, Sarkari *et al.* (2002) described a urinary leishmanial antigen. This is a low-molecular weight, heat stable carbohydrate detected in the urine of VL patients but not in the urine of patients suffering from malaria, schistosomiasis, or non-parasitic diseases including typhoid and brucellosis. An agglutination test for the detection of this urinary antigen has been evaluated in laboratory trials, using urine collected from well-defined cases and endemic and non-endemic controls. The test had 100% (95% CI: 98.8–100.0) specificity and sensitivity between 64 (95% CI: 42.5–82.0) and 100% (95% CI: 47.8–100.0) (Attar *et al.* 2001). In a field trial in Sudan the test was positive in all 15 microscopy-positive kala-azar cases [sensitivity 100% (95% CI: 78.2–95.2)] and was negative in 41 of 45 bone marrow and/or lymph node smear negative clinical suspect cases of kala-azar [specificity 87.2% (95% CI: 78.8–97.5)]*.

We evaluated the diagnostic accuracy and the reproducibility of the KAtex, a urinary leishmanial antigen-based latex agglutination test, in clinically suspect patients in an endemic kala-azar area in Nepal.

Materials and methods

Study population

The study was conducted at the B.P. Koirala Institute of Health Sciences (BPKIHS), a 648 bed University Hospital located in Dharan, in the south-eastern region of Nepal. BPKIHS serves as a reference tertiary level hospital for the eastern region, which includes several kala-azar endemic districts. The research protocol was approved by the ethical committee of BPKIHS in August 2000. Ethical clearance was also obtained from the institutional review board of the Institute of Tropical Medicine in Antwerp (ITMA), Belgium. The patients were prospectively recruited from the outpatient department and the emergency department of BPKIHS between November 2000 and January 2002. Every patient presenting with a history of fever for 2 weeks or more and clinical splenomegaly was considered as a clinical suspect case and was enrolled if he/she gave written informed consent. For paediatric patients, informed consent was sought from the guardian.

Reference standard

Splenic aspiration is considered close to gold standard for VL diagnosis, but as it is an invasive procedure, bone marrow-negative patients often reject it at BPKIHS. Therefore, we used a combination of parasitology and serology as the reference standard for the evaluation of the KAtex. We considered those with positive microscopy in bone marrow or spleen as confirmed kala-azar cases. A non-kala-azar case was somebody with negative parasitology and negative serology (i.e. DAT titre ≤ 3200).

Diagnostic procedures

All patients enrolled were admitted to the medical wards for the diagnostic work up and treatment. On day 0 (admission day), blood was drawn for complete blood count, chemistry, coagulation profile, thick and thin smear for malaria parasite, blood cultures and HIV testing after pre-test counselling. Chest X-ray, abdominal ultrasound and other tests were performed at the physician's discretion. Serum was collected for DAT. A urine specimen was collected in duplicate at day 0 and stored at −70 °C until analysis.

Parasitological diagnosis

All patients had a bone marrow aspiration performed at day 0 or 1 and a microscopic search for the amastigote form of *L. donovani* (LD bodies) was carried out by the Department of Microbiology of BPKIHS. Giemsa-stained smears were designated as positive if LD bodies were seen or negative if no LD bodies were seen in 1000 oil immersion fields. If bone marrow aspiration was negative for LD bodies, spleen aspiration was performed except in those with prolonged prothrombin time, decreased platelets below 50 000/mm^3 or spleen <2 cm palpable below the costal margins. A parasite density score was determined microscopically at magnification objective 100 × eye piece 10 in the Giemsa-stained smear by use of a scale ranging

* Confidence intervals were computed by authors on the basis of published figures.

from 0 (no parasites per 1000 oil immersion fields) to +6 (>100 parasites per field) using the method originally developed for splenic biopsies (WHO 1990) but which has been successfully applied to the quantification of bone marrow smears both in BPKIHS and ITMA.

Two independent readers at BPKIHS read the slides, and, in case of discrepancy, the reading of a third more senior reader was decisive. For quality control, 10% of the positive and 10% of the negative slides were cross-checked in the same way at the Protozoology Unit, ITMA.

Direct Agglutination Test

The DAT was performed by a laboratory technician at BPKIHS who had been previously trained by the chief laboratory technician of the Protozoology Unit of ITMA. The DAT antigen was prepared at ITMA using a modification of the method of El Harith *et al.* (1986) and described by Boelaert *et al.* (1999). The liquid antigen was kept at 4 °C during transport and storage at BPKIHS. The test was carried out on microtitre plates (V-shaped wells) with the necessary positive and negative controls. The test was read visually against a white background and the end titre was read as the dilution immediately before the well with a clear sharp-edged blue spot identical in size to the negative control. For the analysis, a DAT titre >1:3200 was taken as positive.

Urine latex agglutination test

Urine samples were taken on the day of admission and kept frozen until analysis. The KAtex urine latex agglutination test (Kalon Biological Ltd, Aldershot, UK) was performed by one technician at both BPKIHS and ITMA. Both technicians were blinded to the patient's diagnosis. The KAtex kit consists of test latex, a positive and a negative control and a reusable glass test slide with a black background.

As pretreatment, 1 ml of urine was transferred into the sample tube and placed on a boiling water bath for 5 min. This was to inactivate heat labile material capable of causing a false positive reaction. Meanwhile, all test reagents were brought to the ambient temperature. Fifty microlitres of the treated urine sample was placed to a reaction zone in the glass slide and a drop of test latex was added to it. The liquids were stirred to a completely homogenous mixture and rotated continuously for 2 min. For every assay, a negative control in the reaction zone next to the test sample was run. Any agglutination discerned when compared with the negative control was considered as positive. When no agglutination was seen, KAtex was considered negative.

Data analysis

Numerical variables were summarized by mean and SD if normally distributed and if they were not, by median and quartiles. Mean values were compared with Student's *t*-test and medians with the Mann–Whitney *U*-test, at a critical α-level of 0.05. All *P*-values were two-sided. The results of KAtex obtained in ITMA were used to assess diagnostic accuracy of the KAtex. Sensitivity of KAtex was assessed in confirmed kala-azar patients, i.e. those who were parasitologically positive. The specificity of KAtex was assessed in the group of patients with negative parasitology and a negative DAT (i.e. DAT titre ≤1:3200). We excluded patients who could not be categorized according to this reference standard from the data analysis. Exact 95% binomial confidence intervals were computed for the sensitivity and specificity. The association of KAtex positivity in kala-azar patients with size of spleen, duration of fever and the parasite intensity was assessed by Pearson's chi-square for linear trend. Reproducibility between the KAtex performance at ITMA and BPKIHS was assessed by Cohen's kappa. The data were analysed with SPSS for Windows version 10.0.5 (SPSS Inc., Chicago, IL, USA).

Results

A total of 269 kala-azar suspect cases were enrolled between November 2000 and January 2002. Eight cases had to be excluded, as the KAtex could not be performed in Antwerp because urine samples were lost during transport. Of the remaining 261 cases, there were 155 confirmed kala-azar cases (with positive microscopy) and 77 non-kala-azar cases (negative microscopy and negative DAT). Twenty-nine cases were excluded from the analysis, 28 because they could not be classified by our reference standard (negative microscopy but positive DAT) and one because he left the hospital early, against medical advice. The diagnosis of kala-azar in the 155 patients was reached by a positive bone marrow ($n = 152$) or positive spleen aspirate ($n = 3$). The quality control done on the parasitology smears showed perfect concordance for grade 2+ or above, but for smears with scanty parasites (grade 1), there was considerable discrepancy.

Table 1 shows the characteristics of the kala-azar and non-kala-azar cases. There were significant differences between the two groups with respect to the duration of fever, spleen size, haemoglobin percentage, platelet count and the white cell count.

The most frequent discharge diagnosis in the non-kala-azar group was malaria (42 patients, 54.5%). The other diagnoses were haematological malignancy ($n = 8$), tuberculosis ($n = 6$), haemolytic anaemia ($n = 4$), portal

Table 1 Clinical and haematological characteristics of confirmed kala-azar and non-kala-azar patients in Dharan, Nepal

	Confirmed kala-azar ($n = 155$)	Non-kala-azar ($n = 77$)	*P*-value†
Age (years)‡	23 (13; 26)	20 (10; 30)	0.084
Fever duration (weeks)‡	8 (4; 12)	4 (3; 8)	0.001
Spleen size (cm)‡	5 (3; 8)	3 (1; 5)	<0.001
Haemoglobin (gm%)*	8.4 (2.0)	9.5 (2.7)	<0.001
Median WCC (mm^3)‡	3800 (3100; 4400)	6050 (4550; 10 150)	<0.001
Platelets (mm^3)*	140 143 (67 632)	237 947 (131 727)	<0.001

* Mean (SD).
† Mean values were compared with Student's *t*-test, medians with a Mann–Whitney non-parametric test.
‡ Median (quartile 1, quartile 3).

hypertension ($n = 3$), enteric fever ($n = 3$), septicaemia ($n = 3$), HIV ($n = 3$), systemic lupus erythematosus ($n = 1$) and other infections ($n = 4$).

The KAtex performed at ITMA was positive in 74 of the 155 kala-azar patients, sensitivity 47.7% (95% CI: 39.7–55.9) and negative in 76 of the 77 non-kala-azar patients, specificity 98.7% (95% CI: 93–100). The association of the intensity of parasite, size of spleen and duration of fever with KAtex sensitivity in the 155 confirmed kala-azar patients was assessed. As shown in Table 2, the sensitivity of KAtex increased significantly with increasing parasite intensity, spleen size and duration of fever.

In Nepal all urine samples were tested within 3 months of collection. In Antwerp, storage time varied, but all samples were analysed within 26 months. We found no significant difference in the sensitivity of samples stored longer or shorter than 10 months.

Table 2 KAtex sensitivity according to duration of fever, spleen size and parasite grading in 155 confirmed kala-azar patients in Dharan, Nepal, 2001–2002

	N	Number of KAtex-positive	KAtex sensitivity	*P*-value
Duration of fever (weeks)				0.024*
<9	96	39	0.406	
≥9	59	35	0.593	
Spleen size (cm)				0.005**
<4.0	44	13	0.295	
4.0–5.9	36	20	0.556	
6.0–7.9	28	11	0.393	
≥8.0	47	30	0.638	
Parasite grading				<0.001**
1	29	6	0.207	
2	43	11	0.256	
3	41	24	0.585	
4	36	28	0.778	
5	6	5	0.833	

* Pearson χ^2 test; ** χ^2 for linear trend.

In comparing the reproducibility of the tests performed at BPKIHS and ITMA, the test was negative at BPKIHS in 16 patients of the 75 that were positive at ITMA. There were also 16 negatives in ITMA from the 75 that were positive at BPKIHS. The kappa score was 0.685 (95% CI: 0.586–0.784).

Discussion

In this study, the sensitivity of KAtex was found to be low in confirmed VL cases although the specificity was excellent amongst a control group of patients with similar symptoms in whom kala-azar was ruled out. The study design purposefully included this spectrum of patients, as they would represent the persons on whom physicians would use the test for diagnosis in future.

We observed 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 reflect the duration of the illness. Parasite intensity is probably well-correlated with antigen load in urine. The low overall sensitivity is in contrast to earlier results by Attar *et al.* (2001) and unpublished data from Muzzafarpur, India and Sudan. However, Attar *et al.* (2001) reported data from Brazil where only 16 of 25 confirmed kala-azar showed a positive KAtex (64%, 95% CI: 42.5–82).

Low sensitivity in our study could possibly be explained by shorter duration of the disease in the patients presenting. Also, our series of kala-azar patients contained a high number with low parasite intensity, 46.5% had grade 1+ or 2+. On the contrary, smaller sample sizes in the earlier studies lead to wider confidence intervals around those

sensitivity estimates. Deterioration of antigen due to freezing and storage of the urine samples is unlikely to be a major contributor to this low sensitivity, as the detection of antigen was shown to be stable, although at a reduced score, over an 8 year storage period at −20 °C. Also, we observed similar low sensitivity of KAtex in a recent study comparing fresh and frozen urine samples collected from a group of kala-azar patients from Nepal (unpublished data).

The reproducibility of KAtex results was good. The main difficulty in reading the test is the discrimination of a 1+ test result (the test is normally graded from 1+ to 3+) from a negative result; any tendency to interpret the KAtex test result conservatively will decrease the sensitivity of the test.

Sensitivity is one of the crucial parameters in the choice of a diagnostic test for VL. Although not ideal as a diagnostic test in its present format, there are field settings with minimal laboratory infrastructure where the test could be of use. Given its high specificity, the positive predictive value of a positive KAtex result is likely to be high, in this study it was 0.987 (95% CI: 0.928–1.0). One might consider treating a clinical suspect patient with a positive KAtex without the need for a parasitological diagnosis. However, a negative KAtex result is of little value, and such a patient should be referred for further investigation.

A latex agglutination test detecting a heat stable leishmanial antigen from the urine from kala-azar patients presents an interesting technology. It is simple to use, results are immediately available, it does not require any electric appliances and is thus feasible in the rural health centres. Testing the urine is acceptable to the patients especially when compared with the alternative of the invasive bone marrow aspirations. Testing of an antigen has moreover a potential for monitoring response to treatment where the antibody-based tests are of no help. Therefore, the test merits further development and evaluation. However, future assessment of KAtex should carefully document the stage of disease, parasite intensity as well as handling of samples (fresh/stored).

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Authors

Suman Rijal (corresponding author), **Sudhir Regmi**, **Balmansingh Karki**, **Rupa Singh** and **Shekhar Koirala**, B.P. Koirala Institute of Health Sciences, Dharan, Nepal. Tel.: +977-25-525668; Fax: +977-25-520251; E-mail: sumanrijal2@yahoo.com, sudhirregmi@yahoo.com, bmskarki@hotmail.com, rupasingh@hotmail.com, shekharkoirala@hotmail.com

Marleen Boelaert, **Diane Jacquet**, **Patrick Van der Stuyft** and **Dominique Le Ray**, Prince Leopold Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium. Tel.: +32-3-2476283; Fax: +32-3-2476258; E-mail: boelaert@itg.be, djacquet@proto.itg.be, pvds@itg.be, dleray@itg.be

Michael L. Chance and **Marcel Hommel**, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK. Tel.: +44-151-705-3232; Fax: +44-151-705-3371; E-mail: mchance@liv.ca.uk, mhommel@liv.ac.uk

François Chappuis, Geneva University Hospital, 1211 Geneva 14, Switzerland. Tel.: +41-22-3729620; Fax: +41-22-3729626; E-mail: francois.chappuis@hcuge.ch

Phillippe Desjeux, Disease Control, Prevention and Eradication, World Health Organization, 20, av Appia, 1211 Geneva 27, Switzerland. Tel.: +41-22-791-38-70; Fax: +41-22-791-48-78; E-mail: desjeuxp@who.int