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


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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. 1998). Sarkar 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) 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).

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/mm³ 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...
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 modifi-
cation 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
z-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 para-
sitologically 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 posi-
tivity 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 micro-
scopy 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
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.

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

<table>
<thead>
<tr>
<th>Number of KAtex-positive</th>
<th>KAtex sensitivity</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Duration of fever (weeks)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;9</td>
<td>96</td>
<td>39</td>
</tr>
<tr>
<td>≥9</td>
<td>59</td>
<td>35</td>
</tr>
<tr>
<td>Spleen size (cm)</td>
<td></td>
<td></td>
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<tr>
<td>&lt;4.0</td>
<td>44</td>
<td>13</td>
</tr>
<tr>
<td>4.0–5.9</td>
<td>36</td>
<td>20</td>
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<tr>
<td>6.0–7.9</td>
<td>28</td>
<td>11</td>
</tr>
<tr>
<td>≥8.0</td>
<td>47</td>
<td>30</td>
</tr>
<tr>
<td>Parasite grading</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>43</td>
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<td>5</td>
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* Pearson χ² test; ** χ² for linear trend.
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 patient 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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References


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