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Field validity, reproducibility and feasibility of diagnostic tests for visceral leishmaniasis in rural Nepal

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Summary

OBJECTIVES To assess the field accuracy, reproducibility and feasibility of the formol gel test (FGT), the urine latex agglutination test (KAtex) and a rK39 antigen-based dipstick for the diagnosis of visceral leishmaniasis (VL) in rural Nepal.

METHOD Patients with clinical suspicion of VL were recruited at Rangeli District Hospital (DH), a 15-bed government hospital located in south-eastern Nepal. FGT, KAtex and rK39 dipstick tests were performed on site and later repeated at a reference kala-azar diagnostic laboratory to assess reproducibility. Diagnosis of VL was confirmed by either a positive bone marrow aspirate examination or a positive direct agglutination test (DAT titre ≥1:3200) in patients who later responded to anti-leishmanial therapy.

RESULTS Of 155 patients initially recruited, 142 (85 with VL and 57 with another diagnosis) were included in the study. The sensitivity of the rK39 dipstick [89%; 95% confidence interval (CI): 81–94] was significantly higher than that of the KAtex (57%; 95% CI: 46–67) and the FGT (52%; 95% CI: 41–62). All three tests had a specificity of at least 90%. Agreement was higher for the rK39 dipstick ($\kappa = 0.87$) than for the FGT (0.68) and the KAtex (0.43). All tests required ≤20 min of actual work and ≤40 min to obtain the results.

CONCLUSION The rK39 dipstick was easy to do, more accurate and reproducible than other rapid diagnostic tests for VL in a DH of rural Nepal. It should be integrated into the field diagnostic algorithm of VL in this region and mechanisms to secure its availability should be found.

keywords kala-azar, visceral leishmaniasis, diagnosis, serology, antigen detection, Nepal

Introduction

Visceral leishmaniasis (VL) or kala-azar affects an estimated 500 000 persons yearly, predominantly in the poor rural areas of India, Bangladesh, Nepal, Sudan and Brazil (Desjeux 1996). Most patients present with prolonged fever, weight loss and an enlarged spleen. This clinical picture is shared by several other endemic diseases such as malaria, disseminated tuberculosis or enteric fever, which are also commonly seen in the same focus. Laboratory testing is therefore necessary to confirm the diagnosis of VL. A highly sensitive and specific diagnostic approach is needed, because of the fatal evolution of the disease without specific treatment and the serious toxicity of the most widely used first-line drug, sodium stibogluconate (Sundar *et al.* 2000). Most communities affected by VL are poor and located in remote rural areas with limited access to referral hospitals. Therefore, laboratory tests for VL diagnosis need to be cheap, easy to perform and available in peripheral health centres to insure that most patients have an adequate access to diagnosis. The development of diagnostic tests for improved case-management of VL has been rated as one of the most needed among other infectious diseases prevalent in the developing world (Mabey *et al.* 2004).

Direct microscopic examination of spleen aspiration is considered the gold standard for VL diagnosis but the expertise required for the procedure makes it unsuitable for generalized field use. Alternatively, lymph node or bone marrow aspirates are often used but these are substantially less sensitive (Zijlstra *et al.* 1992). Serological tests have been developed to replace parasitological diagnosis in the

field. The direct agglutination test (DAT), developed in the 1980s (Harith *et al.* 1986, 1987), has been validated in several endemic areas (Boelaert *et al.* 1999a, b, c, 2004). Its use has been encouraged by the World Health Organization (WHO) for surveillance and control programs of VL (DAT workshop, Antwerp, 25–27 March 1998). Unfortunately, the relative sophistication of the DAT procedure (e.g. need for micropipettes and microtitration plates) restricts its use to referral hospitals or well-supported health centres.

Simpler tests designed for field use exist. Recently, serological testing based on the detection of antibodies against a recombinant antigen derived from a 39-amino acid repeats in *Leishmania chagasi* (rK39) was developed into a dipstick format. Validation studies have shown variable results depending on the location of the study site, the brand of the dipstick and the study method (Sundar *et al.* 1998; Delgado *et al.* 2001; Zijlstra *et al.* 2001; Chappuis *et al.* 2003; Schallig *et al.* 2002; Carvalho *et al.* 2003; Veeken *et al.* 2003; Boelaert *et al.* 2004). The formol gel test (FGT) is a cheap, easy to perform but poorly sensitive test that is at present the only diagnostic test available for VL in many peripheral health centres in the Indian sub-continent and in East Africa (Chowdhury *et al.* 1992). A urinary antigen-based latex agglutination test (KAtex) has been recently developed and evaluated. The specificity of KAtex was found to be excellent but its sensitivity varied from 48% to 100% (Attar *et al.* 2001; Rijal *et al.* 2004).

In Nepal, where VL is endemic in 12 south-eastern districts located in the Terai region, the DAT, rK39 dipstick, FGT and KAtex have been prospectively evaluated in groups of clinical suspect patients recruited in referral tertiary hospitals (Chappuis *et al.* 2003; Boelaert *et al.* 2004; Rijal *et al.* 2004). However, the performance of the tests might be different in a 'real life' setting of district hospitals (DHs), where most of the VL patients attend, for several reasons. First, patients consulting in a DH may present with a less advanced disease, thus modifying the test's sensitivity and specificity. For example, the KAtex urine test was shown to be significantly less sensitive in patients with a shorter duration of fever and a smaller spleen size (Rijal *et al.* 2004). Second, the prevalence (prior probability) of VL among clinical suspects can differ from the one found in a referral hospital and this would directly influence the positive and negative predictive value of the tests. Third, limited training of the laboratory technicians, their high workload and poor logistic facilities can alter the execution and/or the interpretation of the test. For these reasons, we assessed the validity, reproducibility and feasibility of three candidate tests for decentralized use, the rK39 dipstick test, the FGT and the KAtex, in the setting of a first-referral hospital located in an endemic area of VL in Nepal.

Materials and methods

Study site

The study was conducted at Rangeli DH in Rangeli, a 15 000 inhabitants town of the district of Morang. This rural hospital serves a district with 800 000 inhabitants located in the Eastern Region of Nepal and bordering the Bihar State of India. The Rangeli DH is a 15-bed government hospital that caters to patients from neighbouring villages who may, or may not, have had a prior consultation at the health post in their village. The clinical activities at Rangeli DH are supervised by two medical doctors. The other medical staff includes one clinical officer, four nurses, one laboratory technician and one laboratory assistant. The two laboratory workers underwent a 2-week training before the start of the study at the Kala-azar Laboratory of the B.P. Koirala Institute of Health Sciences (BPKIHS). Both were trained in performing and interpretation of the rK39 dipstick and the KAtex tests and reviewed the procedures of the FGT and the microscopic search for *Leishmania donovani* bodies in bone marrow aspirates. The BPKIHS is a 648-bed teaching hospital with strong expertise in VL diagnostics and research located in Dharan, at 150 km by road from Rangeli DH. The BPKIHS laboratory served as a reference laboratory in this study.

Patients

All patients with a history of 2 weeks or more of fever and a clinically assessed enlarged spleen were defined as suspect VL cases and were eligible for the study. Patient recruitment aimed at enrolling at least 50 VL cases and 50 non-VL cases to achieve sufficient precision for the sensitivity and specificity estimates of the tests. Therefore the total sample size was fixed at 150 patients, assuming a certain degree of imbalance between VL and non-VL cases. Patients were enrolled consecutively until the required sample size was achieved. They were included in the study after informed consent given by the patient or his/her guardian in case of minors. The research protocol was approved by the Ethical Committee of the BPKIHS in July 2001.

Diagnostic work up

All patients included in the study were admitted in the in-patient ward of the Rangeli DH for the initial diagnostic

work up (Figure 1) and treatment. Blood was drawn on the day of admission (day 0) for a complete blood count and a thick and thin smear for malaria parasite. Two filter papers were impregnated with whole blood, allowed to dry and transported to the Kala-azar Laboratory of BPKIHS within 48 h. After elution of the blood from the filter papers, the DAT was performed at the BPKIHS and the result was faxed back to the physician of Rangeli DH. The remaining blood sampled on day 0 was centrifuged at Rangeli DH and the serum was collected to perform the FGT and the rK39 antigen based dipstick. Urine was collected from all patients and stored in the refrigerator between 4 and 8 °C for a maximum of 48 h. A bone marrow aspirate was performed on day 0 for all patients and six slides were prepared and fixed. Slide staining and a microscopic search for the amastigote form of *L. donovani* were carried out independently at the Rangeli DH Laboratory and at the Kala-azar Laboratory of the BPKIHS. Quantification of parasites was graded from 1+ to 6+. Slides with discrepant reading results between the two laboratories were reviewed at the Kala-azar Laboratory of the BPKIHS and the outcome of this additional reading was recorded as final result. Testing for HIV was not performed because of the lack of proper counselling available at Rangeli DH and the expected low prevalence of HIV among the rural population living in the area.

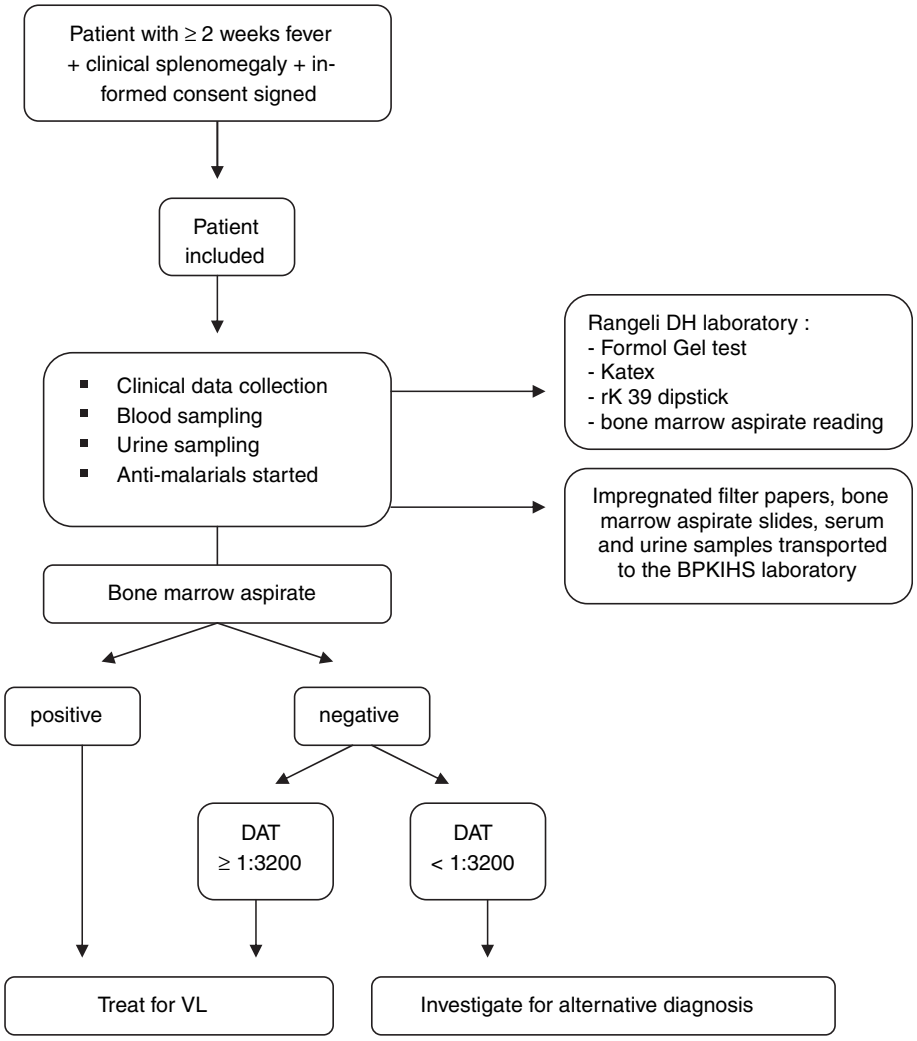

Figure 1 Operational flow-chart for the diagnosis of VL at the Rangeli DH, Nepal, during the study period (September 2001–December 2002).

Test procedures

For the FGT, 20 μl of 40% formaldehyde (Glaxo India Ltd., Bombay, India) was added to 200 μl of the patient's serum in a glass tube. After 20 min, the gelification reaction was visually assessed as positive or negative.

The rK39 antigen-based dipstick (Insure One-Step Rapid Test for VL, InBios International, Seattle, USA) was performed according to the manufacturer's instructions. In brief: 20 μl of the patient's serum was added to the test's strip. The strip was placed in a test tube and two drops of the chase buffer solution were added. The test was read after 5 min and, if negative, after 10 min. Even a weak line was considered as positive.

The Latex agglutination test (KAtex, Kalon Biological Ltd., Aldershot, UK) was performed according to the manufacturer's instructions. As pre-treatment, 1 ml of urine was transferred into the sample tube and placed in a rack immersed in a boiling water bath for 5 min. The sample and the reagents were brought to room temperature before testing. About 50 μl of the treated urine sample were added onto a reaction zone on 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. Any agglutination discerned when compared with the negative control was considered as positive.

Formol gel test, rK39 dipstick and KAtex were all performed on day 0 in Rangeli DH laboratory. Results were kept blinded to the physician in charge of the patient. The remaining serum and urine were stored in the refrigerator and sent in a cool box up to three-time per week to the Kala-azar Laboratory of the BPKIHS where all tests were repeated by a laboratory technician with extensive experience in VL diagnosis within 24 h after reception. The same test procedures were used in both laboratories.

The time interval between the sampling of blood/bone marrow and the test results, as well as the time required to perform the tests, were measured in a subset of nine patients at the Rangeli DH laboratory by an independent observer. The feasibility of the diagnostic tests was also qualitatively assessed with the laboratory workers of the Rangeli DH. The cost per test unit was determined by the price in use at Rangeli DH for the FGT and the bone marrow aspiration and reading. The cost of the rK39 dipstick was recorded from the retail price of the manufacturer. The commercial cost of the KAtex has not been determined yet.

Treatment and follow up

Malaria is most frequently due to *Plasmodium vivax* in this region and blood-circulating parasites are usually scanty, leading to possible false negative blood smear examination. Therefore, all patients included in the study received a full course of anti-malarials on admission day, independently of diagnostic tests results. Early specific treatment also allowed for an easier assessment of response to anti-malarials during the short period of hospitalization. All patients with a positive bone marrow aspirate or a negative bone marrow aspirate but a positive DAT result (end-dilution titre ≥1:3200) were treated with intramuscular sodium stibogluconate (SAG from Albert David Ltd., Calcutta, India) 20 mg/kg/day for 30 days. The initial injections were performed at Rangeli DH until clinical improvement and the remaining injections were completed in the village health post. Patients with a previous history of treatment for VL were considered as relapse cases and were treated with intravenous amphotericin B 0.5 mg/kg/day for 14 days. Patients with a negative initial bone marrow aspirate and negative DAT were further investigated at Rangeli DH (or referred to the BPKIHS) and treated for an alternative diagnosis.

All VL and non-VL patients were scheduled for follow-up visits at 1, 3 and 6 months after hospital discharge. Bone marrow aspiration was repeated at 1 (test of initial cure) and 6 month (test of definite cure) follow-up visit for all patients with a positive initial bone marrow aspirate and at any visit in case of clinical suspicion of VL.

Case definitions

As the sensitivity of bone marrow aspirate is only around 80%, we based our reference standard on a combination of parasitological, serological (DAT) and clinical evidence. A confirmed case of VL was defined as a patient with (1) a positive bone marrow aspirate during initial evaluation or (2) a positive bone marrow aspirate during any follow-up visit of an initial aspirate-negative case or (3) a negative bone marrow aspirate but a positive DAT (titre ≥1:3200) at day 0 and an absence of response to anti-malarial treatment but a successful response to anti-leishmanial therapy (definite cure documented at month 6 of follow up). A confirmed case of non-VL was defined as a patient with a negative bone marrow aspirate at initial and follow-up evaluation with (1) a negative DAT (titre <1:3200) or (2) a positive DAT but a definite cure with anti-malarial or other non-VL specific therapy. All other patients were classified as cases with uncertain disease status and were excluded from the analysis.

Data analysis

Personal and medical characteristics, diagnostic tests' results, follow-up data and patients' outcome were entered

in case-report forms by the study field supervisor. Data from the case-report forms were entered in an Excel data sheet and later checked by two investigators. The data were analysed with SPSS 11.0 for Windows version (SPSS Inc., Chicago, IL, USA). Numerical variables were summarized by mean and SD if normally distributed and if they were not, by median and quartiles. Categorical variables were compared using cross-tabulations and chi-square tests whereas numerical variables (means) were compared with Student's *t*-test, at a critical α-level of 0.05. All *P* values were two-sided. Sensitivity, specificity, positive and negative predictive values and their exact 95% binomial confidence interval were calculated for the FGT, the rK39 dipstick test and the KAtex from the groups of confirmed VL and non-VL patients. Reproducibility between the performance of the FGT, rK39 dipstick, KAtex and bone marrow aspirate at the Rangeli DH and BPKIHS was assessed by Cohen's kappa and interpreted following the grading system described by Landis and Koch (1977). The time to get the test result was measured from the sampling of blood or bone marrow to the availability of the test result. The time of actual work for the nurse or physician (blood or bone marrow sampling) and the laboratory workers (test procedures) was measured, including working times of 2 min for blood centrifugation, 2 min for the FGT procedure and 10 min for staining of bone marrow aspirates.

Results

Between September 2001 and December 2002, 155 patients were admitted to Rangeli DH with a clinical suspicion of VL. Of these, 154 patients were recruited in the study and one patient defaulted. Four patients did not complete the diagnostic work up and were excluded from the analysis. Of the 150 remaining patients, the proportion of patients examined during follow-up 1, 3 and 6 months after hospital discharge was 64%, 55% and 72%, respectively. After completion of the diagnostic work-up, treatment and follow-up, 85 patients (57%) were confirmed with VL whereas 57 patients (38%) had VL excluded and were classified as non-VL patients. Of the 85 patients with VL, the diagnosis was confirmed by a positive bone marrow aspiration in 75 patients during initial evaluation and in four patients during follow-up while six patients had a DAT ≥1:3200 and were definitely cured 6 months after completion of sodium stibogluconate treatment. The final diagnosis of the 57 non-VL patients was malaria (38), enteric fever (10), tuberculosis (4), liver disease (2), haematologic disorder (1) and others (2). Fifty-two (91%) non-VL patients had a negative initial DAT and did not develop VL during follow up. Five (9%) non-VL patients had a positive DAT but did not receive anti-leishmanials and were definitely cured at 6 months after treatment for malaria (3), enteric fever (1) and tuberculosis (1). Eight patients (5%) had an uncertain diagnosis and could not be classified as VL or non-VL: all had a DAT ≥1:3200 but did not receive SAG treatment and/or were lost to follow up. These eight patients were excluded from further analysis.

The general, clinical and biological characteristics of the 85 VL patients and the 57 non-VL patients are presented and compared in Table 1. Weight loss, emaciation and darkened skin were significantly more frequent in patients with VL ($P < 0.05$). VL patients also presented with a significantly longer duration of fever, a larger spleen and lower Hb, WBC and platelet counts ($P < 0.05$).

Test validity

The three tests were performed at the Rangeli DH Laboratory in all 142 patients. Results of the FGT and the rK39 antigen based dipstick were all included in the validation analysis. One month after the start of the study, we found that the reagent initially supplied with the KAtex (Lot No. K11-035/1) had a defect (absence of reaction with the positive control at both Rangeli DH and BPKIHS). The reagent was swiftly replaced by the manufacturer. Only the results of the KAtex from the 124 patients tested with the new lot of reagent (Lot No. K11-064/1) were included in the validation analysis.

The FGT had a 52% sensitivity [95% confidence interval (CI): 41–62], a 97% specificity (95% CI: 88–99), a 96% PPV (95% CI: 86–99) and a 57% NPV (95% CI: 47–67). The rK39 antigen based dipstick test had a 89% sensitivity (95% CI: 81–94), a 90% specificity (95% CI: 79–95), a 93% PPV (95% CI: 85–97) and a 85% NPV (95% CI: 74–92). The KAtex urine test had a 57% sensitivity (95% CI: 44–67), a 98% specificity (95% CI: 88–100), a 98% PPV (95% CI: 89–100) and a 56% NPV (95% CI: 45–67). The performance of the FGT and the rK39 dipstick test, when used in combinations, are also shown in Table 2.

The sensitivity and the specificity of the tests were not significantly modified when the analysis was restricted to the 130 patients with no previous history of VL (data not shown). Interestingly, out of the six non-VL patients with a history of previous VL, all had a negative FGT and KAtex but two patients had a falsely positive rK39 dipstick. These two patients also had a positive DAT with high titres (≥1:102400).

Test reproducibility

The FGT, rK39 dipstick and KAtex urine tests were repeated using the same test batches at the Kala-azar

Table 1 Comparison of the general, clinical and biological characteristics of the 85 VL patients and the 57 non-VL patients at the Rangeli DH, Nepal

Characteristics	VL patients (total: 85) *n* (%) or mean (SD)	Non-VL patients (total: 57) *n* (%) or mean (SD)	*P* value
Gender			
Female	37 (43)	20 (35)	0.31
Male	48 (57)	37 (65)	
Age	22.0 (13.4)	25.5 (15.2)	0.15
Profession			
Farmer	24 (28)	19 (33)	0.34
Student or pre-school	33 (39)	18 (32)	
Housewife	18 (21)	16 (28)	
Others	10 (12)	4 (7)	
Previous treatment for VL	6 (7)	6 (11)	0.47
Symptoms (self-reported)			
Chills	31 (37)	26 (46)	0.28
Skin darkening	27 (32)	3 (5)	<0.001
Anorexia	21 (25)	9 (16)	0.20
Cough	13 (15)	6 (11)	0.41
Weight loss	13 (15)	2 (4)	0.025
Abdominal pain	3 (4)	0 (0)	0.15
Bleeding	0 (0)	0 (0)	–
Duration of fever (weeks)	6.3 (4.8)	3.6 (2.6)	<0.001
Signs			
Emaciation	28 (33)	4 (7)	0.001
Hepatomegaly	79 (93)	49 (86)	0.17
Enlarged lymph nodes	3 (4)	2 (4)	0.99
Spleen size from costal margin (cm)	6.4 (3)	4.5 (2.8)	<0.001
Haematology			
Haemoglobin (g/dl)	9.2 (1.3)	9.7 (1.2)	0.01
White blood cell count (per mm^3)	4270 (1193)	6880 (2180)	<0.001
Platelet count (per mm^3)	126753 (34746)	159404 (31913)	<0.001

VL, visceral leishmaniasis.

Laboratory of the BPKIHS in 135, 142 and 116 patients, respectively. The strength of agreement was almost perfect for the K39 dipstick test ($\kappa = 0.87$), substantial for the FGT ($\kappa = 0.68$) and moderate for the KAtex ($\kappa = 0.43$).

Despite the fact that microscopic reading of bone marrow aspirates was performed on different sets of slides (but sampled at the same time) at Rangeli DH and BPKIHS, we assessed the strength of agreement and we found it to be substantial ($\kappa = 0.71$). Twenty (14%) out of 142 sets of slides examined showed discordant results, of which 17 (85%) were found in slides graded as 1+ by one of the readers.

Test feasibility

The cost per test unit, time of actual work and time to get the results are presented in Table 3. The median time of actual work was below 10 min for the FGT and the rK39 dipstick, 20 min for the KAtex urine test and 50 min for the bone marrow aspirate whereas the median time to get the results was 20 min for the rK39 dipstick, 25 min for the KAtex, 40 min for the FGT and 80 min for the bone marrow aspirate.

Discussion

We found the rK39 antigen-based dipstick (Insure one-step rapid test for VL) to be significantly more sensitive and more reproducible than the FGT and the urine antigen detection test (KAtex). Moreover, the rK39 dipstick required little actual work and gave quick results. The FGT and the KAtex were insufficiently sensitive but showed a non-significant trend towards a higher specificity than the rK39 dipstick.

An accurate validation of diagnostic tests for VL had been previously achieved in several studies conducted in optimally controlled conditions at the BPKIHS, a referral teaching hospital located in Eastern Nepal (Chappuis *et al.* 2003; Boelaert *et al.* 2004; Rijal *et al.* 2004). Our study was conducted in the real conditions of a DH located in the

Table 2 Sensitivity, specificity, positive and negative predictive values of the FGT, the K39 antigen-based dipstick test and the KAtex urine test at the Rangeli DH, Nepal

Diagnostic test	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
Individual tests				
FGT	52 (41–62)	97 (88–99)	96 (86–99)	57 (47–67)
K39 dipstick test	89 (81–94)	90 (79–95)	93 (85–97)	85 (74–92)
Katex urine test	57 (46–67)	98 (88–100)	98 (89–100)	56 (45–67)
Tests in combination				
FGT+and/or K39 dipstick+	93 (85–97)	88 (77–94)	92 (84–96)	89 (79–95)
FGT+ and K39 dipstick+	47 (37–58)	98 (91–100)	98 (87–100)	55 (46–65)

FGT, formol gel test; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

Table 3 Cost per unit, time of actual work and time to obtain results of the diagnostic tests performed at the Rangeli DH, Nepal

Diagnostic test	Cost per unit (US $)	Time of actual work (min)*	Time to obtain results (min)*
rK39 dipstick test	1†	9 (6.5; 9)	20 (15; 25)
FGT	0.25‡	6 (6; 7)	40 (34; 57.5)
Katex urine test	Undetermined	20 (15; 22.5)	25 (20; 27.5)
Bone marrow aspirate	1.5‡	50 (45; 55)	80 (71.2; 97.5)

FGT, formol gel test.
* Median (quartile 1; quartile 3).
† Price communicated by the manufacturer.
‡ Price billed to the patients at Rangeli DH.

same endemic area, and we evaluated the effectiveness rather than the efficacy of those diagnostic tests. No extra-staff was hired for the study and the laboratory technicians received a limited previous training on the diagnostic test procedures. This might have impaired the accuracy of the test validation but we believe that our results showed a more realistic picture of the performance of the tests when used in daily field practice. Our reference standard for the validation was based on a combination of parasitological, serological and clinical evidence, as systematic splenic aspiration of all suspects was not possible in this context. Nonetheless this should not jeopardize the validity of our sensitivity and specificity estimates. Documented response to treatment in chronic febrile splenomegaly patients with a positive DAT should be considered as sufficient evidence of VL (Zijlstra *et al.* 1991), because antimonials have a very narrow therapeutic spectrum.

Despite these limitations, the results of the tests validation were in accordance with previous studies (Boelaert *et al.* 2004; Rijal *et al.* 2004), which equally showed a low sensitivity of FGT and KAtex. This low sensitivity is most likely influenced by patient's delay in presenting at the rural hospital. Indeed, we found no clear differences in the clinical and laboratory parameters when comparing to cohorts of VL patients previously studied at the tertiary care centre BPKIHS (data not shown), reflecting a similar degree of disease progression. However, the sensitivity of KAtex appears better in VL patients co-infected with HIV because of their higher parasite load (Riera *et al.* 2004).

The high specificity of KAtex also confirmed previous reports and highlights the very interesting concept of the test (detection of a specific antigen). Further developments to improve the sensitivity of specific antigen detection in the urine are underway. The high specificity of the FGT was more surprising considering the low intrinsic specificity of the test principle (detection of an increased level of polyclonal immunoglobulins), but confirmed an earlier report from Nepal (Boelaert *et al.* 2004). This should be confirmed in other VL endemic areas such as East Africa where polyclonal hypergammaglobulinemia might be more commonly found with other diseases like *Plasmodium falciparum* infections (Abele *et al.* 1965).

The rK39 dipstick (Insure one-step rapid test for VL) performed well in our study with sensitivity and specificity of respectively 89% and 90%. The same dipstick was previously validated in Nepal and India, showing sensitivity and specificity ranges of 90–100% and 93–100%, respectively (Bern *et al.* 2000; Sundar *et al.* 2002a,b; Boelaert *et al.* 2004). A lower specificity (71%) was found with an earlier generation of the Insure dipstick in Nepal (Chappuis *et al.* 2003). Other brands of rK39 antigen-based dipsticks have been evaluated elsewhere but are either no longer produced (Sundar *et al.* 1998; Zijlstra *et al.* 2001; Veeken *et al.* 2003), or in need of further evaluation (Brandonisio *et al.* 2002; Iqbal *et al.* 2002). RK39 antigen-based dipsticks have so far performed poorly in Sudan (Zijlstra *et al.* 2001; Veeken *et al.* 2003). A newly manufactured rK39 dipstick (Opti-LEISH from Diamed AG, Switzerland) showed promising results in India (Sundar *et al.* 2003), Uganda (personal observation) and, interestingly, in Sudan (Ritmeijer K, Melaku J, Möller M, Kipngetich S, O'Keefe C & Davidson RN, in press).

We found that two out of six non-VL patients with a history of previous treatment for VL had a false positive dipstick result. The rK39 antigen-based dipsticks are not suited for the diagnosis of relapses due to the long persistence of antibodies after initial treatment (Zijlstra *et al.* 2001), as observed with other serological tests for VL such as the DAT (Hailu 1990). For patients with a previous history of VL, parasitological diagnosis remains the approach of choice but antigen detection in the urine could be an interesting alternative, provided that the sensitivity of the test improves.

We found an almost perfect agreement of the rK39 dipstick between the Rangeli DH and the BPKIHS laboratories. Inter-reader concordance was already found to be 100% in a previous study (Schallig *et al.* 2002). This excellent reproducibility most likely reflects the simplicity of the procedure and the easy interpretation of most dipstick results. In contrast, we found only a moderate reproducibility of the KAtex urine test between the two laboratories ($\kappa = 0.43$), lower than previously reported ($\kappa = 0.69$) by Rijal *et al.* (2004). This is likely to be due to the difficulty in interpreting weakly positive agglutinations and to some practical problems occurring during the test procedure such as having the urine sample spoiled by the boiling water during the heating process. Nevertheless, we cannot exclude that the strength of concordance was underestimated by some degree of deterioration of the antigen during storage and transport of urine samples. Discordant results of bone marrow aspirate examinations between the two laboratories were not rare and mostly occurred in patients with low parasite densities, emphasizing the high level of expertise required to perform this test with accuracy.

The time to get the results of the FGT, rK39 dipstick and the KAtex urine test is equal or less than 40 min. The three tests thus deserve to be labelled as 'rapid tests'. They compare favourably with the minimal time of 18 h to get the result of the DAT when performed on site. Their direct cost is low (0.25 US $ for the FGT) to moderate (1 US $ for the dipstick). The time of actual work to perform the KAtex urine test exceeds the time needed to perform the rK39 dipstick and the FGT. This should be taken into account for the calculation of the real cost of these diagnostic tests, bearing in mind that the cost of test-treatment strategies depends mainly on the cost of hospitalization and treatment (Boelaert *et al.* 1999d).

Our findings support the recent yearly distribution of several thousands Insure dipsticks to the DHs of VL endemic areas by the Nepalese Ministry of Health. As recently shown in the neighboring Bihar State of India (Sundar *et al.* 2002b), the rK39 antigen-based dipstick appears as the best first-line single diagnostic test for the diagnosis of VL in this part of the world. It fulfils most of the 'ASSURED' criteria that describe the ideal characteristics of a diagnostic test for resource-limited settings (Mabey *et al.* 2004). The DAT is a well-validated and robust test, especially when freeze-dried antigen is used (Abdallah *et al.* 2004), but it is too sophisticated to be widely used at first line health services in the endemic areas of Nepal and India. Sending blood-impregnated filter papers to a reference laboratory performing the DAT, as done during our study and routinely in some endemic areas (i.e. Somalia), is neither practical nor sustainable. Integrating the FGT in the diagnostic tree as a first-step procedure and restraining the use of the rK39 dipsticks to negative FGT results appears as a promising approach in this endemic region. This approach would slightly improve diagnostic accuracy (cf. Table 2) and would decrease the cost by decreasing the consumption of dipsticks (a 32% decrease in our study). A careful cost-effectiveness analysis remains to be performed before a final diagnostic tree can be proposed to national policy makers. Similar evaluations should be done in other VL endemic areas such as East Africa or South America.

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Validité, reproductibilité et faisabilité sur le terrain des tests de diagnostic de la leishmaniose viscérale en milieu rural au Népal

OBJECTIFS Evaluer sur le terrain la précision, la reproductibilité et la faisabilité du test au Gel de Formol (FGT), du test de l'agglutination urine-latex (KAtex) et du test sur bandelette à l'antigène rK39, pour le diagnostic de la leishmaniose viscérale (LV) en milieu rural du Népal.

MÉTHODE Les patients avec une suspicion clinique de la LV ont été recrutés à l'hôpital du district de Rangeli, un hôpital gouvernemental de 15 lits, situé dans le sud-est du Népal. Les tests FGT, KAtex et au rK39 ont été appliqués sur place et répétés ultérieurement dans le laboratoire de diagnostic de référence du Kala-azar pour évaluer leur reproductibilité. Le diagnostic de la LV a été confirmé soit par examen positif d'aspiration de moelle osseuse ou par un résultat positif du test d'agglutination direct (titre DAT ≥1:3200), chez les patients qui, plus tard ont répondu au traitement anti-leishmaniose.

RÉSULTATS Sur 155 patients initialement recrutés, 142 (85 avec la LV et 57 avec un autre diagnostic) ont été inclus dans l'étude. La sensibilité pour le test au rK39 (89%, IC 95%: 81–94) était significativement plus élevée que celle du KAtex (57%, IC95%: 46–67) et celle du FGT (52%, IC95%: 41–62). Tous les trois tests avaient une spécificité d'au moins 90%. La concordance était plus élevée pour le test au rK39 ($\kappa = 0.87$) que pour le FGT ($\kappa = 0.68$) et le KAtex ($\kappa = 0.43$). Tous les tests nécessitaient ≤20 min de travail effectif et ≤40 min au total pour l'obtention des résultats.

CONCLUSION Les bandelettes au rK39 étaient d'utilisation facile avec des résultats plus précis et plus reproductibles que les autres tests de diagnostic de la LV dans un hôpital de district dans le Népal rural. Ce test devrait être intégré dans cette région dans l'algorithme du diagnostic sur le terrain de la LV et les mécanismes pour assurer sa disponibilité devraient être trouvés.

Mots clés kala-azar, leishmaniose viscérale, diagnostic, sérologie, détection d'antigènes, Népal

Validez en el campo, reproducibilidad y factibilidad de test diagnósticos para leishmaniasis visceral en Nepal rural

OBJETIVOS Evaluar la exactitud en el campo, la reproducibilidad y la factibilidad del test de gel de formol (FGT), el test de latex en orina (KAtex) y la tira diagnóstica basada en el antígeno rK39 para el diagnóstico de leishmaniasis visceral (LV) en el Nepal rural.

MÉTODO Pacientes con sospecha clínica de LV fueron reclutados en el hospital distrital de Rangeli, un hospital gubernamental de 15 camas, localizado en el sur occidente de Nepal. Pruebas con FGT, KAtex y las tiras diagnósticas rK39 se realizaron *in situ* y posteriormente repetidas en un laboratorio de referencia para el diagnóstico de kala-azar con el fin de evaluar su reproducibilidad. El diagnóstico de LV se confirmó, bien por aspirado de médula ósea positivo o por test de aglutinación directa (titulo DAT ≥1:3200) en pacientes que luego respondieron a terapia anti-leishmania.

RESULTADOS De los 155 pacientes inicialmente reclutados, se incluyeron 142 (85 con LV y 57 con otros diagnósticos). La sensibilidad de la tira diagnóstica rK39 (89%; 95% CI: 81–94) fue significativamente más alta que la del KAtex (57%; 95%CI: 46–67) y la del FGT (52%; 95%CI: 41–62). El acuerdo fue más alto para la tira diagnóstica rK39 ($\kappa = 0.87$) que para el FGT (0.68) o el KAtex (0.43). Todos los test necesitaron ≤20 min de trabajo real y ≤40 min para obtener resultados.

CONCLUSIÓN La tira diagnóstica rK39 fue más fácil, más exacta y reproducible que los otros test rápidos utilizados en un hospital distrital del área rural de Nepal para el diagnóstico de la LV. Se debería integrar dentro del algoritmo diagnóstico de esta región, así como encontrar los mecanismos para asegurar su disponibilidad.

Palabras clave kala-azar, leishmaniasis visceral, diagnóstico, serología, detección antigénica, Nepal