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Prospective evaluation and comparison of the direct agglutination test and an rK39-antigen-based dipstick test for the diagnosis of suspected kala-azar in Nepal

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Summary The diagnosis of visceral leishmaniasis (kala-azar) remains difficult in rural endemic areas and practical and reliable tests are badly needed. Two serological tests, the Direct Agglutination Test (DAT) and an rK39-antigen-based dipstick test, were compared to parasitological diagnosis in a group of 184 patients presenting at a tertiary care centre in south-eastern Nepal with a history of fever ≥14 days and splenomegaly; 139 patients had a parasitologically proven kala-azar and 45 patients had a negative parasitological work-up. The rK39 dipstick showed a sensitivity of 97% and a specificity of 71%. The DAT was up to 99% sensitive with a low cut-off titre (1:400) but its specificity did not exceed 82% even with a high cut-off titre (1:51 200). Both tests could be used for screening suspect patients in endemic areas. However, their use as confirmatory tests should be restricted to situations where the proportion of kala-azar among clinical suspect patients is high. The rK39 dipstick is cheaper and easier to use than the DAT and could be used widely provided that both its performance and production remain stable.

keywords visceral leishmaniasis, kala-azar, diagnosis, direct agglutination test, k39 antigen, dipstick, Nepal

Introduction

An estimated 500 000 persons are affected by visceral leishmaniasis (VL, also known as kala-azar) every year worldwide. The vast majority of these cases (90%) occurs in poor rural areas of Sudan, India, Bangladesh, Nepal and Brazil (Desjeux 1996). Kala-azar is usually fatal if left untreated but the use of sodium stibogluconate, the most frequently prescribed first-line therapy generally considered to be relatively safe, can have severe side-effects (Sundar *et al.* 2000). It is, therefore, of prime importance to have accurate and practical diagnostic methods available in endemic areas. In most countries, diagnosis relies on microscopic examination of lymph nodes, bone marrow or spleen aspirates. Lymph node and bone marrow aspirations are safe procedures but their sensitivity for diagnosing kala-azar is only 58–64% and 70–86%, respectively (WHO 1984; Zijlstra *et al.* 1992). Spleen aspiration is generally considered as the gold standard for kala-azar diagnosis (Ho *et al.* 1948; Zijlstra *et al.* 1992) because of its high sensitivity and specificity (both close to 100%). However, it is contra-indicated in quite a number of kala-azar suspect patients, although it is a safer procedure than thought by many physicians. When performed properly, the death rate due to severe bleeding is 0.1% as reviewed by Kager & Rees (1983). One of the major drawbacks of parasitological diagnosis is the expertise required from both the physician to perform the procedures and the laboratory technician to stain and read the slides accurately. This expertise is very difficult to obtain in practice outside reference tertiary hospitals or specialized treatment or research centres.

Research has focused on the development of cheap, simple and reliable serological tests for kala-azar, which could replace parasitological diagnosis in the field. The Direct Agglutination Test (DAT) was first described by Allain & Kagan (1975) and the method was then adapted by El Harith *et al.* (1986, 1988). The DAT proved to be a

very valuable tool for field workers especially when high numbers of patients present to health facilities, as during the disastrous epidemics raging in Sudan for the last 15 years. The DAT is very accurate under laboratory conditions (El Safi & Evans 1989; Singla *et al.* 1993; Joshi *et al.* 1999) with high specificity when control groups were composed of healthy persons from endemic areas (Schaefer *et al.* 1995; Boelaert *et al.* 1999a). In field conditions, the DAT is very sensitive, but low specificity values were reported in series of clinical suspect patients, with a range depending on the reference test used, between 58% (Boelaert *et al.* 1999b) and 72% (Zijlstra *et al* 1991). Use of the DAT has been encouraged by WHO for surveillance and control programmes of VL (DAT workshop, Antwerp, 25–27 March 1998).

More recently, serological testing against a recombinant antigen derived from a 39-amino acid repeat in *Leishmania chagasi* (rK39) was developed. It is very accurate when used in an ELISA format (Singh *et al.* 1995; Badaro *et al.* 1996; Zijlstra *et al.* 1998). It was later developed as a dipstick format that showed to be 100% sensitive and 98% specific in India (Sundar *et al.* 1998). As Zijlstra *et al.* (2001) found a sensitivity of only 67% in Sudan these results require further confirmation.

Within this context of new diagnostic development, this study aimed at evaluating and comparing the validity of the DAT and an rK39-antigen-based dipstick for the diagnosis of VL among clinically suspect patients in Nepal.

Materials and methods

Study site

This study took place at the B.P. Koirala Institute of Health Sciences (BPKIHS), a 650-bed University Hospital located 2 km away from the town of Dharan, Sunsari District, Eastern Region of Nepal. The BPKIHS serves as a reference tertiary hospital for the eastern region, which includes several kala-azar endemic districts. Recruitment of patients took place in the Outpatient Department (OPD) and the Emergency Room of BPKIHS from July 1999 to August 2000. The research protocol was approved by the Ethical Committee of BPKIHS on May 1999.

Inclusion and exclusion criteria

All patients coming to BPKIHS with a history of fever for 14 days or more and clinical splenomegaly were eligible for the study and were included after informed consent was given by the patient or his/her closest relative (for unconscious or paediatric patients). Only clinically suspect patients were thus included in the study. Indeed, as emphasized by Sackett *et al.* (1991), we considered it essential to validate both diagnostic tests on a patient group representative for those persons on whom physicians will use the tests in the future. Patients with prior treatment for kala-azar were excluded from the study. Serological diagnosis is known to be unreliable in such patients because of the long persistence of antibodies against *Leishmania donovani* after treatment.

Diagnostic procedures

All patients included were admitted to the medical ward for the initial diagnostic work-up and treatment. On day 0 (admission day), blood was drawn for complete blood count, chemistry, coagulation profile, thick and thin smear, blood cultures and HIV testing after pre-test counselling (Vironostica® and Recombigen® ELISA tests). Chest X-ray, abdominal ultrasound and other tests were performed at the physician's discretion.

rK39 dipstick test

The rK39-antigen-based dipstick test (InSure Rapid Test for Visceral Leishmaniasis® from InBios International, Seattle, USA) was performed using patient serum on day 0 or 1 by the same house officer throughout the study and the results were kept blinded to the physician in charge of the patient. The procedure was as follows: after allowing the serum specimen and the dipstick to reach room temperature, 20 µl of serum were added on the dipstick, which was then placed vertically in a test tube. Two drops of the chase buffer solution provided with the dipstick kit were added in the test tube. The results were read after 5 min and, if still negative, after 10 min. Even a weak band in the test region was considered as a positive result. The test was repeated if the control line remained negative after 10 min.

Direct agglutination test

Patients sera were kept frozen at −70 °C and the DAT was performed at BPKIHS every 3–6 months by a laboratory technician (M.L.D.) previously trained on-site by the chief laboratory technician of the Protozoology Unit of the Prince Leopold Institute of Tropical Medicine in Antwerp (ITMA). Results of the DAT were thus not used for clinical decisions. Crosschecking of the DAT was performed at ITMA from blood impregnated on filter papers on day 0 and stored in sealed plastic bags. 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.* (1999c). The liquid antigen was kept at 4 °C during transport and storage at BPKIHS. The test was carried out

in microtitre plates (V-shaped wells) with the necessary positive and negative controls. The test was read visually against a white background and the end-point titre was taken as the last well where agglutination was seen. A first serum dilution was tested at 1:400; when positive, full titration was performed (1:400 to 1:204 800).

Parasitological diagnosis

All patients underwent bone marrow aspiration on day 0 or 1. Careful microscopic search for the amastigote form of *L. donovani* (LD bodies) was carried out independently at both the Department of Microbiology and the Department of Pathology of BPKIHS by two examiners. When results were discordant, both examiners met in order to reach a consensus. Moreover, slides from discordant results were reviewed at the Parasitology Laboratory of the University Medical Centre of Montpellier (Dr J. Dereure). If bone marrow aspiration was negative for LD bodies, spleen aspiration was performed unless one or more of the following condition was present: coagulation disorder or platelet count $\leq 50\,000$ mm^{-3}, refusal of the patient or her/his physician, alternative diagnosis ascertained by initial work-up with a clear clinical response to specific therapy or spleen size ≤ 3 cm under the left costal margin.

Case definitions

A confirmed case of kala-azar was defined as a patient with positive parasitology on either bone marrow or spleen aspiration smear. A control was defined as an eligible patient in whom kala-azar was excluded by a negative bone marrow aspiration and by a subsequent negative spleen aspiration. Also, we considered as a control a patient with a negative bone marrow smear in whom a firm alternative diagnosis was reached. In addition, all control patients were carefully followed-up for 6 months. Patients showing any clinical or parasitological evidence of kala-azar during this 6-month follow-up have been excluded from our control group.

Case management

All patients were hospitalized until clinical improvement. Kala-azar patients with no prior history of complete anti-leishmanial treatment were treated with generic sodium stibogluconate (SAG from Albert David Ltd – Calcutta) 20 mg/kg/day for 30 days.

Statistical analysis

Data were entered at BPKIHS in an Excel data sheet and electronically sent on a weekly basis to the principal investigator. Kala-azar and control patients were compared on socio-demographic, clinical and laboratory characteristics, using cross-tabulations and chi-square tests for categorical variables and comparison of means and *t*-test for continuous variables. Sensitivity, specificity, positive and negative predictive values were calculated for the rK39-antigen-based dipstick test and for each dilution of the DAT, as well as their 95% confidence intervals. The performance of the two DATs (BPKIHS and ITMA) was analysed by comparing the area under the curve (AUC) of the corresponding receiver-operating characteristics curves with a statistical software (Statistical Package for Social Sciences, v. 10.1.0). All statistical tests were two-tailed, with a significance level of 0.05.

Results

A total of 227 clinically suspect patients were admitted to BPKIHS during the 14-month study period. Of these, 195 patients were included in the study after exclusion of three patients because of early defaulting and 29 patients (25 kala-azar cases, four controls) because of previous treatment for kala-azar. Seven patients were later excluded from analysis because their physician treated them for kala-azar despite the absence of parasitological evidence and four patients were excluded from the control group because kala-azar was diagnosed during follow-up.

The analysis of the validity of the rK39 dipstick and the DAT was performed in the remaining 184 patients: 108 males and 76 females with a mean age of 23 years. There were 139 kala-azar patients and 45 controls. The proportion of kala-azar among clinical suspects was 76%. The diagnosis of the 139 kala-azar patients was made by a positive bone marrow (131 patients) or spleen (eight patients) aspiration.

All 45 controls had a negative bone marrow aspiration but spleen aspiration was performed for only six patients. The main reasons for not performing spleen aspiration were a clear initial response to treatment of an alternative diagnosis (17 patients), a refusal of the patient or his physician (13 patients), a spleen too small to be punctured (eight patients) and a coagulation disorder (one patient). The discharge diagnosis of the 45 controls was malaria (20 patients), disseminated tuberculosis (five patients), enteric fever (four patients), leukaemia (three patients), other haematological disorders (three patients), infectious endocarditis (two patients), sepsis (two patients), solid malignancy (one patient) and other infections (five patients). Follow-up was completed at 1, 3 and 6 months for 29 of 45 controls (64%).

The presence of weight loss, skin darkening and the absence of cervical lymph nodes were significantly more

frequent in kala-azar patients and the mean platelet count was significantly lower in kala-azar patients (data not shown). HIV testing was negative for all 184 patients.

The InSure® dipstick was performed on the 184 patients. The result was positive in 148 patients and negative in 36 patients. The sensitivity of the InSure® dipstick is 97% (95% CI: 92–99%) and the specificity is 71% (95% CI: 56–81%). The positive and negative predictive values of the dipstick test are 91% (95% CI: 85–95%) and 89% (95% CI: 73–95%), respectively. The diagnosis of kala-azar would have been missed in four patients. Three out of the four patients had also a low DAT titre (≤1:800) but one patient had a DAT titre of 1:25 600. The dipstick was positive in 13 controls. Final diagnosis of these patients was malaria (10 patients), enteric fever (two patients) and disseminated TB (one patient). These patients were treated according to their clinical diagnosis and had a good initial response. Nine out of the 13 patients were followed-up at 6 months and were all considered as definitely cured.

The DAT was performed on 183 patients. One serum tube from a kala-azar patient was lost. The performance of the DAT depends on the cut-off titre chosen (Table 1). When compared with the DAT performed at BPKIHS, the DAT performed at ITMA shows similar sensitivity values but a trend towards higher specificity (data not shown). The AUC of the DAT performed at BPKIHS is 0.858, as compared to 0.890 for the DAT performed at ITMA (Figure 1). This difference is not significant ($P = 0.25$).

The predictive values calculated for both the rK39 dipstick and the DAT would vary greatly according to the proportion of kala-azar in clinical suspect patients (Figure 2). For a prevalence of 50%, for example, the InSure® dipstick would have a positive predictive value (PPV) of 77% and a negative predictive value (NPV) of 96%, the DAT 1:400 a PPV of 60% and a NPV of 96%, the DAT 1:6400 a PPV of 74% and a NPV of 88% and the DAT 1:51 200 a PPV of 82% and a NPV of 81%.

The four patients initially classified as controls but later excluded because they developed kala-azar during follow-up interestingly had all a positive dipstick and, for three of them, a DAT > 1:6400 between 3 and 6 months before kala-azar was diagnosed.

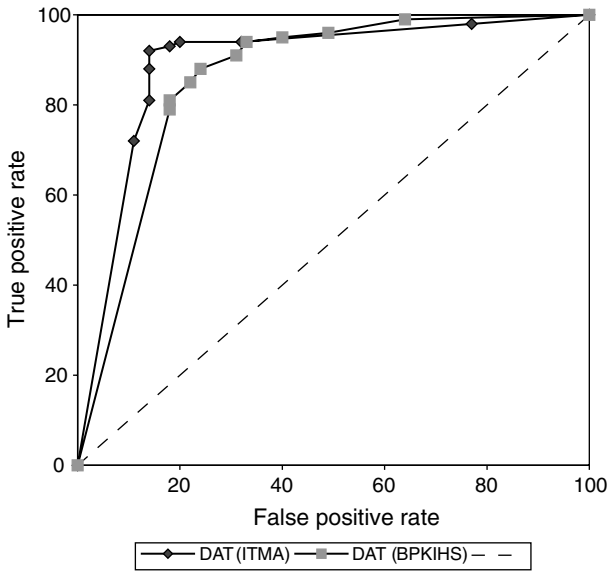

Figure 1 Receiver-operating characteristics curves of the DAT performed at ITMA and BPKIHS.

Table 1 Sensitivity, specificity and predictive values of the DAT performed at BPKIHS*

DAT cut-off titre	Kala-azar, no. of cases†	Controls, no. of cases	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
<1:400	2	16				
1:400	4	7	99 (94–99)	36 (22–49)	82 (76–87)	89 (64–96)
1:800	1	4	96 (90–98)	51 (36–64)	86 (79–90)	79 (60–89)
1:1600	1	3	95 (89–97)	60 (44–72)	88 (81–92)	79 (62–89)
1:3200	5	1	94 (89–97)	67 (51–78)	90 (83–93)	79 (62–88)
1:6400	4	3	91 (84–94)	69 (53–80)	90 (83–94)	70 (55–81)
1:12 800	4	1	88 (81–92)	76 (60–85)	92 (85–95)	67 (52–77)
1:25 600	5	2	85 (77–90)	78 (63–87)	92 (86–95)	63 (49–73)
1:51 200	3	0	81 (73–87)	82 (67–90)	93 (87–96)	59 (46–69)
1:102 400	109	8	79 (71–85)	82 (67–90)	93 (87–96)	56 (43–67)

* Values in parentheses are 95% confidence limits.
† One serum sample was lost.

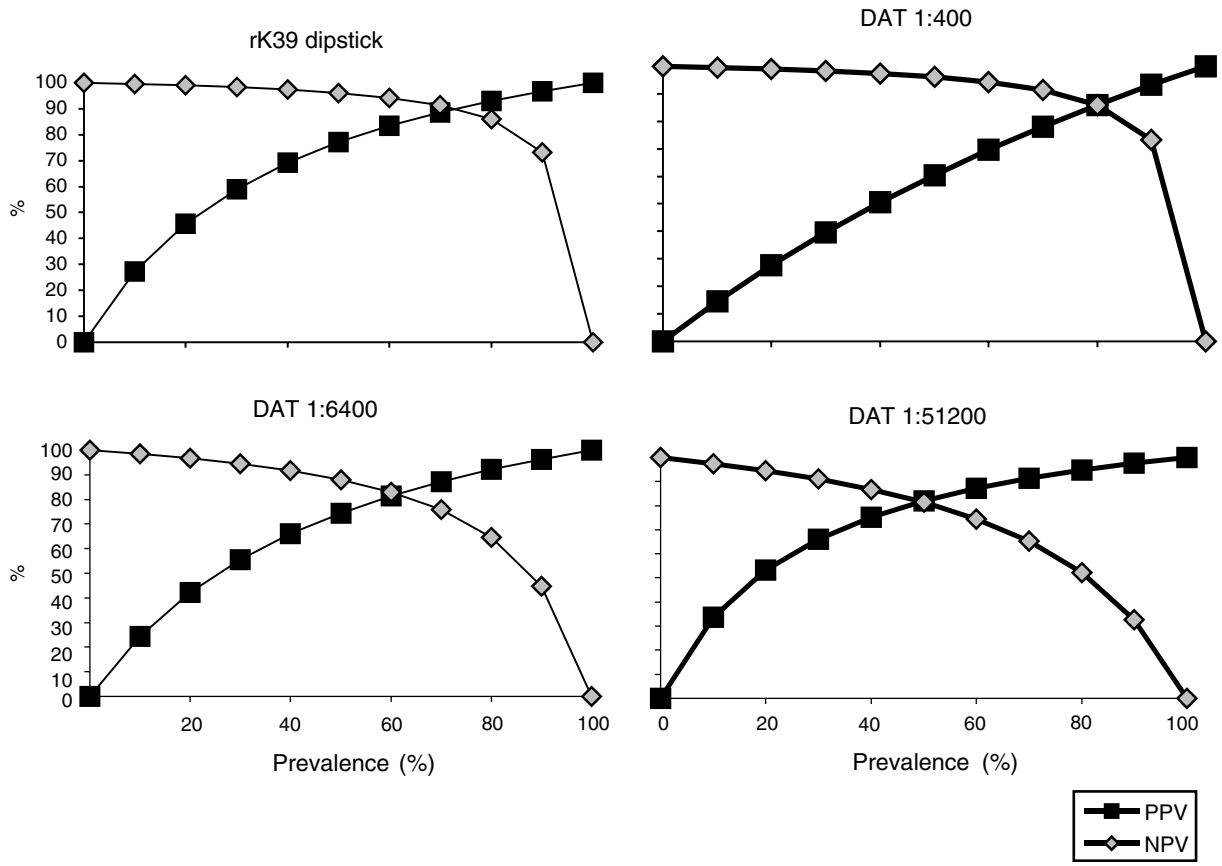

Figure 2 Positive (PPV) and negative (NPV) predictive values of the rK39 dipstick and the DAT with cut-off titres of 1:400, 1:6400 and 1:51 200 depending on the prevalence of kala-azar among clinical suspect patients.

Discussion

This validation study, conducted in a tertiary hospital of South-East Nepal where kala-azar is endemic, evaluated and compared the performance of the DAT and the rK39-based InSure® dipstick test for the diagnosis of VL among a group of 184 clinical suspect patients.

When interpreted with a cut-off titre of 1:6400, corresponding to the titre of 1:3200 found in the original report of El Harith *et al.* (1986), the DAT was 91% sensitive and 69% specific. When setting a higher cut-off titre (1:51 200), DAT specificity would increase only moderately to 82% but at the cost of lower sensitivity (81%). There was a non-significant trend towards a better specificity when the test was performed in the reference laboratory. The relative lack of reproducibility of the DAT, previously described by Boelaert *et al.* (1999c), did not significantly alter the results in our study considering the good conditions of transport and storage of the liquid antigen and the specific training received on site by the laboratory technicians.

The sensitivity of the DAT is invariably recorded above 90% in the literature when low or intermediate cut-off titres are used. However, its specificity varies greatly and depends to a large extent on the choice of the control group. DAT specificity is reported well above 90% when control groups are composed of non-symptomatic people or patients with known other diseases (except African trypanosomiasis), coming either from kala-azar endemic or non-endemic areas (El Harith *et al.* 1987; Sinha & Sehgal 1994; Schaefer *et al.* 1995; Boelaert *et al.* 1999a). When only clinically suspect patients are studied, a much more realistic situation for a physician, the reported DAT specificity falls to 58% (Boelaert *et al.* 1999b) and 72% (Zijlstra *et al.* 1991). Our findings of a specificity of 69% for a cut-off titre set at 1:6400 are consistent with these previous reports.

The DAT is a well-validated test for the diagnosis of kala-azar and has been widely used for more than 15 years. Because of the quantitative results obtained by gradual dilution, the cut-off titres to use can be (and should be) tailored for each epidemiological situation. The main limitation of the DAT is its relatively sophisticated procedure, which impairs its wide application in peripheral health structures and the fragile nature of the antigen in its liquid form, which is a source of logistic problem for transport and the likely cause of lack of reproducibility. The latter problems could be solved in the future by the use of freeze-dried antigen (Meredith *et al.* 1995; Zijlstra *et al.* 1997; Oskam *et al.* 1999). The relative high price of the DAT liquid antigen (4.5 US$ per test) is also a constraint but Boelaert *et al.* (1999d) showed that currently the cost of test-treatment strategies depend mainly on the cost of hospitalization and treatment.

The rK39-antigen-based InSure® dipstick showed a sensitivity of 97% and a specificity of 71% in our study. It proved to be very easy to perform with minimal training. Reproducibility of the dipstick was not assessed here but is clearly an issue to be considered in future studies. Indeed, the test line can be sometimes very faint and the distinction from the white background of the dipstick can be difficult. It is difficult to compare these results with those obtained from previous studies assessing the value of serology against rK39 antigen. The initial studies showed excellent sensitivity and specificity but used an ELISA format that is not practical to use in most health facilities in kala-azar endemic areas (Badaro *et al.* 1996; Zijlstra *et al.* 1998). A well-designed study from Sundar *et al.* (1998) showed excellent results (sensitivity: 100%; specificity: 98%) of an rK39-antigen-based test on a dipstick format in India but these results were challenged by a low sensitivity (67%) found by Zijlstra *et al.* (2001) in Sudan. The manufacturer of the dipstick tested in the two latter studies has now abandoned its production. A second generation of InSure® dipstick is currently produced and commercialized and is designed to be more specific than the first-generation dipstick tested in our study. A preliminary retrospective study in Nepal showed a sensitivity and specificity of 100% of this second-generation dipstick but only 14 VL patients were included and the control group was composed of non-symptomatic villagers with no personal or household history of VL (Bern *et al.* 2000).

The transition of rK39-antigen-based serological test from its very accurate ELISA format to a very practical to use dipstick format is not without difficulties. With the current format expressing only a qualitative result (positive or negative), it will be difficult for manufacturers to produce a dipstick with both high sensitivity and specificity applicable in all kala-azar endemic areas. An alternative approach would be to produce a dipstick with two test lines (or two separate dipsticks): one detecting low antibody titre for screening and one detecting high antibody titre for potentially confirming the diagnosis. The dipstick tested in this study has a high potential for use as a screening test for kala-azar in peripheral health structures where parasitological diagnosis and the DAT cannot be used for technical reasons. This will apply to the currently produced second-generation InSure® dipstick only if its sensitivity remains unchanged. Another constraint for the InSure® dipstick is the sustainability of production by a private company of a diagnostic test for kala-azar, a neglected disease with low profit potential.

The specificity of the InSure® dipstick and the DAT might have been underestimated in our study because of the imperfection of our gold standard. Indeed, only six of the 49 patients with an initial negative bone marrow underwent spleen aspiration. Because of the limited sensitivity of bone marrow aspiration, some diagnosis of kala-azar might have been missed. We believe that the number of missed cases is small considering that only four of the 49 (8.2%) initial controls were diagnosed with kala-azar during follow-up and that 6-month follow-up was completed in two-thirds of the patients. Boelaert *et al.* (1999b) showed in a group of 149 clinical suspect patients that specificity of the DAT raised from 68% to 85% when a mathematical model (Latent Class Analysis) was applied to correct for the imperfection of the parasitological gold standard (which did not include spleen aspiration). Furthermore, the confidence intervals of the specificity values are relatively wide in our study because of the limited number of patients included in the control group.

The specificity of serological tests for VL is anyway affected by the long persistence of antibodies after *L. donovani* infections, which are most frequently sub-clinical. Hailu (1990) showed in Ethiopia that the DAT remained positive in most kala-azar patients up to 7 years after treatment. Antibodies targeted against the more specific rK39 antigen have been shown to be detected by a strip test significantly less frequently than antibodies detected by the DAT 12 months after completion of treatment of kala-azar patients in Sudan (Zijlstra *et al.* 2001). However, the strip test used in this study was lacking sensitivity and IgG directed against rK39 have been detected up to 24 months post-treatment in Sudan by the more sensitive ELISA technique (Zijlstra *et al.* 1998). Because of this long persistence of antibodies, clinical suspect patients with a positive rK39 dipstick or an elevated DAT titre who have a prior history of kala-azar should have a confirmatory bone marrow or spleen aspiration performed.

Despite the relative lack of specificity of both the InSure® dipstick and the DAT, both tests can be used as fairly reliable confirmatory tests at BPKIHS with positive predictive values of, respectively, 91% and 90% (cut-off titre: 1:6400). This would not apply in areas where the proportion of kala-azar among clinically suspect patients is lower. In these areas, spleen puncture could be used for confirmation of the diagnosis or decision analysis strategies aiming at increasing the pre-test probability of kala-azar before initiating treatment could be evaluated and applied.

Both the InSure® dipstick and the DAT with a very low cut-off titre (1:400) have a negative predictive value of 89% and can be used at BPKIHS as fairly reliable screening tests. The proportion of kala-azar among clinical suspects is high at BPKIHS (76%) because of its status as a reference tertiary hospital and its situation in a low endemic area for malaria, a usual alternative diagnosis. Both tests are very sensitive and would perform much better as screening tests in areas where the proportion of kala-azar within patients tested is 50% or lower, a situation found in East Africa, for example, where malaria is much more frequent. For a proportion of 50%, for example, the negative predictive value of the dipstick and the DAT (cut-off titre 1:400) would be 96%.

None of the 184 patients included in this study were infected with HIV. The sensitivity of several serological tests being decreased in HIV–kala-azar co-infected patients (Rosenthal *et al.* 1995; Medrano *et al.* 1998), the performance of the DAT and the Insure® dipstick shown here should not be extrapolated to settings where HIV–kala-azar co-infection rate is high.

In summary, our results show that in Nepal, the potential of the first-generation Insure® dipstick test to be used as a screening test for VL in peripheral health structures is excellent. Patients with a positive dipstick could then be referred to secondary or tertiary hospitals for diagnosis confirmation (by bone marrow or spleen aspiration or by the DAT with a high cut-off titre) and for specific treatment.

Before definite guidelines of utilization of these tests in Nepal can be drawn, the performance of the currently produced second-generation Insure® dipstick needs to be prospectively assessed in clinical suspect patients. Such evaluation has just been completed in Nepal and results should be published soon. Moreover, the feasibility, reproducibility and predictive values of these tests, when performed in more peripheral health structures, are being assessed in a district hospital based study in the same region.

The ongoing fight led by numerous medical organizations for a better access in developing countries to essential drugs for diseases like AIDS, multidrug resistant tuberculosis, African trypanosomiasis or leishmaniasis should include the support of development and production of standardized, affordable, practical and reliable diagnostic tests. Both the lack of diagnosis of kala-azar, a deadly disease if left untreated, and the overdiagnosis of this condition, exposing patients to relatively toxic drugs, can have fatal consequences for the patients.

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