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A COMPARATIVE STUDY OF THE EFFECTIVENESS OF DIAGNOSTIC TESTS FOR VISCERAL LEISHMANIASIS

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Abstract. We compared the validity of pancytopenia, the formol-gel test (FGT), the indirect fluorescence antibody test (IFAT), the direct agglutination test (DAT), and the rK39 dipstick test as diagnostic criteria for visceral leishmaniasis (VL) in Nepal. Between September 2000 and January 2002, 310 clinical suspects had a bone marrow aspirate, and if negative, a spleen aspirate smear examined for *Leishmania donovani*. Sensitivity and specificity of all tests were determined compared with parasitology and by latent class analysis (LCA). Compared with parasitology, the sensitivities of the other tests were as follows: pancytopenia = 16.3% (95% confidence interval [CI] = 11.3–22.5%), FGT = 39.9% (95% CI = 32.7–47.4%), IFAT = 28.4% (95% CI = 22.0–35.5%), DAT = 95.1% (95% CI = 90.8–97.7%), and the rK39 dipstick test = 87.4% (95% CI = 81.7–91.9%). Sensitivity estimates obtained by LCA were similar, but specificity estimates were substantially higher (DAT = 93.7% versus 77.8%; rK39 dipstick test = 93.1% versus 77.0%). The DAT or the rK39 dipstick test can replace parasitology as the basis of a decision to treat VL in Nepalese peripheral health services.

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is considered as one of the most neglected tropical diseases.[1] Communities affected by kala-azar often live in remote areas and have poor access to health services. The need for innovation in VL chemotherapy and diagnosis was recently highlighted.[2] Treating patients on clinical presumption is inadequate because of the potentially serious side effects of current chemotherapy. Direct microscopic examination and/or culture of spleen tissue aspirate is the reference test for diagnostic confirmation, but this technique is hardly recommendable in the first-line health services in endemic areas. Alternatively, bone marrow and lymph node aspirates are used, but these methods are substantially less sensitive.[3] Past research aimed at developing a cheap and reliable serologic test that could replace parasitology. El Harith and others developed a direct agglutination test (DAT) in the 1980s and proposed it as a test for field use.[4] However, some investigators disagreed because of a presumably low specificity in the clinical setting.[5] The effectiveness of the DAT proved satisfactory in a large multi-center study.[6] More recently, high validity was reported for the rK39 dipstick test,[7] but this was contested soon after the initial report.[8,9] Moreover, commercial production of the dipstick test was interrupted several times.[9]

Diagnostic research in VL has been hampered by the lack of a gold standard. As Staquet and others[10] showed, a flawed reference test can substantially affect validity estimates of new tests under scrutiny. Thus, the published sensitivity and specificity estimates of serologic tests might be biased to some extent, depending on the controls and reference test used. Parasitology for VL is very specific, but unless spleen aspirates can be taken, its sensitivity is less than 90%.[3] The allegedly low specificity of serologic tests for VL, along with problems of regular supply, have so far prevented their introduction into routine health service practice. For example, in rural Nepal, most VL patients in peripheral health facilities are still treated on the basis of clinical suspicion and/or the result of a formol-gel test (FGT).[11]

To avoid biased estimates and needless discussions, a better and more standardized validation methodology is needed. Hadgu and Qu[12] suggested latent class analysis (LCA) could be a potential solution to the gold standard problem. This method of analysis is a mathematical technique that models associations between observed variables that imperfectly measure a non-observable (latent) variable.[13,14] When a diagnostic test is validated in a group of people, their true disease status can be considered as a latent variable with two mutually exclusive and exhaustive classes or categories: diseased and non-diseased. The LCA model estimates disease prevalence and sensitivity and specificity of all the diagnostic tests. So far, LCA has been used extensively in psychiatric research and in the social sciences, but experience in biomedical research is still limited. To clarify the current options in VL diagnostics in endemic areas, we validated three existing (FGT, DAT and an indirect fluorescence antibody test [IFAT]) and one novel serologic test (rK39), as well as a hematologic sign (pancytopenia) in a clinical care setting. We used classic contingency table analysis as well as LCA to corroborate the findings.

METHODS

Patients. The data were collected at the B.P. Koirala Institute of Health Sciences (BPKIHS) in Dharan in the Morang District of Nepal. This is a 648-bed tertiary care center serving the eastern region of Nepal, which includes VL-endemic areas. Patient recruitment aimed at enrolling at least 100 true cases of VL and at least 100 true non-cases to achieve adequate precision for the sensitivity and specificity estimates of the tests. Therefore, the total sample size to enroll was fixed at 300 clinical suspects given the expected prior probability of 70% VL cases in a group of presenting clinical VL suspects.[9] Patients were enrolled consecutively until the required sample size was achieved.

Clinical suspicion for VL was defined as a history of fever of 14 days or more and splenomegaly. Children of very young age were excluded. All clinically suspected adult and pediatric patients who presented to the Out-Patient Department and Emergency rooms between September 2000 and January 2002 were enrolled on the condition that they (or their guardian) gave informed consent.

A questionnaire with clinical and epidemiologic data was filled out for each patient at enrollment and diagnostic procedures were performed: bone marrow aspiration and the leishmanin skin test. Also, a venous blood sample and a urine sample was taken. If the bone marrow aspirate was negative, and malaria had been ruled out, a spleen aspirate was proposed. All VL patients were offered free treatment in accordance with current policy at BPKIHS and World Health Organization guidelines. The Institutional Review Board of the BPKIHS and of the Prince Leopold Institute of Medicine in Antwerp (ITMA) gave ethical clearance for the study.

Diagnostic criteria and tests. *Pancytopenia.* Pancytopenia was defined as a hemoglobin level < 11 g/dL, a white blood cell count < 4×10^9/L, and a platelet count < 100×10^9/L.

Formol-gel test. Twenty microliters of 40% formaldehyde (Glaxo India, Ltd., Bombay, India) was added to 200 μL of serum in a glass tube, and after 20 minutes the gelification reaction was visually assessed as positive or negative.

Indirect fluorescence antibody test. The IFAT was carried out at the Unidade de Leishmanioses of the Institute of Hygiene and Tropical Medicine in Lisbon, Portugal according to the procedure of Lanotte[15] using cultured promastigotes of *Leishmania (Leishmania) infantum* MON-1 as antigen. Washed promastigotes were dispensed on immunofluorescent microscope slides, air-dried and kept at -70°C until use. Total anti-human immunoglobulins conjugated with fluorescein isothiocyanate and diluted 1:100 (BioMérieux, Lyon, France) was used. A cut-off value ≥ 1:32 was used to establish a positive IFAT result for a child < 15 years old and a value ≥ 1:128 was used for adults.

Direct agglutination test. The DAT was performed at BPKIHS on 1 μL of serum according to standard procedures.[4,6] The antigen was produced at ITMA as follows. Promastigotes of the reference batch *L. (L.) donovani* 1-S were cultivated in a temperature-controlled cell shaker in glucose lactalbumin serum hemoglobin.[16] Log phase promastigotes were harvested and treated with 0.4% trypsin, fixed with 4% formaldehyde, stained with 0.025% Coomassie blue, and stored at 4°C until use. This DAT antigen was mixed in V-shaped well microtiter plates (Greiner-Bio One GmbH, Frickenhausen, Germany) with patient serum dilutions between 1:200 and 1:204,800, and after 12 hours the agglutination reaction was visually assessed against a white background. The end titer 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 titer > 1:3,200 was considered positive.

rK39 dipstick test. The rK39 dipstick test (lot numbers AF1008 and BF1019, InSure Rapid Test for Visceral Leishmaniasis®; InBios International, Seattle, WA) was performed at BPKIHS.[17] At room temperature, 30 μL of serum was added to 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 to the test tube. The results were read after five minutes and, if still negative, after 10 minutes. Even a weak band in the test region was considered a positive result. The test was repeated if the control line remained negative after 10 minutes.

Parasitology. Two independent readers at BPKIHS performed direct semi-quantified microscopic examination of methanol-fixed (10 minutes), Jenner-stained (five minutes), Giemsa-stained (15 minutes) smears of bone marrow or spleen aspirate. Two independent readers at ITMA performed quality control on 10% of the slides.

Statistical analysis. The data were processed with SPSS for Windows version 10.0.5 (SPSS, Inc., Chicago, IL), as well as with Latent Gold® version 2.0.18 (Statistical Innovations, Inc., Belmont, MA). Sensitivity and specificity were estimated by the classic validation method as well as by LCA modeling. We used parasitology as the reference test in the 2 × 2 contingency table: a patient with a positive bone marrow aspirate and/or spleen aspirate was considered a kala-azar case. In LCA, we started by fitting the basic two latent class model. Given a group of individuals with unknown disease status, for whom results from several diagnostic tests are available, LCA will model the probability of each combination of test results conditional on latent class, i.e., disease status. In basic latent class models, tests are taken to be conditionally independent (conditional on latent class), i.e., there are no associations between test errors within each category of the latent variable (diseased/not-diseased). Subsequently, more advanced models were fitted in which this condition was relaxed.[12,18] Model fit was assessed on the basis of the Bayes information criterion.[19] We report sensitivity and specificity of tests with an approximate 95% confidence interval (1.96 × SE) as estimated by the best model.

RESULTS

Between September 6, 2000 and December 23, 2001, 310 patients with febrile splenomegaly syndrome were enrolled in the study. Bone marrow aspirates were negative for 132. However, spleen aspirates could not be obtained in 101 (76.5%) because of contraindications or patient refusal. A diagnosis of kala-azar was reached by parasitologic confirmation in 184 (59.4%) of the 310: 178 by positive bone marrow and six by spleen aspirate.

Three confirmed VL syndromes were re-admissions of relapse cases. The 181 confirmed new kala-azar patients had a median age of 25 years (25th percentile = 13 years, 75th percentile = 36 years). The male:female ratio was 1.7:1. The median duration of fever at first admission was eight weeks (25th percentile = 3.5 weeks, 75th percentile = 11 weeks) and mean ± SD spleen size was 6.1 ± 3.7 cm below the costal margin. Anemia was present in 166 (91.7%) of 181, leukopenia in 104 (57.5%), and thrombocytopenia in 41 (22.7%). Pancytopenia was present in 29 (16%) of the 181 patients.

There were 126 febrile splenomegaly syndromes with a negative parasitology. For 13 (10.3%) of them, the clinicians indicated that kala-azar was the most probable diagnosis on the basis of the overall case history and clinical picture. In 91 (72.2%), an anatomopathologically or microbiologically confirmed alternative diagnosis was reached. For 20 (15.9%) patients, such an alternative diagnosis was deemed most likely on clinical grounds. Two patients left against medical advice before a final clinical diagnosis was reached.

The validity of pancytopenia and serologic tests as diagnostic markers for VL in the 310 febrile splenomegaly syndromes is shown in Table 1. When compared with the results of parasitology as the reference test, pancytopenia, the FGT, and the IFAT had low sensitivity, but very high specificity. The DAT and rK39 dipstick test had high sensitivity, but moderate specificity. The quality control done on the parasitology smears showed perfect concordance for grade 2+ or higher, but for smears with scanty parasites (grade 1), there was considerable discrepancy.

The validity of pancytopenia and diagnostic tests (including parasitology) as estimated by the best LCA model is shown in Table 1. This best model was reached in a modeling strategy comparing nine models (Table 2). A basic two latent class model did not fit the data very well because there was considerable residual correlation between the FGT and IFAT, between the IFAT and pancytopenia, and between the FGT and parasitology. Therefore, we fitted several other two latent class models including direct effects between pairs of tests.[18] Model 5, which included a direct effect between the FGT and IFAT, gave the best fit. The expected frequencies of diagnostic test patterns, as well as the probability of belonging to latent class VL predicted by this model, are shown in Table 3.

The LCA estimates for sensitivity of pancytopenia, the FGT, and IFAT, as estimated by the best-fitting LCA model, were low, whereas the DAT showed high sensitivity and the rK39 dipstick test and parasitology showed good sensitivity (Table 1). Almost exactly the same sensitivity estimates were obtained by both validation methods. The specificity of DAT and the rK39 dipstick test was good and substantially higher in LCA than when estimated by classic validation. The discriminatory power of each test based on LCA results is shown in Figure 1. The estimated prevalence of VL in this group of patients was 65.4% (n = 309) in LCA versus 59.2% (n = 310) according to parasitologic results only and 64.0% (n = 308) according to the clinicians' final diagnosis.

DISCUSSION

The main finding in our study was that the sensitivity and specificity of the DAT and rK39 dipstick test compared favorably in the diagnosis of VL with that of parasitologic examination of bone marrow combined with spleen aspirates. Moreover, our study illustrates the problems related to parasitology as a reference test for the validation of VL diagnostics.

To our knowledge, no study so far has addressed the performance of VL diagnostics by a comparative assessment on the same patients from an endemic area. Although our patients were certainly representative for the clinical suspects presenting to referral hospitals in eastern Nepal, this does not necessarily imply that frequency and stage of VL would be the same in patients presenting to first-line health services. Serologic test performance is known to be dependent on stage of the disease, so one must be careful in extrapolating results. The rK39 dipstick test format evaluated in this study was a prototype version distributed in 2000 by the company for research purposes only. Although similar to the currently commercialized version (Raychaudhury S, 2003, personal communication), discrepancies between our findings and those of other investigators[9] might be explained by the fact that they evaluated earlier prototypes.

Validation studies of VL diagnostics are usually based on parasitologic examination as a reference test. This should not be too problematic if spleen aspirates can be used for case ascertainment in all subjects, but this is rarely the case. In this tertiary care setting, it was not possible to obtain spleen aspirates for all patients with a negative bone marrow because of contraindications or patient refusal. When a reference test with sub-optimal sensitivity for case ascertainment is used, true VL cases are missed and therefore included in the group of controls. They will generate a positive result in any new test one wishes to evaluate (assuming this new test is 100% sensitive). For those cases, the new test is actually right while the reference test is wrong, and the specificity of the new test will thus be systematically underestimated. To prevent this kind of bias, our study included a multivariate analysis of the data by LCA, a technique based on loglinear modeling. LCA confirmed that parasitology, even though optimized by the combined use of bone marrow and spleen aspirates, had a lack of sensitivity as a reference test in this setting. As anticipated, the specificity estimates of the DAT and rK39 dipstick test were considerably higher in LCA than in classic validation. The specificity estimates of the IFAT and FGT were less affected because these tests were also less sensitive. Remarkably, LCA did not estimate the specificity of parasitology as 100% as one would expect on theoretical grounds. The reading of smears for *L. donovani* bodies is not easy. Our quality control showed discrepancies in the low-graded smears (grade 1), and some smears might actually have been wrongly taken as positive. This demonstrates again that parasitology cannot

TABLE 1

Validity of pancytopenia and diagnostic tests compared to parasitology, and as estimated by the best model in latent class analysis (CA)*

	Compared to parasitology†		LCA‡ (n = 309)§	
	Sensitivity % (n = 183)§	Specificity % (n = 126)	Sensitivity %	Specificity %
Pancytopenia	16.3 (11.3–22.5)¶	96.8 (92.1–99.1)	16.0 (10.9–21.1)	98.4 (95.8–100.0)
FGT	39.9 (32.7–47.4)	95.2 (89.9–98.2)	33.7 (26.5–40.9)	98.5 (95.8–100.0)
IFAT	28.4 (22.0–35.5)	94.4 (88.9–97.7)	30.0 (22.9–37.0)	98.3 (92.0–100.0)
DAT	95.1 (90.9–97.7)	77.8 (69.5–84.7)	96.9 (94.1–99.8)	93.7 (88.0–99.4)
RK39 dipstick test	87.4 (81.7–91.9)	77.0 (68.6–84.0)	90.1 (85.7–94.6)	93.1 (87.5–98.6)
Parasitology	Reference	Reference	88.1 (83.2–92.9)	94.8 (89.9–99.7)

* FGT = formol-gel test; IFAT = indirect fluorescence antibody test; DAT = direct agglutination test.
† Estimate with 95% exact binomial confidence interval.
‡ Estimate with approximate 95% confidence interval (1.96 × SE).
§ One serum sample was missing in the group with positive parasitology.
¶ n = 184.

TABLE 2
List of models fitted to the data*

Models	Number of latent classes†	LL	BIC	df	Model *P*
1 A, B, C, D, E, F	1	607.918	281.118	57	3.60×10^{-93}
2 X, A|X, B|X, C|X, D|X, E|X, F|X	2	85.675	–200.992	50	0.0013
3 X_3, A|X_3, B|X_3, C|X_3, D|X_3, E|X_3, F|X_3	3	44.6516	–201.882	43	0.4
4 X_4, A|X_4, B|X_4, C|X_4, D|X_4, E|X_4, F|X_4	4	30.6552	–175.745	36	0.72
5 X, A|X, BC|X, D|X, E|X, F|X	2	55.6076	–225.326	49	0.24
6 X, AC|X, BC|X, D|X, E|X, F|X	2	50.3012	–224.899	48	0.38
7 X, AC|X, BC|X, BF|X, D|X, E|X	2	46.0745	–223.393	47	0.51
8 X, AC|X, BC|X, BF|X, CE|X, D|X	2	39.7886	–223.945	46	0.73
9 X, AC|X, BC|X, BE|X, BF|X, CE|X, D|X	2	37.3878	–220.613	45	0.78

* LL = loglikelihood; BIC = Bayes information criterion; df = degrees of freedom; A = pancytopenia; B = FGT; C = IFAT; D = DAT; E = rk39 dipstick test; F = parasitology; X = latent variable (disease status); A|X = probability of test result A depends on disease status; BC|X = probability of test results B and C depends on disease status but also on direct effect between B and C. For definitions of other abbreviations, see Table 1.

claim the gold standard role, and the usefulness of complementary approaches such as LCA for validation.

Which of the five diagnostic markers we evaluated could then play a role in the first-line health services in this region of Nepal? Clinicians in tertiary hospitals rely on pancytopenia as a sign for VL when present in a case of febrile splenomegaly. Our data showed its specificity was extremely high, but unfortunately, its sensitivity was not. Furthermore, since laboratory infrastructure for hematology is limited in peripheral health services, this criterion might not be very helpful in the field. Interestingly, the specificity of the FGT was high in this study, but its sensitivity was low. The FGT is known to show positive results quite late in the disease process, and requires at least three months evolution, which may explain part of the low sensitivity in this study.

The performance of a MON-1/*L.infantum*-based IFAT using the same diagnostic cut-off titer as the reference values of the Portuguese laboratory showed low sensitivity in this area endemic for *L. donovani*, an observation that was already made in Sudan.[20] However, a receiver-operator characteristic analysis of the IFAT at several age-independent cut-off values showed good discrimination. An age-independent IFAT cut-off value for positivity ≥ 1:16 had a sensitivity of 87.4% (95% confidence interval [CI] = 81.7–91.9%) and a specificity of 77.0% (95% CI = 68.6–84.0%). Other investigators have reported comparable sensitivity but higher specificity of

TABLE 3
Observed and expected frequencies of tests patterns and probability of visceral leishmaniasis (VL) as estimated by latent class analysis model 5*

Pancytopenia	FGT	IFAT	DAT	Rk39 dipstick	Parasitology	Observed frequency	Estimated frequency	Probability of VL as estimated by model 5
–	–	–	–	–	–	88	85.9766	0.0004
–	–	–	–	–	+	4	4.9455	0.0479
–	–	–	–	+	–	6	6.7107	0.0436
–	–	–	–	+	+	2	2.5157	0.8602
–	–	–	+	–	–	6	6.8152	0.1477
–	–	–	+	–	+	13	7.7639	0.959
–	–	–	+	+	–	14	9.628	0.9549
–	–	–	+	+	+	63	68.0188	0.9997
–	–	+	–	+	–	1	0.0974	0.5228
–	–	+	+	–	–	1	0.2173	0.8064
–	–	+	+	+	+	12	11.8322	1
–	+	–	–	–	–	1	0.921	0.0125
–	+	–	+	–	+	2	2.6889	0.9987
–	+	–	+	+	–	2	3.3207	0.9986
–	+	–	+	+	+	30	24.5246	1
–	+	+	–	–	+	1	0.0919	0.9763
–	+	+	–	+	–	1	0.1138	0.9739
–	+	+	+	+	–	2	3.4831	0.9999
–	+	+	+	+	+	26	25.7573	1
+	–	–	–	–	–	1	1.3781	0.0044
+	–	–	–	–	+	1	0.1204	0.3759
+	–	–	–	+	+	1	0.4191	0.9866
+	–	–	+	–	+	1	1.4278	0.9964
+	–	–	+	+	–	1	1.7637	0.9961
+	–	–	+	+	+	10	12.9926	1
+	–	+	+	+	–	2	0.3058	0.9998
+	–	+	+	+	+	3	2.2608	1
+	+	–	+	+	+	4	4.686	1
+	+	+	+	–	+	1	0.5389	1
+	+	+	+	+	+	9	4.9216	1

* For definitions of other abbreviations, see Table 1.

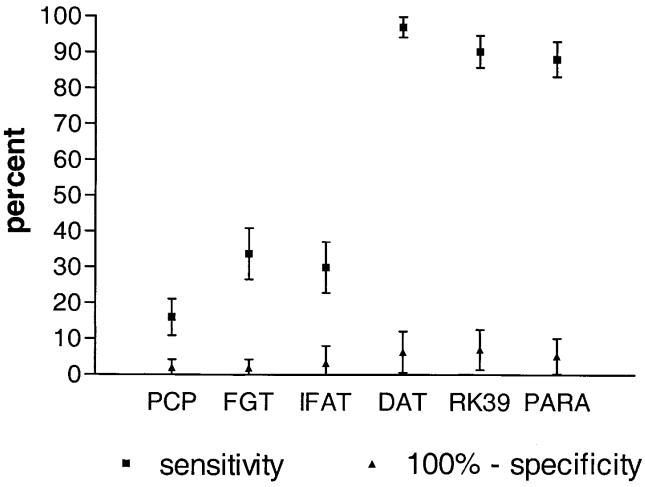

FIGURE 1. Sensitivity (%) and 100% - specificity of several diagnostic markers for visceral leishmaniasis. PCP = pancytopenia; FGT = formol-gel test; IFAT = indirect fluorescence antibody test; DAT = direct agglutination test; RK39 = rK39 dipstick test; PARA = parasitology. Bars show the 95% confidence intervals.

the IFAT when used in areas endemic for *L. infantum*.[21,22] Because the IFAT cannot be regarded as an option for peripheral health services in Nepal because of technologic constraints, we did not explore this further.

This study corroborated the known high sensitivity and the more than acceptable specificity of the DAT.[6] The features of the rK39 dipstick test format and of parasitology were found comparable to DAT in this study. In peripheral health services, the DAT or rK39 dipstick test could replace parasitology as the basis of a decision to treat, if one looks only at the validity criteria, while simplicity of use favors the dipstick format. However, deciding which test to include in diagnostic algorithms for VL should include other criteria, such as disease prevalence and health service context, as well as test reproducibility, cost, and sustainability.

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