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Latent class analysis permits unbiased estimates of the validity of DAT for the diagnosis of visceral leishmaniasis

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Summary

BACKGROUND Substantial uncertainty surrounds the specificity of the Direct Agglutination Test (DAT) for visceral leishmaniasis (VL) in clinical suspects, since no good gold standard exists for unequivocally identifying diseased subjects. We explored the Latent Class Analysis (LCA) modelling technique to circumvent this problem.

PATIENTS AND METHODS Data on 149 clinical suspects recruited in 1993–96 during a multicentre study in Sudan were re-examined. Clinical data, lymph node and bone marrow aspirate and DAT results were available. IFAT was performed in 1997 on stored filter paper blood of 80 individuals. Classical Validity Analysis (CVA) in a 2×2 contingency table with parasitology as a gold standard was compared with the parameter estimates produced by the best fitting LCA model.

RESULTS The sensitivity estimates of DAT produced by CVA (98% (89%-100%)) were almost exactly reproduced by LCA. The specificity estimates by LCA were substantially higher than those obtained in CVA. Specificity of DAT depended, however, on whether the subject was treated for VL before. In subjects without prior treatment, CVA estimated DAT specificity at 68% (56% – 79%), whereas LCA estimated it at 85% (63% – 100%).

CONCLUSION LCA modelling proved a useful tool, as it gave consistent estimates of test characteristics and allowed for control of confounding factors and interaction effects. Since VL is a life-threatening disease for which expensive but effective and safe treatment exists, a clinical suspect in an endemic area should be treated on the basis of a positive DAT result.

keywords visceral leishmaniasis, direct agglutination test, sensitivity, specificity, latent class analysis

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Introduction

The current diagnostic reference test for Visceral Leishmaniasis (VL) is parasitological evidence obtained in direct microscopic examination or culture of tissue aspirates (Bryceson 1996; World Health Organization and Overseas Development Administration 1996). Conditional on flawless technical execution, the parasitological tests are very specific (100%), but their sensitivity varies between 50% and 60% in aspirates of enlarged lymph nodes, between 60% and 80% in bone marrow aspirates to 95% in the less innocuous splenic aspirate (Zijlstra *et al*. 1992; Bryceson 1996; Thakur 1997). Clinicians are urged to repeat aspirations from different tissues before withholding treatment from a clinical suspect (Zijlstra *et al*. 1991). In endemic areas, however, multiple aspirates and particularly splenic aspirates are hardly feasible. The latter require a prior check of prothrombin time and platelet count, and after the procedure the patient has to be observed in the recumbent position for at least 8 h in a facility where blood transfusion and splenectomy is readily available (Bryceson 1996). Moreover, splenic aspiration is not possible in individuals without palpable spleen (Zijlstra *et al*. 1992)

or restless children, and contraindicated in very anaemic persons.

A sensitive serological test with sufficiently high specificity to make it fit to replace parasitology would thus be a major advance for clinical practice and for VL control, with the additional advantage of being less invasive for the patient. The Direct Agglutination Test (DAT) seemed promising in this respect by virtue of its high sensitivity and ease of use under field conditions (El Harith *et al*. 1986; 1988) Several authors also reported high specificity of DAT (in the range of 90%–100%) on healthy controls from endemic areas (Abdel Hameed *et al*. 1989; Schaefer *et al*. 1995; Shiddo *et al*. 1995; Boelaert *et al*. 1999a). The healthy controls in these studies were, however, not representative for the clinical setting. In clinical suspects with negative parasitology, Zijlstra *et al*. (1991) estimated the specificity of DAT at 72%. As a consequence, clinicians do not consider a positive DAT result sufficient evidence for starting treatment. Even during epidemics, when the positive predictive value of the test is higher compared to the one in endemic areas (due to the higher prevalence) and the workload is huge, empirical diagnostic strategies combining DAT and parasitology have been used for fear of reliance on DAT only (Seaman, personal communication).

DAT validation studies in clinical suspects suffer from another inherent methodological problem: parasitology is not a gold standard for VL (Boelaert *et al*. 1999a). Whereas parasitological evidence can be considered 100% specific, its sensitivity is definitely below 100%, and a negative parasitological result in a clinical suspect does not declare a person unequivocally negative for VL. Validation studies based on parasitology as a reference test thus suffer from bias, as parasitologically negative VL cases in the control series cause systematic underestimation of the specificity of the diagnostic tests under scrutiny.

We explored different methods to estimate the specificity of DAT in a sample of clinical VL suspects. Our objective was to assess whether a mathematical modelling technique, Latent Class Analysis (LCA), could circumvent the gold standard problem, and thus reduce uncertainty regarding DAT specificity in the clinical setting.

Patients and methods

Patients and technical examinations

A detailed description of the methods can be found in Boelaert *et al*. (1999b). In summary, we enrolled, in 4 successive rounds between 1993 and 1996, 149 clinical suspects at the Ban Dighaiw study site in Gedaref State, Sudan (Boelaert *et al*. 1999a). The definition of 'clinical suspect' was: 'a person with fever of more than two weeks duration with either enlarged lymph nodes or splenomegaly at the exclusion of thick-film confirmed malaria cases'. For each clinical suspect, clinical history and examination was recorded prior to bone marrow and lymph node aspiration and a sample of finger-prick blood was collected on Whatman 3 filter paper. Age was recoded in three groups: 0–4 years, 4–14 years and 15 and above. 'Prior treatment' was defined as reportedly ever having been treated for visceral leishmaniasis, regardless of drug, dose and duration of treatment.

Parasitology smears were examined at the laboratory of the University of Khartoum. A parasitologically positive person was defined as 'a clinical suspect with a positive lymph node and/or bone marrow smear'. DAT with *Leishmania donovani* 1-S was performed on whole blood eluted from the filter paper samples at the Protozoology Laboratory of the Institute of Tropical Medicine in Antwerp (Boelaert *et al*. 1999a) and a cut-off of $> 1:4000$ final blood dilution (corresponding to 1 : 6000 serum dilution) was used to establish a positive diagnosis. The Immunofluorescence Antibody Test (IFAT) (Abranches 1984) (Pellegrino & Brener 1958), using cultured promastigotes of *Leishmania infantum* MON-1 as antigen, was performed on eluted filter paper blood at the Unidade de Leishmanioses, Instituto de Higiene e Medicina Tropical, Lisboa, Portugal. A cut-off of $\geq 1:50$ was used to establish a positive IFAT result. The cut-off points for DAT and IFAT were based on the cut-offs recommended in the literature.

Data analysis

First we estimated sensitivity and specificity of DAT in a 2×2 contingency table with parasitology as the reference test and computed exact binomial 95% Confidence Intervals (CI). Subsequently, we analysed the data set with Latent Class Analysis (LCA), assuming that the clinical suspects belonged to either one of two latent classes: persons with VL and persons without VL.

LCA is a mathematical technique that models associations between observed variables that imperfectly measure a nonobservable (latent) variable (Goodman 1974b; Heinen 1996). In basic latent class models, the observed variables are assumed to be independent conditional on latent class, i.e. there are no associations between the observed variables within each category of the latent variable. More advanced models exist, where this condition is relaxed (Hagenaars 1988; Hadgu & Qu 1998).

When attempting to validate a diagnostic test on a group of people, their true disease status can be considered a latent variable with two mutually exclusive and exhaustive classes or categories, 'diseased' and 'nondiseased'. Given a group of individuals with unknown disease status, for whom results from several diagnostic tests are available, LCA will model

Table 1 Frequency of test results in clinical suspects* according to reported prior treatment (regardless of dose and duration)

Parasitology		Not reporting prior treatment Positive	Not reporting prior treatment Negative	Reporting prior treatment Positive	Reporting prior treatment Negative	All suspects Positive	All suspects Negative
DAT Positive							
IFAT	Positive	7	2	5	4	12	6
	Negative	15	13	5	7	20	20
	Missing	8	8	5	7	13	15
	Subtotal	30	23	15	18	45	41
DAT Negative							
IFAT	Positive	–	–	–	–	–	–
	Negative	1	19	–	2	1	21
	Missing	–	30	–	7	–	37
	Subtotal	1	49	—	9	1	58
Total		31	72	15	27	46	99

*Case definition see text.

the probability of each combination of test results conditional on latent class, i.e. disease status. The LCA model hence produces an estimate of disease prevalence and of sensitivity and specificity of all the diagnostic tests.

A series of multigroup loglinear latent class models were fitted with the LEM package (Vermunt 1997, unpublished), including the latent variable 'VL disease' (X), 3 observed diagnostic test variables: IFAT, parasitology (para), DAT and, to control for possible confounding, three observed external variables: prior treatment, sex and age group. Models were compared by the difference in likelihood statistic (G^2) if nested (Heinen 1996), and by Akaike's Information Criterion (AIC) (Akaike 1987). Goodman's convention was applied in case boundary parameter estimates (0 or 1) were obtained: the number of degrees of freedom was increased with the number of estimated boundary parameters (Goodman 1974a). An approximate 95% CI was computed for the sensitivity and specificity LCA estimates as the interval lying within ± 1.96* Standard Error of the estimate.

For this analysis, 4 clinical suspects were excluded: one because of a missing parasitology result and three others because they were found parasitologically positive at a follow-up visit. IFAT results were not available for 65 filter papers of the first lot collected. The hypotheses that IFAT data were missing completely at random (MCAR), missing at random (MAR) or were missing because of a nonignorable mechanism of nonresponse were tested through a comparison of the corresponding loglinear models.

Results

Forty-six of the 145 clinical suspects retained in the analysis (31.7%) had positive parasitology, and 45 out of these 46 (97.8%) were positive on DAT (Table 1). Only 58 of 99 suspects with negative parasitology (58.6%) were also negative on DAT. Of 41 parasitologically negative but DAT positive clinical suspects, 6 were IFAT positive, 20 were IFAT negative and for 15 no IFAT results were available. Tables 2 and 3 show the distribution of separate test results according to sex and age. No significant association was found between any of the test results and sex or age. Forty-three (29.7%) of the clinical suspects reported prior treatment for VL, and prior treatment was homogeneously distributed across sex and age groups.

Table 2 Positive test results and prior treatment according to sex

	Percentage positive		
	Male	Female	*P*-value *
Parasitology	31.4% ($n = 86$)	32.2% ($n = 59$)	1
DAT	59.3% ($n = 86$)	59.3% ($n = 59$)	1
IFAT	15.7% ($n = 51$)	34.5% ($n = 29$)	0.09
Prior Treatment	29.1% ($n = 86$)	30.5% ($n = 59$)	0.86

* *P*-value for two-sided Fisher Exact test.

Table 3 Age distribution according to test results and prior treatment

	Median Age (quartile 1, quartile 3)		
	Test Positive	Test Negative	*P*-value *
Parasitology ($n = 145$)	10 (5, 12)	6 (3, 10)	0.08
DAT ($n = 145$)	7 (4, 11)	7 (3, 11)	0.48
IFAT ($n = 80$)	10 (4, 13)	7 (4, 10.5)	0.24
Prior Treatment ($n = 145$)	8 (4.5, 11)	6 (3, 10)	0.35

**P*-value for two-sided Mann–Whitney test.

Table 4 Distribution of DAT positivity by sex, age, prior treatment and IFAT result in the group of parasitologically negative subjects ($n = 99$)

	Frequency of DAT positivity	RR	*P*-value
Sex			
Male ($n = 59$)	42%	1.1	0.81
Female ($n = 40$)	40%		
Age group			
> 5 years ($n = 59$)	42%	1.2	0.30
0–4 years ($n = 34$)	35%		
Prior treatment			
Yes ($n = 72$)	67%	2.1	0.002
No ($n = 27$)	32%		
IFAT			
Positive ($n = 6$)	100%	2.0	0.002
Negative ($n = 41$)	49%		

Investigating the group of parasitologically negative clinical suspects, we found no association between DAT positivity and sex or age, but the phenomenon was twice as frequent in subjects who reported prior treatment for VL ($P = 0.002$) (Table 4).

A selection of the best fitting LCA models is shown in Table 5. Model 1 assumes a 2 Latent Class model making the strongest possible assumption on the missing IFAT data, i.e. that they were missing completely at random (MCAR). However, the MCAR assumption was rejected by the data, as several missing at random (MAR) models (2–4) provided a significantly better fit than model 1. On the basis of lowest AIC, the MAR pattern of model 3 was used in the subsequent modelling. This pattern states that there was a different proportion of DAT-positive subjects in the group without IFAT results ($n = 65$) compared to the group with ($n = 80$), but that the event of a missing IFAT result was ignorable, i.e. did not depend on the actual value of the IFAT result.

Model 3 explained the associations in the data set remarkably well (*P*-value for the G^2 test: 0.96), but a significant improvement in fit was obtained by including a term for the dependence of the (prevalence of) disease on prior treatment (model 5). The difference in G^2 between model 5 and 3 was 8.1 for 1 d.o.f.; $P < 0.05$. Models specifying that the disease was dependent on sex or age provided no better fit (not shown).

As the bivariate analysis indicated twofold higher IFAT positivity in females compared to males, we fitted a model specifying that the sensitivity and specificity of IFAT depended on sex (model 6). This model was not significantly better than model 5. In the same way, we specified model 7, indicating that the sensitivity and specificity of DAT depended on whether the subject had prior treatment or not. Model 8 combined both the dependence of IFAT on sex and the dependence of DAT on prior treatment. Although model 7 did not fit the data significantly better than model 5 (difference in $G^2 = 2.6$ for 1 d.o.f., p 0.11) and model 5 remained statistically speaking preferable on the basis of AIC, we decided to retain model 7 as the 'best' one, because it included the dependence of DAT on prior treatment, which was detected in the bivariate analysis and is furthermore biologically very plausible since DAT becomes very slowly negative after treatment.

Table 6 gives an overview of test characteristics as estimated in classical validity analysis (CVA) in 2×2 tables with parasitology as a gold standard and in LCA (model 7). The CVA sensitivity estimates of DAT and IFAT were almost exactly reproduced in LCA, whereas the specificity estimates for DAT as well as for IFAT were substantially higher. LCA estimated specificity of DAT at 85% (63%–100%) for subjects without prior treatment and at 58% (15%–100%) for subjects with prior treatment. The sensitivity of parasitology in LCA was estimated at 64% (42%–86%).

Table 5 Comparison of fitted models

Model	G^2	D.o.f.	*P*-value	AIC
1. X, IFAT|X, para|X, DAT|X, treatment, sex, age, R	117.3	133	0.832	1059.4
2. X, IFAT|X, para|X, DAT|X, treatment, sex, age, R{para}	109.6	132	0.923	1053.7
3. X, IFAT|X, para|X, DAT|X, treatment, sex, age, R{DAT}	104.3	132	0.964	1048.4
4. X, IFAT|X, para|X, DAT|X, treatment, sex, age, R{para, DAT}	103.2	131	0.963	1049.2
5. X| treatment, IFAT|X, para|X, DAT|X, treatment, sex, age, R{DAT}	96.2	131	0.990	1042.3
6. X| treatment, IFAT|X*sex, para|X, DAT|X, treatment,sex, age,R{DAT}	93.5	129	0.992	1043.6
7. X| treatment, IFAT|X, para|X, DAT|X* treatment, treatment,sex, age,R{DAT}	93.6	130	0.993	1043.7
8. X| treatment, IFAT|X* sex, para|X, DAT|X*treatment, treatment, sex, age, R{DAT}	90.9	129	0.996	1045.0

X, latent variable (VL disease); para, parasitology; R, Missing Completely at Random – pattern; R {}, Missing at Random pattern; G^2, goodness of fit likelihood ratio chi square statistic; D.o.f., degree of freedom, number adjusted for number of estimated boundary parameters; AIC, Akaike's Information Criterium.

Table 6 Test characteristics (95% C.I.) as estimated by classical validity analysis (CVA)* and through final LCA -model 7 ($n = 145$)

	Prior treatment	DAT	IFAT	Parasitology
Sensitivity				
CVA	Without	1 (0.88–1)		
	With	0.94 (0.70–1)		
	Both strata	0.98 (0.89–1)	0.37 (0.20–0.55)	1**
LCA	Without	1 (1–1)		
	With	0.95 (0.85 –1)		
	Both strata	0.98	0.36 (0.20–0.53)	0.67 (0.46–0.85)
Specificity				
CVA	Without	0.68 (0.56–0.79)		
	With	0.33 (0.17–0.54)		
	Both strata	0.59 (0.48–0. 68)	0.87 (0.74–0.95)	1**
LCA	Without	0.85 (0.63–1)		
	With	0.58 (0.15–1)		
	Both strata	0.79	1 (1–1)	1 (1–1)

* in 2 × 2 contingency tables taking parasitology results as the gold standard ** by definition.

Discussion

The issue of DAT specificity is crucial for clinicians who have to decide on whether or not to administer a potentially life-saving but cumbersome and costly treatment to a person in whom they suspect VL on clinical grounds. Whereas the specificity of DAT established on a series of 176 healthy controls living in the same area as the parasitologically proven cases was high, 99% (97%–100%) (Boelaert *et al*. 1999a), it gave remarkably lower results, 59% (48%–68%), when assessed with classical validation analysis on the present series of parasitologically negative clinical suspects. Although the spectrum of patients is known to influence the characteristics of a test (Begg 1987; Valenstein 1990), specificity of DAT in clinical suspects is necessarily underestimated when comparing it to parasitology as a reference test. While the sensitivity of a diagnostic test will be correctly estimated when validating it against a 100% specific reference test, its specificity will be underestimated if the reference test is less than 100% sensitive (Staquet *et al*. 1981). Staquet *et al*. (1981) also showed that the real specificity of a diagnostic test in such a situation should be somewhere between the observed specificity, 59% in this case, and 100%. Without knowledge of the sensitivity of parasitology one can, however, not directly estimate the true specificity of DAT.

Researchers have tried to address the gold standard problem through discrepant analysis. Hadgu and Miller convincingly demonstrated it to be a biased method that should not be used in test validation (Hadgu 1996; Miller 1998). In this study, we circumvented the problem with LCA. The parameter estimates obtained in the different models we fitted were quite stable, which coincided with earlier observations on LCA robustness (Boelaert unpublished observation). All fitted models produced DAT sensitivity estimates near 100% and specificity estimates that were in the range of 78–90%. The final selection of the 'best' model was based on statistical criteria and on biological plausibility. It included dependence of both the disease and the DAT result on prior treatment. Though statistically not better-fitting than the model without the dependence of DAT on disease, it was preferred because while all serological tests have been shown to slowly become negative over the first months after treatment, this phenomenon lasts longer for DAT (Hailu 1990).

The final model reproduced almost exactly the estimates of DAT and IFAT sensitivity obtained in classical validation: excellent for DAT but low for IFAT. The latter problem might be related to the use of a heterologous antigen, or aged filter paper blood samples, or possibly to nonspecific immunosuppression in patients suffering of malnutrition and/or infections. In fact, in Sudan, *Leishmania* strains isolated from VL patients were identified as zymodemes MON-18, MON-30 and MON-82 (Ashford *et al*. 1992; Ibrahim *et al*. 1995; Oskam *et al*. 1998) and not *L. infantum* MON-1 used as antigen in this study. Also, in previous studies IFAT showed a sensitivity of 83.3% in immunocompetent people with VL, but in children and immunocompromised patients sensitivity values decreased to 65.0% and 36.8%, respectively (Campino & Abranches 1991; Campino *et al*. 1997).

The LCA specificity estimates of both serological tests were higher than those obtained in the contingency table analysis, which is in accordance with theoretical expectations. Specificity of DAT in clinical suspects with prior treatment was rather low, but it proved high in those without prior treatment. As the Ban-Dighaiw community had almost no access to professional VL treatment before the study, reports of prior VL treatment have to be interpreted with caution.

The area lacks a permanent health facility or VL control program, there is a high demand for VL treatment, but the price of a full drug course imposes a financial barrier and expired, diluted or counterfeit stibogluconate circulates. This leads us to attribute the positive association between reported prior treatment and DAT (as well as between prior treatment and positive parasitology) as most likely due to inadequate previous treatment. Notwithstanding, our estimates of DAT specificity in the subgroup who did not report prior treatment coincide remarkably well with findings reported in the study cited above (Zijlstra *et al*. 1991): he estimated DAT specificity at 72% (47/65) in a group of parasitologically negative clinical suspects. Zijlstra's gold standard was a combination of evidence from all possible smears, and his specificity estimate must hence be an underestimate. Indeed, he investigated the 18 DAT positive/parasitologically negative subjects further after treating them anyhow and subsequently reported that 3 persons showed a dramatically positive response to treatment and that one developed parasitologically proven PKDL. We can thus safely assume that these 4 clinical suspects were true VL cases. Furthermore, the group of 18 contained 6 leishmanin skin test positive persons, which indicates past or subclinical infection. Exclusion of these 4 + 6 individuals leads to an estimate of DAT specificity of 85% (47/55) in clinical suspects without evidence of past infection.

We acknowledge that the data available in our study had limitations: the relatively small sample size and randomly missing information for part of the IFAT results, but LCA provided a way to control for this as well as for confounding factors and produced consistent estimates of the test characteristics. Sample size, at any rate, only influences precision but not the validity of the estimates, which proved quite robust over models. Nevertheless, it seems worthwhile to corroborate our findings on a larger and fully documented study group, preferably in another ecological context, and to pay particular attention to the influence of prior treatment and past infection with *Leishmania sp*. on the specificity of DAT.

In conclusion, mathematical modelling through LCA proved a useful tool for validation research. Our results indicate that DAT is 100% sensitive and 85% specific in clinical suspects who did not report prior VL treatment. The current diagnostic-therapeutic algorithm for VL in endemic areas is based on parasitology that is either unfeasible in the peripheral health service (splenic aspirate) or lacks sensitivity (bone marrow/lymph node aspirate). Since VL is a life-threatening disease for which an expensive but effective and safe treatment exists, serological tests such as the DAT, with very high sensitivity and sufficient specificity, would constitute a valuable alternative for parasitology. We recommend that the diagnostic-therapeutic guidelines for VL be revised in the light of this evidence.

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