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Comparative evaluation of parasitology and serological tests in the diagnosis of visceral leishmaniasis in India: a phase III diagnostic accuracy study

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Summary In this phase III trial for diagnostics for visceral leishmaniasis (VL) in India, we compared parasitological diagnosis with several serological tests: direct agglutination test (freeze dried; DAT-FD), rK-39 strip test, rK-26 strip test and a latex agglutination test for antigen detection in urine (KAtex) in 452 subjects from the endemic regions of Bihar, India. The subjects were segregated into four categories: 230 confirmed patients, 52 probable cases, 70 non-cases and 100 healthy endemic controls. The first two groups were used for estimating sensitivity, the latter two for specificity. Sensitivity of DAT-FD was 98.9%, rK-39: 98.9%, KAtex: 67.0% and rK-26: 21.3%. Sensitivity of DAT-FD on blood taken on filter paper (DAT-FDF) was 99.3%, which was comparable with that using serum. Specificity of serological tests was comparable and high (DAT-FD and DAT-FDF: 94%, rK-39 strip test: 97%, KAtex: 99% and rK-26 strip test: 100%). The classical 'gold standard' parasitological demonstration in splenic smear performed poorly as it missed 18.4% of cases that benefited from VL treatment. Reproducibility of the serological tests between field and central laboratories was excellent ($\kappa = 1.0$, 0.99, 0.96 and 0.94 respectively for microscopy, DAT-FD, rK-39 strip test and rK-26 strip test). A high degree of agreement was observed between DAT-FD and rK-39 strip test ($\kappa = 0.986$). Although DAT-FD and rK-39 strip test were highly sensitive with excellent specificity, the ease of use of the latter makes it most suitable for the diagnosis of VL in the field conditions.

keywords visceral leishmaniasis, direct agglutination test, rK-39 strip test, rK-26 strip test, KAtex, diagnostic accuracy

Introduction

The governments of three visceral leishmaniasis (VL) affected countries in the Indian subcontinent have recently joined forces in the elimination of the disease from the region by 2015 (TDR 2005). Because of the less optimal health infrastructure and lack of tools for diagnosis and treatment, this task seems daunting. The primary health centres or subcentres are ill-equipped; skilled personnel is often not available, and laboratory diagnosis suffers from an erratic electricity supply with hours, sometimes days, of power cuts. Adequate diagnostic technology is critical in these difficult areas as the toxicity and complexity of current VL drugs makes an accurate diagnosis mandatory (Sundar *et al.* 1991).

Demonstration of parasites in splenic or bone marrow smears is considered the gold standard. However, splenic smears – though considered to be highly sensitive – are fraught with the danger of serious/fatal haemorrhage and the sensitivity of bone marrow smear is quite low. For want of a simple test, the diagnosis of VL is often delayed in routine clinical care in endemic areas (Sundar & Rai 2002). Introduction of sound diagnostic algorithms using tools applicable under difficult field conditions of endemic regions is of paramount importance for achieving control of the disease (Guerin *et al.* 2002).

Several less invasive methods for diagnosis of VL have been developed in the recent past. These include an improved version of the direct agglutination test (DAT) using freeze dried antigen (Meredith *et al.* 1995; Sundar *et al.* 2006) and a rapid immunochromatographic test based on a recombinant 39-amino acid repeat antigen, conserved in the kinesin region of *Leishmania chagasi* and *Leishmania donovani* (rK-39 strip test) (Sundar *et al.*

1998a; Goswami *et al.* 2003), a similar strip test based on a recombinant 26 kDa protein (rK-26 strip test) (Bhatia *et al.* 1999), and a latex agglutination based on the detection of a heat stable carbohydrate leishmanial antigen in urine of VL patients (KAtex) (Attar *et al.* 2001; Hommel *et al.* 2001). However, none of these tests have been properly evaluated side by side to provide conclusive evidence for adoption in the field. The UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR/WHO) commissioned two studies, one in Africa involving Ethiopia, Kenya and Sudan, and another in the Indian subcontinent in India (multicentre) and Nepal for comparative evaluation of the serological tests (named above) with parasitological diagnosis in field settings. As the aim of the study was to produce recommendations for field use of these diagnostics, a phase-3 design for diagnostic accuracy studies was adopted, requiring to recruit patients as representative as possible for the clinical situation in which the diagnostics should be used in the future, in this case patients with clinical signs and symptoms of VL disease, further called 'clinical suspects' (Zhou *et al.* 2002). We report the results of this multicentre study from India.

Methods

Study site and sample size

The study was conducted in two endemic areas in Bihar state: Muzaffarpur and Patna. All 'clinical suspect' patients were consecutively recruited at the outpatient departments of the Kala-azar Medical Research Center (KAMRC), Rambag, Muzaffarpur and at the Rajendra Memorial Research Institute of Medical Sciences (RMRI), Patna between May 2005 and August 2005 until the sample size ($n = 350$) had been reached. Healthy controls ($n = 100$) with no obvious signs and symptoms of any disease, and living in the endemic regions for VL, were recruited at KAMRC, Muzaffarpur only. The Ethics Committee of the respective institutions and of TDR approved the protocol, and a written informed consent was obtained from every subject included in the study.

Inclusion and exclusion criteria

From May 2005 onwards all eligible adult and paediatric patients who gave informed consent were consecutively enrolled in the study until completion of prior determined sample size. All clinically suspects presenting to the outpatient departments of KAMRC or RMRI with a history of fever of 2 weeks duration or more and with splenomegaly were considered as eligible for the study. Patients below the age of 1 year, with past kala-azar history, positive HIV serology or positive pregnancy test and those who refused to give consent for study were excluded.

Sample and data collection

For every patient enrolled, the following laboratory investigations were performed: haemoglobin, white blood cell count, platelets, prothrombin time with International Normalized Ratio (INR), peripheral blood smear, serum bilirubin, alanine aminotransferase, aspartate aminotransferase, serum creatinine, serum sodium and potassium, thick film and thin smear for malaria, electrocardiogram and acid fast staining of sputum if pulmonary tuberculosis was suspected. After baseline diagnostic work up, patients with suspected VL underwent splenic aspiration and smear examination. Blood was collected for serological tests and 5 ml urine for KAtex® (Kalon Biological, Aldershot, UK).

Serum (1.5 ml) was collected in a 1.8-ml cryogenic vial (Corning®) and frozen for testing at the central laboratory at Banaras Hindu University (BHU), Varanasi. One spot of blood was collected on a filter paper and sent to the BHU laboratory for an assessment of the reproducibility of the DAT-FDF executed on serum compared with filter paper blood. No repeat examination of KAtex was contemplated and thus urine was not preserved. Data were handled confidentially.

Laboratory procedures

The direct microscopic examination of the tissue aspirate, DAT-FD, rK39 and rK26 strip tests and the urine test were performed at the peripheral study site under the field conditions. Splenic smears were stained with Giemsa stain, and read in a standardized way under 10×100 magnification, and graded on a scale from 0 to 6+. If the number of amastigotes counted per field was >100, 10–100 or 1–10, it was graded as 6+, 5+ and 4+, respectively. Similarly, 1–10 amastigotes in 10, 100 or 1000 fields were graded as 3+, 2+ and 1+, respectively (Chulay & Bryceson 1983).

Freeze-dried DAT (DAT-FD) kits were procured from the Koninklijk Instituut voor de Tropen, Amsterdam to be used at all centres and performed as per standard protocol. DAT-FDF was performed according to the protocol of DAT-FD where a round 5-mm punched filter paper with dried blot blood was kept overnight in DAT diluent for elution. A sample was considered positive if it had a titre ≥1:3200, the cut off value of DAT. rK-39 and rK-26 strips (InBios®, Seattle, USA) tests were performed on 30 μl of serum or one drop of blood (fingerstick). For latex antigen agglutination test in urine, the KAtex test was performed as per manufacturer instructions.

The DAT-FD, the rK-39, rK-26 strip and the KAtex tests were done twice independently on each patient sample in the standardized conditions in the field laboratories, by senior laboratory technicians who were blinded to all the previously obtained data on the patients.

Reference standard for data analysis

Sensitivity. As sensitivity of parasitological smears is known to be suboptimal, we used a composite reference standard for sensitivity (Alonzo & Pepe 1999). We defined as a VL case two classes of patients: (i) clinical suspects with positive parasitology in splenic smears were considered as 'confirmed VL cases'; and ii) clinical suspects with typical features of VL (fever, splenomegaly and pancytopenia) but negative parasitology. If the latter had a positive DAT performed with aqueous antigen and showed response to antileishmanial treatment (resolution of fever, spleen regression and improvement of haematological parameters within 2 weeks from the onset of treatment) they were considered as 'probable VL cases'. Both categories qualified as cases and were included in the group to estimate the sensitivity. All the patients were treated with 15 infusions of amphotericin B deoxycholate in the doses of 1.0 mg/kg diluted in 5% dextrose solution administered on alternate days.

Specificity. Specificity was estimated in two different control groups: the non-cases (i.e. those clinically suspect patients from the consecutively enrolled series in whom the disease was ruled out) and the healthy endemic controls. The following definitions apply.

Non-cases. Clinical suspects with negative parasitology and with alternative diagnosis who stay asymptomatic for VL during 6 months of follow up were the 'non-cases' in this study.

Healthy controls. Fellow villagers, friends or family members visiting the patients in the hospital were informed about the study. If they consented to participate in the study, after a thorough physical examination, healthy subjects, without any signs and symptoms of any illness and living in the endemic region, were enrolled.

Statistical analysis

Sensitivity was calculated as the percentage of those test positive in the group of VL cases [i.e. those parasitology positive (confirmed) and in those with positive response to therapy (probable)]. Specificity was calculated separately in the group of healthy endemic controls as well as in those clinical suspect patients that were diagnosed as not having VL (non-cases). In the latter, a negative follow up of 6 months was required. 95% confidence interval (CI) was calculated using Fleiss Quadratic method for proportions. The reproducibility of a test between field and central laboratories was determined by calculating kappa values with 95% CI using Epi-info version 6. Kappa values express the agreement beyond chance. A kappa-value of 0.60–0.80 represents substantial agreement beyond chance, and a kappa-value of >0.80 represents almost perfect agreement beyond chance (Landis & Koch 1977).

Results

At the two field centres at Muzaffarpur and Patna of Bihar, India, 452 subjects were enrolled, comprising 282 true cases of VL, 70 non-cases. Additionally 100 healthy endemic controls were recruited at KAMRC as described above. The sensitivity and the specificity are shown in Tables 1 and 2. The sensitivity of all tests on sera was highest with DAT-FD and this test was even marginally more sensitive when executed on blood collected on a filter paper (Table 1). For parasitologically confirmed cases, the sensitivity of DAT-FD was perfect. rK-39 strip tests performed equally well (sensitivity 98.6%). Surprisingly, splenic smear failed to detect 18.4% of all cases who benefited from VL therapy. KAtex and rK-26 strip test were also less sensitive.

A high degree of agreement was observed between DAT-FD and DAT-FDF ($\kappa = 0.986$), DAT-FD and rK-39 strip test ($\kappa = 0.98$), DAT-FDF and rK-39 strip test ($\kappa = 1$), and microscopy and KAtex ($\kappa = 0.858$). High Kappa values ($\kappa = 1.0$, 0.99, 0.96 and 0.94 respectively for microscopy, DAT-FD, rK-39 strip test and rK-26 strip test) indicated excellent reproducibility between field and the central laboratories.

Discussion

This study was different from the conventional ones for the fact that a composite reference standard was used that allowed us not to be completely dependent on the diagnostic performance of splenic smears (Lightner *et al.* 1983; Gatti *et al.* 2004). In contrast to previous reports (Zijlstra *et al.* 1992; Sundar & Rai 2002), we found good but not perfect sensitivity of splenic smears. As observed by other authors (Haque *et al.* 1993; Sundar *et al.* 1998b), splenic aspirates missed out on several true kala-azar cases that were parasitologically negative but responded very well to kala-azar treatment. The decision to give anti-leishmanial treatment to these patients was based primarily on clinical and haematological features within a specific

Table 1 Sensitivity of different diagnostic tests for VL

	VL cases					
	Confirmed ($n = 230$)			Probable ($n = 52$)		
	No. of positive	Sensitivity (%)	95% CI	No. of positive	Sensitivity (%)	95% CI
Microscopy	230	100.0	98–100	0	NA	NA
DAT-FD	227	99.1	96–100	50	96.2	86–99
DAT-FDF	230	100.0	98–100	50	96.2	86–99
rK39	230	100.0	98–100	47	90.4	78–96
rK26	52	22.6	17–29	8	15.4	7–29
KAtex	169	73.5	67–79	20	36.5	26–53

CI, confidence interval; DAT-FD, direct agglutination test-freeze dried; NA, not applicable; VL, visceral leishmaniasis.

Table 2 Specificity of different tests in the diagnosis of VL

	Healthy endemic controls ($n = 100$)			Non-cases($n = 70$)		
	No. of negative	Specificity (%)	95% CI	No. of negative	Specificity (%)	95% CI
Microscopy	NA	NA		70	100.0*	
DAT-FD	94	94.0	86.9–97.5	65	92.9	83.3–97.3
DAT-FDF	94	94.0	86.9–97.5	64	91.4	81.7–96.5
rK39	97	97.0	90.8–99.2	60	85.7	74.8–92.6
rK26	100	100.0	95.4–100	70	100	93.5–100
KAtex	99	99.0	93.7–99.9	62	88.6	78.2–94.6

*By definition.
CI, confidence interval; DAT-FD, direct agglutination test-freeze dried; NA, not applicable; VL, visceral leishmaniasis.

epidemiological context. Positivity with DAT liquid antigen was used as supportive evidence in this study, but has been used by others as the sole serological criterion for diagnosis and treatment of VL (Zijlstra *et al.* 1991; Hailu & Berhe 2002; Veeken *et al.* 2003; Sundar *et al.* 2006b). We would have missed about one-fifth of the patients if we had used parasitology as the sole diagnostic criterion.

Of the serological tests, DAT-FD either using blood on filter paper or sera and rK-39 strip test performed with almost perfect sensitivity. The only drawback for both these diagnostic systems was their lack of specificity, with three (3%) and six (6%) of 100 healthy endemic controls, and five (7.1%) and 10 (14.3%) of 70 non-cases being positive. These proportions could even be higher for healthy endemic controls depending upon the level of transmission. However, in a well-defined clinical suspect i.e. fever of greater than 2 weeks duration of non-malarial origin with splenomegaly, and anaemia and leucopenia, these tests would provide diagnosis with a very high degree of probability.

KAtex had a sensitivity of 67% and most of these positive patients turned negative after treatment. Though the sensitivity was unacceptably low, a perfect specificity indicates that there is a scope for developing KAtex further by changing its format to improve the sensitivity (Rijal *et al.* 2004). KAtex has the unique advantage of being leishmania antigen based, and thus better in predicting the active disease. Several other drawbacks of KAtex were observed: its current format lacks objectivity in the interpretation of agglutination, while the need to boil the urine would seriously hamper its application in the endemic regions especially if it was to be used by paramedics. rK-26 strip test detected only a fifth of active patients, and thus was not useful in the diagnosis of VL.

Of the two tests DAT-FD and rK-39 strip test, which performed equally well, DAT has the advantage of being a quantitative test, but it has several drawbacks that make it less suitable as a test for the field. It requires multiple pipetting and several hours of incubation. Once you dissolve the freeze dried antigen, it has to be kept refrigerated till the entire vial is consumed and this may be problematic in peripheral health centres as patients are not likely to present in groups. rK-39 strip test can be stored at room temperature and has a shelf life of 18 months, it is simple enough for a trained paramedic to perform without

any equipment and the results are unambiguous. The price of the rK-39 strip test is in the range of US$1.00 per test and comparable to that of DAT-FD, (US$1.25–2.25 per test). Given the conditions prevailing in the endemic region, rK-39 appears currently to be the best available option for the field diagnosis of VL. In regions where laboratories are equipped to do DAT successfully, it can also be used. Both tests, if positive in a clinical suspect person, warrant treatment for VL.

However, while making these recommendations, one should keep in mind that depending on the intensity of transmission, up to 32% of healthy people living in the endemic region could be seropositive (Sundar *et al.* 2006a,b), and one has to be careful while making a decision to treat seropositive patients to meticulously look for clinical features and supportive laboratory evidence. Furthermore, in patients with previous kala-azar, in whom a relapse is suspected, only parasitological diagnosis can be helpful in making a decision, as antibody tests remain positive for years after treatment. Notwithstanding the excellent results of this study, there is a need to develop markers of active disease which will be devoid of the handicaps associated with the detection of antibodies. Nevertheless, of the currently available tools, rK-39 rapid strip test appears to be the best for field use for the diagnosis of VL.

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Evaluation comparative de la parasitologie et des tests de diagnostic sérologiques de la leishmaniose viscérale en Inde: phase III d'étude sur la précision du diagnostic

Dans cette phase III de l'étude sur diagnostic pour la leishmaniose viscérale en Inde, nous avons comparé le diagnostic parasitologique à plusieurs tests sérologiques: essai d'agglutination directe (lyophilisé; DAT-FD), test sur bandelette rK-39, test sur bandelette rK-26 et un test d'agglutination sur latex pour la détection d'antigène dans l'urine (KAtex) chez 452 sujets des régions endémiques de Bihar en Inde. Les sujets ont été groupés en 4 catégories: 230 patients confirmés, 52 cas probables, 70 qui n'étaient pas des cas et 100 contrôles sains de zone endémique. L'estimation de la sensibilité a été basée sur les deux premiers groupes et la spécificité sur les deux derniers groupes. La sensibilité du test DAT-FD était de 98.9%, celle du test rK-39 était de 98.9%, celle du KAtex de 67.0% et celle du test rK-26 de 21.3%.La sensibilité du test DAT-FD sur le sang prélevée sur papier filtre (DAT-FDF) était de 99.3%, ce qui était comparable à celle du test sur sérum. La spécificité des essais sérologiques était comparable et élevée (DAT-FD et DAT-FDF: 94%, test sur bandelette rK-39: 97%, KAtex: 99% et test sur bandelette rK-26: 100%). Le test parasitologique standard sur frottis splénique s'est avéré peu performante en ratant 18.4% des cas. La reproductibilité des tests sérologiques entre les laboratoires centraux et sur le terrain était excellente (R = 1.0; 0. 99; 0.96 et 0.94 respectivement pour la microscopie, le test DAT-FD et les tests sur bandelettes rK-39 et rK-26). La concordance entre le test DAT-FD et celui sur bandelette rK-39 était élevée (0.986). Bien que le test DAT-FD et celui sur bandelette rK-39 soient tous deux très sensibles avec une spécificité excellente , la facilité d'utilisation du test sur bandelette rK-39 la rend plus appropriée pour diagnostic de la leishmaniose dans les conditions de terrain.

mots clés leishmaniose viscérale, test d'agglutination directe, test sur bandelettes rk-39, test dur bandelettes rk-26, Katex, précision du diagnostic

Evaluación comparativa de pruebas parasitológicas y serológicas en el diagnóstico de la leishmaniasis visceral en India: - Estudio de precisión diagnóstica de Fase III

En este ensayo de fase III de diagnóstico de la leishmaniasis visceral en India, hemos comparado el diagnóstico parasitológico con varias pruebas serológicas: prueba de aglutinación directa (liofilizado; PAD-L), tiras diagnósticas rK-39, tiras diagnósticas K-26 y prueba de aglutinación en látex para detección del antígeno en orina (KAtex) en 452 sujetos provenientes de áreas endémicas de Bihar, India. Los sujetos fueron separados en 4 categorías: 230 pacientes confirmados, 52 casos probables, 70 'no-casos' y 100 controles sanos pertenecientes a un lugar endémico. Los dos primeros grupos fueron utilizados para estimar la sensibilidad, mientras que con los últimos dos se estimó la especificidad. La sensibilidad de PAD-L fue del 98.9%, de rK-39 del 98.9%, de KAtex 67.0%, y de rK-26 del 21.3%. La sensibilidad de PAD-L, en sangre tomada en papel de filtro (PAD-LF), fue del 99.3%, lo cual es comparable con los resultados obtenidos utilizando suero. La especificidad obtenida con las pruebas serológicas era comparable y alta (PAD-L y PAD-LF: 94%, tiras diagnósticas rK-39: 97%, KAtex: 99% y tiras diagnósticas rK-26: 100%). La clásica demostración parasitológica en un frotis esplénico dio resultados pobres, ya que se perdió un 18.4% de los casos. La reproducibilidad de las pruebas serológicas entre los laboratorios de campo y centrales fue excelente ($R = 1.0$, 0.99, 0.96 y 0.94 respectivamente para microscopía, PAD-L, tiras diagnósticas rK-39 y tiras diagnósticas rK-26). La concordancia entre PAD-L y las tiras diagnósticas rK-39 (0.986) fue alta. A pesar de que tanto el PAD-L como las tiras diagnósticas rK-39 eran altamente sensibles y específicas, la facilidad de uso de las tiras diagnósticas rK -39 las hace mucho más convenientes para el diagnóstico de LV en condiciones de campo.

palabras clave leishmaniasis visceral, prueba de aglutinación directa, tiras diagnósticas rk-39, tiras diagnósticas rk-26, Katex, precisión diagnóstica

