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Leishmania donovani infection in Eastern Sudan: Comparing direct agglutination and rK39 rapid test for diagnosis−a retrospective study

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ABSTRACT

Objective: To compare diagnostic accuracy and agreement between direct agglutination test and rK39 rapid tests for diagnosis of visceral leishmaniasis in an endemic area, the Doka area in Eastern Sudan.

Methods: Stored sera of confirmed visceral leishmaniasis cases, unconfirmed visceral leishmaniasis-suspects and negative controls were tested by direct agglutination test and rK39 rapid test. The sera were collected from the Doka area in Eastern Sudan. Diagnostic accuracy of direct agglutination test and rK39 rapid test was assessed in terms of sensitivity, specificity, positive predictive value and negative predictive value, estimated at 95% confidence interval (*CI*). Agreement between the two tests was determined by the kappa (κ) value.

Results: Taking lymph node aspiration of *Leishmania* as a gold standard, direct agglutination test showed 91.0% sensitivity, 99.3% specificity, resulting in a positive and negative predictive value of 99.3% and 91.0%, respectively. In contrast, the sensitivity of rK39 rapid test was 85.2% and specificity 98.6%, resulting in a positive and negative predictive value of 98.5% and 85.9%, respectively. Most (81.3%) of the confirmed visceral leishmaniasis sera revealed strong antibody titers (≥1:6 400). Some sera (*n*=5) that were positively tested with rK39 rapid test were negative in direct agglutination test (≤1:800); in contrast, direct agglutination test was positive in 12 confirmed visceral leishmaniasis sera that were negatively tested with rK39 rapid test. There was moderate to good agreement between direct agglutination test and rK39 rapid test for confirmed visceral leishmaniasis patients (κ=0.42, 95% *CI*=0.21-0.63) and control sera (κ=0.80, 95% *CI*=0.41-1.00).

Conclusions: Both direct agglutination test and rK39 rapid test are satisfactory test systems for visceral leishmaniasis diagnosis in East Sudan. Their simplicity makes them ideal for first healthcare in rural areas. These data are relevant also for other East African endemic countries because of the geographical and overlapping distribution of the *Leishmania* parasite.

KEYWORDS: DAT; rK39; *Leishmania donovani*; Eastern Sudan

1. Introduction

Leishmaniases are a group of infectious diseases caused by parasites belonging to the *Leishmania* genus. *Leishmania* parasites are transmitted through the bite of an infected female sandfly. The disease occurs in three clinical forms, among which, visceral leishmaniasis (VL) is the most severe form with 100% mortality rate if left untreated[1]. VL is caused by species belonging to *Leishmania (L.) donovani* complex. The disease is spread over the world with major endemic regions in South-West Asia, East Africa, South America and Mediterranean countries. However, more than 90% of all cases occur in Sudan, South Sudan, Ethiopia, India, Bangladesh and Brazil[2]. East Africa is of particular interest because Leishmaniasis co-exists with other infectious diseases and is expanding into so far non-endemic areas[3–5].

In Sudan, VL is endemic mainly in the East and Central regions where thousands of cases are reported annually[6,7]. It is caused by *L. donovani* and transmitted by the sandfly vectors, *Phlebotomus orientalis* and *Phlebotomus martini*[8]. The country has experienced several epidemics, with several thousand fatalities. There is a public health concern about re-occurrence of VL epidemics in Sudan. A prime goal for endemic countries is to establish easy-to-use and reliable VL-diagnostic tests to support early detection of the disease and thus to prevent further outbreaks[9]. Till today, parasitological detection of *Leishmania* from organ aspirates and *in vitro* cultivation are considered to be the reference methods of VL diagnosis. However, these methods have several drawbacks: their application

in the field is limited; their sensitivity can be affected by type of the organ, from which samples are collected, with high numbers of parasites in spleen and low in lymph node samples. Because of the low sensitivity of most commercially available tests, the absence of parasites in organ aspirates doesn't rule out the infection[10,11]. Due to these limitations, parasitological detection is not widely used in many countries.

Sero-diagnostic is an alternative method for VL detection. It is more sensitive than classical parasite isolation and culture methods[12]. Several serological tools are now available and used in endemic countries. In Sudan, VL diagnosis is primarily based on direct agglutination test (DAT) and to some extent, on rK39 rapid tests (RT). These tests, however, may not be specific enough to differentiate between previous exposure and current active disease and may give false positive results in some other diseases such as tuberculosis and malaria[13–15]. Here, we report on a study which compared the accuracy and concordance of DAT and rK39 rapid tests for VL diagnosis in the Doka area, a major endemic region in Eastern Sudan. The comparison between DAT and rK39 rapid was performed with stored serum samples from a serum bank facility in Sudan.

2. Materials and methods

2.1. Ethical approval

Human sera used in this study were randomly selected and tested anonymously without referring to the patients' information. The sera were collected in endemic area of Eastern Sudan-Doka after filling in consent forms. These sera were used previously in other studies[16–18]. This study was ethically approved by the Ethical Review Committee of the Federal Ministry of Health in Sudan (23-06-2005).

2.2. Characteristics of sera and study design

This is a retrospective study performed with human sera (n=494) obtained from the serum bank at the Laboratory of Biomedical Research of Ahfad University for Women. These samples were collected as part of a previous project at Doka region, Eastern Sudan. VL suspicion was based on clinical signs and symptoms according to clinical practice guidelines of the Sudanese Ministry of Health[19]. Patients from different health centers were sent to Doka hospital for diagnosis and treatment from February 2006 and October 2007. Major signs and symptoms of these patients included: fever, hepatomegaly, splenomegaly, lymphadenopathy and abnormal complete blood count including leukopenia, anemia

and thrombocytopenia. Lymph node aspirates and blood samples were collected for VL diagnosis. In this study, we selected 155 sera of confirmed VL cases (positive lymph node samples) and 197 sera of un-confirmed VL suspects (negative lymph node samples). The classification was based on the presence of classical signs and symptoms combining with lymph node results. The patients were not tested for other diseases, as they did not show any other clinical signs. As controls, we selected a group of 142 sera of individuals from the same VL-endemic area that were lacking disease symptoms (apparently healthy), or patients suffering from other diseases rather than visceral leishmaniasis. The control group included VL-endemic controls (n=50), non-endemic controls (n=20), cases with malaria (n=20), pulmonary tuberculosis (n=20), leukemia (n=20) and African trypanosomiasis (n=12). The diagnosis of diseases was performed according to the national guideline of the Sudanese Ministry of Health.

2.3. Serological tests

We used two serological tests, DAT, and rK39 RT based on the recombinant protein of *L. infantum*.

2.3.1. Direct agglutination test (DAT)

DAT test is based on *L. donovani* isolated from a VL patient in Eastern Sudan and was prepared at the Laboratory for Biomedical Research, Ahfad University for Women according to the standard procedure, as described previously[20]. Briefly, sera were serially diluted (1:100-1:102 400) in 0.2% gelatin and physiological saline solution (0.9% g/v). *Leishmania* antigen (50 μL) was mixed with 50 μL diluted sera in V-shaped microtiter plates (Greiner Bio-One, Frickenhausen, Germany) and incubated overnight at room temperature. Antibody titers were determined as the highest dilution of sera showing agglutination reaction with a cut-off titer of 1:3 200. Lower titers were considered borderline (1:1 600) or negative (≤1:800)[20].

2.3.2. rK39 RT

Rapid test kits using the recombinant protein K39[21] was purchased from Bio Rad (France). Tests were performed as recommended by the manufacturer. Briefly, 20 μL of the serum sample was added to the test device. Following incubation the test was read and interpreted. Results were recorded as positive if two lines appeared, or negative when only one line appeared. If no lines appeared, the test was considered invalid and was repeated.

2.4. Statistical analysis

The diagnostic accuracy of DAT and rK39 rapid test was compared

in term of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) and estimated at 95% confidence interval (*CI*). Sensitivity was presented as percent through the ratio of positive sera divided by confirmed VL cases; specificity in percentage as ratio of all control cases with negative test results. The latter group included sera from patients with malaria, pulmonary tuberculosis, leukaemia, African trypanosomiasis as well as negative controls from VL-endemic and non-endemic areas. PPV was estimated as the number of true positives over the number of true positives and false positives. NPV was estimated as the number of true negatives over the number of true negatives and false negatives. Agreement between the two tests was done by kappa (κ) value using Graph Pad available at https://www.graphpad.com/quickcalcs/ kappa1.cfm. The level of agreement between the two tests was done as described earlier[22] as follows: κ<0.4, bad; 0.41≤κ≤0.60, moderate; 0.61≤κ≤0.80, good, and κ>0.8, excellent.

3. Results

A total of 494 serum samples including 155 parasitologically confirmed VL cases, 197 clinically suspected but un-confirmed VL cases and 142 negative controls were used in this study. Most sera were originally collected at Doka Hospital, Eastern Sudan. Serum samples were tested by DAT produced at Ahfad University and the commercial rK39 RT.

Out of the 155 confirmed VL positive sera, the DAT truly identified 141 positive samples and missed 14 sera. Sensitivity for DAT was 91.0%. Out of the 142 control sera, the test revealed only one false positive result (a VL-endemic negative control) that was negative with the rK39 RT. The test demonstrated 99.3% specificity.

Accordingly, PPV was 99.3% and NPV was 91.0% (Table 1). For the confirmed VL group, the sensitivity of rK39 RT was 85.2%. Out of 155 VL confirmed positive sera, the test correctly identified 132, but failed in 23 cases. Out of the 142 control sera, rK39 RT identified 140 sera, demonstrating 98.6% specificity. The test was false positive in two cases, one patient suffering from malaria and the other from leukaemia. These two sera showed negative results with DAT. The PPV and NPV were 98.5% and 85.9%, respectively (Table 1).

Table 1. Accuracy for DAT and rK39 rapid test in diagnosing (VL) in Eastern Sudan.

Diagnostic test	TP	FN	TN	FP	%Sensitivity (n=155)	%Specificity (n=142)	PPV (%)	NPV (%)
DAT (LBR, Ahfad University)	141	14	141	1	91.0 (85.0-94.8)	99.3 (95.6-100)	99.3	91.0
rK39 RT (Bio Rad)	132	23	140	2	85.2 (78.3-90.2)	98.6 (94.5-99.8)	98.5	85.9

Serum samples were obtained from serum bank facility of Laboratory for Biomedical Research of Ahfad University for Women. TP, true positive cases; FN, false negative cases; TN, true negative cases; FP, false positive cases; PPV, positive predictive value; NPV, negative predictive value. Values are at 95% *CI*.

Out of the 155 confirmed VL sera, 126 (81.3%) showed strong antibody titres (≥1:6 400) with DAT (Table 2). The test recorded negative antibody titers (≤1:800) in 1 serum positively tested with rK39 RT and in 5 sera that were also negative with rK39 RT. Likewise, DAT recorded weak positive antibody titers in 12 confirmed VL sera that were positive with rK39 RT and 3 sera negative with rK39 RT. There was moderate agreement between DAT and rK39 RT (κ=0.42, 95% *CI*=0.21-0.63) for confirmed VL patients (Table 2) and control sera (κ=0.80, 95% *CI*=0.41-1.00) (Table 3).

Table 2. Comparison of reactivity for *Leishmania* antibody detection by DAT and rK39 RT.

			1:100-1:800 (negative)	1:1 600 (borderline)	1:3 200-1:6 400 (weak positive)	≥1:6 400 (positive)	Total	κ value (95% *CI*)
VL	rK39 RT	+	1	4	12	115	132	0.42 (0.21-0.63)
		-	5	4	3	11	23	
	Total		6	8	15	126	155	

Header spanning: "DAT titer" spans the four titer columns; "Agreement" spans the κ value column.

Sera of VL patients were serially diluted from 1:100 to 1:102 400 and classified into four groups based on DAT antibody titers. Sera with different DAT titers were compared to the reactivity of rK39 RT. κ=0.42; SE of κ=0.11; 95% *CI* from 0.21 to 0.63. The strength of agreement is considered to be 'moderate'.

Table 3. Results of rK39 RT and DAT for the control group.

			1:100-1:800 (negative)	1:1 600 (borderline)	1:3 200-1:6 400 (weak positive)	≥1:6 400 (positive)	Total	κ value (95% *CI*)
Controls	rK39 RT	+	2	0	0	0	2	0.80 (0.41-1.00)
		-	138	1	1	0	140	
	Total		140	1	1	0	142	

Control sera were diluted from 1:100 to 1:102 400 and classified into four groups based on DAT antibody titers. The reactivity of sera with different DAT titers was compared with the results of rK39 RT. κ=0.80; SE of κ=0.20; 95% *CI* from 0.41 to 1.00. The strength of agreement is considered to be 'good'.

In the 197 sera of VL-suspected (Table 4), 13 were positive by the two tests and 175 were negative. Four sera were positive by DAT and negative by rK39 RT while five were positive by rK39 and negative by DAT. The agreement between these two tests in the VL-suspected group with unconfirmed diagnosis was also good (κ=0.72, 95% *CI*=0.54-0.89).

Table 4. Comparison of rK39 RT and DAT in unconfirmed VL-suspects group.

			DAT		Total	Agreement
			+	-		κ value (95% *CI*)
VLS	rK39	+	13	5	18	
		-	4	175	179	0.72 (0.54-0.89)
	Total		17	180	197	

Sera of VL suspects were tested in DAT and compared with results of rK39 RT. κ=0.72; SE of κ=0.10; 95% *CI* from 0.54 to 0.89. The strength of agreement is considered to be 'good'. VLS: VL-suspects.

4. Discussion

In this study, we compared the diagnostic accuracy and agreement between DAT and rK39 rapid tests for VL diagnosis in sera from patients originating from a known endemic region in Eastern Sudan. This region is similar to many other regions in East Africa where the disease has been identified. The assessment of diagnostic tests of VL in these regions is important, as the disease remains a major health problem and many environmental factors favour the disease transmission[23–25].

DAT and rK39 RT have been previously evaluated in several studies from different countries with considerable variable results[26,27]. The present study provides evidence of good agreement between DAT and rK39 RT for VL diagnosis in Eastern Sudan. It is essential to validate diagnostic tests in areas where it will find it´s application. Here we show that the sensitivity and specificity of DAT and rK39 are acceptable but not optimal. Previously, we have conducted multicenter study, which revealed that compared to other tests, DAT has a better sensitivity for VL detection in immunocompetent HIV-negative patients in Sudan and India but not in France (94%, 92.4% and 88.5%, respectively). On the other hand, sensitivity of rK39 RT was better in India (96.2%) than Sudan (88%) and France (85%)[18]. Here, we used different DAT (DAT prepared at Laboratory for Biomedical Research-Ahfad University) and rK39 RT obtained from the same source as in the multicenter study (Bio Rad-France). This study confirms variations in the diagnostic accuracy of DAT and rK39 RT, as reported previously[18]. Indeed, HIV can lower sensitivity of the test[27]. This is particularly important, since there is high prevalence of VL/HIV co-infection in East Africa[28–30]. Heterogeneity in *Leishmania* parasites is an additional factor that complicates diagnosis of VL[27–31]. Indeed, combining results of more than one test would increase sensitivity of the final diagnosis.

However, in resource-limited countries like East Africa, this approach seems not to be possible.

To compare accuracy of DAT and rK39 RT with regard to specificity, we extended our study design by also including patients suffering from malaria, pulmonary tuberculosis, leukaemia and African trypanosomiasis, all being confirmed non-VL cases. Of particular importance was the concordance of DAT and rK39 RT in healthy controls and unconfirmed VL-suspected cases. As the incidence of VL is very high in East Sudan[32,33], serological tests can be used to discriminate between these two important groups without the necessity to perform invasive parasitological diagnostic methods. This approach is used in many health centres in Sudan because it allows quick and easy interpretation of test results[34,35]. As reported previously, some individuals from endemic areas are positive in serological tests without any signs and symptoms of VL, while some true positive patients don't have detectable antibody titres[27]. These results may explain the false positive and negative results in our study. It is known that the majority of these patients were not HIV co-infected but other immunocompromising factors cannot be ruled out; *e.g.*, the high prevalence of malnutrition in East Africa may influence the immunity, a question that needs urgent attention in order to reinterpret a low or absent antibody responses in infected patients[36].

All this underscores the need for a combined use of DAT and rK39 RT for diagnosis of VL in rural settings at Eastern Sudan. However, additional methods are required to confirm highly suspected cases with negative serology. ELISA tests based on *Leishmania* recombinant proteins such as the KLO8 might be good alternatives[18].

It is important to continue developing new diagnostics for VL in Sudan and other endemic countries. Recombinant proteins are very promising to improve sero-diagnosis and discern between false negative and false positive test results. With the high prevalence of asymptomatic cases and co-existence with other diseases such as malaria, care should be taken to identify true VL infected patients to avoid undesired effects of the drugs, including pancreatitis, liver and heart problems and cardiac arrhythmia[37,38].

The limitations of the study are related to its retrospective nature based on previous findings. The sera of this investigation were already used in previous studies, collected from patients, transported and stored for years under freezing condition. The sera had exposed to freezing and thawing. This may affect the quality and test results.

The accuracy and agreement between DAT and rK39 RT were satisfactory for diagnosis of VL in East Sudan. Their simplicity and fastness make both tests suitable for primary healthcare in rural areas. These data are relevant also for other endemic countries in East Africa because of the overlapping distribution of the *L. donovani* parasite.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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