Assessment of the direct agglutination test, fast agglutination screening test, and rK_{39} dipstick test for the sero-diagnosis of visceral leishmaniasis in Syria

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ABSTRACT

الأهداف: تقييم فاعلية ثلاث اختبارات مصلية (rK_{39}, FAST, DAT)، في تشخيص داء الليشمانية الحشوي (VL) في سورية.

الطريقة: أجريت هذه الدراسة على 267عينة مصلية مأخوذة من مصابين أو مشكوك بإصابتهم بداء الليشمانية الحشوي (VL)، ومن مصابين بالداء الجلدي، والتوكسوبلاسما، إضافة على عينات مصلية من أشخاص أصحاء في سورية خلال عام 2007م، في مختبرات جامعة دمشق، حيث طبقت الاختبارات الثلاث (rK_{39}, FAST, DAT) على هذه العينات في آن واحد.

النتائج: أظهرت نتائج الدراسة أن جميع الاختبارات المطبقة تتمتع بدرجة حساسية مرتفعة، في حين كانت درجة نوعيتها متفاوتة.

خاتمة: يمكن اعتبار هذه الاختبارات الثلاث (rK_{39}, FAST, DAT) ذات أهمية في الكشف المبكر عن داء الليشمانية الحشوي (VL) في سورية.

Objective: To evaluate the performances of 3 serological assays (direct agglutination test [DAT], fast agglutination screening test [FAST], recombinant protein [rK_{39}] dipstick) test for use in primary care, for the diagnosis visceral leishmaniasis (VL) in Syria.

Methods: We utilized 267 serum samples obtained during 2007 from patient groups (confirmed and suspected VL, confirmed cutaneous leishmaniasis, toxoplasmosis) from endemic areas in Syria and control samples, and applied the 3 serological tests in the Damascus University, Damascus and Health laboratories at the same time, on these samples.

Results: Our data show that the tests were very sensitive, where the DAT was the most specific followed by FAST, then rK_{39} dipstick.

Conclusion: Our study confirmed that all the tests performed well, and proved to be very important sero-diagnosis tools for visceral leishmaniasis.

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Leishmaniasis is caused by an intracellular protozoan parasite belonging to the genus *Leishmania*. This disease ranges from self-healing cutaneous lesions to fatal systemic disease, depending on the parasite and the host immune response. A common estimate of the worldwide annual incidence is 600,000 newly reported clinical cases. The overall prevalence of Leishmaniasis was 12 million cases in the world, and the estimated population at risk is approximately 350 million.[1,2] Visceral leishmaniasis (VL) caused by *Leishmania donovani (L. donovani)*, *Leishmania infantum,* and *Leishmania chagasi*, is a serious illness that can give rise to an epidemic and provoke high mortality rate, if left untreated.[3] The clinical symptoms of VL are similar throughout the world. Large numbers of amastigote infected mononuclear phagocytes in the liver and spleen, result in progressive hypertrophy. In sub-acute or chronic cases, symptoms are splenomegaly, prolonged irregular fever and weakness, loss of appetite, weight loss, pallor, anemia, and diarrhea.[4-6] At present, routine diagnosis of VL is carried out by direct microscopic examination of patient material, or by culture. However, sample retrieval is inconvenient for the patients, and parasite isolation by culture is time consuming, expensive and

difficult.[7,8] Sero-diagnosis tests have been widely utilized as anti-leishmanial antibody titers are high, especially during the acute phase, and have been developed to replace parasitological methods for the diagnosis of VL. The indirect fluorescence technique (IF), Western blot (WB), enzyme-linked immunosorbent assay (ELISA), direct agglutination test (DAT), and others can be used for the sero-diagnosis of VL.[9-13] In addition to serologic tests, polymerase chain reaction (PCR) based assays have been used for detecting *Leishmania* DNA in diagnostic samples.[14,15] The aim of this study was to evaluate the performance of the DAT, fast agglutination screening test (FAST), and recombinant protein (rK_{39}) dipstick in detecting anti- *Leishmania* antibodies for use in primary care in Syria, where a total of 193 VL human cases were reported by the Department of Disease Control during the last 8 years (2000-2007). There were 98 males (50.8%), and 95 females (49.2%) that harbored the disease. By age, children under 6 years old constituted 78.8% (152 cases) of the reported cases. By provinces, the highest number of reported cases was 129 from Aleppo and Idlep, 52 from Lattakia and Tartous, and 8 from Daraa governorates over the period of 8 years.

Methods. We utilized serum samples (267 samples) from patient groups from endemic areas, and control samples from other regions in Syria during 2007. A questionnaire with clinical and epidemiological data (age, gender, address, symptomatology, and so forth) was filled out for each patient. The patients were notified on all the procedures and signed informed consent was obtained. The Ethics Committee of the Damascus University Science Faculty, Damascus, Syria approved the study. The serum samples used were obtained from the following: 1. Visceral leishmaniasis patients clinically and parasitologically confirmed (fever with splenomegaly and demonstrated to have *Leishmania* parasites in bone marrow aspirate smears) (n=30). 2. Patients clinically suspected of VL (fever, weight loss, enlarged lymph nodes, and sometimes enlarged spleen), however, no parasitological assessment was carried out (n=85). 3. Patients presenting cutaneous leishmaniasis (CL) lesions (n=110). 4. Patient with proven toxoplasmosis (n=7), both of patient's groups 3 and 4 are from non-endemic areas. 5. Healthy individuals from VL endemic areas (n=25). 6. Healthy individuals from non-endemic regions (n=10). The presence of *Leishmania* antibodies in the serum samples was determined by DAT, FAST, and rK_{39} dipstick test that were performed in the Damascus University and Health laboratories, Damascus, Syria. The DAT was performed as described previously.[16,17] In brief, the samples were diluted in physiological saline [0.9% sodium chloride (NaCl)] containing 0.78% β-mercaptoethanol. Two-fold dilution series of the sera were carried out (in V-shaped micro-plate) starting at a dilution of 1:100, and going up to maximum serum dilution of 1:102400. Well 12 was used as a negative control. Freeze-dried DAT antigen (*L. donovani* promastigotes) produced by Kit Biomedical Research, Amsterdam, Netherlands, was reconstituted with physiological saline according to the manufacturer's instructions. Fifty µl DAT antigen (5×10^7 promastigotes/ml) was added to each well containing 50 µl diluted serum, and results were read after 18 hours of incubation. A sample is considered positive if it has a titre ≥1:800, the cut-off value of the DAT. The FAST test was performed according to the protocol described.[18] In brief, serum samples were diluted 1:100 in saline solution (0.85% NaCl) to which 0.78% β-mercaptoethanol was added in a V-shaped micro-plate (Grenier, Germany), then 20 µl of the 1:100 dilution was transferred to another well of the same plate, and 20 µl FAST antigen (2×10^8 promastigotes/ml) was added. The plate was gently tapped by hand, and then incubated for 3 hours at room temperature, after which the results were read. The rK_{39} dipstick was obtained from InBios International, Seattle, Washington, USA. Procedures of the dipstick assay involve loading 20 µl of serum sample onto a test strip, which was placed vertically in a test tube. Two-three drops of the chase buffer solution provided in the kit were added to the test tube. The results were read after 5-10 minutes. A dipstick was considered positive if both the internal control band and the test band were stained. A dipstick was considered negative, if only the internal control band was visible. The sensitivity and the specificity were calculated as follows: sensitivity=TP/(TP+FN)×100%, and specificity=TN/(TN+FP)×100%, where TN represents true negative, TP true positive, FN false negative, and FP false positive. The sensitivity of the 3 tests was assessed with sera from confirmed VL patients (n=30). Sera of healthy controls from VL endemic and non-endemic regions (n=35), and sera of patients with confirmed other diseases (n=117) were used to determine the specificity of DAT, FAST, and rK_{39} dipstick test.

The degree of agreement between FAST, DAT, and rK_{39} dipstick test was determined by Fisher exact test with 95% confidence intervals. Data were analyzed using the open Epi info software. The calculation of the degree of agreement between DAT and FAST was based on all serum samples, whereas the *p* values for DAT-rK_{39}, and FAST-rK_{39} were based on the results obtained, with the confirmed and suspected VL serum samples.

Results. The results of DAT, FAST and rK_{39} testing of positive and negative controls, and of the serum samples of patients with other confirmed infectious diseases are presented in **Table 1**. The sensitivity of the

DAT, FAST, and rK_{39} in the present study was calculated to be 100%. The calculated specificity of the DAT was 98.7%, FAST was 94.7% and rK_{39} was 88.2% on the basis of the results obtained. The results of DAT, FAST, and rK_{39} testing of serum samples from confirmed and suspected patients (115), are summarized in **Tables 2-4**, in order to evaluate the efficacy of these tests in detecting the VL antibodies especially in the suspected persons. A high degree of agreement (97%, p=0.0001) between the DAT and FAST was observed (**Table 2**). In addition, important agreement between DAT and rK_{39} (95%, p=0.0001, **Table 3**), or FAST and rK_{39} (97%, p=0.0001, **Table 4**) was also observed.

Discussion. In view of the public health importance of VL and the inherent difficulties of conventional

Table 1 - Comparison results between DAT, FAST, and rK_{39} dipstick using serum from confirmed and suspected VL patients, healthy endemic and non-endemic controls, and samples from patients with other confirmed diseases.

Tests	DAT^+	DAT^-	$FAST^+$	$FAST^-$	rK_{39}^+	rK_{39}^-
	n (%)					
Confirmed VL patient sera (n=30)	30	0	30	0	30	0
Suspected VL patient sera (n=85)	61	24	63	22	67	18
Confirmed CL patient sera (n=110)	2	108	6	104	13	97
Confirmed toxoplasmosis sera (n=7)	0	7	0	7	0	7
Healthy non-endemic controls (n=10)	0	10	0	10	0	10
Healthy endemic controls (n=25)	0	25	2	23	5	20

DAT - direct agglutination test, FAST - fast agglutination screening test
rK_{39} - recombinant protein, VL - visceral leishmaniasis, CL - cutaneous leishmaniasis,
DAT^+ = 34.8%; DAT^- = 65.2%, $FAST^+$ = 37.8%, $FAST^-$ = 62.2%, rK_{39}^+ = 43.1%; rK_{39}^- = 56.9% at 95% confidence interval

Table 2 - Comparison results between DAT and FAST dipstick using sera from suspected visceral leishmaniasis patients.

Tests	$FAST^+$	$FAST^-$	Total
	n (%)		
DAT^+	93 (35)	0 (0)	93 (35)
DAT^-	8 (3)	166 (62)	174 (65)
Total	**101 (38)**	**166 (62)**	**267 (100)**

DAT - direct agglutination test, FAST - fast agglutination screening test,
$FAST^+$ = 37.8%, $FAST^-$ = 62.2% at 95% confidence interval

Table 3 - Comparison results between DAT and rK_{39} dipstick using sera from suspected visceral leishmaniasis patients.

Tests	rK_{39}^+	rK_{39}^-	Total
	n (%)		
DAT^+	91 (79)	0 (0)	91 (79)
DAT^-	6 (5)	18 (16)	24 (21)
Total	**97 (84)**	**18 (16)**	**115 (100)**

DAT - direct agglutination test, rK_{39} - recombinant protein,
rK_{39}^+ = 84.4%, rK_{39}^- = 15.6; at 95% confidence interval

Table 4 - Comparison results between FAST and rK_{39} dipstick using sera from suspected visceral leishmaniasis patients.

Tests	rK_{39}^+	rK_{39}^-	Total
	n (%)		
$FAST^+$	93 (81)	0 (0)	93 (81)
$FAST^-$	4 (3)	18 (16)	22 (19)
Total	**97 (84)**	**18 (16)**	**115 (100)**

FAST - fast agglutination screening test, rK_{39}^- recombinant protein,
rK_{39}^+ = 84.4%, rK_{39}^- = 15.6% at 95% confidence interval

diagnosis techniques in Syria, we tried in this study to evaluate the performance of sero-diagnostic tests. The 3 assays displayed a very high sensitivity (100%), whereas the specificity was 98.7% (DAT), 94.7% (FAST), and 88.2% (rK_{39} dipstick). The specificity of these tests was assessed by using sera from healthy endemic and non endemic controls, and from patients with other confirmed infectious diseases. Several samples of healthy endemic controls and confirmed human cutaneous leishmaniasis (CL), tested positive with FAST and rK_{39} dipstick, whereas, only 2 positive sample was obtained with DAT. These results corroborate with many previous studies.[9,17-23] It is noted that the sensitivity and specificity of these tests, observed in the present study were determined on a limited number of healthy controls, patients with 2 other infectious diseases, and patients with confirmed VL. Therefore, 100% sensitivity is not claimed. However, there was a good agreement between the performances of the 3 tests with regard to the sero-diagnosis of VL. In contrast, both tests, FAST and rK_{39} dipstick, found only a very limited number of CL sero-positive. In conclusion, the findings further confirmed earlier reports that DAT, FAST, and rK_{39} dipstick are suitable tools for the sero-diagnosis of VL. The 3 tests are easy to interpret, and are sensitive and specific, as well. The rK_{39} dipstick is a rapid and simple test not requiring extensive training of the operator. However, this test requires cold storage of the chase buffer, and the test strips cannot be stored at high ambient temperature. The DAT, using the freeze-dried *Leishmania* antigen, is very practical under field or rural conditions, as no equipment is required nor cold storage is necessary for reagents. A limitation of the DAT is the relatively long incubation period (18 hours), and the serial dilutions of the samples that must be made. Finally, the FAST is also practical under field conditions, and it can be used for screening large populations as well, as it requires only one serum dilution, and the results can be read in 3 hours. Therefore, if only validity criteria are considered for the 3 tests, then they are all applicable, however, the simplest is the dipstick test. It is necessary to mention that the serological tests are an aid in the diagnosis of VL. The results of rK_{39} dipstick, or FAST, or DAT should always be correlated with clinical, epidemiological, and other diagnostic test data.

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