Performance of Latex Agglutination Test (KAtex) In Diagnosis of Visceral Leishmaniasis in Iran

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ABSTRACT

Background: Visceral leishmaniasis (VL) is an endemic disease in some parts of Iran. Many techniques have been used for diagnosis of VL, among which the urine based latex agglutination test (KAtex) is a promising one. **Objective**: To compare three diagnostic tests of VL including KAtex, ELISA and Direct Agglutination Test (DAT) in VL patients and healthy controls in the south west of Iran. **Methods:** Serum (n = 29) and urine samples (n = 31) were collected from parasitologically confirmed VL patients. Control samples were obtained from healthy individuals (n = 61) and also from patients with infectious diseases other than VL. The collected serum samples were tested by DAT and ELISA using crude antigen from promastigotes of *Leishmania infantum* and the urine samples were tested by KAtex. **Results:** Sensitivity and specificity of KAtex for diagnosis of VL was found to be 83.9% and 100%, respectively. Sensitivities of DAT and ELISA were 93.1% and 86.2% and their specificities were 100% and 90.5%, respectively. **Conclusion:** KAtex yielded a satisfactory sensitivity and specificity in diagnosis of VL in Iran and can be recommended as a rapid, field applicable and reliable test for diagnosis of VL in this region.

Keywords: Visceral Leishmaniasis, ELISA, Agglutination Test

INTRODUCTION

Leishmaniasis is a protozoan disease caused by the members of the genus *Leishmania*, infecting numerous mammalian species including humans (1). Visceral leishmaniasis (VL) is caused by *Leishmania donovani* complex including *L. donovani* in the Indian subcontinent and Eastern Africa, *L. infantum* in Mediterranean area and Middle East and *L. chagasi* in Latin America (2).

In VL, the clinical manifestation may be confused with other similar conditions such as malaria, shistosomiasis, American trypanosomiasis, etc. and the diagnosis cannot be

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confirmed merely based on the signs and symptoms (3). Demonstration of the presence of parasite in the spleen or bone marrow smears is considered the gold standard for the diagnosis of VL. However its use is limited due to its invasiveness and contradiction in severe anemia, bleeding tendency and in restless children (4).

Several antibody detection methods have been developed over the past decade for the diagnosis of VL. The direct agglutination test developed in the 1980s by Harith (5) presented 90 to 100% sensitivity and 72 to 100% specificity in different studies (6-12). ELISA is another approach for VL diagnosis with reasonable sensitivity and specificity. The commonly used antigen for ELISA is a crude soluble antigen, while purified antigens such as glycoprotein 63 (gp63), rK39, H2B histone proteins, and selective antigen masses have also been used (13-15). The test for detection of anti-rk39 antibodies showed promising results in the early studies (8). Subsequent studies showed low sensitivities for this test including a 67% in Sudan (16), 71.4% in Southern Europe (17) and 60% in Brazil (18). More recent studies showed a higher sensitivity for this test in India and Tunisia (19-20).

Antigen detection is a relatively new approach for the diagnosis of VL. Using urine collected from VL-confirmed cases and controls from Brazil, Yemen and Nepal, the recently developed latex agglutination test (KAtex) showed 100% specificity and 68-100% sensitivity in the first trial (21). The test detects a low molecular weight antigen in the urine of VL patients (22). The KAtex has been evaluated in different VL endemic areas, showing different sensitivities and specificities including: 95.2% and 100% in Sudan (23), 57% and above 90% and 47.7% and 98.7% in two different studies in Nepal (24-25), 67% and 99% in India (9), and 73.9% and 82.4% in Ethiopia (26), respectively. KAtex is a simple, easy to perform, inexpensive, field applicable and rapid test which can be performed at the patient bedside. It is a suitable test for diagnosis of VL in areas where laboratory facilities are not available and this is the case in most of VL endemic areas in the developing countries.

The current study was conducted to evaluate the performance of KAtex for the diagnosis of VL in an area in southwestern Iran where VL is endemic. The study also aimed to compare the validity of this test with those of ELISA and DAT.

MATERIALS AND METHODS

Study population. Urine and sera were obtained from 31 parasitologicaly confirmed VL patients, based on detection of amastigotes in Geimsa stained samples of their bone marrow or spleen aspirates, from Shiraz hospitals during 2005 to 2007. Control samples (n = 30) were collected from healthy individuals referred to medical laboratories in Shiraz for check up. Urine and serum samples were also obtained from non-VL patients (n = 31) including those with cutaneous leishmaniasis (n = 4, caused by L. major), leukemia (n = 7), chronic heart failure (n = 1), renal failure (n = 2), tuberculosis (n = 3), fever with unknown origin (FUO, n = 3), brucellosis (n = 2), typhoid fever (n = 1), sepsis (n = 1), lymphadenopathy (n = 1), lung abscess (n = 1), CVA (n = 2), malaria (n = 1), liver cancer (n = 1) and lung cancer (n = 1). The healthy controls and non-VL cases were mainly from non-endemic areas and had no history or clinical signs and symptoms of VL. All the samples were kept at -70ºC until use. The collected sera were tested by DAT and ELISA and the urine samples were tested by KAtex. Ethical approval of the

study was given by the Ethics Committee of Shiraz University of Medical Sciences and consent was obtained from the participants for taking blood and urine samples.

Latex Agglutination Test (KAtex). KAtex was purchased from Kalon Biological LTD, England. The test was performed for all the collected urine samples according to the manufacturer's instruction. Briefly, urine was boiled for 5 min at 100°C in a water bath and after cooling, 40 µl was mixed with an equal volume of the latex reagent on a ceramic slide. Agglutination was checked under bright light.

Direct Agglutination Test (DAT). DAT was performed to detect anti-*Leishmania* antibody in the sera of VL patients and in the controls. DAT antigen was kindly provided by professor Mohebali of the parasitology department of school of health at Tehran University of Medical Sciences. The test was performed as described before (27). Briefly, the serum samples were diluted in normal saline containing 0.78% of 2- mercaptoethanol and 0.2% gelatin in V-shaped microtiter plates, starting from 1/100 to 1/6400 dilution. After 10 minutes of incubation at 37˚C, 50 µl of DAT antigen was added to each well containing 50 µl of diluted serum. After gentle shaking, the plates were incubated in a wet chamber for 18-24 hours before reading the results. Using positive and negative samples, DAT cut off was defined as the highest dilution at which agglutination was still visible in comparison with negative control samples. The positive control samples were from parasitologicaly confirmed VL patients and the negative samples came from individuals living in non endemic areas. Considering the results of these experiments and also the results of the available literature in the region (12), a titer of ≥ 1:3200 was considered as positive DAT.

Enzyme-linked Immunosorbent Assay (ELISA). The ELISA was performed as described previously (27). Briefly, flat-bottomed ELISA microtiter plates were coated with 5 µg/mL (100 µL per well) of the crude antigen of a native strain of *L. infantum* isolated from a VL patient from the region. The antigens were extracted from the in vitro cultivated parasites by washing the parasites three times in PBS, followed by three freeze and thawing cycles, and four pulses of 5-sec sonications. The extract was then centrifuged at 1300 g for 15 min. The supernatant was removed and the protein content was estimated.

The antigen-coated plates were incubated overnight at 4°C. The wells were washed three times with 0.5% Tween 20 in PBS (PBST, pH = 7.2) washing buffer. Blocking was performed using 3% skimmed milk for one hour at room temperature. Serum (100 µL of 1:100 dilutions in PBST) was added to the wells of the plates followed by incubation for 1.5 hours at room temperature. After five washes with PBST, the plates were incubated with peroxidase-conjugated anti-human IgG (Sigma) (1:4000 dilution in PBST) for one hour. After washing as before, 100 µL of substrate was added to each well and the plates were incubated for another hour at room temperature. Finally the optical densities were measured at 490 nm. The cut off point was set at mean ± 2 SD.

RESULTS

From 31 VL patients, 26 cases (83.9%) had positive KAtex results whereas only 5 had negative results (16.1%). None of the 61 healthy and non-VL patients (controls) had positive KAtex results. Accordingly, a sensitivity of 83.9% (95% CI = 65.5%-93.9%) and a specificity of 100% (95% CI = 92.6%-100%) was found for KAtex. ELISA on the

sera of 29 VL patients yielded a sensitivity of 86.2% (95% CI = 67.4%-95.4%) while only 4 cases of VL patients were negative by this system. Nine out of 61 (14.7%) cases of the control individuals had a positive reaction in ELISA system which resulted in a specificity of 85.2% (95% CI = 73.3%-92.6%) for this test.
When serum samples were tested with the DAT, 27 out of 29 of VL patients (93.1%) had a reactivity above the cut off (1:3200) while none of the control samples had a reactivity equal or above the cut off value. Thus sensitivity and specificity of the test were 93.1% (95% CI = 75.7%-98.7%) and 100% (95% CI = 92.6%-100%), respectively. Table 1 summarizes the performance of KAtex, ELISA and DAT for the diagnosis of VL in this study. Statistical analysis of the data showed an almost perfect agreement (kappa=0.869) between Katex and ELISA, whereas a moderate agreement (kappa=0.522) was found between Katex and DAT. Substantial agreement (kappa=0.633) was also found between DAT and ELISA.

Table 1. Comparison of the performances of KAtex, ELISA and DAT for the diagnosis of VL in southwest of Iran.

Test	VL patients			Controls			Sensitivity (percent)	Specificity (percent)
	Positive	Negative	Total	Positive	Negative	Total		
DAT	27	2	29	0	61	61	93.1	100
ELISA	25	4	29	9	52	61	86.2	85.2
KAtex	26	5	31	0	61	61	83.9	100

DISCUSSION

Currently available serodiagnostic techniques based on anti-*Leishmania* antibody detection are not entirely satisfactory, because they do not differentiate between disease and symptomatic infection. Moreover, the antibody detection assays are not suitable for diagnosis of VL in immunocompromised patients like HIV/VL co-infected cases where low antibody titer is present (28). The use of an antigen detection system, in serum or urine, can distinguish between the past and the current infections (28,29).
The newly developed latex agglutination test for diagnosis of VL (KAtex), as an antigen detection approach, has some advantages over serological and parasitological methods. It is a non-invasive method in which sampling is from voided urine, while splenic aspiration may result in fatal hemorrhage and is contraindicated in some conditions like severe anemia and bleeding tendency.
KAtex has been evaluated in different VL endemic areas. It seems obvious that KAtex shows different sensitivities in the diagnosis of VL in different regions. EL-Safi et al. reported 95.2% sensitivity and 100% specificity for KAtex from Sudan (23). Diro et al. reported 73.9% sensitivity and 82.4% specificity for KAtex in Northwest Ethiopia (26). An excellent performance with 100% sensitivity and 96% specificity has been reported for KAtex in diagnosis of VL in HIV/VL coinfected patients in a study by Vilaplana et al. (29).
Mazloumi Gavgani has shown that KAtex can be used as an effective screening test for the latent infection of VL in Iran (30). Molaei et al. evaluated the performance of KAtex for the diagnosis of VL in an area with a high prevalence of zoonotic visceral leishmaniasis in Iran. A sensitivity of 82.7% and a specificity of 98.9% were reported for KAtex (31).

Disappointing results about KAtex came from Nepal study where a sensitivity of 47.7% and a specificity of 57% were reported for KAtex (24-25). Although the results of a few trials on KAtex are not encouraging, some are quite promising.

Different studies have been conducted to evaluate the performance of KAtex in comparison with other currently available tests for the diagnosis of VL including DAT, IFAT, ELISA, polymerase chain reaction (PCR) and rk39 (6, 23-26). In a comprehensive multi-central study conducted by Boelaert in East Africa and the Indian subcontinent, KAtex was compared with FD-DAT, rk39 strip test and a parasitological method for diagnosis of VL where low sensitivity but high specificity was reported for KAtex (6). Higher sensitivity was reported for rK39 in comparison with KAtex in the diagnosis of VL in a study by Chappuis et al. (24). In a study by Cruz et al., KAtex was performed for 23 patients and 16 (69%) gave positive results. The KAtex along with bone marrow culture and microscopy gave negative results for all patients during a post-treatment follow up (28).

The high specificity (up to 100%) of KAtex in most of the studies is a valuable feature of the test. Furthermore, KAtex is a simple, inexpensive, easy to perform test which does not need sophisticated tools for its performance.

Two main drawbacks of KAtex i.e. low sensitivity in some studies and boiling of the urine are the major obstacles for using this test as the main diagnostic tool for VL, and further studies are needed to overcome these shortcomings. A current study showed that false positive reaction in KAtex can be removed to some extent by treatment of the samples by dithiothreitol (32).

Taken together, results of this study showed that KAtex has satisfactory specificity and sensitivity, in comparison with DAT and ELISA, for the diagnosis of VL in Iran. Although sensitivity of KAtex was lower than that of DAT in our study, KAtex as a simple, inexpensive and easy to perform test can be used to support the data obtained by DAT in the field or by ELISA method in the laboratory for the diagnosis of VL in Iran.

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