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Improvement of the Newly Developed Latex Agglutination Test (Katex) for Diagnosis of Visceral Lieshmaniasis

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Introduction: Different methods are available for diagnosis of visceral leishmaniasis (VL), among them the urine-based antigen detection assay, latex agglutination test (Katex), is a recently developed one. The main drawback of the test is false-positive reactivity in some of healthy individuals. The false positivity of the test can be removed by boiling the urine sample for 5 min before testing. In this study an attempt was made to improve Katex by removing unpleasant boiling process, which also decreases field applicability of the test.

Methods: False-positive and true-positive urine samples were collected from VL patients and healthy individuals. Both samples were then treated by reagents including, sodium dodecyl sulfate, trichloroacetic acid, dithiothreitol (DTT), sulphosalicylic acid and also heating at 56°C.

Results: Findings of this study showed that DTT pretreatment significantly reduced the rate of false-positive reactivity of Katex where 73% of false-positive urine samples changed to negative after DTT treatment. However, the DTT treatment reduced the rate of true positivity by 14%.

Conclusion: These data indicate that DTT can be used to eliminate nonspecific reactivity in the Katex. This will improve the performance of Katex and make it a more convenient and field applicable test. J. Clin. Lab. Anal. 23:202–205, 2009.

Key words: Katex; visceral leishmaniasis; improvement; diagnosis

INTRODUCTION

Leishmaniasis is a protozoan disease caused by the members of the genus *Leishmania*, parasites infecting numerous mammal species, including humans (1). Visceral leishmaniasis (VL), which has been known as kala-azar, 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 the Latin America (2). VL is endemic in 62 countries, 200 million people are at risk and 500,000 new cases occur annually worldwide (3).

For diagnosis of VL, a highly sensitive and specific diagnostic approach is needed, as the mortality rate of the disease is quite high if left untreated. Demonstration of the parasite in splenic or bone marrow smears is considered as the gold standard with high sensitivity. Although spelnic smear is highly sensitive (95%), its usage is limited owing to being invasive and is contraindicated in severe anemia, bleeding tendency, and restless children cases.

Several leishmanial antibody detection methods including direct agglutination test (DAT) and ELISA have been developed over the past decades (4).

The relative sophistication of the DAT procedure (e.g. need for micropipettes and microtitratin plates), nonstandardizing of test reading, batch-to-batch variation and need to cold chain restrict its use to referral hospitals or well-supported health centers (5,6).

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An antigen detection test should provide a better means of diagnosing VL, as antibody detection tests cannot readily distinguish between current, subclinical, and past infection. They are not reliable for immunocompromised patients like HIV/VL coinfected cases who present lower antibody responses (7).

Several studies have demonstrated leishmanial antigen in the urine of VL patients. Sarkari in 2002 detected a 5–20 kDa carbohydrate-based antigen in the urine of VL patients, which was target of the previously designed latex agglutination test for kala-azar (8).

A latex agglutination test (Katex) as a new test for detection of leishmanial urinary antigen in VL patients was developed by Attar et al., in 2001, showing 100% specificity and 68–100% sensitivity using urine collected from confirmed cases and controls from Brazil, Yemen, and Nepal (9). Other studies have reported different sensitivities and specificities for Katex, including: 95.2 and 100% in Sudan, 67 and 99% in India, 57 and above 90% and 47.7 and 98.7% in two studies in Nepal, 73.9 and 82.4% in Ethiopia, and 82.7 and 98.9% in Iran (10–14). The Katex has been used for diagnosis of VL in 49 *Leishmania*/HIV patients in Riera et al.'s study. A sensitivity of 85.7% was reported in their study for Katex and they came to the conclusion that Katex is an appropriate method for primary diagnosis and monitoring the efficacy of treatment in *Leishmania*/HIV coinfected cases (7).

Katex is a simple, easy performing, inexpensive, field applicable, and rapid test, which can be performed at the patient's bedside. It is a suitable test for routine setting where laboratory facilities are poor. One of the main drawbacks of the test is that the samples need to be treated to remove the false-positive reactivity in some of healthy individuals. The false positivity of the test can be removed by boiling the urine sample for 5 min before testing. Boiling declines field applicability of the test. Furthermore, inconvenient odor of urine during boiling process in closed spaces like laboratory is usually a subject of complaint by those who are performing the test. In this study an attempt was made to improve Katex by removing unpleasant boiling treatment with a physical or chemical treatment.

MATERIALS AND METHODS

Urine Samples

One hundred urine samples were randomly collected from healthy individuals and non-VL patients, and tested by Katex. Table 1 shows the details of non-VL patients.

The healthy controls and non-VL cases were mainly from nonendemic areas and had no history or clinical sign and symptoms of VL. Moreover, the serum of control samples was tested by ELISA (using *L. infantum* crude antigen) and DAT to rule out *Leishmania* infection (DAT antigen was kindly provided by Prof. M. Mohebali, from School of Health, Tehran University of Medical sciences). Among them 30 false-positive urine samples (from both healthy controls and non-VL patients), which were positive by Katex (degree of agglutination: +++or ++++) without boiling but changed to negative after boiling, were selected. Moreover, 20 urine and serum samples were collected from parasitologicaly (in bone marrow aspiration) confirmed VL patients admitted in infections pediatric ward of Shiraz hospitals, southern Iran. 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.

TABLE 1. Details of Non-VL Patients

Patients	Number	Percent
Cutaneous leishmaniasis	4	12.8
FUO[a]	3	9.6
Leukemia	7	22.6
Chronic heart failure	1	3.2
Renal failure	2	6.6
Brucellosis	2	6.6
Sepsis	1	3.2
Tuberculosis	3	9.6
CVA[b]	2	6.6
Malaria	1	3.2
Liver cancer	1	3.2
Lung cancer	1	3.2
Lung abscess	1	3.2
Typhoid fever	1	3.2
Lymphadenopathy	1	3.2
Total	31	100

[a]Fever with unknown origin.
[b]Cerebrovascular attack.

Katex Kit

Katex kit was purchased from Kalon Biological LTD, Guilford, England. Latex agglutination for kala-azar was performed according to the manufacturer's instruction.

Trichloroacetic Acid (TCA) Treatment of Urine

Different concentrations of TCA (20, 10, 5, and 2% w/v) was prepared in double distilled water (ddH2O). The urine samples were mixed with an equal volume of different concentrations of TCA and incubated for 30 min followed by centrifugation (13,000*g*, 10 min). The supernatant was removed and tested by Katex.

Exton Solution Treatment

Exton solution (C7H $6O6S_2H_2O$, 254.229 g/mol and Na_2SO_4, 142.04 g/mol) was prepared by adding 50 g

sulphosalicylic acid (SSA) and 88 g sodium sulfate in 1 l of ddH2O. The urine samples were mixed with an equal volume of different concentrations of SSA (20, 10, 5, and 2%) and incubated for 30 min followed by centrifugation (13,000*g*, 10 min). The supernatant was removed and tested by Katex.

Sodium Dodecyl Sulfate (SDS) Treatment

Different concentrations of SDS (10, 5, 1, and 0.5%) were prepared in phosphate-buffered saline. The urine samples were mixed with an equal volume of different concentrations of SDS, incubated for 10 min and tested by Katex.

Heat Treatment

The urine samples were incubated for 15, 30, 45, and 60 min in 56°C temperature in a water bath before testing by Katex.

Dithiothreitol (DTT) Treatment

DTT solution was prepared by dissolving 1.39 g DTT in 6 ml of ddH2O (22.6% w/v). Different volumes of prepared DTT solution were mixed with 50 µl of urine samples and incubated for different times before testing with Katex. Table 2 shows the details of DTT treatment of urine samples.

RESULTS

Treatment of urine samples by TCA did not remove the false-positive reactions in tested samples and all the false-positive specimens remained positive by Katex. Same result was obtained when the urine samples were treated with exton solution. When the samples, after 10 min of incubation at room temperature, were treated with SDS, the positive reactions in urine samples from controls were still noticeable.

Heat treatment of samples, 56°C for 5 min, did not remove the false positivity from the urine samples when tested by Katex.

TABLE 2. Details of Treatment of Urine by DTT

Time (min)	Volume of urine sample (µl)	PBS volume (MI)	DTT volume (MI)
30	50	5	5
15	50	5	5
10	50	5	3
10	50	5	2
10	50	10	2
10	50	10	1.5
10	50	10	1

DTT with a concentration that is usually being used in SDS-page for breaking the protein disulfide bands was used for treatment of the urine samples.

The DTT solution was mixed with an equal volume of urine and after 8–10 min incubation was tested by Katex. The result was promising as the true-positive sample showed a weak agglutination, whereas no agglutination was seen in false-positive samples. When lower concentrations of DTT (3, 2, 1.5, and 1 µl DTT from the stock solution) were applied to the urine samples, stronger agglutination appeared in true-positive samples, whereas the false-positive samples remained negative.

By changing the concentration of DTT and incubation time, it was found that 1.5 µl of DTT (from stock solution) and incubation time of 10 min are the suitable conditions where a prominent positivity in true positive and negative state (no agglutination) in false-positive sample can be seen.

For further evaluation of the DTT treatment, Katex was performed on 30 false positive and 20 true VL positive urine samples after treatment with DTT. Results showed that DTT treatment has significantly reduced the proportion of false-positive reactivity where 73% (22/23) of false-positive samples changed to negative after treatment. However, the DTT treatment reduced the rate of true positivity by 14% (3/20). It means 14% of true-positive samples changed to negative by this treatment.

Considering the background diseases in a few of control samples, it was found that most of long-lasting false-positive samples were from patients with autoimmune diseases. These diseases included rheumatoid arthritis and systemic lupus erythromatous.

DISCUSSION

Katex is a recently developed urine-based antigen detection assay for diagnosis of VL (9). It detects a 5–20 KDa carbohydrate low molecular weight antigen that is released from *Leishmania* parasite into patient's urine (8). Katex is a specific (specificity up to 100%) test with the ability of discriminating between active and past infections, predictive of response to treatment and valid as a *Leishmania* detection test in HIV/VL coinfected patients (7,10–14).

Furthermore, Katex is an inexpensive, easy to perform, field applicable, and electricity independent test. All of these features make Katex as a suitable diagnostic approach for diagnosis of VL in many of developing countries where VL is a serious health problem.

One of the main drawbacks of Katex is the need for boiling the urine for performing the test. This is necessary to remove the false-positive reactivity in the

urine sample. It is obvious that boiling of the urine sample is an inconvenient step in performing the Katex and this in turn affects the Katex field applicability. Moreover, spending time for boiling of sample and awkward odor of vaporized urine reduce the intention of the user to make use of this test in routine setting.

Having these problems with Katex performance, an attempt was made in this study to improve the test performance by omitting the boiling step from the test.

Different chemical and physical reagents have been used by researchers to remove the false positivity from the latex agglutination test. These include pretreatment of the specimens with heat (at 56°C), EDTA (ethylene diamin tetra acetic acid), trypsin, pronase, and 2ME (2 mercaptoethanol) (15–17). With respect to not knowing the exact composition of the carbohydrate antigen in urine, which is the target of Katex, we had to treat the sample with different chemical and nonchemical reagents such as TCA, SSA, which precipitate the urine proteins, SDS, which works by disrupting the noncovalent bonds in the proteins, denaturing them, and causing the molecules to lose their native shape (conformation), and DTT that acts like 2ME to break disulfide bands in protein and glycoprotein.

Although TCA, SDS, SSA, and 56°C heat treatment were not beneficial for the improvement of Katex, the DTT treatment of urine samples could remove false positivity from unboiled urine samples, whereas true positivity was almost saved.

The DTT treatment increases the positive predictive value of the test as it increases the probability of detecting the patient who has the disease, but reduces its sensitivity as some true-positive cases might be missed by this pretreatment of the sample.

Although the exact cause of the original cross-reactivity in Katex remains undefined, when the background diseases in a few of the control samples was evaluated, it was found that a few of false-positive samples were from patients with autoimmune diseases, such as rheumatoid arthritis. This is not surprising as rheumatoid factor is a well-documented cause of false positivity in latex reactions (18,19). Further studies is needed to find out the reasons for false positivity in Katex and also to apply more reagents to urine sample for completely removing unknown substances that is present in urine and interfere with Katex reaction.

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