

A Capture ELISA for the Diagnosis of Visceral Leishmaniasis Using a Monoclonal Antibody against a Leishmanial Urinary Antigen

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Received 14 September 2004; revised 15 January 2005; accepted 30 January 2005

ABSTRACT

A capture ELISA system was developed for diagnosis of visceral leishmaniasis (VL) using a monoclonal antibody raised against an antigen previously detected in the urine of VL patients. Urine samples from confirmed VL cases from Yemen, Nepal, Spain, Sudan and Brazil were tested in the capture ELISA in comparison with urine samples from endemic and non-endemic areas along with urine samples from patients with malaria, brucellosis, schistosomiasis and patients with non-infectious diseases. All of VL patient samples from different geographical areas (apart from 2 samples from Brazil) gave a positive result, while no cross-reaction was found with the control samples. The results obtained with the capture-ELISA were compared to those obtained with KAtex, a previously described latex agglutination test, showed that the KAtex and the new ELISA are comparable in terms of specificity (100%) but a better sensitivity (94.1%) was found for the capture-ELISA. Moreover, the capture-ELISA adds a useful quantitative dimension to antigen detection. In addition, the boiling of urine samples, which is necessary for KAtex, was not required in the capture-ELISA. These results suggest that the antigen detection in urine by the new capture ELISA system provides a useful method for diagnosis of VL and fulfils the requirements of a non-invasive method for diagnosis of VL. *Iran. Biomed. J. 9 (3): 117-122, 2005*

Keywords: *Leishmania donovani*, Visceral leishmaniasis (VL), ELISA, Urinary antigen, Antigen detection

INTRODUCTION

Visceral leishmaniasis (VL) is a disease caused by protozoan parasites of the genus *Leishmania*, in particular *L. donovani* and *L. infantum*. VL remains a serious public health problem in many tropical and subtropical regions of the world. Despite enormous efforts directed towards the development of a suitable test for the serological diagnosis of VL, the parasitological diagnosis of VL which relies on detection of parasite in bone marrow or spleen aspirate still remains the "gold standard" method.

The serodiagnosis of leishmaniasis (based on antibody detection) appears to be an alternative to parasitological diagnosis. A number of serological techniques have been developed for diagnosis of

VL including ELISA [1-3], dot ELISA [4, 5] and direct agglutination test [6-9]. The sensitivity and specificity of such diagnostic methods depends on the type, source and purity of antigen employed, as some of *Leishmania* antigens have common cross-reactive epitopes shared with other microorganisms [10-12]. Detection of antibody in urine has been used for diagnosis of VL. Islam *et al.* [13] reported the detection of IgG in urine of VL patients using ELISA system with 95% sensitivity and specificity. However, there are various problems with antibody detection assays including: (i) they may be positive in asymptomatic infections, and (ii) as with many serological tests, they are not easily distinguished between current, sub clinical, or past infections.

An alternative to antibody detection is antigen detection in urine or serum, which is a recent

approach in diagnosis of parasitic infections. Antigen detection in urine as a non-invasive technique has been used for diagnosis of several parasitic diseases [14-16].

In leishmaniasis, the first detection of *L. donovani* soluble antigen in the urine of VL patients was reported by Kohanteb *et al.* [17], using double countercurrent immunoelectrophoresis. In this study, urinary antigen was detected in 19 out of 21 VL cases. Recently, work in the field of antigen detection in leishmaniasis carried out by Attar *et al.* [18] resulted in the demonstration of an antigen in the urine of VL patients by a latex agglutination test ("KAtex"). The sensitivity and specificity of KAtex was found to be 70-80% and 100%, respectively if the sample was boiled for 5 minutes before testing. Further work showed that the target antigen for this agglutination test is a low molecular weight (5-20 kDa) carbohydrate antigen [19].

In this study, we report an antigen-detection capture ELISA system for diagnosis of VL, using a monoclonal antibody raised against our previously characterized leishmanial urinary antigen [19].

MATERIALS AND METHODS

Parasites. *L. donovani* promastigotes (MHOM/ET/67/HU3; LV9) were cultured in serum-free M199 defined medium [20] in a 25-cm² tissue culture flask at 26°C.

Urine samples. Urine samples from confirmed VL patients were obtained from Yemen (12 samples from parasitologically confirmed cases), Nepal (8 samples), Brazil (8 samples), and Spain (6 samples). Control urine samples were obtained from VL endemic (Yemen and Nepal) and non-endemic (from Royal Liverpool University Hospital, UK) areas along with urine samples from patients with different microbiological infections, but not leishmaniasis, (from Yemen) as well as patients with non-infectious diseases (from Royal Liverpool University Hospital, UK).

Production of polyclonal antibody. Rabbits were immunized with live *L. donovani* promastigotes as described by Attar *et al.* [18]. Briefly, rabbit was immunized IV repeatedly with promastigotes of *L. donovani*, collected from stationary phase cultures. An IgG fraction of rabbit anti-promastigote antibody was prepared by protein A-Sepharose affinity chromatography (CL4B; Pharmacia).

Purification of the leishmanial urinary antigen.

The urinary antigen was purified from the urine of VL patients by phenol-water extraction (Westphal method, [21]) or affinity purification as described before [19]. Briefly, equal volumes of urine and 88% phenol (both pre-warmed to 68°C in water bath) were mixed and stirred at 68°C for 15 min. Sample was cooled to 10°C on ice and then spun at 4°C for 45 min at 1500 ×g. The aqueous phase was removed and the phenol layer was extracted one more time. The aqueous phase was dialyzed against PBS overnight and the extracted antigenic fraction (for convenient called 'phenol extracted antigen') was concentrated by ethanol precipitation. Affinity purification of the urinary antigen was performed using an activated cyanogen bromide column coupled with rabbit anti-promastigote IgG. Two ml of processed urine (e.g. phenol-extracted urine) was loaded onto a 3.5-ml column, the flow-through was collected and the column was washed with PBS to remove unbound antigens. The bound antigen was eluted with fresh elution buffer (50 mM diethylamine in saline, pH 11.5) and collected in tubes containing 100 µl of 1 M phosphate buffer, pH 6.8. The eluted fractions were tested by KAtex and the positive ones were pooled and dialyzed against PBS overnight.

Production of monoclonal antibody against the urinary antigen.

Mice were immunized, intravenously, with 0.2 ml of cell suspension of *L. donovani* promastigotes containing 5×10^7 cells. The immunization schedule was continued with four more injections at 4-5-day intervals. Five days after the last injection, the spleen of the mouse was removed, spleen cells from the immunized mice were isolated and fused with NS-1 myeloma cells (at a ratio of 1 NS-1 cell to 10 spleen cells) using polyethylene glycol. Monoclonal antibody production against the urinary antigen was assessed after 10 to 14 days. Hybridoma cells secreting specific antibody were expanded and cloned by limiting dilution.

Isotyping of monoclonal antibody. Isotyping of monoclonal antibody was performed using monoclonal antibody typing kit (The Binding Site, Birmingham, UK) by placing 75 µl of hybridoma culture supernatant into the central well of the immunodiffusion gel and 10 µl of mouse antiserum (IgM, IgA, IgG1, IgG2a, IgG2b and IgG3) in the peripheral wells. Precipitation line was checked after 48 hours.

Labelling of the monoclonal antibody with horseradish peroxidase. Horseradish peroxidase (4 mg) was dissolved in 1 ml of ddH₂O and mixed with 200 µl of freshly prepared 0.1 M sodium periodate at room temperature for 20 min. After dialysis (against 1 mM acetate buffer, pH 4.4, at 4°C overnight), the pH of the mixture was raised to 9.5 by the addition of 20 µl of 0.2 M sodium carbonate/bicarbonate buffer, pH 9.5. Immediately, 8 mg of purified monoclonal antibody, which had been dialyzed against 50 mM sodium carbonate/bicarbonate buffer (pH 9.5), was added to the solution and mixed for two hours at room temperature. Then, 100 µl of freshly prepared sodium borohydride (4 mg/ml) were added and the solution was mixed again at 4°C for 2 hours. Finally, the solution was dialyzed against PBS overnight and the conjugated antibody was stored at -20°C in small aliquots until use.

Capture ELISA with the produced monoclonal antibody for the detection of the urinary antigen.

Capture ELISA was carried out in flat-bottom 96-well microtiter plates (Immulon II, Dynatech). The plates were coated with 5 µg/ml of rabbit anti-promastigote IgG (100 µL/well) in coating buffer (0.05 M carbonate bicarbonate buffer, pH 9.6) and incubated at 4°C overnight. The plates were washed three times with PBS buffer containing 0.05% Tween 20, pH 7.4 (PBST) every 5 minutes. Urine samples (100 µL) from VL patients along with endemic and non-endemic controls were applied to the plate and incubated for 1.5 hours at room temperature. The plates were washed three times as before and horseradish peroxidase conjugated monoclonal antibody (100 µL, 1/1000 dilution in PBST) was added to the plates and incubated for one hour at room temperature. After being washed three times as before, the plates were incubated with chromogen/substrate (100 µL/well of 0.1 mg/ml ABTS, 0.025% H₂O₂ in 0.1 M citrate buffer, pH 5) for 45 min at room temperature and the OD was measured at 415 nm.

RESULTS

Production of monoclonal antibody against the leishmanial urinary antigen. It was found that the phenol-extracted urinary antigen or the affinity-purified antigen does not stick to the ELISA plate, mainly because of its carbohydrate nature. Therefore, indirect ELISA could not be used for screening of culture supernatant antibody. An

alternative method was developed using rabbit anti-promastigote antibody to capture the urinary antigen and then using the ELISA plate with captured antigen for screening of the hybridoma culture supernatant. This system was optimized and was used for the screening of the antibody-producing hybridomas.

Using this system, more than ten monoclonal antibodies were identified. The performance of one of these monoclonal antibodies, BS5-IVD3, in detecting of the urinary antigen was found to be much better than the others; therefore further work was focused on this monoclonal antibody. The isotype of BS5-IVD3 monoclonal antibody was IgG2a. Purification was performed by a protein A column and the purified antibody was labelled with horseradish peroxidase to be used in ELISA assay.

Table 1. Urine samples of VL patients and controls from Yemen tested by capture ELISA.

Sample	Capture ELISA	Bone marrow aspiration
VL1	0.67	+
VL 2	0.83	+
VL 3	0.83	+
VL 4	0.73	+
VL 5	0.90	+
VL 6	0.88	+
VL 7	0.63	+
VL 8	0.98	+
VL 9	0.61	+
VL 10	0.35	+
VL 11	0.59	+
VL 12	0.69	+
Urinary schistosomiasis ¹	0.12	*
Schistosomiasis ²	0.09	*
Malaria ³	0.08	*
Normal human ⁴	0.11	*

¹, *S. haematobium* (3 samples, mean OD); ², *S. mansoni* (2 samples, mean OD); ³, *P. falciparum* (2 samples, mean OD); ⁴, Normal human urine (9 samples, mean OD); *not done.

ELISA with BS5-IVD3 monoclonal antibody for diagnosis of VL.

All urine samples of VL patients from different VL-endemic areas including: Yemen (12 parasitologically confirmed cases), Nepal (8 samples), Brazil (8 cases) and Spain (6 cases) along with control samples (104 samples) from endemic and non-endemic areas and samples from malaria, schistosomiasis, typhoid, and brucellosis patients were tested by the capture ELISA. The urinary antigen has been detected in all of parasitologically confirmed VL cases from Yemen and all of VL cases from Spain and Nepal (Table 1, Figs. 1 and 2). Same result was obtained with the urine

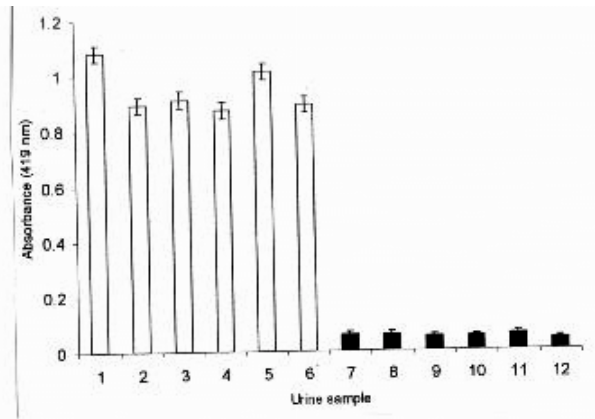


Fig 1. Absorbance values of urine samples from *Leishmania*/HIV coinfected patients (from Spain) and endemic controls tested by capture ELISA. 1-6, *Leishmania*/HIV coinfected patient with active leishmaniasis; 7-9, endemic controls; 10, urine from *Leishmania*/HIV coinfected patient with history of leishmaniasis; but no more information is available; 11 and 12, urine from *Leishmania*/HIV coinfected patient after chemotherapy with anti-leishmanial agents.

samples from Brazil. Using these collections of samples, the test had 100% specificity since there was no cross-reaction with the endemic control samples and all of the samples from VL patients were positive. However, when the ELISA cut off was set at 2SD above the mean of control samples, two cases from Brazil had borderline (1 case) or lower (1 case) OD value. Considering these two samples as negative, a sensitivity of 94% can be estimated for the system.

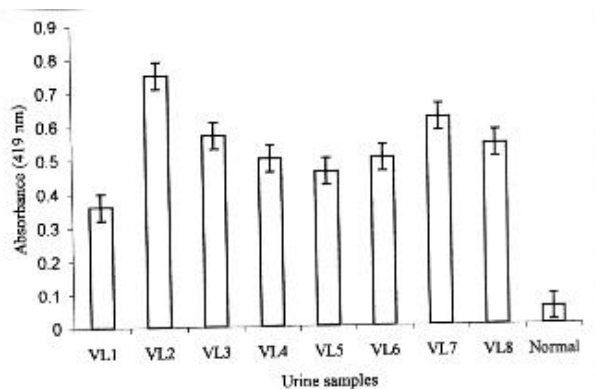


Fig. 2. Urine samples from VL patients from Nepal tested by capture ELISA.

DISCUSSION

Presently available serodiagnostic techniques based on anti-*Leishmania* antibody detection are not entirely satisfactory because they do not discriminate between disease and asymptomatic

infection. The use of an antigen detection system, in serum or urine, can distinguish past and current infection. Moreover, the antigen detection seems to be a method of choice in *Leishmania*/HIV coinfected patient where the antibody detection has limited value.

It seems that antigen detection in urine has many advantages over antibody or even antigen detection in serum. It uses urine that is non-hazardous and can be collected easily on several occasions without causing inconvenience to the patient, and is collected by non-invasive procedures which minimise the risk of transmission of blood-borne infections (e.g. hepatitis B and HIV).

Antigen detection in urine has been used for diagnosis of several parasitic infections including schistosomiasis [22] malaria [14], trypanosomiasis [23, 24] and filariasis [25]. Azazy *et al.* [26] reported a 45 and 58 kDa *Leishmania* urinary antigens in the urine of experimentally infected animals using anti-amastigote IgG and De Colmenares *et al.* [27] demonstrated two polypeptide molecules of 72-75 kDa in the urine of 14 out of 15 VL patients. Recently, Attar *et al.* [18] demonstrated an antigen in the urine of both experimentally infected cotton rats and VL patients using a latex agglutination test. The test had a sensitivity of 75-80% and was highly specific for VL cases.

In the present study, we produced a monoclonal antibody against the previously characterized leishmanial urinary antigen [19] and used it in a capture ELISA for diagnosis of VL. The specificity of our capture ELISA system appears to be 100% since no cross-reaction was found with the urine samples from different VL-endemic areas (Yemen, Spain, Brazil and Nepal) and also non-endemic controls.

The specificity of our ELISA assay seems to be equivalent to that of previously described latex agglutination test [18, 28]. However in the latex agglutination test the sample needs to be treated (heating at 100°C for 5 min) before testing. The new ELISA system circumvented this problem since the urine sample does not need any treatments before testing.

Based on results obtained from testing of the urine samples from confirmed VL patients (26 cases) it seems that the introduced captures ELISA has got a sensitivity as high as 94%. However, it might be too early to draw this conclusion since the system must be checked in a larger field condition with a relatively large sample population.

Our results of capture ELISA, using samples of different VL endemic areas indicate that the test

performance is well regardless of geographical source of sample. Taken together, antigen detection in urine of VL patients with capture ELISA is promising since it uses urine specimens, rather than serum, which reduces the risk of infection to the person carrying out the test.

ACKNOWLEDGEMENTS

This work was supported in part by the Ministry of Health and Medical Education (Iran). We thank Dr. J. Carney (Kalon Biological Ltd.) for providing the latex agglutination kit.

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