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# Detection of urinary antigens and their seroreactivity with serum of patients in *Leishmania donovani* infection

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## ARTICLE INFO

## ABSTRACT

**Objective:** To detect leishmanial antigens in pre and post treated urine of visceral leishmaniasis (VL) patients. **Methods:** Urine and serum sample from three VL patients were collected. Ammonium sulphate precipitation and purification of urine sample was done for proteins isolation. SDS PAGE of proteins was done followed by western blotting, with the patient's pre and post treatment serum. **Results:** Eight proteins of molecular weights 17 kDa, 25 kDa, 28 kDa, 42 kDa, 47 kDa, 54 kDa, 60 kDa and 85 kDa were detected in the urine of VL patients before treatment. After treatment with miltefosine, none of the above proteins was detected in urine samples. The western blot analysis with pre treatment serum confirmed the antigenicity of four urinary proteins of molecular weights 25 kDa, 28 kDa, 54 kDa and 60 kDa. The seropositivity with 25 kDa and 28 kDa antigens was negative with serum obtained after the completion of treatment. **Conclusions:** In the context to unavailability of a prognostic tool, urinary leishmanial antigens may offer a better choice and may also be useful as immunoprophylactic candidates.

## 1. Introduction

The leishmaniasis has been considered as tropical affliction that together constitutes one of the six entities on the World Health Organization tropical disease research list of most important disease[1]. It occurs in 88 countries in tropical and temperate regions, 72 of them developing or least developed. The annual global prevalence of all form of leishmaniasis is approximately two millions. However, there is a gross under reporting of the cases from endemic regions in developing countries like India, Nepal and Bangladesh. New cases are often being reported from newer areas mainly due to population migration from endemic to non−endemic regions [2]. Indian kala−azar or visceral leishmaniasis, caused by *Leishmania donovani (L. donovani)*, is a potentially fatal disease. It is endemic in eastern part of India and often turns epidemic. Out of 500 000 new cases of VL that occurs every year worldwide, about 100 000 cases of VL are estimated to occur annually in India[3].

The state of Bihar accounts for more than 90% of the cases and other affected area includes the states of Assam, West Bengal and eastern part of Uttar Pradesh[4].

The successful management of leishmaniasis requires an early and accurate diagnosis as well as a test of cure to confirm the final status of disease. Various serological tests based on serum antibodies detection like rk39 strip test, ELISA and direct agglutination test (DAT) are being used to diagnose VL with high sensitivity but their specificity is very poor in disease endemic areas[5]. Moreover, the prognostic capabilities of the current methods is highly compromised because anti−leishmanial antibodies may persist in the serum for extended period even after successful treatment, which limit their use in the diagnosis of past infections, reinfections, relapse and as a test of cure[5, 6]. So a specific test that could be used for both, diagnostic and prognostic purpose is still required.

The presence of parasitic antigens released in urine can be linked to the level of renal physiology as well as status of infection present in the host. In addition, the urinary antigens may be also useful in diagnostic and prognostic purposes. The diagnostic capabilities of urinary antigens have been reported in various infectious diseases like schistosomiasis[7], malaria[8], Chagas disease[9] and rever blindness[10]. The presence of soluble urinary leishmanial antigens in visceral leishmaniasis has been successfully

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reported in various studies however, their characterization has not been done in most of the cases[11–14]. This study was done to detect the antigens of leishmanial origin in pre and post treatment urine samples of VL patients and to evaluate their seropositivity.

## 2. Materials and methods

### 2.1. Patients

Three confirmed cases of VL [mean age (46.00±1.73) years] admitted to village primary health center, Baniyapur of district Saran, State of Bihar, India were included in this study. This study area is hyper−endemic region for VL. These patients were clinically correlated for VL before the start of treatment. The pretreatment basal characteristics viz. weight, spleen size, WBC count, hemoglobin and platelet counts were (50.33±1.52) kg, (8.07±1.17) cm, (4.3±0.1) x10$^3$ mm$^3$, (8.37±0.32) g/dL and (83 000±11269)/$\mu$L respectively. They were treated with miltefosine, available at the primary health center, as per recommended guidelines. After completion of treatment, increase in weight [(50.66±1.53) kg], WBC count [(5.43±0.38)×10$^3$ mm$^3$], hemoglobin[(11.000±0.260) g/dL], platelet counts (140666±5033) /$\mu$L] and decrease in spleen size [(3.13±0.12) cm] were observed. For control, one healthy individual (one of the author of this study) from non−endemic region and one from endemic region without any past history of VL were used for urine collection.

### 2.2. Sample collection

The twenty four hour urine samples were collected for isolation of proteins. On the same day samples were brought on ice to Molecular Immunology Laboratory, Department of Biochemistry, Banaras Hindu University, Varanasi, and processed for isolation of urinary antigens (ULAs). The sera were obtained from blood that was collected for routine laboratory analysis.

### 2.3. Isolation of ULAs

The urine samples were centrifuged at 5 000 rpm for 10 min at 4 ℃ to remove sediment and debris. The clear urine samples were subjected to ammonium sulphate precipitation (90%) by adding calculated amount of powdered ammonium sulphate according to their percentage saturation directly into the urine samples with constant stirring on magnetic stirrer at 4 ℃ overnight followed by centrifugation at 10 000 rpm for 10 minutes at 4 ℃. Pellets containing the proteins were dissolved in small volume of phosphate buffer saline (PBS). The isolated proteins were purified by Amicon Ultra (Millipore, USA), a 3 kDa molecular weight cutoff membrane filtration device. The purified proteins were quantified by Lowry method and stored at −80 ℃ till further use.

### 2.4. SDS−PAGE

Purified proteins were resolved by SDS−PAGE analysis of

that was carried out using 12% non gradient slab gel with a 5% stacking gel as per standard protocol at 100V for 3.5 hours by using gel electrophoresis system (Klever Scientific, UK).The protein bands were stained with commassie brilliant blue (CBB). The gels were visualized and analyzed by gel documentation system (Alpha Innotech Corporation, USA) using gel densitometry software against known molecular weight markers.

### 2.5. Immunoblotting

In order to confirm the antigens of leishmanial origin, western blot analysis was done. Proteins were transferred onto PVDF membrane according to the standard protocol in a mini trans−blot electrophoresis transfer cell (Klever Scientific, UK) following manufacturer's protocol. Briefly, the membranes were soaked in blocking buffer (5% non−fat dried milk in a PBS (0.02 M, 0.9% NaCl, 0.1% Tween 20) for three hours at room temperature [(22± 2) ℃]. After three times wash in PBS/Tween 20, the membranes were incubated with sera (1:100 dilutions) obtained from patients before and after the treatment blocking buffer for 2 hours at RT. The membranes were again washed for three times to remove unbound antibodies and incubated with a 1:3000 dilution of AP−conjugated anti−human immunoglobulin G (Sigma, Aldrich, USA) in blocking buffer followed by three additional washes. Color was developed by using the BCIP/NBT as substrate for alkaline phosphate and reaction was stopped by distilled water. The developed membranes were photographed after drying and analyzed by Gel documention system (Alpha Innotech Corporation, USA) using gel densitometry software.

## 3. Results

Eight proteins of molecular weights 17 kDa, 25 kDa, 28 kDa, 42 kDa, 47 kDa, 54 kDa, 60 kDa, and 85 kDa were detected in pretreated urine samples of VL patients. (Figure 1, lane A–C) Although, all proteins were detected in all three samples but their excretion in urine was differential in terms of quantity of proteins. The 85 kDa protein was less excreted in first patients. The excretion of 25 kDa antigen was highest in all patients.

The western blot analysis with patients serum obtained prior to treatment confirmed the antigenicity of total four urinary proteins of mw 25 kDa, 28 kDa, 54 kDa, and 60 kDa. (Figure 2 lane A–C). The antibodies against proteins of molecular weight 17 kDa, 42 kDa, 47 kDa and 85 kDa were not detected in pretreated serum samples. The blot analysis with serum samples obtained a day after the completion of treatment showed that antibodies against 54 kDa and 60 kDa were persistent in serum even after successful treatment (Figure 2 lane D–F). These urinary proteins were not reactive with serum samples obtained from healthy endemic and non−endemic controls. (Figure 2 lane G, H)
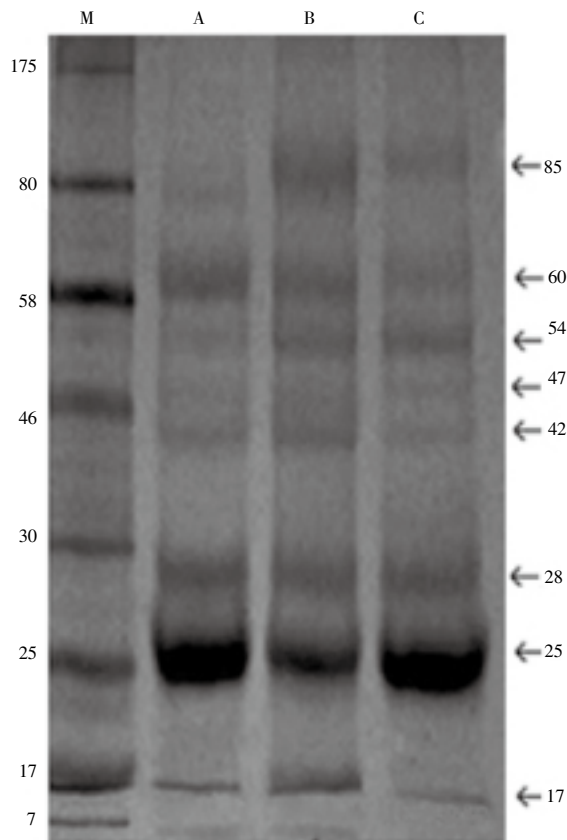
**Figure 1.** Urinary proteins on 12% SDS–PAGE. Lane M– molecular weight marker (7–175kDa); lane A–C: VL patients.
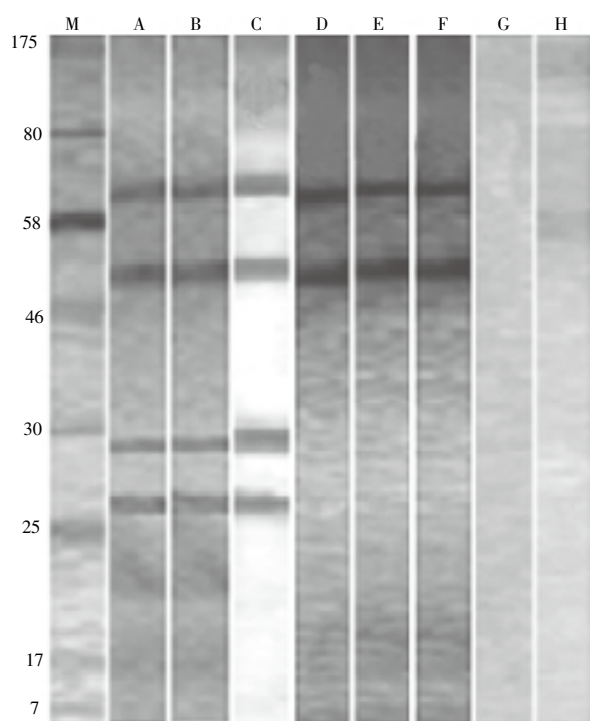


**Figure 2.** Immunoblot of urinary proteins with pre and after treatment serum samples of patients and controls.

Lane M: marker (7–175kDa); lane A–C: pretreatment serum, lane D–F: post treatment serum, lane G: endemic control, lane H: non–endemic control.

## 4. Discussion

A common symptom of the renal lesion due to protozoan infection is immunodeposits in granular pattern [15], and it also results in parasitic antigen release in urine[16]. The studies on urinary antigens can be correlated to immunopathogenesis & immunoprophylaxis, and it can also be beneficial to develop diagnostic and prognostic assays with excellent sensitivity & specificity. In spite of availability of various serological tests, the major problem of current tools for VL is their cross reactivity with other diseases like malaria, tuberculosis, sleeping sickness, amoebiasis etc, which are co–endemic in affected region[5, 17].

In our previous studies, we have found that up to 32% of healthy people without any sign and symptoms of VL could be seropositive by these test[5]. In these situations, and because of the serious therapeutics implication related to an incorrect or late diagnosis of VL, an accurate laboratory test is required to confirm the clinical diagnosis[18]. To the best of our knowledge, this is the first study that reports severe urinary proteinuria in VL patients and implicate their circulatory antibodies in patients blood. This study further demonstrates the differential excretion of urinary proteins that may be correlated with disease severity. Following treatment with miltefosine the proteins were disappeared in urine, which confirms the temporary presence of urinary antigens due to the leishmaniasis.

Immunoblot analysis using patient's serum revealed that four urinary proteins are immunogenic in nature. This is an interesting finding and we are evaluating their immunoprohylactic capabilities in context to unavailability of a vaccine candidate and a true drug target. Further the disappearance of antibodies for 25 kDa and 28 kDa antigens offers a greater probability for development of a prognostic serological test based on these antigens. However, the disappearance of urinary proteins after successful treatment seems to be more reasonable to develop diagnostic and prognostic tools as the urine based assays are noninvasive and facilitates compliance of people in field activities.

To conclude and in the context to unavailability of a perfect diagnostic and prognostic tool, the urinary leishmanial antigens (ULAs) offer a better prospective. However, more detailed investigations are required for better understanding to develop an accurate diagnostic and prognostic tool, which is crucial to establish and implement measures to control the spread of this disease in disease endemic countries.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgements

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