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ORIGINAL ARTICLE

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ELISA and western blotting for the detection of Hsp70 and Hsp83 antigens of *Leishmania donovani*

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Abstract Visceral leishmaniasis (VL) is endemic in the tropical, sub-tropical regions of Asia, Africa, the Mediterranean, Southern Europe and South and Central America. Approximately 500,000 new cases are reported annually. Classically the diagnosis of VL is confirmed by the demonstration of the parasite in aspirates of spleen, bone marrow or liver which can yield false negative results, also these methods are invasive. In this study, we aimed to evaluate the serodiagnostic potential of two heat shock proteins (Hsps) i.e. Hsp70 and Hsp83 in combination by ELISA and western blotting in mouse model. Parasite proteins were separated by SDS-PAGE and transferred to PVDF membranes which were further incubated with serum samples of infected mice for visualizing different bands. Both the bands i.e. Hsp70 and Hsp83 were simultaneously visualized in all infected groups on different post infection days. The presence of both the antigens was also detected by sandwich ELISA. The results suggest that the simultaneous occurrence of both the antigens Hsp70 and Hsp83 may be useful in serodiagnostic assay of VL as it may reduce the need for traumatic bone marrow sampling and risky spleen aspiration.

Keywords Hsp70 · Hsp83 · ELISA · Western blotting

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Introduction

Since its resurgence, the increasing incidence of kala-azar remains unabated despite aggressive treatment and control measures. Routine diagnosis of the acute disease relies on classical microbiological methods like splenic/bone marrow aspirates which are cumbersome, invasive and painful (Sundar and Rai 2002). These classical methods are also limited by low sensitivity, requiring repeated tissue sampling and trained laboratory staff (Badaro et al. 1996). Frequently, false positive results are obtained when the sera from the chagas disease, leprosy, tuberculosis and malaria patients are assayed (Zijlstra et al. 1992). Therefore, there is a need to develop a more rapid, inexpensive and simple assay for diagnosis of visceral leishmaniasis (VL). Since high titres of antileishmanial antibodies are detected in the sera of VL patients, serodiagnosis may be a better alternative to the parasite detection in biopsy samples. Current serologic tests such as DAT, IFAT and ELISA use crude antigen preparations and are limited in terms of specificity as well as assay reproducibility (Kar 1995). In order to improve the specificity of the serodiagnostic tests a search for defined *Leishmania*-specific antigens have been undertaken and various candidates have been proposed (Ashford et al. 1992; Kar 1995; Jaffe et al. 1990). Two heat shock proteins, Hsp70 and Hsp83 have previously been reported to induce a strong cell mediated and humoral immune responses during leishmanial infections (Kaur et al. 2011a). Hsp70 is a major target of immune responses to a wide variety of pathogens including bacteria, fungi, helminths (worms) and protozoan parasites (Young and Elliot 1989; Kaufmann 1990; Young et al. 1990; Young 1990). It has been reported that parasite-derived Hsp70 plays an important role in the host-parasite interactions (Polla 1991). Hsp70 of *Trypanosoma cruzi*, the causative

agent of chagas disease, has been reported to be a major target of humoral immunity in human infections. Despite its high degree of evolutionary conservation, antibodies are highly specific for the parasite Hsp70 and do not have cross-reactivity to the human Hsp70 (Engman et al. 1990). A sero-specific epitope has been identified in rHsp70 of *Leishmania donovani* (Arora et al. 2000). Hsp70 has also been found to enhance the immunogenicity of gp63 based protein against *Leishmania donovani* in mice (Kaur et al. 2011b). Hsp83 also has been shown to be an immunodominant antigen recognized by sera from diffuse cutaneous leishmaniasis patients (Skeiky et al. 1997). In an earlier study, Hsp83 of *L. infantum* has been found to be useful for serodiagnostic assays for canine leishmaniasis (Angel et al. 1996). Therefore, Hsp70 and Hsp83 might be good candidates as diagnostic antigens. Although various serologic techniques can detect active leishmaniasis when a high level of specific antibodies is present, they do not distinguish between various phases of the disease when levels of antibodies are at or near the cut-off level. Western blot analysis of whole parasite antigens is considered to be sensitive when low serum antibody titers are present (Riera et al. 2004). Western blotting is not preferred in case of cutaneous leishmaniasis because antileishmanial antibodies are present in low titers. Therefore most of the work concerning the diagnosis of leishmaniasis has been done on visceral leishmaniasis. In a study by Talmi-Frank et al. (2006)12, 14, 24, 29, 48, and 68 kDa bands were found to be immunodominant during experimental canine visceral leishmaniasis. Keeping in view the above background the present study was designed to evaluate the serodiagnostic potential of two defined *Leishmania*-specific antigens i.e. Hsp70 and Hsp83 in combination using sandwich ELISA and western blotting.

Materials and methods

Parasite

The Indian strain of *Leishmania donovani*, viz; MHOM/IN/80/Dd8, originally obtained from the London School of Hygiene and Tropical Medicine, U.K, was used for doing the present experimental work.

In vitro culture of parasites

The promastigotes of *L. donovani* were grown at 22 ± 1 °C in RPMI-1640 supplemented with 10 % FCS. The promastigotes were examined by wet mount preparation and the promastigotes were seen as motile, spindle shaped, fast swimming organisms with long anterior flagellum. The strain was maintained by serial subculture after every 48–72 h. The amastigotes were grown in the same medium at a temperature of 37 °C and a pH of 5.5 with 5 % CO_2 and 90–95 % relative humidity.

Animals

5–6 weeks old inbred BALB/c mice of either sex, weighing 20–25 g were used for this work. These were obtained from the central animal facility NIPER Mohali, Punjab. They were fed with water and mouse feed ad libitum in the Central Animal House, Panjab University Chandigarh. The ethical clearance for conducting the experiments was obtained from the Institutional Animal Ethics Committee, Panjab University, Chandigarh, India.

Identification of promastigote antigen by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970). Parasites were harvested from the culture, dissolved in sample buffer and boiled for two minutes in boiling water bath. After loading the antigen the electrophoresis was carried out at 90 V from cathode to anode till the bromophenol blue tracer reached the bottom of the running gel. In one lane, molecular weight markers were also run. The gels were stained in Coomassie blue for 4 h and then destained in order to visualize the protein bands.

Electroelution of antigen band from SDS-PAGE gels

The proteins with a molecular mass of Hsp70 and Hsp83 were visualized, localized in gels and eluted by electrophoresis in running buffer (0.025 M Tris, 0.192 M glycine, 1 % SDS) at 10 mA for 5 h. After elution the proteins were dialyzed, lyophilized, suspended in PBS and filtered for further utilization. The proteins were quantified by Lowry method (1951).

Raising of antisera in rabbit against specific antigens

20 μg of purified antigen was injected into the New Zealand white rabbit along with complete Freund's adjuvant subcutaneously. Two booster injections of same amount of antigen in Freund's incomplete antigen were given at an interval of 2 weeks. Western blotting was carried out to check the antisera. It was found to be positive and the rabbits were given a booster dose and bled after 1 week. The blood was kept at 4 °C for 12–14 h and then centrifuged at 5,000×*g* for 15 min for collection of antisera (Azazy et al. 2008).

Detection of antigens in serum samples by ELISA

ELISA for detection of antigen was done by double antibody sandwich method (Voller et al. 1976). Wells of plates were coated with antisera raised against specific antigen made up to 5–10 mg/ml in coating buffer. Coating was done at 37 °C for 5 h. Plates were washed with PBS Tween-20. Test serum diluted serially with PBS Tween-20 was added to each well. Incubation was done at 4 °C overnight. Plates were washed again. HRP conjugated antimouse immunoglobulins were then added and incubated at 37 °C for 3 h. After washing, substrate and chromogen were added and absorbance was read on an ELISA plate reader at 450 nm.

Detection of Hsp70 and Hsp83 in serum samples by western blotting

Immunoblot assay was performed according to the method of Rolland-Burger et al. (1991)with slight modifications. For analysis the leishmanial antigen run on SDS-PAGE was transferred to a PVDF membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol and 0.038 % SDS) for 90 min at 250 mA. The blots were then incubated with blocking buffer (4 % BSA 100 mM NaCl, 25 mM Tris pH 7.6, 0.1 % Tween-20) overnight at room temperature. Now the blots were incubated with sera from different groups of mice. The blots were then washed and incubated with anti-mouse IgG–HRP conjugated in blocking buffer for 1 hour at room temperature. Enzymatic activity was revealed with 3,3′-diaminobenzidine tetrahydrochloride in tris buffered saline (TBS) containing 30 % H_2O_2.

Results and observations

Identification and purification of proteins

The parasite proteins were identified by running them in SDS-PAGE. After electrophoresis, the protein bands of 70 and 83 kDa were visualized (Fig. 1d). The bands were excised from the gel and submitted to electroelution (Fig. 1b, c). During electroelution a constant voltage of 50 V for 5–6 min was applied through the gel.

Sandwich ELISA

Antibodies against Hsp70 and Hsp83 were raised in rabbit by subcutaneous administration of these antigens. Two booster doses were given and blood was collected after 1 week of second booster dose and then serum was separated. The specificity of antigen was checked by western blotting. Two bands (83 and 70 kDa) were observed as depicted in Fig. 1b, c. For ELISA 96 well plate was coated with antiserum (overnight at 4 °C) raised in rabbit. Antiserum was decanted and plate was incubated with serum samples of infected groups of animals collected at different days post infection. Thereafter the plate was incubated with secondary antibody conjugated with horseradish peroxidase (HRP). After adding substrate and stop solution the absorbance was read at 450 nm.

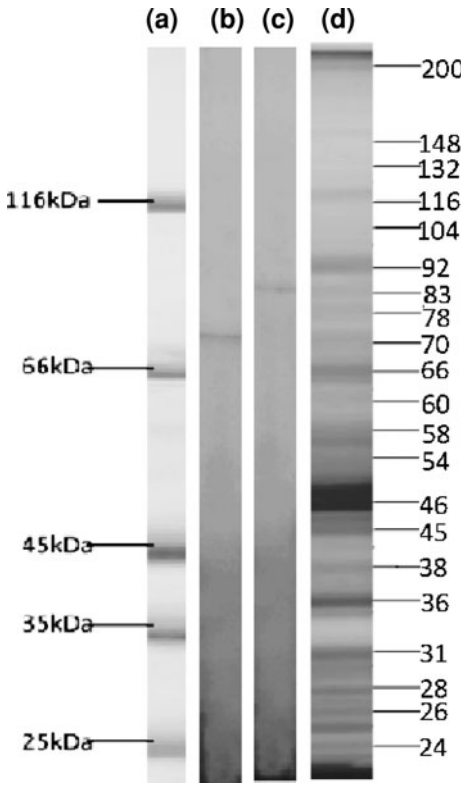

Fig. 1 SDS-PAGE profile of *L. donovani* and electroeluted proteins. *Lane* (*a*) Molecular weight markers; *Lane* (*b*) Hsp70; *Lane* (*c*) Hsp83 and *Lane* (*d*): Parasite antigens

The absorbance values observed were 0.638 ± 0.059, 0.456 ± 0.042 and 0.408 ± 0.035 on 30, 60 and 90 days of post infection. The absorbance value for normal uninfected mice was 0.078 ± 0.004. The difference in absorbance values of infected and normal uninfected groups was significant ($p < 0.001$). The difference in absorbance values was also significant from 30 to 60 days.p.i. ($p < 0.005$). However it was not significant from 60 to 90 days.p.i. (Fig. 2).

Identification of Hsp83 and Hsp70 in infected mice and expression of other antigens by western blotting

The parasite proteins were separated electrophoretically using SDS-PAGE. Then they were transferred to PVDF membrane. The membrane was cut into strips. The strips

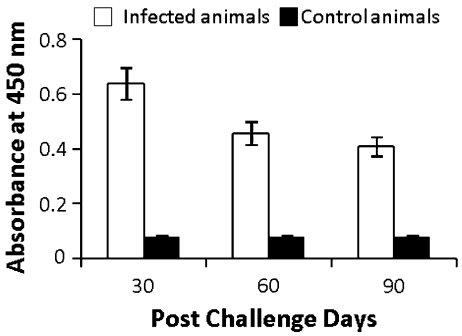

Fig. 2 Detection of Hsp83 and Hsp70 antigens in serum samples of infected mice on different days post infection by sandwich ELISA. *p* value: Infected mice versus normal mice $*p < 0.001$

were incubated with serum samples of different groups of animals. The bands appeared after incubation with secondary antibody and substrate. The antigen profiles in different groups were analyzed.

In the sera of infected control group many antigens were identified. Their molecular weight ranged from 36 to 104 kDa. The antigens which showed reactivity with secondary IgG antibody were 36, 46, 54, 58, 66, 70, 78, 83, 100 and 104 kDa. The antigenic profile was similar on different days post infection (Fig. 3).

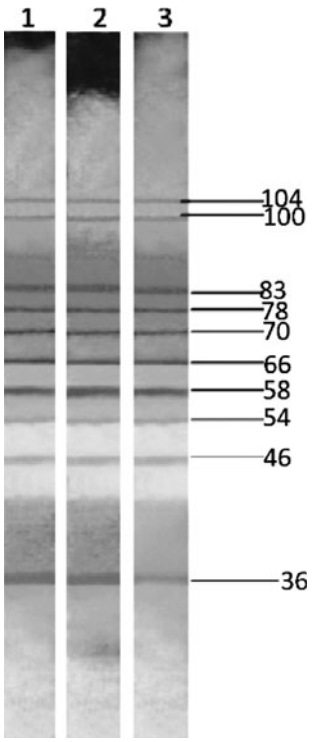

Fig. 3 Detection of Hsp70 and Hsp83 antigens in the serum samples of infected mice on different post infection days by Western blotting. *Lane* 1, 2 and 3 shows blotting analysis with sera of mice on 30, 60 and 90 days post infection

Discussion

There are many challenges in the diagnosis of the leishmaniasis. Early diagnosis of the disease is difficult because the clinical and epidemiological findings in various forms of leishmaniasis resemble several other diseases. Untreated VL patients also act as a reservoir for parasites and therefore contribute to disease transmission in anthroponotic VL areas. Therefore, early case-finding is considered an essential component of VL management.

ELISA has shown high diagnostic accuracy in several studies (Iqbal et al. 2002; Sinha and Sehgal 1994; Sreenivas et al. 2002). In order to develop specific assays for the serodiagnosis of leishmaniasis, several promastigote and amastigote antigens, purified antigens such as FML, defined, synthetic peptides or recombinant antigens have been characterized and evaluated. Among the many antigens used in ELISA for the diagnosis of leishmaniasis are total soluble antigen (Hommel et al. 1978), dp72 (Jaffe and Zalis 1988), gp 63 (Okong'o-Odera et al. 1993), 78 kDa (Ravindran et al. 2004) and rK39, a recombinant and a kinesin-related protein (Burns et al. 1993). Some other antigens from *L. infantum* such as the acidic ribosomal proteins P2a and P2b, the ribosomal protein P0, the histones H2A (Soto et al. 1995) and H3 (Soto et al. 1996) were isolated and characterized for diagnosing canine VL. Similarly, the heat shock proteins Hsp70 and Hsp83 have shown the serodiagnostic potential against experimental murine visceral leishmaniasis.

An antigen detection test would, in principle, provide better means of diagnosis since antigen levels are expected to broadly correlate with the parasite load. Antigen detection systems are also an ideal alternative to the antibody detection systems in immunocompromised patients and more particularly with the growing number of HIV co-infected cases, especially in advanced cases where the immune response is impaired (Rosenthal et al. 1995). A latex agglutination test detecting a heat-stable, low-molecular-weight carbohydrate antigen in the urine of VL patients has shown promising results (Attar et al. 2001; Sarkari et al. 2002). Several studies conducted in East Africa and the Indian subcontinent showed good specificity but only low to moderate (48–87 %) sensitivity (Rijal et al. 2004; Sundar et al. 2005; Sundar et al. 2007). Apart from its low sensitivity, there are two practical limitations: the urine must be boiled to avoid false-positive reactions and it is difficult to distinguish weakly positive from negative results, which affects the reproducibility of the test (Chappuis et al. 2006; Rijal et al. 2004). Therefore, no satisfactory antigen detection test is currently commercially available. In the present work, an effort was made to develop an antigen detection test. For this the simultaneous occurrence of two heat shock proteins Hsp70 and Hsp83

was analyzed. A sensitivity of 100 % was observed in *L. donovani* model of experimental murine leishmaniasis. The absorbance value was 8.18-fold on 30 p.i.d in infected controls as compared to normal uninfected controls. However it decreased on 60 (5.850-fold) and 90 (5.23-fold) days post infection. Several other investigators have also reported heat shock proteins (Hsp) from *Leishmania* belonging to the 60, 70, 83, and 90 families that have been tested for the serodiagnosis of leishmaniasis. *L. major* Hsp 60 was tested with cutaneous leishmaniasis sera (Rey-Ladino et al. 1997) and *L. braziliensis* Hsp83 and Hsp70 with cutaneous, mucocutaneous and diffuse cutaneous leishmaniasis sera (Skeiky et al. 1997). In another study, two polypeptide fractions of 72–75 kDa were detected in the urine of 14 of 15 patients with visceral leishmaniasis (VL) and another fraction of 123 kDa was found in 10 of the 15 patients with 96 % sensitivity and 100 % specificity (De Colmenares et al. 1995).

Western blotting was used to identify the parasite antigens recognized by serum samples from the experimentally *L. donovani* infected BALB/c mice. The main advantage of western blotting over other serologic techniques that use whole *Leishmania* antigen is its capacity to discriminate early infections. The results showed that bands of both heat shock proteins (Hsp70 and Hsp83) were simultaneously present in all the infected animals on all post infection days. Other antigens frequently recognized were those with molecular weights of 36, 46, 54, 58, 78, 100 and 104 kDa. Therefore, the results suggest that Hsp70 and Hsp83 in combination may be considered good candidates for diagnosis of visceral leishmaniasis. Therefore, these studies must be further followed for diagnostic purposes in human patient's sera.

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