Identification of An Antigenic and Potential Diagnostic Marker of *Leishmania donovani* Infection by Immunoblot Assay

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Abstract

Leishmania donovani parasites from a sodium antimony gluconate (SAG) refractory patients of endemic region were cultured for extraction of crude soluble antigen and performed Western blot with sera from 138 subjects of different groups for diagnosis of Kala azar. The pretreated patients, however, frequently showed 18 bands of different molecular weight, the 85, 74, 63, 31, and 28 kDa were the major proteins against which more than 75% patients developed antibodies. The 74 kDa protein fraction band, which was consistently present in all the pretreated patients(n=35), completely waned out or found in state of virtually absent gloomy band in the six-month follow up patients (n=24). Furthermore, the band was absent in 91% of healthy controls from endemic population(n=33) and was absolutely absent in non-endemic(n=13) and other diseased(n=19) subjects. Although the band was present in 86% of Day 31 subjects(n=14), the average integrated density value (5110±634) of the paired samples was significantly less (p value, <0.0001) than that of Day 0 (11385±1230). The nature of the antigen showed its diagnostic and prognostic importance along with marker for kinetic recovery. Sensitivity and specificity of the protein were absolute in patients and non-endemic population, however, sensitivity was 94% in endemic population. Moreover, it did not show cross-reactivity to different disease groups. Hence, it could be a good diagnostic tool for Kala-azar disease.

Key words: antigenic fraction, diagnosis, immunoblot, Kala-azar, leishmaniasis, SDS-PAGE

Introduction

Visceral leishmaniasis(VL) is caused by *Leishmania donovani,* which comes under an important disease as it causes absolute death if untreated. It accounts for 75,000 deaths per year (Ashford *et al.* 1992, Desjeux 2004) and is clearly a poverty-related disease characterized by prolong fever (often with chills and rigors), splenomegaly, hypergammaglobinaemia, hypoalbuminemia, weight loss, lymphadenopathy and pancytopenia (Berman 1997). It has been a major problem in the affected regions of the Indian subcontinents (India, Nepal and Bangladesh), Brazil and Sudan with 90% of the total outbreak for which high priority is given by their respective governments (Yao *et al.* 2007). In Nepal, 13 districts from southeastern Terai region are endemic zone for VL and more than 6 million people are at risk and average toll rate for it is around 1341 cases annually (Pun *et al.* 2011). The real figure of its impact is beyond the official data as many peoples in rural areas are dying without proper diagnosis before they come for treatment.

L. donovani composed of many soluble protein components of glycoproteins and lipoproteins make the parasites antigenically complex albeit only a limited number of antigens evoke protective host immunological responses (Manuel & Behin 1987). The polyclonal activation of host immune response against putative leishmanial proteins lead to enormous production of IgG, the major class of antibodies present in the sera of Kala-azar patients (Ghose *et al.* 1980). Although the life cycle of the parasites involve an extracellular motile promastigote in sandfly and an intracellular non-motile amastigote within the

mononuclear phagocytes of vertebrate host, there are many immunogenic proteins, which are common in both stages (Handman *et al.* 1984). These proteins have been shown to play important roles in parasite virulence both in vector and the mammalian host and are very useful clinically (Mengeling *et al.* 1997, Ilg *et al.* 1998, Ilg *et al.* 1999, Piani *et al.* 1999, Stierhof *et al.* 1999). Exploitation of these antigens would thus be the better means for diagnosis.

For the study of antigens and selection of appropriate diagnostic marker, Western blotting (Immunoblotting) is being used as an effective and simple technique. Immunoblots from SDS-PAGE comprehensively documents the antigenicity of the parasites and the range of the specificities of antibody responses against these parasites (Forgber *et al.* 2006). The usefulness and high sensitivity of immunoblotting methods and its superiority over serodiagnostic methods like IFA, ELISA for diagnostic purpose have been well established (Mary *et al.*1992, Albertini *et al.*1993, Badaro *et al.* 1996, Salotra *et al.* 1999). Reports on a number of *Leishmania* antigens of molecular weight ranging from 14-76 kDa and a few higher molecular weight antigens have been reported as diagnostic marker for symptomatic as well as asymptomatic VL (Tiwari *et al.* 1995, Da Costa *et al.* 1996, Aisa *et al.*1998, Fernandez-Perez *et al.* 1999, Rami *et al.* 2005).

This part of the study has tried to exploit the antibody response in identification of antigens (diagnostic marker) which is responsible for the symptomatic VL cases. A prominent band recognized by active patient sera has been identified which in the course of treatment gradually fades-out and finally disappears or virtually disappear in six month follow up. This band was found absent in all other study groups except in few endemic healthy control subjects (6%) indicating its potential as a marker of VL patients.

Methodology

Parasites and crude soluble antigen (CSA)
L. donovani parasites (MZP 151) drawn from splenic aspirates of sodium antimony gluconate (SAG) refractory VL patients from endemic region (Muzaffarpur, Bihar, India) were inoculated in biphasic medium in NNN tube. After 7 days of incubation the culture was grown at 25^0 C in RPMI–1640 medium (HyClone) supplemented with 0.2 % $NaHCO_3$, 2.05 mM

L-Glutamine, 12 mM HEPES buffer (HiMedia, India), 15% (v/v) heat inactivated FBS (Gibco, Germany) and 50mg/l Gentamycin and subculture at an average density of 5X 10^7 cells/ml. Parasites of stationary phase were pelleted at 6000 rpm for 20 minutes at 4^0C. Pellets were pooled and washed for 4 times in PBS and stored at -70^0. Approximately equal volume of the lysis buffer (7.4 pH, Tris HCl 20 mM, NaCl 40 mM, EDTA 10 mM, PMSF prepared in methanol 2 mM, leupeptine 10 µg/ml, Nonidet-40 2%, 10 % SDS 0.4% maintained in TDW) was added to the pellet and mixed thoroughly. The solution was then sonicated (Soniprep 150) at 12 amplitude for 10 cycles 30 sec. each in cold-chain. Further, the suspension was centrifuged at 3000 rpm and 4^0C for 20 minutes. The supernatant, crude soluble antigen (CSA). was collected and stored at -70^0 C until use. The protein concentration in the CSA was quantified by dye binding method using albumin as the standard protein (Bradford 1976).

Human sera
Altogether 138 blood samples from different study subjects were collected for analysis of antibodies in the sera. Sera from 35 microscopically confirmed Kala-azar cases (Day-0), 14 post treated (Day-31) with complete course of amphotericin B deoxycholate (infusion of 1 mg/kg on alternate days for 1 month), 24 follow up (Day-180), and 33 healthy attendants of the patients from the endemic region who were either positive or negative to the rK39 strip test (EC) were collected from study site. For the non-endemic healthy control, blood samples from 13 volunteers were collected from Varanasi who had no history of kala-azar (NEHC). Samples from 19 patients of different diseases other than Kala-azar (DD) were collected from Sir Sundar Lal Hospital, BHU, Varanasi, which comprised malaria (n=5), tuberculosis (n=6), amoebic liver abscesses (n=2), enteric fever (n=4) and meningitis (n=2). The sera were collected by centrifuging the blood samples at 3000 rpm at room temperature for 5 minutes and were stored at -70^0 C until use.

Sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting
SDS-PAGE was run as described by (Laemmli 1970). Briefly, a mixture of equal volume of CSA (~25 µg/5mm well length) and sample buffer (Genei, Mumbai) was boiled for 5 minutes and loaded on to 12% polyacrylamide gel and electrophoresed at constant

current of 25 mA for about 5 hour. Molecular weight markers (Fermentas) ranging from 11-170 kDa were also run on both sides of the gel. Protein bands of CSA were visualized by staining with Comassie Brilliant Blue (CBB) (0.025% w/v in 50% methanol and 10% acetic acid).

The immunoblotting of CSA was performed according to Towbin *et al.*1979 with some modifications. Briefly, the CSA (~45µg/well) in a broad well and prestained protein ladder to 5 mm well on both sides were electrophoresed in 12% SDS PAGE. The fragmented proteins were then transferred to the polyvinylidene difluoride (PVDF) membrane (0.45 µm pore size, Millipore, USA) in transblotting unit (Amersham) containing transfer buffer (Tris base 25mM, glycine 192mM, methanol 5%). The unit was run at constant voltage of 60 V for 2½ hr. The membrane was stained with 0.5 % Ponceu red dye for five minutes and immediately destained after confirming the transfer of protein. The membrane was incubated in blocking buffer (1% BSA, 0.1% Tween-20 in PBS) overnight. The lane containing molecular weight markers were separated and the remaining membrane was cut into longitudinal strips of 5 mm. These strips were incubated in sera (1:100 in PBS) of different study groups for 2 hours at room temperature. After washing (3 times15 minutes each) with washing buffer (0.1% BSA, 0.1% Tween-20 in PBS), the strips were incubated with alkaline phosphatase enzyme conjugated goat antihuman IgG (1:1200) (Corredor Arjona.,*et al.*1999). Washing was repeated for thrice, twice with washing buffer and once with PBS. Colour was developed by using BCIP-NBT (5-Bromo-4-Chloro-3-indolylphosphate + Nitro Blue Thiazole) as substrate (Promega, USA) until blue bands were appeared.

Analysis
The image of protein bands developed in SDS-PAGE and Western blot were captured and analysed the unknown molecular weight and average intergrated density value (AIDV) by using Alpha Imager™ 2200.

Results and Discussion
Protein profile of *L. donovani*: The CSA, which is a heterogenous mixture including both cytoplasmic and membrane bound component of virulent *L. donovani* promastigotes, run for SDS-PAGE expressed the protein profile of the parasites. The CSA from SAG resisted *L. donovani* showed about 32 prominent and

many low intensified polypeptide bands ranging from 14 – 147 kDa **[Fig 1.]**. The bands of 110, 100, 85, 74, 63, 59, 50, 44, 36, 31, 28, 22, 20 and 18 kDa were found with high intensities as confirmed by AIDV.

Immunoblotting: The peptides bands appeared in the SDS-PAGE were later found to be transferred to PVDF membrane treated with sera of Day-0 patients. The analysis of the Western blot profiles of different study groups revealed that highest number of bands (~19±2) were present in the strips interacted by antibodies of Day-0 sera. The numbers of bands were found reduced by 30% (~13±3) just after the completion of treatment in Day-31 group, however, the number appeared were not considerably less in the 6 month follow up (~11±2).
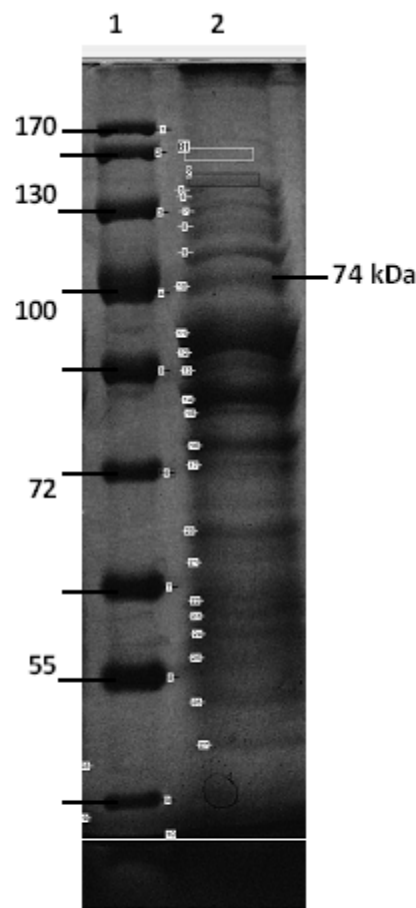
- **Fig.1.** CBB-stained SDS-PAGE gel. Lane 1, prestained mol wt. marker and lane 2 *Leishmania* antigen.
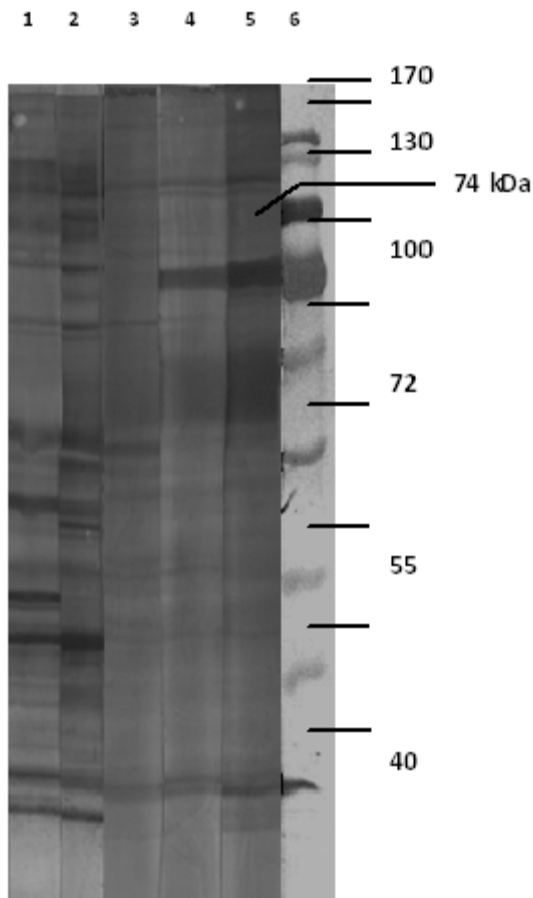
Fig. 3. Comparison of Western blot profiles; Lane 1, Non-endemic Healthy Control (NEHC); Lane 2,Endemic Control(EC); Lane 3, Patient after 6 month of successful treatment (Day-180); Lane 4, Patient after a month long complete treatment (Day-31); and Lane 5, Active Visceral leishmaniasis patient (VL); and Lane 6, prestained protein marker
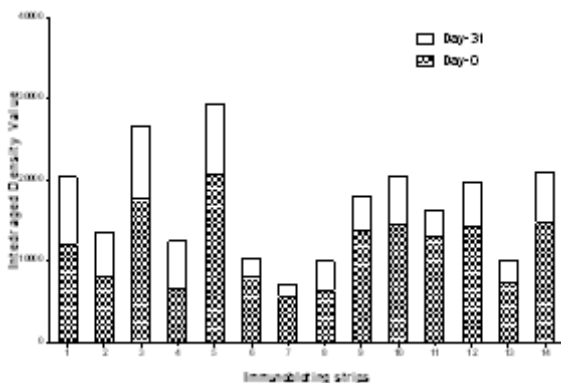
Fig. 2. Comparative average integrated density values of 74 kDa band in immunoblot profiles of Day 0 and Day 31.

Additions to it, the intensities of the bands were also not reduced except in some. The other control groups had comparatively less number of average bands (d" 9). Most of the bands were not consistently appeared. Eighteen bands were found in more than 50% profiles of Day-0 group and none of them were completely absent in all the profiles of Day-31 group. The patients of other disease group did not show cross reactivity to 74, 31, 28 and 16 kDa proteins (Table I).

74 kDa Protein fraction: It was interesting to note that intensified 74 kDa protein fraction was detected by the 100% sera of Day-0 and its intensity was found consistently low when interacted with sera of Day-31 [Fig 2]. The AIDV in Day 31 (5110 ± 634) was 45% less than in Day-0 (11385 ± 1230). Although, the band was present in 86% strips of Day-31 group, the intensity was decreased significantly (p value, <0.0001). The progressive decrease on intensity of the band was either vanished (n=20) or very dimly recognized (n=4) in Day-180 strips. This band was found only in virtually absent state of 6% strips of the EC group. Furthermore, it had neither shown cross-reactivity with NEHC nor any other non-Kala-azar disease group (Table 2).

Sensitivity, specificity and cross-reactivity of 74 kDa protein fraction: All the 35 sera of pretreated patients found interactive with the 74 kDa protein fraction expressing its absolute sensitivity. It declined to 86% when interacted with sera collected just after completion of one month long treatment by amphotericin B deoxycholate to the patients. The sensitivity percentage further declined to 17 with sera from six-month follow up. Although the 17% sera samples (n=4) showed interactive to the protein, they could be considered as negative on the basis of AIDV (Table 2).

All most all sera collected from the relatives inhabiting in close vicinity to patients who were expected to be exposed to sandfly vectors, did not show interaction with the 74 kDa antigen and had 94% specificity in endemic region. However, the bands seen in 2 strips treated with sera from EC were dimly recognized. Therefore, if the bands were considered as negative, it will have 100% specificity. Precisely, it showed 100% specificity with sera collected from Varanasi, a non-endemic population.

Table 1. Average number of bands and percentages of some notable major protein fractions developed in the immunoblot after interaction of protein profile of crude soluble antigen transferred to PVDF membrane with the sera of different study groups

	Day – 0 (n=35)	Day-31 (n=14)	Day-180 (n=24)	EC (n=33)	NEHC (n=13)	DD (n=19)
Average nos. of band seen in PVDF	19	13	11	9	8	9
Std Dev	±2.04	±3.18	±2.08	±8.73	±4.90	±2.78
Bands of interest (kDa)	Percentages of bands appeared					
120	74	71	82	52	38	53
85	80	43	26	18	15	16
74	100	86	17	9	0	0
70	54	50	75	30	23	58
63	86	14	60	9	0	11
53	51	36	43	0	0	5
51	71	36	43	12	54	5
50	57	50	68	6	8	47
37	57	64	81	61	15	26
36	57	21	33	27	8	16
34	54	50	54	24	62	42
31	80	29	20	9	15	0
28	80	79	66	42	23	0
20	66	29	64	21	23	37
18	51	21	50	18	0	5
16	69	57	78	64	8	0
14	63	36	22	12	0	11

Moreover, the protein fraction did not interacted with antibodies of sera from patients of other diseases such as malaria, tuberculosis, amoebic enteric fever and meningitis. This result clearly approved its nil cross-reactivity with many common diseases (Table 1).

Parasitological method for diagnosis of VL is still the gold standard technique with 100% specificity. However, splenic smear remained failure to detect 18.4% of all cases who were benefited from VL therapy (Sundar *et al.* 2007). In many cases like in cutaneous and visceral leishmaniasis, the demonstration of parasite becomes very difficult in the aspirate samples and serodiagnosis becomes the alternative method for the purpose (Kar 1995, Zeyrek *et al.* 2007). Immunochromatographic strip test (rK39) is one of the valuable achievements in VL diagnostic array. There are some constraints in this tool, for instances, this strip remains many times failure to evaluate cure patients, relapse cases and discriminate the symptomatic and asymptomatic cases in endemic population (Boelaert *et al.* 2007).

Table 2. Recognition of 74 kDa protein fraction of *L.donovani* by human serum antibody of different study groups and analysis of sensitivity, specificity and cross-reactivity

Study Group	No. of samples	No. of positive/negative	%
Sensitivity[a]			
Day-0	35	35	100
Day-31	14	12[d]	86
Day-180	24	4[e]	17
Specificity[b]			
EC	33	31[d]	94
NEHC	13	13	100
Cross-reactivity[c]			
Malaria	5	0	0
Tuberculosis	6	0	0
Amoebic Liver Abscesses	2	0	0
Enteric Fever	4	0	0
Meningitis	2	0	0

Day-0, Pretreatment; Day-31, After completion of treatment of 31 days; Day-180, six month after the treatment; EC, Endemic Control Population; NEHC, Non Endemic Healthy Control Population.

[a] Confirmation of positive to confirmed patients; [b] Confirmation of negative to non-VL cases; [c] Positive reactivity by non-VL ailments; [d] Positivity expressed by low intensified bands; [e] Virtually absent bands

In this study, we found 32 prominent peptide bands ranging from 14 to 147 kDa from leishmanial crude soluble antigen of MZP151 in a SDS-PAGE stained with CBB (Fig 1.). In similar pattern, Afrin *et al.* (2002) reported 33 distinct polypeptides ranging in molecular mass from 18 to 153 kDa and 17-20 bands of 14-66 kDa by Singh *et al.* (1995). The leishmanial proteins when transblotted to PVDF membrane [Fig. 2], 41 bands of different molecular mass in 35 profiles were appeared variably in Day-0 group due to the inconsistent antigen binding response of IgG class antibodies of the sera to *L. donovani* polypeptides. Thirty-six polypeptides, with molecular weights ranging from 14 to 123 kDa, were demonstrated by dos Santos *et al.* (1987). The blots obtained with patient sera revealed a broad range of immune specificities and extensive heterogeneity of the serological anti-LD responses in the individual patients (Forgber *et al.* 2006). It has also been known for decades that sera of all vertebrate taxa have natural anti-*Leishmania* antibodies (Rezai *et al.* 1975,

Ulrich *et al.* 1986). Therefore, disagreement to the report of Kumar *et al.* (2002), the reduction in number of bands after the treatment was not drastic but found in average of 19±2, 13±3 and 11±3 in Day-0, Day-31 and Day-180 respectively. The reduction of bands would be useful tool to study the kinetics of recovery.

In contrast to 100% reactive 65 kDa (Kumar *et al.* 2002), and 120, 91, 72, 63, 51, 34 and 31 kDa (Ravindran *et al.* 2004) protein component of *L. donovani*, this study found only the 74 kDa protein fraction against which consistent antibodies were developed. In the case of *L. d. infantum* 100% reactivity was reported with 94 kDa polypeptide (Rolland *et al.* 1994). The antibody responses to the parasites show highly individuality (Forgber *et al.* 2006) which could be the reason for dissimilar result.

In this study, some bands including 74 kDa fraction appeared in Day-0 group were waned out after completion of treatment. It is due to the fall of the level

of circulating immune complexes after treatment as analysed by antigen specific ELISA (Srivastava 1989). Similarly, the decreases of titer level of the antibodies in cured patient than in the active VL patients have been reported by Kumar *et al.* (2002) and Atta *et al.* (2004), and negativity ranged to 50% and 89% after treatment up to 1 year and 2-8 year respectively according to Hailu (1990). The drug pressure might also have suppressed most of the antibodies against the *Leishmanial* antigens (Khan *et al.* 1998). The disappearance or virtually disappeared band of 74 kDa protein fraction is directly proportionate to recovery from disease, suggesting no more antibodies are being developed against the fraction of the protein due to destruction of the same. Hence, it can be concluded that the 74 kDa protein fraction is playing role in conversion to symptomatic subjects.

Serologically, cross-reactivity between *Leishmania* species and other pathogens has been reported for whole-cell lysates (Smrkovski and Larson 1977, dos Santos *et al.* 1987). Molecular weights 72, 56, 50, and 40 kDa were found to be cross reactive with sera of patients of other diseases (Tiwari *et al.* 1995). In this study 125, 120, 107, 70, 38, and 30 kDa showed the cross–reactivity in >50% of strips which expressed their meaningless role in diagnosis of VL. But 16, 28, 31 and 74 kDa protein fractions did not show any cross-reactivity to the other diseases. However, the 74 kDa became the only one which was also not reacted with sera of NEHC and least with EC. Furthermore, though, the dim band was appeared in the EC population, it was virtually absent indicating its potential as a diagnostic marker in Indian population. In Spanish population 14 and/or 16 kDa proteins were reported as diagnostic marker (Riera *et al.* 2004, Garcia-Garcia *et al.* 2006) for the active VL patients.

There is great lack of diagnostic tool for this neglected tropical disease which is able to avoid false positivity in the endemic population and successfully treated patients. The very popular field friendly, rK39 strip test, though has absolute sensitivity and specificity, it is non predictable to cured patients as it still shows positive result after successful treatment and even after six-month follow up cases (Singh *et al.* 2009). Similarly, in the endemic population it missed to predict 3% endemic population and 14.3% non cases as negative (Sundar *et. al.* 2007). In this research work, 74 kDa protein was found able to evaluate the buffer population promptly by Western blot technique with its 100% sensitivity, and 100% and 94% specificity in NEHC and EC population respectively (Table 1). Though, 74 kDa protein was previously reported from *L. donovani* (C-II strain) (Khan *et al.* 1998), Indian PKDL as ~72-74 (Singh *et al.* 2003) and American VL (Evans *et al.* 1989), so far in our knowledge, this is the first time to report it with high potential diagnostic marker. This fraction of the protein was found highly conserved in SAG resistant strain of *L. donovani* from endemic area. Its intensity found to be decreased by 45% just after treatment, absent in all cases of NEHC and non-Kala-azar disease group, and virtually absent in EC. These pictures clearly demonstrate that 74 kDa protein fraction of *L. donovani* could be a biological marker of Indian Kala-azar which is being in search for a long time. Furthermore, the gradual significant decrease (p=0.0001) of band intensities of in the course of treatment are an additional usefulness of 74 kDa protein fraction for prognosis and kinetic study of recovery from Kala-azar.

Acknowledgements

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