Chapter 4
Detection of immune complex from Kala-azar patients’ serum.

Introduction

Blood plasma proteome has been recognized as a valuable source to discover biomarkers for diagnosis and prognosis of disease and also the disease processes (Kakisaki et al., 2007). Plasma is the preferred specimen because the samples are easily available by less invasive methods. It is also thought that it contains subsets of other tissue proteomes as well as hepatically secreted plasma proteins. Loss of function and change in plasma proteins can cause or result from disease processes (Smithies et al., 1956). Using the proteomics based dissection of sero specificities of *leishmania* patients, researchers have reported detection of 330 different leishmanial antigens and also observed an antigenic drift in the *leishmania* parasite (Forgber et al., 2006). The plasma proteome information of kala-azar patients will provide important clues to initiate better diagnostic strategies and also identification of leishmaniasis specific proteins. Differential protein expression at different stages of infection and comparison with data of normal/healthy individual will provide useful information for vaccine development.

Proteomics can be seen as a mass-screening approach to molecular biology, which aims to document the overall distribution of proteins in cells, identify and characterize the individual proteins of interest, and ultimately to elucidate their relationships and functional roles. Such direct protein-level analysis has become necessary because the study of genes by genomics cannot adequately predict the structure or dynamics of proteins, since most regulatory processes take place at the protein level. Also this is where diseases processes primarily occur and where most drug targets are found. Proteomics is therefore instrumental in diagnosis and for discovery of biomarkers, such as markers that indicate a particular disease, and can be used as drug targets. Plasma proteome analysis will provide a good baseline for examining the overall profile of the types of immune proteins that are quickly secreted or processed following infection that would not be detected by transcript analysis.

Infection of an individual with LD induces vigorous serological and cellular immune responses, (Croft et al., 2006) and in VL, there is a high level of *Leishmania*-specific antibodies (Reiner et al., 1990, Bray et al., 1976) which appear soon after the infection and before development of cellular immunologic abnormalities. The role of these elevated antibodies in resolution of the disease and protective immunity is mostly unknown. To worsen matters, in the endemic areas in India, treatment is increasing failing due to resistance of the parasites to the most common anti-leishmanial drug; pentavalent antimony (Croft et al., 2006).
The aim of the study is to generate the proteome profile of kala-azar patient’s sera and compare the result with normal/healthy individual, with the hope of discovering Leishmaniasis specific biomarkers that can provide clues towards vaccine development and better diagnosis of the disease.

**Layout of the study**

*Sample*

(Anticoagulated blood)

- Centrifuge at 12,000 X g for 12 min at 20°C

pellet (stored in liquid nitrogen for latter analysis)

supernatant (plasma)

fractionation (affinity column)

depletion of high abundant plasma proteins

bound flow through depleted

normal/healthy individual

kala-azar patients
The clinical data from the study subjects are summarised in Table 1. All VL samples were collected from RMRI, Patna. Analysis of the age distribution between the two groups showed that there was

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**Result**

**Clinical data**

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no significant difference among the patient and (p value > 0.05). Patients study group had an established diagnosis of visceral leishmaniasis as evidenced by the presence of the parasite in the bonemarrow aspirates and presence of anti rK39 antibody. Validation cohort consisted of patients with clinical diagnosis of visceral leishmaniasis supported by the presence of anti rK-39 antibody.

**Table 1.Clinical Data of study subjects**

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Age in yrs (Mean±SD)</th>
<th>Sex Male/Female</th>
<th>LD in bone marrow</th>
<th>Presence of anti rK39 antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>VL group</td>
<td>5</td>
<td>30.5± 14.3</td>
<td>4/1</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Healthy Control</td>
<td>5</td>
<td>29.6± 22.2</td>
<td>3/2</td>
<td>NA</td>
<td>0/5</td>
</tr>
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</table>

**PEG precipitation of immune complex**

Previously it has been reported that PEG can be used for the precipitation of protein form plasma. Moreover there are also reports that PEG can be also used for depletion of protein like albumin (Donald et al., 1981). One of the difficulties to detect immune complex in the sera of LD infected individuals is the presence of high abundant proteins like albumin that can mask low abundant protein in the sera of infected individuals. In order to deplete the albumin in the sample as well as to precipitate out the immune complex we use different concentration of PEG (1%, 2%, 3%, 4%, 6%, and 8%) in the sample and pass them through albumin removal column (IgY-R7-LC2 affinity column). Depleted plasma samples were initially collected at 0.1ml/min for 10 min and then for an additional 7 min at 0.2 ml/min. The column was washed for 5 min at 1 ml/min. Bound plasma proteins were eluted with 14 ml of 0.1 M glycine solution (pH 2.5). Elution was monitored at 280 nm. Plasma fraction were pooled and concentrated. Both the bound and the eluted fraction was resolved in SDS-PAGE as previously described (Schagger et al., 1987) and our result showed that 3% PEG concentration is optimum for maximally depleting albumin amount as well as also to precipitate out most amount of low abundant proteins (Fig 1A, 1B). Thus for further analysis 3% PEG was used both for healthy and infected sample.
Figure 1: PEG precipitation of immune complex. Representative SDS-PAGE gels of precipitate fraction and supernatant fraction of normal serum after precipitation using different concentrations of PEG (1%, 2%, 3%, 4%, 6% and 8%).

Two-dimensional gel electrophoresis of infected and normal sample

Three hundred μg of precipitated samples of both normal and LD infected individual were diluted in 200μl sample rehydration buffer and incubated for 16 h with an IPG strip (ReadyStrip™, pH 3-10, 11 cm). Following rehydration, IPG strips were focused for a total of 35,000 V-hr at 20°C in a PROTEAN IEF cell (Biorad). Prior to 2nd-dimension SDS-PAGE, focused IPG strips were first reduced with DTT in the equilibration buffer and then alkylated with iodoacetamide for 10 min each at room temperature. Second-dimension separations were carried out on 4-12% Bis-Tris gradient gels in a MES buffer system. Two-dimensional gels were stained with colloidal Coomassie Blue, or silver stain, scanned and analyzed with the Imagemaster 2D Platinum 6.0 software package. From the image analysis of normal and infected sera we were able to identify two distinct spots that were present at least in four independent LD infected individual, but was absent in normal healthy counterpart (Fig 2A, 2B). These two spots are dissected out and subsequent analysis was done.
Figure 2: Analysis of serum proteome by 2D-gel electrophoresis. Representative gels of normal sera and infected sera in 2D-gel electrophoresis. Arrows indicating spots that were present in infected sera but absent in normal sera. The range of the horizontal dimension is isoelectric point (from pI = 3 to pI = 10); the range of the vertical dimension is molecular weight (from approx. 150 to 10 kD)

2D-DIGE analysis of normal and LD infected sample

In order to reduce the gel to gel variation, and also to increase the detection sensitivity of low abundant proteins, 2D-DIGE was performed keeping internal standard as control. Equal content of normal and infected sample are pooled together, labeled with Cy2 and used as internal standard. Normal sample was labeled with Cy3 dye and infected with Cy5. The labelled proteins are then mixed and separated simultaneously on the same 2D gel.

The Cy2, Cy3, and Cy5 dye images are scanned sequentially with 488, 532, and 633 nm lasers, respectively, and emission filters of 520 (band pass 40), 580(band pass 30), and 670 nm (band pass 30), respectively to obtain the final image (Fig 3). The CyDye DIGE dye filter and laser combinations are selected to give the optimum results with minimal crosstalk between fluorescent channels.
Figure 3: Analysis of serum proteome by DIGE. A representative DIGE image (grey scale) showing the serum protein profile. Proteins identified as differentially expressed are shown by arrows in the 2D-Master analysis. Patient and control sera were labelled with Cy3 and Cy5 respectively, and Cy2 is used as internal control (IS) in this gel. The range of the horizontal dimension is isoelectric point (from $pI = 3$ to $pI = 10$); the range of the vertical dimension is molecular weight (from approx. 150 to 10 kD).

**In-gel protein digestion and Mass spectrometry**

For identification, the two protein spots were excised from the silver-stained gel and the proteins fragmented in the gels with trypsin. The tryptic fragments were extracted and analyzed by mass spectrometry. The resulting mass pattern of these fragments (peptide mass fingerprints, PMF) is shown in Figure 4A, 4B for the 2antigens. These patterns were used for MASCOT database searches for corresponding PMF pattern generated from the sequence database entries. The searches identified the antigens as Envelope glycoprotein, C2-V5 region (Fragment) of Human immunodeficiency virus 1 (spot1) and Envelope glycoprotein (Fragment) Human immunodeficiency virus 1(spot 2). The percentages of matched masses and sequence coverage are listed for each protein in Table 2. Our result although fail to identify any known leishmanial antigen, it probably indicated towards the presence of a probable HIV co-infection infection in LD infected patients.
Table 2. Antigens in human visceral leishmaniasis identified by proteome serology

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein name</th>
<th>Accession no</th>
<th>Appearance in gels (n = 4)</th>
<th>Mascot Score</th>
<th>Peptide matches</th>
<th>Protein Score C. I. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Envelope glycoprotein, C2-V5 region of HIV virus</td>
<td>Q74052_9HIV</td>
<td>4</td>
<td>88</td>
<td>6</td>
<td>74.8</td>
</tr>
<tr>
<td>2</td>
<td>Envelope glycoprotein</td>
<td>Q9JDS1_9HIV1</td>
<td>4</td>
<td>84</td>
<td>6</td>
<td>73.2</td>
</tr>
</tbody>
</table>

**Figure 4:** Identification of leishmanial antigens by mass spectrometry. The protein spots in the silver-stained gel those were present at least in four individual patient sample but not in normal sera were destained and incubated with trypsin. The resulting fragments were extracted from the gel pieces and analyzed by MALDI-TOF-MS. Panels A and B show the peptide mass fingerprints (PMF) of the proteins in spots 1 and 2, respectively. Upon processing via MASCOT, the antigens were identified as Envelope glycoprotein, C2-V5 region (Fragment) of Human immunodeficiency virus 1 (spot 1) and Envelope glycoprotein (Fragment) Human immunodeficiency virus 1 (spot 2).

**Discussion**

Serum is a rich source of disease-related information especially in a systemic infection like visceral leishmaniasis. Since the dynamic range of human serum proteome is large, we chose to deplete seven high abundant proteins from serum. Of all the methods employed for depletion, PEG precipitation followed by immunoaffinity chromatography (albumin removal column IgY-R7-LC2 affinity column) is more effective in removing targeted albumin, with minimal carryover, high longevity, minimal nonspecific binding and high reproducibility (Steel et al., 2003). However, there remains a possibility of losing some proteins by protein-protein interaction. Since the advantage conferred by depleting the high abundant proteins was deemed to be of more value in discovering low abundant proteins, we chose to deplete the serum. These seven abundant proteins make up 85-90% total protein in serum and hence, their depletion yielded a highly resolved profile of serum proteome on 2D gels enabling the analysis of low abundant proteins. According to a recent statistical study, a minimum of four biological replicates are needed to identify at least two fold difference in
DIGE studies employing immune-depleted serum (Corzett et al., 2006). The alteration in total protein in sera visceral leishmaniasis is a well-known phenomenon (Shanker et al., 1959). The use of proteomics to explore the plasma proteome of related infectious diseases like human African trypanosomiasis (Papadopoulos et al., 2004), tuberculosis (Agranoff et al., 2006) and leprosy (Gupta et al., 2006) has been reported previously. These studies reported the differential expression of many acute phase proteins in the plasma in these conditions. In this study, as expected, we found many acute phase proteins being differentially expressed. 2D gel analysis identified two spots in infected serum which were absent in normal serum in four independent experiments. MALDI-TOF analysis of these two spots however failed to identify any leishmanial antigen but identified these two spots as: spot 1 envelope glycoprotein, C2-V5 region of HIV virus, spot 2 envelope glycoprotein of HIV. Assuming similar experimental conditions, our DIGE study was sufficiently powered as we used four biological replicates. Although DIGE reviled nine spots that were differentially expressed between infected and normal serum, however due to some technical problem we failed to identify these differentially expressed proteins. In this context it should be mentioned these leishmania infected serum were obtained RMRI, Patna, where there are several reports of Leishmania HIV co-infection (Sinha et al., 2011, Mathur et al., 2006). It is quite possible that clinical samples used in this study may have HIV infection along with leishmaniasis. Moreover, since these proteins are related to the inflammatory process, they will serve as good biomarkers for monitoring response to therapy. Longitudinal studies are needed in this regard to evaluate their utility as prognostic biomarkers. Since visceral leishmaniasis is endemic in resource constrained areas, simple and low cost methods need to be developed to use these results in the clinical setting. Development of simpler dipstick assays will enable such a possibility of testing these proteins in field conditions. In conclusion, DIGE based proteomic analysis showed that several proteins are differentially expressed in the sera of visceral leishmaniasis. The nine spots identified here have potential, either independently or in combination, for prognostic biomarkers. Further studies are suggested to establish their application potential.
References


