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Clinicopathological manifestation of Falciparum malaria in Bikaner (Northwest Rajasthan) population and proteomic investigation for identification of potential serum marker proteins

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Abstract
Aims & objectives: To study the biochemical and hematological parameters of blood of patients suffering from different levels of severity of falciparum malaria and proteomic analysis of these patients. The ultimate objective of this research plan was to utilize the proteomic platform for discovery of falciparum malaria biomarkers for diagnosis of asymptomatic and low-parasitemic falciparum malaria patients.
Material and Methods: After implication of inclusion and exclusion criteria, 52 cases were selected for study, out of which 17 cases were in severe category and 35 were non severe. Comparative proteomic analysis of malaria and controls, Immunoassay-based validations, Proteins networks and functional analysis, and PCR confirmation of diagnosis was done. The comprehensive serum proteome analysis was done on these samples using 2D-Electrophoresis.
Results: This study has identified a number of differentially expressed serum proteins, 5 proteins were found to be significant between healthy and non-severe malaria serum samples, 5 between healthy and severe malaria serum samples and 3 between severe and non-severe serum samples.
Conclusion: Differentially expressed proteins in the context of *P. falciparum* malaria will be valuable in understanding the basis for the disease pathogenesis and may ultimately help to establish potential surrogate markers aiding in diagnosis, prognosis, monitoring disease progression.

Keywords: Clinicopathological manifestation, falciparum malaria, population, proteomic investigation

Introduction
Malaria has emerged as one of the top 10 killer diseases around the globe. It is the major cause of mortality in various tropical and subtropical regions. Approximately half of the world population is vulnerable to malaria, may be more than that. The most important of parasitic disease of humans, it is transmitted in 106 countries with 3 billion people at risk and causes 2000 deaths per day [1]. The total number of reported malaria cases in india in the year of 2015 was 1126661 leading to 287 deaths. [National Vector Borne Disease Controle Programme] [2]
Malaria is caused by six species[1] of the genus *Plasmodium* namely, *Plasmodium vivax*, *P. falciparum*, *P. malariae*, two morphologically identical sympatric species of *P. ovale (P. ovale curtisi and P. ovale wallikeri)* and *P. knowlesi*. *P. falciparum* and *P. vivax* account for over 90% of the total malaria cases worldwide. While almost all deaths are caused by falciparum malaria, occasionally *p. vivax* also can cause severe illness and death [1]. Recently, life threatening complications with *P. knowlesi* infection has been reported in humans [3].
During last 2-3 decades there is a changing trend not only in the clinical manifestations but also the pattern of complications in malaria. During the last decade of 20th century, cerebral malaria was the predominant manifestation of severe malaria, whereas in the recent past the combination of jaundice and renal failure are predominant severe manifestations [4]
Several clinical complications have been described in malaria caused by *Plasmodium falciparum*, including severe anaemia, cerebral malaria, acute pulmonary oedema(ARDS), acidosis, renal failure, hypoglycemia, hypotension /shock, bleeding, DIC, convulsions, hemoglobinuria, extreme weakness and jaundice [2, 5].

Proteomics

The spectacular advancements, achieved in the preceding decade with the completion of genome sequence of different species of *Plasmodium* and its insect and vertebrate hosts, have propelled the growth of proteomics into different arenas of malaria research. Recently, comprehensive proteomic analyses of different biological fluids such as serum or plasma, urine, saliva etc. have attracted considerable attention for the identification of protein biomarkers as early detection surrogates for diseases.

The overall objective of this research plan was to utilize the proteomic platform for discovery of falciparum malaria biomarkers for diagnosis of asymptomatic and low-parasitemic falciparum malaria patients. Identification of biomarkers with diagnostic or prognostic utility for falciparum malaria will have great clinical relevance.

Materials and methods

This study was conducted as a Prospective observational study at tertiary care center in North West Rajasthan India. All cases of fever were screened and evaluated to detect malarial parasites, and out of that, 52 cases were included in study. Proteomic and immunoassay-based analysis of the clinical samples was carried out at Department of Biosciences and Bioengineering, Indian Institute of Technology, Mumbai.

Sample size

This study was conducted on 24847 cases of fever examined for malaria parasite during 2012-2013. Among these 1340 cases were diagnosed as malaria, out of these 78 cases were *P. falciparum*, 1242 were *P. vivax* and 20 were mixed malaria.

After implication of inclusion and exclusion criteria, who have not received antimalarials before sample collection, 52 cases were included in study, out of which 17 cases were in severe category and 35 were non severe.

Diagnosis of falciparum malaria was confirmed by PBF (peripheral Blood Film) and/ or RDT (Rapid Diagnostic Test). Malaria patients with history of significant systemic diseases like autoimmune disorders, chronic liver diseases, psychiatric illness and bleeding disorders; dengue, leptospirosis or any other infectious disease as well as pregnant female were excluded from the study.

Age and sex matched healthy persons from same geographical areas was selected as control. Blood samples (5.0 mL) were collected from the antecubital vein of the malaria patients and healthy subjects using serum separation tubes. Immediately after blood collection the tubes were kept in ice for 30 mins for clotting. Serum separation was performed as mentioned by Ray et al [8]. Collected serum was divided into multiple aliquots and stored at -80 ^{0}C until time of analysis to prevent protein degradation. Prior to analysis, maximum 3 freeze/thaw cycles were allowed for any serum sample to reduce pre-analytical variations. 1ml of venous blood/drop on filter paper were collected for PCR confirmation.

Proteomic analysis was done in total of 15 patients with nonsevere *P. falciparum* malaria and 12 patients with severe *P. falciparum* malaria and 18 healthy subjects for comparative proteomic analysis.

Results

This study was conducted on 24847 cases of fever admitted in PBM Hospital, Bikaner, during the year 2012 and 2013.

Our study do not reflect the true prevalence of *P. falciparum* and *P. vivax* malaria in the community as most of our patients were from Bikaner city and others were serious and complicated cases referred from primary and secondary health centers situated in rural areas with limited medical facilities. A total 1340 were positive for malarial parasite, out of which 78 (5.8%) cases were infected with *P. falciparum*, 1242(93.7%) cases were of *P. vivax*, and 20(1.5%) were due to mixed malaria. Two parasite species can develop in the same vector species and infection can be transferred with a single bite or may be caused by two different bites. In such cases clinical picture gets mixed up and fever might occur daily. In India, about 70% of the infections are reported to be due to *P. vivax*, 25–30% due to *P. falciparum* and 4–8% due to mixed infection[9].

Table 1: Distribution of Patients According To Clinical and Laboratory Parameters

	Non severe (n =35)		Severe (n = 17)	
	No.	**%**	**No.**	**%**
Hb (gm%)				
<5	-	-	3	5.77
5 – 7	10	19.23	-	-
7 – 11	28	53.85	-	-
>11	11	21.15	-	-
S. bilirubin (mg/dl)				
<3	42	80.77	-	-
3 – 5	-	-	3	5.77
5 – 10	-	-	1	1.92
>10	-	-	6	11.54
S. Creatinine (mg/dl)				
<3	46	88.46	-	-
>3	-	-	6	11.54
CEREBRAL MALARIA	-	-	1	
Hypoglycemia	-	-	-	-
Convulsions	-	-	-	-
Bleeding/DIC	-	-	-	-
ARDS	-	-	-	-
Acidosis	-	-	-	-
Platelets				
<20,000	1	2.86	1	5.88
20,000 – 50,000	12	34.29	5	29.41
50,000 – 100,000	11	31.43	5	29.41
100,000 – 150,000	9	25.71	3	17.65
>150,000	2	5.71	3	17.65
Total	35	67.31	17	32.69
Patients with Organomegaly				
Hepatomegaly	2	5.71	2	11.76
Splenomegaly	9	25.71	8	45.06
Hepatosplenomegaly	2	5.71	2	11.76
Total	13	37.14	12	70.58

The distribution of thrombocytopenia in severe and non-severe falciparum malaria cases shows that thrombocytopenia is very common both in severe and non-severe cases. Most of the patients with *P. Falciparum*. Malaria were anaemic (78.9%). Percentage of severe anemia (Hb<5 gm %) was (5.77%).

The involvement of liver and spleen was more in severe cases. 10 out of 52 patients (21.5%) had elevated levels of serum bilirubin. 6 out of 52 patients (11.5%) had elevated levels of serum creatinine. (Table - 1)

Table 2: Distribution of Patients with One or More Organ Dysfunction (N = 17)

	No.	%
Anemia (Hb<5gm%)	3	17.64
Jaundice (S.Bil.>3mg%)	5	29.41
Renal Failure (S.Cr.>3mg%)	1	5.88
Cerebral Malaria	1	5.88
Anemia + Jaundice	2	11.76
Anemia + Renal Failure	2	11.76
Jaundice + Renal Failure	3	17.64

3 patients had severe Anemia (17.64%), 5 patients had Jaundice (29.41%), 1 patients had Renal failure (5.88%). 2 patients had anemia and jaundice (11.76%), 2 patients had anemia and renal failure, 3 patients had jaundice and renal failure (17.64%). (Table - 2)

Comparison of serum proteome profile of *P.falciparum* malaria and healthy subjects using 2-DE (2 Dimensional Electrophoresis)

The principal aim of this study was to perform a comparative serum proteome analysis of *P. falciparum* malaria patients and healthy subjects to identify the alteration in expression pattern of various serum proteins in response to *P. falciparum* infection. A comprehensive serum proteome analysis was performed on 45 human subjects (15 *P. falciparum* non-severe malaria patients, 12 *P. falciparum* severe malaria patients and 18 healthy subjects) using 2-DE. Over 400 protein spots were detected reproducibly in each gel by IMP7 software. In order to study the variations in protein expression between healthy subjects and *falciparum* malaria patients as well as between severe and non-severe patients, individual gel images (healthy controls (n = 3), severe samples (n = 3) and non-severe samples (n = 3)) were grouped and analyzed using IMP7 software. 5 proteins were found to be significant between healthy and non-severe malaria serum samples, 5 between healthy and severe malaria serum samples and 3 between severe and non-severe serum samples.

Identification of Differentially Expressed Proteins in *P. falciparum* Malaria Using MALDI-TOF/TOF MS

Every protein spot in 2-DE gels exhibiting differential expression and fulfilling the statistical criteria was selected and excised for further MS analysis. Excised spots were subjected to in-gel trypsinization followed by mass spectrometric analyses in both MS and MS/MS. The results indicate that the 5 significant spots obtained for healthy and non-severe malaria samples comprised of 4 proteins (haptoglobin, apolipoprotein A-I, serum albumin precursor and one protein for which MS ID was not available. All the above proteins were found to be down regulated in the *P. falciparum* non-severe malaria cases with high statistical significance (Student's t-test; $p<0.005$) as compared to healthy controls. Similarly 5 significant spots obtained for healthy and severe malaria samples comprised of 4 proteins (haptoglobin, apolipoprotein A-I, haptoglobin β chain and Plasma retinol-binding protein precursor). All the proteins were found to be down regulated in the *P. falciparum* severe malaria cases as compared to healthy controls. Both haptoglobin and apolipoprotein A- I were also found to be down-regulated in case of severe malaria as compared to non-severe malaria samples.

In a few cases, MS (Mass spectrometry) analysis revealed the same identity for multiple protein spots appearing as separate entities in 2D gels, indicating the presence of various isoforms of those particular proteins. Presence of such several isoforms is quite expected since many of the serum/plasma proteins exhibit complex combinations of post-translational modifications (specifically involving glycosylation) leading to the multiple distinct post-translationally modified protein spots for the same candidate [10, 11]. Alternatively, the similar identity of multiple protein spots might be due to the cleavage or degradation of proteins generating different proteolytic fragments discriminated by 2D electrophoretic separation.

Discussion

Severe malaria is defined by WHO [12, 13] as the presence of one or more pernicious syndromes in a patient with asexual forms of plasmodium species in his blood. Depending on the severity and rapidity of parasite infection and immune system of the host the patient may have different types of clinical presentations.

The work presented here demonstrates the use of differential proteomic analysis to elucidate the host immune responses against the *P. falciparum* infection. This study has identified a number of differentially expressed serum proteins with different biological functions in patients with *P. falciparum* malaria. Acute phase proteins (APPs) are group of serum proteins that exhibit differential expression during various acute phase responses. In malaria, changes in the concentration of different APPs have long been demonstrated [14, 15, 16]

Our proteomic analysis revealed a decrease in the expression levels of acute phase protein such as haptoglobin, retinol-binding protein precursor and serum albumin precursor. Serum haptoglobin (Hp) levels in the *P. falciparum* malaria patients were found to be significantly lower in comparison to those of the normal healthy donors. Hp is an acute phase protein that removes free hemoglobin (Hb) released during hemolysis. In malaria infection, red blood cell rupture releases abundant amounts of Hb. Free haptoglobin would subsequently disappear as the haptoglobin- Hb complexes are formed [17, 18].

The serum concentration of retinol, the alcohol form of vitamin A, reduces during malaria as a direct consequence of the inflammatory response to the *Plasmodium* infections [14, 19, 20]. Retinol binding protein (RBP) exhibits steady association with serum retinol since their metabolism is intimately linked to that of vitamin A [15]. The decreased level of RBP and the retinol in malaria could be due to the suppressing activity of IL during the infection and/or due to the increased transport of retinol to the tissues [19]. Moreover, RBP, being low molecular weight protein, might escape from the vascular system as a result of the increased capillary permeability during the acute phase response and excreted out in the urine since it can easily be filtered in glomerulus tubules [20].

Levels of Apo-AI, a protein having a specific role in lipid metabolism was found to be significantly lower in malaria patients. Apo-AI is known to have anti-inflammatory effects and thus apoA-1 reductions may contribute to the inflammatory processes that result in severe anemia, a common consequence of *Plasmodium* infection. Similarly the expression levels of serum albumin precursor were also down-regulated. This could be due to the fact that parasites

utilize albumin during the process of infection as a source of amino acids.

In conclusion, 5 proteins were found to be significant between healthy and non-severe malaria serum samples, 5 between healthy and severe malaria serum samples and 3 between severe and nonsevere serum samples.

5 significant spots obtained for healthy and non-severe malaria samples comprised of 4 proteins (haptoglobin, apolipoprotein A-I, serum albumin precursor and one protein for which MS ID was not available). All the above proteins were found to be down regulated in the *P. falciparum* non-severe malaria cases with high statistical significance (Student's t-test; $p<0.005$) as compared to healthy controls.

Similarly 5 significant spots obtained for healthy and severe malaria samples comprised of 4 proteins (haptoglobin, apolipoprotein A-I, haptoglobin β chain and Plasma retinol-binding protein precursor). All the proteins were found to be down regulated in the *P. falciparum* severe malaria cases as compared to healthy controls.

3 spots were found to be significant for *P. falciparum* severe and nonsevere malaria cases. These spots comprised of 3 proteins (haptoglobin, apolipoprotein A-I and one protein for which MS ID was not available). Both haptoglobin and apolipoprotein A- I were also found to be down-regulated in case of severe falciparum malaria as compared to non- severe malaria samples.

We have conducted comprehensive analysis of the human serum proteome to understand the host immune response in *P. falciparum* malaria to identify the unique proteomic pattern or potential biomarkers for *P. falciparum* infection. In seeking an alternative diagnostic approach in malaria apart from clinical symptoms and microscopic observation of a blood smear, protein biomarkers will be interesting candidates. In this discovery-driven approach, we have identified 10 differentially expressed, statistically significant (Student's t-test; $p<0.05$) serum proteins, in falciparum malaria patients relative to healthy subjects. Further exhaustive analysis of the functional properties of these differentially expressed proteins in the context of *P. falciparum* malaria will be valuable in understanding the basis for the disease pathogenesis and may ultimately help to establish potential surrogate markers aiding in diagnosis, prognosis, monitoring disease progression. However, further validation of the identified proteins using immunoassay-based approaches like ELISA and/or western blotting employing a greater numbers of patients and healthy controls is required to confirm the findings obtained in this proteomic analysis prior to any clinical implication.

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