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SERUM PROTEOME ANALYSIS AND IDENTIFICATION OF SURROGATE PROTEIN MARKERS IN MALARIA

General Medicine

Tundwal Vijay Kumar Associate Professor, Department Of Medicine, S.P. Medical And P.B.M. Associated Group Of Hospitals, Biknaer (Rajasthan)

Satyaveer Singh Resident, Department Of Medicine, S.P. Medical And P.B.M. Associated Group Of Hospitals, Biknaer (Rajasthan)

Somveer Resident, Department Of Medicine, S.P. Medical And P.B.M. Associated Group Of Hospitals, Biknaer (Rajasthan)

Jinger Naveen Kumar* Resident, Department Of Medicine, S.P. Medical And P.B.M. Associated Group Of Hospitals, Biknaer (Rajasthan).*Corresponding Author

Meena Manoj Kumar Medical Officer , Department Of Medicine, S.P. Medical And P.B.M. Associated Group Of Hospitals, Biknaer (Rajasthan)

Kochar Aditya Medical Student, Department Of Medicine, S.P. Medical And P.B.M. Associated Group Of Hospitals, Biknaer (Rajasthan)

Tundwal Divyanshi Medical Student, Department Of Medicine, S.P. Medical And P.B.M. Associated Group Of Hospitals, Biknaer (Rajasthan)

Kochar Sanjay Kumar Senior Professor, Department Of Medicine, S.P. Medical And P.B.M. Associated Group Of Hospitals, Biknaer (Rajasthan)

ABSTRACT

Aim and objective: Serum proteome analysis of patients suffering from severe and non-severe vivax and falciparum malaria infections to get mechanistic insights about disease pathogenesis and host immune response. Validation of candidate serum protein biomarkers for P. vivax and P. falciparum infections malaria.

Material & Methods: This study was carried out as a hospital – based, observational case-control study and conducted in total 8449 cases of acute febrile illness with symptoms in the Department of Medicine. Proteomic and MRM assay based analysis of the clinical samples will be conducted in Department of Biosciences and Bioengineering, Indian Institute of Technology, Bombay, Powai, Mumbai. The study was conducted in two steps. The first step was to identify patients with *P. vivax and falciparum* malaria, collect clinical data and serum samples while the second step was to subject the serum samples to proteomic study with appropriate controls.

34samples were collected out of 74 for research purpose, rest of the cases were excluded due to patient refusal for consent and concomitant illnesses. 7 cases were further excluded from our study due to improper sample transportation and handling. 27 samples were processed and enrolled in our study.

Results: In our study serum proteome analysis of patients suffering from severe and non-severe vivax and falciparum malaria infections was done to get mechanistic insights about disease pathogenesis and host immune response. Validation of candidate serum protein biomarkers was done for P. vivax and P. falciparum infections. Inter-alpha-trypsin inhibitor was found to be significantly down-regulated in P. vivax malaria cases. Whereas ceruloplasmin was found to be up-regulated and Cystatin-C protein was down-regulated in vivax malaria cases. Catalase, ComplementC1r subcomponent-like protein, Complement component C8 beta chain was up-regulated in P.falciparum cases. Out of these three, catalase was found to be significantly up-regulated.

Conclusion: Several proteins were identified in our study, Inter-alpha-trypsin inhibitor was found to be significantly down-regulated in P. vivax malaria cases. Whereas ceruloplasmin was found to be up-regulated and Cystatin-C protein was down-regulated in vivax malaria cases. Catalase, ComplementC1r subcomponent-like protein, Complement component C8 beta chain was up-regulated in P. falciparum cases. Out of these three, catalase was found to be significantly up-regulated. Including a larger number of patients to confirm the findings obtained in this analysis is required so as to confirm the findings.

KEYWORDS

Serum Proteome, P. falciparum, P. vivax, Ceruloplasmin, Catalase

INTRODUCTION

According to the World Health Organization (WHO), malaria is a significant public health problem in more than 100 countries and causes an estimated 200 million infections each year, with more than 500 thousand deaths annually. Over 90% of these deaths occur in sub-Saharan Africa[1]. Despite previous elimination in regions like the United States and Western Europe, the phenomenon of "imported malaria" introduced by immigrants and travellers, still contributes with sporadic cases in these regions[2].

Very few studies have been conducted to identify surrogate protein markers for vivax and falciparum malaria in serum, plasma or other types of biological fluids. Advanced mass spectroscopy based strategies have facilitated the identification of novel proteins for malaria research. Current proteomic tools have allowed the analysis of different human body fluids including saliva, urine and serum for the discovery of potential disease related markers[7-9].

Once potential candidates are recognized, the key step becomes *validation*. At the validation stage, hundreds of samples must be analysed, however, only a small number of analytes are measured. In validation, where the goal is to focus on a handful of potential biomarkers, the confidence level in the ability to quantify these targets needs to be very high. Validation of potential biomarkers requires methods capable of quantitating these proteins across thousands of clinical samples. The analytical characteristic of mass spectroscopy (MS) makes it ideal for quantitating the absolute amounts of molecules in complex mixtures. The mass spectrometer provides a direct signal for each metabolite in the mixture ensuring the targeted compound is being measured. Antibody based assays are not readily available for discovered biomarkers. Hence, the validation process depends on the ability to develop antibodies or other assays which limits number of potential biomarkers for validation. For some potential biomarkers, antibodies are available to test their sensitivity and specificity within a clinical sample cohort. Due to their simplicity and throughput, high quality antibodies should be considered first for validation across a large number of samples in case of proteins for which no useful

antibody exists, a targeted MS approach could be considered and applied to test the validity of a putative biomarker. Methods for absolute quantitation of peptides {e.g. - multiple reaction monitoring (MRM)} require significant development time and only a limited number of peptide quantitation assays can be developed and utilized concurrently.

MATERIALS AND METHODS

This study was carried out as a hospital – based, observational case-control study and conducted in the Department of Medicine. Proteomic and MRM assay based analysis of the clinical samples will be conducted in Department of Biosciences and Bioengineering, Indian Institute of Technology, Bombay, Powai, Mumbai.

The study was conducted in two steps. The first step was to identify patients with *P. vivax and falciparum* malaria, collect clinical data and serum samples while the second step was to subject the serum samples to proteomic study with appropriate controls.

Analysis performed at S.P. Medical Collage, Bikaner

1) Disease diagnosis (microscopy and RDT)
2) Sample collection and serum separation
3) Complete blood count
4) Liver and kidney function tests
5) Biochemical and rediological tests for other complications (where indicated)
6) Urine analysis
7) Tests to rule out Dengue, Leptospirosis, Typhoid fever, viral hepatitis etc.

Analysis performed at Indian Institute of Technology Bombay, Powai, Mumbai

1) PCR confirmation of diagnosis.
2) Comparative proteomic analysis of malaria and controls.
3) Immunoassay-based validations.
4) Proteins networks and functional analysis.
5) Multivariate statistical analysis.

Sample size

This study was conducted on total 8449 cases of acute febrile illness with symptoms admitted in PBM Hospital, Bikaner Sept 2018-November 2019 total 74 cases were found positive for malaria, out of which 6 were P. falciparum and 68were P. vivax cases. 34samples were collected out of 74 for research purpose, rest of the cases were excluded due to patient refusal for consent and concomitant illnesses. 7 cases were further excluded from our study due to improper sample transportation and handling. 27 samples were processed and enrolled in our study.

RESULTS

The present study was conducted in the Department of Medicine with the collaboration of Department of Biosciecne and Bioengineering, IIT Bombay. The following points were drawn:

Serum proteome analysis of patients suffering from severe and non-severe vivax and falciparum malaria infections was done to get mechanistic insights about disease pathogenesis and host immune response. Validation of candidate serum protein biomarkers was done for P. vivax and P. falciparum infections.

The clearance from Institutional research board of S.P. Medical College, Bikaner was taken before starting our study.

Anemia (Hb< 11gm%) was seen in 33.33% of patients and severe malaria (Hb <7 gm%) was seen in 14.8% of patients. Thrombocytopenia was seen in 100% patients in P. falciparum and 88% in P. vivax infected malaria patients.

Serum proteome analysis, their comparison with healthy controls, immunoassay based validations, protein networks and functional analysis and PCR confirmation of diagnosis was done at IIT Bombay, Powai, Mumbai.

The validation of potential biomarkers was done using MRM immunoassay. Inter-alpha-trypsin inhibitor was found to be significantly down-regulated in P. vivax malaria cases. Whereas ceruloplasmin was found to be up-regulated and Cystatin-C protein was down-regulated in vivax malaria cases. Catalase, ComplementC1r subcomponent-like protein, Complement component C8 beta chain was up-regulated in P.falciparum cases. Out of these three, catalase was found to be significantly up-regulated.

Accurate diagnosis and prognosis of malaria is challenging even in today time due to changing spectrum of malaria presentation. Identification of proteins in the serum of malaria infected patients has been done through proteomics to understand the host immune response in P. vivax and P. falciparum malaria. Through this study we aim to identify the differentially expressed proteins and validate them through MRM immunoassay to establish potential surrogate markers which might aid in better understanding of malaria pathology, early diagnosis, prognosis and monitoring disease progression. Several proteins were identified in our study, Inter-alpha-trypsin inhibitor was found to be significantly down-regulated in P. vivax malaria cases. Whereas ceruloplasmin was found to be up-regulated and Cystatin-C protein was down-regulated in vivax malaria cases. Catalase, ComplementC1r subcomponent-like protein, Complement component C8 beta chain was up-regulated in P. falciparum cases. Out of these three, catalase was found to be significantly up-regulated. Including a larger number of patients to confirm the findings obtained in this analysis is required so as to confirm the findings. A follow up analysis of the patients after therapeutic interventions may provide additional insights regarding the correlation of the identified markers with disease progression and their efficacy as disease monitoring or prognostic markers.

DISCUSSION

Plasmodium falciparum infection represents the major cause of malaria associated mortality worldwide. This lethal species of malaria parasite is responsible for approximately 247 million cases and around one million deaths each year, particularly in the sub Saharan Africa[10]. India notably contributes to the global malaria burden and has the largest population in the world at risk of malaria[11].

27 samples were processed and enrolled in our study**.** Since sample size was too small, most of the patients were from Bikaner city; so this study could not be used for estimating true prevalence of P. falciparum and P. vivax cases in our community.

A study by Kochar et al[12] in 2007 titled " Dynamics of Malaria in Bikaner, Rajasthan, India (1975-2006)" showed that the prevalence of P. falciparum malaria in Bikaner region is <10% except in 1992 when this region witnessed a major epidemic of P. falciparum malaria and the incidence in that year was 50%. The incidence of P. vivax malaria was always >90% in this region. This is in contrary to rest of the country but it might be due to genetic mutations in P. vivax species which have favoured its multiplication and transmission, local environmental factors which might be more favourable for this particular species.

In the present study the 63% patients had raised bilirubin levels (1-3 mg/dl) and S. bilirubin >3 was not present in any patient. In the studies conducted by Kochar et al[12], Abro et al[13], Patwari et al[14] liver dysfunction was seen in 47.89%, 23% and 8.7% of patients respectively.

Kochar et al[12] studied 86 patients of malarial hepatitis, out of which 29 had S. Bilirubin >10 mg%. Maximum value of S. Bilirubin was 48.2%. In our study in which 27 patients were included out of which 2 had falciparum and 25 had vivax malaria. 13 patients had anemia (48.14 %), 17 patients had jaundice (63%), 3 patients had renal dysfunction (11.1%), 9 patients had anemia and jaundice (33.33%). 2 patients had anemia and renal dysfunction (7.4%), no patient had anemia and cerebral malaria, no patient had jaundice and cerebral malaria and 2 patients had anemia, jaundice and renal dysfunction (7.4%).

The present study demonstrates the application of diagnostic proteomics to decipher host responses against the human malaria parasites Pf and Pv, and identifies potentialcandidate biomarkers for these two plasmodial infections. Differentially expressed serum proteins are involved in different physiological pathways, including acute phase response signaling, interleukin signaling, complement and coagulation cascade and vitamin D metabolism pathway, with plausible potential functional role in the disease pathogensis, have been discussed in the context of malaria.

In this study we have identified multiple differentially expressed proteins in Pf and Pv cases. We assume that this study will provide

valuable insight into molecular mechanisms of malaria and may help establish early detection surrogates for better diagnosis and effective therapy. Some of the identified proteins in our study were Leucine-rich alpha-2-glycoprotein, Alpha-1-antichymotrypsin, Alpha-1-acid glycoprotein, Plasma protease C1 inhibitor, Alpha-2-HS-glycoprotein, Hemoglobin subunit alpha, Hemoglobin subunit beta, Complement C3, Cytochrome C, Complement C1r subcomponent, Fibrinogen alpha chain, Histidine-rich glycoprotein, Inter-alpha-trypsin inhibitor heavy chain H4, Complement component C8 beta chain, Complement C1r subcomponent-like protein, Galectin-3-binding protein, Serum amyloid A-1 protein, Insulin-like growth factor-binding protein 3, Vitamin D-binding protein, Cartilage oligomeric matrix protein, Alpha-2-macroglobulin, Clusterin, Apolipoprotein A-I, Gelsolin, Complement C1s subcomponent, Ceruloplasmin, Catalase.

Inter-alpha-trypsin inhibitor was found to be significantly down-regulated in P. vivax malaria cases. Whereas ceruloplasmin was found to be up-regulated and Cystatin-C protein was down-regulated in vivax malaria cases.

Catalase, complement C1r subcomponent-like protein, Complement component C8 beta chain was up-regulated in P. falciparum cases. Out of these three, catalase was found to be significantly upregulated.

In a similar study conducted by Ray et al[15] in 2012 titled Proteomic Investigation of Falciparum and Vivax Malaria for Identification of Surrogate Protein Markers , Validation of a few differentially expressed proteins was performed using different immunoassay-based methods including immunoturbidimetric assay, ELISA and western blotting to confirm the results of proteomic analysis. In their study Western blot analysis showed up-regulation of serum amyloid A and down-regulation of haptoglobin, clusterin and retinol-binding protein in FM and VM patients ($p<0.01$) compared to the healthy and febrile controls.

CONCLUSION

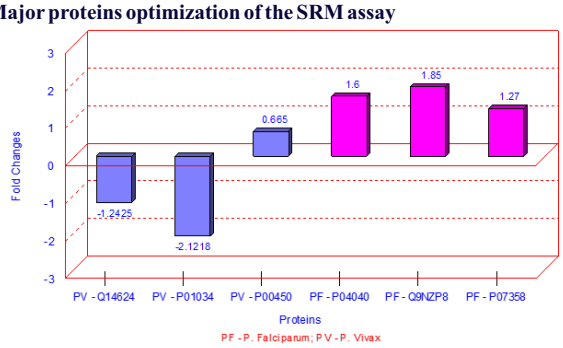
Identification of proteins in the serum of malaria infected patients has been done through proteomics to understand the host immune response in P. vivax and P. falciparum malaria. Through this study we aim to identify the differentially expressed proteins and validate them through MRM immunoassay to establish potential surrogate markers which might aid in better understanding of malaria pathology, early diagnosis, prognosis and monitoring disease progression. Including a larger number of patients to confirm the findings obtained in this analysis, is required so as to confirm the findings. A follow up analysis of the patients after therapeutic interventions may provide additional insights regarding the correlation of the identified markers with disease progression and their efficacy as disease monitoring or prognostic markers.

Top three proteins taken for optimization of the SRM assay

S.no.	Protein ID	Protein name	Adjusted p value	FC
Vivax Malaria				
1	Q14624	sp|Q14624|ITIH4_HUMAN	0.0525	-1.2425
2	P01034	sp|P01034|CYTC_HUMAN	0.0629	-2.1218
3	P00450	sp|P00450|CERU_HUMAN	0.0629	0.665
Falciparum Malaria				
1	P04040	sp|P04040|CATA_HUMAN	0.0482	1.6
2	Q9NZP8	sp|Q9NZP8|C1RL_HUMAN	0.0687	1.85
3	P07358	sp|P07358|CO8B_HUMAN	0.0798	1.27

FC – Fold change

Major proteins optimization of the SRM assay



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