Comparison of protein expression pattern between the *Plasmodium falciparum* chloroquine-resistant RKL9 and chloroquine-sensitive MRC2 strains

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**ABSTRACT**

**Objective:** The objective of this study was to compare the protein expression patterns of *Plasmodium falciparum* extracellular and intracellular proteins separated by two-dimensional electrophoresis (2-DE) from the chloroquine-sensitive (CQS) MRC2 strain and chloroquine-resistant (CQR) RKL9 strain. Materials and Methods: Both the extracellular protein (ECP) and intracellular protein (ICP) were extracted and solubilized. The proteins were separated by 2-DE, first based on their charges using isoelectric focusing and then their sizes by electrophoresis. The separated protein spots were detected by silver staining, and further, the protein spot density was analyzed by an image analysis software. **Results:** 2-DE separated the proteins extracted from the CQS and CQR strains based on their differentially expressed protein patterns. **Extracellular Protein Analysis:** A total of 109 and 77 protein spots were detected by image analysis of ECP extracted from MRC2 and RKL9 strains, respectively. There was a marked reduction in protein expression pattern in the CQR strain when compared with the CQS strain. Interestingly, 50 and 18 protein spots were uniquely expressed in MRC2 and RKL9 strains, respectively. When MRC2 strain-expressed proteins were taken as the control, 12 upregulated and 14 downregulated protein spots were observed in the RKL9 strain-extracted proteins. **Intracellular Protein Analysis:** ICP extracted from MRC2 and RKL9 strains showed 187 and 199 protein spots by an image analysis software, and a small enhancement of protein expression was measured when comparing the CQR strain with CQS strain. There were 67 and 79 unique protein spots detected in MRC2 and RKL9 strains, respectively. A total of 120 protein spots were similar when MRC2 proteins were taken as the control; among these protein spots, 40 upregulated and 22 downregulated protein spots were detected in RKL9 strain-expressed protein. **Conclusions:** Both these unique and matched protein spots might be molecularly potent drug targets for chloroquine resistance in *P. falciparum*. Further identification of these proteins by mass spectrometry/peptide sequencing is essential to clearly understand the mechanism of resistance.

**KEY WORDS**

Chloroquine, drug resistance, extracellular protein, intracellular protein, molecular target, *Plasmodium falciparum*

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INTRODUCTION

Malaria is a serious public health burden in tropical and subtropical regions of the world.\[^1,2\] It is caused by the apicomplexan parasite, Plasmodium, which is transmitted to humans by the infected Anopheles mosquito bite. Among the human malarial parasites, Plasmodium falciparum is the most virulent and the deadliest species with a high mortality and morbidity rate. However, resistance to antimalarial drugs is one of the major concerns for treatment failure and malaria eradication programs across the globe.\[^3\] With the advancement in deciphering the complete genome sequence of P. falciparum 3D7 clone, efforts are being put to identify the potential, novel targets for the diagnosis and effective antimalarial drugs/vaccine development, which in turn facilitate malaria eradication globally.\[^3\]

Chloroquine (CQ), for decades, has been used as the primary therapeutic drug for malaria treatment and control.\[^4,5\] CQ accumulates in the digestive vacuole of the parasite and inhibits the heme detoxification pathway.\[^6,7\] Resistance to CQ was reported first in 1980,\[^8\] and since then, the need to diagnose the drug resistance in an early stage and development of new antimalarial drugs for the effective treatment and control of falciparum malaria are highly in demand. The mutation in P. falciparum chloroquine-resistant transporter (PfCRT) and multidrug-resistant transporter 1 (pfmdr1) genes mainly contribute to CQ resistance. Both the PfCRT and PfMDR1 proteins are membrane proteins spanning the digestive vacuole membrane of the parasite and belong to the drug/metabolite transporter family. Mutation in PfCRT protein is the primary determinant for CQ resistance,\[^9\] whereas the mutation in PfMDR1 does not confer resistance on its own. Recent studies have shown that there is neither upregulation nor downregulation of PfCRT genes between the chloroquine-sensitive (CQS) (3D7) and chloroquine-resistant (CQR) (Dd2) clones of P. falciparum.\[^9\] In both CQR clones, Dd2 and 7G8, the efflux rate of CQ out of the digestive vacuole is higher due to the mutation in PfCRT protein in comparison with the 3D7-sensitive clone and K76T mutant clone.\[^10\] There is no change in the coding sequence of PfCRT protein, and no gene amplification was observed in these clones. Since the resistance to CQ drug pressure could not be explained based on mutation in PfCRT gene alone, it is important to determine whether the CQ drug induces differential changes in expression level of other proteins apart from PfCRT.

The aim of this study is to determine whether other potential protein targets might be involved in CQ resistance of P. falciparum and whether it might help in early detection and treatment for CQR falciparum malaria. Studying the extracellular and intracellular antigens of CQS and CQR strains will give valuable information for identifying potent biomarkers and therapeutic targets for CQ resistance in P. falciparum. Thus, in this study, the extracellular protein (ECP) and intracellular protein (ICP) expression patterns of P. falciparum CQS and CQR strains were analyzed and compared after separation by two-dimensional electrophoresis (2-DE).

MATERIALS AND METHODS

Parasite culture

The CQS and CQR strains of P. falciparum, MRC2 and RKL9, respectively, were obtained from the Malaria Parasite Bank, National Institute of Malaria Research, New Delhi. These two isolates were collected from endemic regions of India and were adapted to laboratory conditions. P. falciparum strains were maintained in continuous culture as described elsewhere,\[^12\] in “O”-positive red blood cells at 1–5% parasitemia and 5% hematocrit in RPMI 1640 medium (Gibco) supplemented with 200 µg/ml gentamycin, 2.1 g/L sodium bicarbonate, 25 mM HEPES buffer, 5% Albumax (Gibco), and 0.05% hypoxanthine (Sigma) at 37°C. When the parasitemia reaches around 10%, synchronization of parasite was performed using 5% D-Sorbitol (Sigma) solution. The synchronized parasite culture was seen in their ring stage mostly and maintained further until approximately 10–15% parasitemia was reached.

Extraction of extracellular protein

The ECPs were collected from the parasite cultures after pelleting the infected red blood cells (iRBCs), initially by centrifugation at 1500 rpm for 5 min, and then at 5800 rpm for 15 min to remove any debris as described.\[^12\] The pelleted iRBCs and supernatants were stored at −80°C and −20°C, respectively, until further use. The ECPs were purified from the supernatant by acetone precipitation, and the pellets were air-dried. Lysis buffer (8 M urea, 2 M thiourea, and 4% CHAPS) was added to the precipitated pellets and resuspended with the addition of 1X protease and phosphatase inhibitor cocktail (Roche Diagnostics). The protein concentration was quantified using Bradford assay and 2-D Quant Kit (GE Healthcare, Amersham).

Extraction of intracellular protein

The pelleted iRBCs were lysed with 0.15% saponin treatment to remove the red blood cell ghost. The cell pellet was separated by centrifuging at 13,000 rpm for 1 min at room temperature (RT) and washed with 1X phosphate-buffered saline buffer and centrifuged till the supernatant became clear. The pellet was stored at −80°C until further use. The frozen cell pellet was suspended in lysis buffer (described above with 1X protease and phosphatase inhibitor cocktail) and disrupted by three
cycles of repeated freezing/thawing, followed by sonication on ice over 10 min (1 s on, 1 s off) at 25% amplitude, as explained. The insoluble material/cell debris was removed by centrifuging at 13,000 rpm for 30 min at 4°C. The ICPs were extracted from the supernatant by acetone precipitation; the pellets were air-dried and resuspended in lysis buffer. The protein concentration of the extracts was estimated as described above.

**Two-dimensional gel electrophoresis**
The protein samples were dissolved in rehydration buffer and loaded onto the 3–10 pH linear, 17 cm isoelectric focusing (IEF) strip (Biorad) by passive rehydration and kept for IEF (PROTEAN IEF Cell, Bio-Rad, USA) at 20°C. The IEF-focused strips were first equilibrated in equilibration buffer 1 (1% dithiothreitol) at RT for 20 min and further incubated in equilibration buffer 2 (2.5% iodoacetamide) for 20 min at RT. The second dimension of separation was carried out by placing the strips on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Ettan DALTsix Electrophoresis System, GE Healthcare, Sweden). After electrophoresis run, the gels were fixed, and the protein bands were visualized by silver staining.

**Two-dimensional image analysis**
The gels were scanned using both the Fujifilm FLA-9000 Scanner, and HP Scanjet G4010 and the gel images were then analyzed and compared using the ImageMaster 2D Platinum 7.0 software (GE Healthcare, Sweden). The software performs gel alignment, spot averaging, and normalization between the control and test gel to determine the difference in expressed protein spots. Protein spots with $P < 0.05$ were considered differentially expressed. The fold change in protein expression spots was determined by comparing the ratio between the percentage volume of RKL9 and MRC2 strains.

## RESULTS

### Analysis of extracellular protein
The ECPs extracted from the MRC2 (CQS) and RKL9 (CQR) strains of *P. falciparum* were analyzed by 2-DE. The proteins were separated using IEF strip (3–10 pH linear, 17 cm strip) initially and then by 10% SDS-PAGE. The gels were visualized by silver staining, as shown in Figure 1. The 2D gel images of MRC2 and RKL9 ECPs were analyzed and compared using the software ImageMaster 2D Platinum 7. A total of 109 and 77 protein spots were detected in the ECP extracts of MRC2 and RKL9 strains, respectively. Marked reduction in the density of protein expression pattern in CQR (RKL9) strain was seen when compared to the CQS (MRC2) strain. Interestingly, 50 and 18 unique protein spots were detected in the protein extracts of MRC2 and RKL9 strains, respectively. On comparing the protein expression patterns of MRC2 and RKL9 strains, 59 matched protein spots were found with 63% similarity. When MRC2 strain-expressed proteins were taken as the control, a total of 26 protein spots exhibited at least 2-fold difference in density, in which 12 proteins were upregulated and 14 proteins were downregulated.

### Analysis of intracellular protein
ICPs extracted from MRC2 and RKL9 strains of *P. falciparum* were analyzed by 2-DE as described previously. The 2D gels were stained using silver stain [Figure 2], and the gel images were compared using the ImageMaster 2D Platinum 7 software. The separation of ICPs of the MRC2 and RKL9 strains showed 187 and 199 protein spots, respectively, indicating small increment in the density of protein expression pattern in CQR (RKL9) strain in comparison with the CQS strain. Expression pattern of MRC2 and RKL9 strains revealed 67 and 79 unique protein spots, respectively; also, 120 matched protein spots were detected with 62% similarity. Out of these matched spots, 62 protein spots were measured with a 2-fold difference in protein density, indicating 40 upregulated and 22 downregulated protein spots in RKL9 strain-expressed proteins considering MRC2 as control.
DISCUSSION

2-DE is an important tool in the field of proteomics to better understand the overall protein expression pattern of eukaryotes and find the variation in expression pattern among the samples. In this study, we have compared the CQS (MRC2) strain with CQR (RLK9) strain of *P. falciparum* for their differential expression of proteins by 2-DE. We have identified a total of 109 and 77 ECP spots and 187 and 199 ICP spots in MRC2 and RLK9 strains, respectively. Most of the proteins were distributed mainly in the pH gradient range of 4–8 and their molecular weight ranged from 10 to 100 kDa. Few proteins were identified above pH 8 and of >100 kDa molecular weight.

By comparing the gel images by ImageMaster 2D Platinum 7, 50 and 18 unique spots were detected in the ECP extracts of MRC2 and RLK9 strains. Similarly, 67 and 79 unique protein spots were observed in the ICP extracted from the MRC2 and RLK9 strains. In addition, 59 and 120 matched protein spots were detected with upregulation and downregulation of ECP and ICP expression pattern by comparing MRC2 strain (as control) with RLK9 strain (test). Out of these 59 matched spots of ECP expression, 14 spots were downregulated and 12 spots were upregulated with at least 2-fold difference in density between them. Of these 26 protein spots, a 10-fold reduction in the density of protein was observed in RLK9 strain when compared with the CQ5 MRC2 strain. Similarly, 40 upregulated and 22 downregulated protein spots were detected with at least 2-fold difference in the ICP expression pattern in RLK9 strain, taking MRC2 as the control. In addition, a 10-fold increase in the density of protein was found in RLK9 ICP expression compared to MRC2 CQ5 control strain. Further analysis of these spots identified in our study will help in the detection of potential protein targets for the differential identification of CQ5 and CQR strains, and also in therapeutics.

In a similar study, the extracellular secretory antigens (ESA) of *P. falciparum* were subjected to 2-DE and visualized by autoradiography and silver staining. About 28 protein spots matched well in both were further identified by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) method, in which only eight spots detected were parasite protein. In addition, ESA samples were analyzed by another proteomic method known as liquid chromatography MS/MS, and around 25 proteins were identified by this approach. Of these 33 proteins identified, 27 proteins were found to be novel extracellular antigens of *P. falciparum*. This extracellular domain of *P. falciparum* ESA protein plays an important role in protein-protein interaction, as well as in host–parasite interaction and new drug targets.[23]

In a quantitative 2-DE gel-based study, *P. falciparum* response to artemether and lumefantrine drugs, respectively, indicated 29 and 2 protein spots, with a 3-fold reduction in their signal intensity. In addition, 22 and 41 protein spots were upregulated, with >3-fold increase following the artemether and lumefantrine treatment, respectively. These deregulated ICPs were identified further by MALDI-TOF MS, and it was found that drugs induce a specific alteration in the pattern of proteome expression.[14]

In another study, different stages of *P. falciparum* were subjected to CQ treatment, and the changes in the oxidized proteins expressed were observed by 2-DE and further identified by MALDI-TOF/TOF-MS. In the nontreated parasites, a relative constant number of oxidized protein spots were found across the different stages of parasites with 122 spots for ring, 132 for trophozoite, and 141 for schizont. However, in the CQ-treated parasites, the number of oxidized proteins increased as the parasite cycle progressed, with 80 spots in ring, 185 in trophozoite, and 260 in schizont stage of the parasite.[13]

Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and isobaric tagging reagents for relative and absolute quantification (iTARQ) methods have explicated the identification of potential drug targets of doxycycline resistance to *P. falciparum* and also discovery of the mechanisms of resistance. A total of 150 ICP spots were deregulated in the soluble and membrane proteins, based on the comparison of three gels with varying linear pH gradient, exhibiting upregulation of 95 protein spots and downregulation of 55 spots in response to doxycycline treatment. These proteins were further identified by MS, correlated with iTARQ results, and revealed that 19% of the identified proteins by DIGE were deregulated in accordance with iTARQ.[16]

In another study, the differential ICP expression pattern in resistant clone T9/94-M1-1(b3) and parent clone T9/94 by 2-DE gel analysis helps to identify potential target for pyrimethamine resistance in *P. falciparum*. A total of 223 and 134 protein spots were detected in the parent and resistant clone, with 25 spots showing at least 2-fold difference in their protein densities. Among the 25 protein spots detected, one spot of mutant clone exhibits a 10-fold difference in their density when compared to the parent clone. Further identification of these protein spots by MALDI-TOF or tandem mass spectrometry could have elucidated in determining potential drug target to detect pyrimethamine resistance.[22]

The differentially expressed trophozoite proteins of *P. falciparum* under various antimalarial drug pressures
were identified through 2-DE and MALDI-TOF MS. These differentially expressed proteins were related to general stress response involved in redox and energy metabolism, synthesis, and transport when induced by quinine, mefloquine, and the natural compound, diosgenone.[27]

In a stable isotope labeling experiment of *P. falciparum* after treatment with CQ and artemisinin, 1211 and 1165 proteins were identified, respectively, with 889 and 814 proteins overlapping with the control (without treatment). Of these, 41 and 38 proteins only were upregulated, and 14 and 8 proteins were downregulated under CQ and artemisinin treatment, respectively.[18]

Thus, proteomics is an important field in eukaryotes to understand the expression pattern and differential expression among the sample studied. Further identification and characterization of the protein detected by 2-DE will help us to unravel the function of unknown proteins and aid more knowledge for known proteins.

**CONCLUSION**

The ability to determine the protein expression levels following drug pressure is one of the primary research areas in malaria proteomics. Identification and quantification of proteins from these differentially expressed protein patterns could be performed by predicting these protein spots in comparison with those reported in the SWISS-PROT database, based on the molecular weight and isoelectric points of the proteins. However, to clearly explicate the mechanism of CQ resistance of *P. falciparum*, further identification of these proteins, either by peptide mass fingerprinting using MALDI-TOF/TOF-MS or peptide sequencing by tandem mass spectrometry and Edman degradation, is required.

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**Conflicts of interest**

There are no conflicts of interest.

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