Detection of glycoproteins from human erythrocytes of different ABO blood groups infected with *Plasmodium falciparum*

Ramon Chin Beng Ong, Patricia Kim Chooi Lim, Joon Wah Mak

Background: Many proteins released by cells to the blood and other fluids are glycoproteins. One set of glycoproteins carry the ABO blood group determinants and glycoproteins have been shown to be vital in determining the structure and organization of plasma membranes. There is evidence suggesting their important role in cell-to-cell contact, adhesion, hormone interaction and vital transformation. Differences in proteins and glycoproteins in the different human blood groups may influence the invasion process of *Plasmodium falciparum*. The objectives of the study were to determine whether there are any changes in proteins and glycoproteins of red blood cells upon infection by *P. falciparum* and whether these protein and glycoprotein changes differ in the various ABO blood groups.

Methods: A Malaysian strain of *P. falciparum* was cultured *in vitro* in red blood cells from A, B, O and AB blood groups. Protein and glycoprotein profiles of uninfected and *P. falciparum-* infected red blood cells from the different human ABO blood groups were analyzed by SDS-PAGE. For protein bands, the gels were stained with Coomassie blue while glycoproteins were visualized following staining of gels using GelCode® Glycoprotein Staining Kit.

Results: Cell membranes of *P. falciparum* infected erythrocytes from different ABO blood groups have different glycoprotein profiles compared to uninfected cells. All the infected samples showed a prominent protein band of molecular weight 99 kDa which was not present in any of the uninfected samples while a 48 kDa band was seen in four out of the seven infected samples. The erythrocyte cell membranes of A and AB blood groups showed different glycoprotein profiles upon infection with *P. falciparum* when compared to those from blood groups B and O.

Conclusion: The two glycoproteins of molecular weights 99 kDa and 48 kDa should be further studied to determine their roles in the pathogenesis of malaria and as potential targets for drug and vaccine development.

IeJSME 2011: 5 (2): 18-28

Key Words: Plasmodium falciparum, proteins, glycoproteins, SDS-PAGE, ABO blood groups

Introduction

Malaria continues to be an important public health problem with half of the world's population at risk. In 2010, there was an estimated 216 million cases that led to nearly 655,000 deaths[1]. Various control methods which include the use of artemisinin-based combination therapy, long-lasting insecticidal nets and indoor residual spraying of insecticide provide new opportunities for large-scale malaria control. Five species of malaria parasites from the genus *Plasmodium* affect humans namely *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and more recently, *Plasmodium knowlesi* which is "now established as the fifth *Plasmodium* species to cause malaria in humans"[2]. Major clinical complications which include cerebral malaria, severe anaemia, jaundice, renal failure, metabolic acidosis, hypoglycemia and acute respiratory distress syndrome occur mainly in severe *P. falciparum* malaria. The pathophysiology of complications involves the sequestration of infected erythrocytes containing mature forms of the parasite in deep vascular beds of vital organs, due to increased cytoadherent properties of infected erythrocytes[3,4]. Severe malaria is associated with the ability of erythrocytes infected with *P. falciparum* to bind to uninfected erythrocytes and epithelial cells[5-9]. A number of glycoproteins have been reported to play a role in sequestration of malaria-infected erythrocytes such as CD36, a leukocyte differentiation antigen which is widely expressed on endothelium, platelets and leucocytes[10]. In fact, a nonsense mutation in the CD36 gene has been shown to protect against severe and cerebral malaria[8]. Another receptor on endothelial cells that has been reported to bind to *P. falciparum* infected erythrocytes is CD31 or platelet/endothelial cell adhesion molecule 1

International Medical University, 126, Jalan Jalil Perkasa 19, Bukit Jalil, 57000 Kuala Lumpur, MALAYSIA.

Address for Correspondence:

Assoc Prof Dr Patricia Lim Kim Chooi, International Medical University, 126, Jalan Jalil Perkasa 19, Bukit Jalil, 57000 Kuala Lumpur, MALAYSIA.

Email: kimchooi_lim@imu.edu.my

(PECAM-1)[10]. *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is reported to be involved in binding to intracellular adhesion molecule-1 (ICAM-1)[7,11] as well as to syncytiotrophoblasts during pregnancy and to placental chondroitin sulphate A and hyaluronic acid[9,12]. It has also been postulated that the ring surface protein 2 (RSP-2), which is expressed during the ring stage of the parasite allows binding of erythrocytes to endothelial cells in the brain, lung and syncytiotrophoblasts[9]. Thus infected erythrocytes are able to bind to endothelial cells throughout the blood-stage cycle. Increased expression of cerebrovascular endothelial expression of cytoadherence receptors ICAM-1, VCAM-1, E-selectin and chondroitin sulphate has been reported in patients who had died of cerebral malaria[8].

Many proteins released by cells to the blood and other fluids are glycoproteins. One set of glycoproteins also carry the ABO blood group determinants and glycoproteins have been shown to be vital in determining the structure and organization of plasma membranes[13-16] and there is evidence suggesting their important role in cell-to-cell contact, adhesion, hormone interaction and vital transformation[17-19]. The ability of *P. falciparum* infected red blood cells to form rosettes is a known virulence factor[20]. The rosetting of *P. falciparum* infected red blood cells with uninfected red blood cells was found to enhance microvascular obstruction under flow conditions, thus suggesting that rosetting plays an important role in the microvascular sequestration of *P. falciparum* infected erythrocytes[21]. Studies have reported that *P. falciparum* rosettes that form in group O cells are smaller and more easily disrupted than those formed in groups A, B and AB erythrocytes[22]. Rowe *et al*.[23] reported that blood group O protects against severe *P. falciparum* malaria through reduced rosetting mechanism. Glycoproteins in erythrocyte membranes are readily detected with using sodium-dodecyl-sulphate polyacrylamide electrophoresis (SDS-PAGE) stained with periodic acid Schiff and three main species of glycoproteins have been reported in human erythrocytic membranes[24]. In this study, the protein and glycoprotein profiles of uninfected erythrocytes from individuals of blood groups A, B, O and AB will be compared to erythrocytes from the same individuals infected *in vitro* with *P. falciparum*. This will allow the identification of proteins and glycoproteins that may be specific to different ABO blood groups which are involved in the invasion process by *P. falciparum* blood stages.

Materials and Methods

Plasmodium falciparum (Gombak A strain)

Plasmodium falciparum (Gombak A strain) was originally isolated from an Orang Asli patient in Gombak hospital, Selangor Darul Ehsan, Peninsular Malaysia. The strain was adapted to *in vitro* culture using the candle-jar method at 37°C[25] and was obtained from the Institute for Medical Research, Kuala Lumpur, Malaysia. Parasites were grown and maintained in RPMI 1640 culture medium (GIBCO, USA) supplemented with 10% heat-inactivated human serum + 100 µg/ml gentamycin. The human sera used to supplement the culture medium were pooled human sera obtained from the National Blood Bank in Kuala Lumpur, Malaysia. The *in vitro* cultures were performed in 6-well sterile tissue culture plates.

Human ABO blood samples

The blood samples used in this study were obtained from seven healthy volunteers, one was from AB blood group and two each from A, B and O blood groups (Subjects AB, A1 and A2, B1 and B2, O1 and O2). No distinction of race, age or gender of the volunteers was considered. The blood sample (20 ml each bleed) was drawn via aseptic venipuncture into tubes containing ethylenediamine tetraacetic acid (EDTA) and the volunteers were bled once a week throughout the study period the *P. falciparum* was being cultured to provide fresh erythrocytes. Before use, the blood samples were centrifuged at 3000 rpm for 10 minutes, the plasma and buffy coat discarded and the pelleted cells resuspended

in 15 ml RPMI 1640 + gentamycin. The cell suspension was again centrifuged, supernatant discarded and the cells washed again. The harvested red blood cells were then stored at 4°C in RPMI 1640 + gentamycin until used.

In vitro culture of _P. falciparum_ in different ABO group erythrocytes

The growth of *P. falciparum* in the 6-well tissue culture plates was monitored daily by making thin blood films. The thin blood films were fixed with methanol, then stained in 1% Giemsa stain for 30 minutes and examined under a bright field microscope under 1000X magnification. When the parasitaemia was high (12%), a portion of the infected cells were mixed with uninfected red blood cells from each volunteer to obtain a starting parasitaemia of about 2% and the cultures grown as mentioned above.

Preparation of uninfected and infected red blood cells for SDS-PAGE analysis

Both uninfected and infected red blood cells (parasitaemia ≥12%) were harvested, centrifuged at 3000 rpm for 10 minutes, supernatant discarded and the red blood cells were then washed two times with sterile normal saline. The packed red blood cells were then lysed with 1% saponin in distilled water at a ratio of 0.4 ml of saponin solution to 1 ml packed red blood cells. Sterile normal saline was then added to make up the volume to 10 ml. The lysed suspension was then incubated at 37°C for 20 minutes, centrifuged at 3000 rpm for 15 minutes and the pellet washed three times with sterile normal saline. The pellet was then resuspended in a very small volume of phosphate buffered saline, pH 7.2 (Takara Biomedicals). The cell suspension was then placed in an ice bath and sonicated at 7 kilocycles per second at I minute intervals for 5 minutes to disrupt the cell membranes. The suspension was then left overnight at 4°C to allow elution of proteins, then centrifuged at 30,000 rpm in an ultracentrifuge for 10 minutes. A small volume of the supernatant was taken for protein estimation using a protein bioassay (Bio-Rad Laboratories kit). The absorbance of the samples was measured at 595 nm using a Perkin-Elmer Lambda Bio 20 spectrophotometer. The remaining supernatant was collected and stored at -20°C until used in SDS-PAGE and glycoprotein analysis.

Sodium- dodecyl-sulphate polyacrylamide electrophoresis (SDS-PAGE) analysis

SDS-PAGE analysis was performed using a discontinuous system as described by Laemmli *et al*.[26]. Samples of the uninfected and infected erythrocytes from different ABO blood groups were thawed and denatured by heating at 95°C for 5 minutes in Laemmli sample buffer containing 0.06% Tris-HCl, pH 6.8, 10% glycerol, 0,02% SDS. 0.05% 2-mercaptoethanol and bromophenol blue dye. The samples were cooled on ice for 5 minutes. For each sample, 20 µl were loaded into the wells in the stacking gel. Known standard molecular weight markers (Bio-Rad Laboratories) were also loaded into the same gel to be used for estimation of the molecular weights of the proteins in the samples after electrophoresis. Electrophoresis using electrophoresis buffer, pH 8.3 consisting of 0.025M Tris base, 0.129M glycine and 0.1% SDS was performed at room temperature at a constant voltage of 200V until the gel front reached within 1 cm from the bottom of the gel. The gels were stained with Coomassie blue to visualize the protein bands.

Detection of glycoproteins by SDS-PAGE and staining

Before SDS-PAGE electrophoresis, the uninfected and infected red blood cell samples were diluted in normal saline to obtain a solution containing 1 mg/ml concentration. 800 µl of each sample were mixed with 200 µl of bromophenol blue dye and 20 µl of the mixture were loaded into the gel. For each gel, a positive control (horseradish peroxidase) and a negative control (soybean trypsin inhibitor) were included. After electrophoresis, the SDS-PAGE gels were stained according to manufacturer's instructions using the

GelCode® Glycoprotein Staining Kit (Pierce, USA) to detect glycoproteins.

Results

Protein profiles of uninfected and infected human red blood cells on SDS-PAGE

Figure 1 shows the SDS-PAGE patterns obtained for uninfected and infected red blood cell extracts from the 4 healthy human subjects with different ABO blood groups viz. 1 subject with blood group AB (AB_c and AB_t), 2 subjects with blood group A ($A1_c$ and $A1_t$; $A2_c$ and $A2_t$) and 1 with blood group B ($B1_c$ and $B1_t$). Figure 2 shows the SDS-PAGE patterns obtained for uninfected and infected red blood cell extracts from 3 other healthy subjects, 1 more subject with blood group B ($B2_c$ and $B2_t$) and 2 with blood group O ($O1_c$ and $O1_t$; $O2_c$ and $O2_t$). Most of the uninfected samples showed similar protein patterns when compared to each other. Samples AB_c, $A1_c$, $B2_c$ and $O1_c$ had similar patterns with bands at 93 kDa, 73 kDa, 63kDa, 28 kDa, 19 kDa and 17 kDa while Samples $A2_c$ and $B1_c$ had bands only at 19 kDa and 2 kDa. Samples $O2_c$ had bands at 63kDa, 28 kDa, 19 kDa and 17 kDa. It could be that the missing bands from $A2_c$, $B1_c$ and $O2_c$ were too faint to be detected. Other than these major bands, there were several other faint bands observed throughout the gels. For the infected samples, most of them showed similar protein profiles when compared to each other and strong bands observed included proteins of molecular weights 110 kDa, 87 Da, 45 kDa and 18 kDa.

Glycoprotein profiles of uninfected and infected human red blood cells on SDS-PAGE

Figure 3 shows the glycoprotein patterns seen on SDS-PAGE gels for uninfected and *P. falciparum* infected red blood cells of the 4 healthy human subjects viz. 1 subject with blood group AB (AB_c and AB_t), 2 subjects with blood group A ($A1_c$ and $A1_t$; $A2_c$ and $A2_t$) and 1 with blood group B ($B1_c$ and $B1_t$). Figure 4 shows the SDS-PAGE glycoprotein patterns obtained for uninfected and infected red blood cell extracts from the 3 other healthy subjects, 1 more with B blood group B ($B2_c$ and $B2_t$) and 2 with blood group blood O ($O1_c$ and $O1_t$; $O2_c$ and $O2_t$). For the uninfected red blood cell samples $A1_c$, $B1_c$, $B2_c$ and $O2_c$, prominent bands seen were proteins of molecular weight 81 kDa and 33.6 kDa but no prominent bands were observed for samples AB_c and $O2_c$ although there were a few faint bands. Sample $A2_c$ had an extra dominant band at 53 kDa. All the infected samples showed a prominent protein band of molecular weight 99 kDa which was not present in any of the uninfected samples. Furthermore, a 48 kDa band was seen in four out of the seven infected samples viz. $B1_t$, $B2_t$, $O1_t$ and $O2_t$ but not in $A1_t$, $A2_t$ and AB_t infected samples. Thus it is noted that the erythrocyte cell membranes of A and AB blood groups showed different glycoprotein profiles upon infection with *P. falciparum* when compared to blood groups B and O.

Discussion

Protein composition changes in the cell membranes of *P. falciparum* infected and uninfected erythrocytes have been reported in several studies, for example, *P. falciparum* erythrocyte membrane protein, PfEMP1, which is a malaria variant antigen and adherence receptor found on the surface of parasitized erythrocytes[27,28]. Other proteins associated with malaria parasites in infected erythrocytes include ring–infected surface antigen (RESA)[29,30], apical merozoite antigen (AMA-1)[31]; (histidine-rich protein-2 (HRP-2)[32] and sequestrin[33]. However, there is no substantiative study comparing the protein profiles of infected erythrocytes from different ABO blood groups. This study did not show any prominent differences in the protein profiles of the different uninfected and *P. falciparum* infected ABO groups but this does not rule out the possibility of there being differences. No major differences were observed probably due to the lack of sensitivity of SDS-PAGE. The focus of this study however, is to determine whether there are differences in glycoprotein profiles in the erythrocytic membranes between different ABO groups.

As the parasite stages involved in the present study are mainly asexual stages in the erythrocytes, the polypeptides identified from SDS-PAGE and the glycoproteins demonstrated in this study are most likely from these sources. The asexual stages involved are ring, trophozoites, schizonts and merozoites and it is well known that the pathogenesis of malaria including the severe complications are due to these stages leading to disease manifestations. It is for these reasons that active research has been carried out on different polypeptides in respect to their roles in pathogenesis, induction of host immune responses, as targets of chemotherapy and target epitopes for the design of potential malaria vaccines. It has been shown that antibodies are directed against a number of identified proteins on the parasite itself or against parasite-derived proteins expressed on the surface of infected erythrocytes[34]. There are many different proteins so expressed, each with defined functions. A merozoite surface protein, for example, the erythrocyte binding antigen 175 (EBA-175), is involved in the attachment of merozites to specific receptors on erythrocytes[35]. The merozoite surface protein, MSP-1, a 195-kDa protein synthesized during the development of the schizont is recognized by serum from immune individuals and is present as a complex of proteolytic fragments on the merozoite surface[36,37]. Some fragments of this polypeptide are shed during erythrocytic invasion by the merozoite but a 119 kDa-fragment (MSP-119) is retained within the newly invaded cell. The importance of this polypeptide is shown by the fact that immunization of *P. falciparum* MSP-1 protects Saimiri monkeys[38,39] and Aotus monkeys[40] from infection. It is not known whether this 119-kDa protein is related to the 99 kDa glycoprotein identified in the present study but it is certainly close enough in its molecular mass to be considered a possibility.

Another protein with a relative molecular weight close to the 99 kDa protein identified in this study is the serine repeat antigen (SERA) which is found in the parasitophorous vacuole of infected erythrocytes. In *P. falciparum* infection, the antigen is a 111-113 kDa protein with a serine content of 11%[41,42]. Experimental immunization of Saimiri monkeys gave protection against subsequent challenge[43]. The merozoite surface protein 2 (MSP-2) is a 45-kDa protein anchored in the merozoite membrane[44,45] which has been identified as a glycoprotein. Studies on different *P. falciparum* isolates showed significant sequence variations in about 50% of the protein with the N- and C-terminal regions well conserved. At present, it is unknown whether the 48 kDa glycoprotein identified in this study is the same as the 45 kDa glycoprotein reported by these researchers.

This study has shown that cell membranes of *P. falciparum* infected erythrocytes from different ABO blood groups have different glycoprotein profiles compared to uninfected cells. Two prominent glycoprotein bands of 99 kDa and 48 kDa appeared in infected cells while two other glycoproteins, 81 kDa and 33.6 kDa disappeared from the uninfected cells upon infection. Further studies would be required to determine whether the 99 kDa and 48 kDa glycoproteins are parasite-derived or parasite modification of host proteins. As mature erythrocytes do not have organelles or bio-synthetic capability, it is therefore very likely that the parasite itself is responsible for the loss and/or gain of glycoproteins in the erythrocytic cell membrane after infection. This would suggest that the parasite has the capability to modify or digest glycoproteins in the cell membrane of infected erythrocytes to escape host defenses or increase infectivity and the change in glycoprotein profile in erythrocyte membranes after infection could play a significant role in the sequestration of erythrocytes leading to complications of falciparum malaria.

To-date, there have been few studies on the role of glycoproteins involved in malaria infection. Friedman[46] reported that during malaria and other infections, the plasma concentration of alpha 1-acid glycoprotein (AGP) increases 3 to 4-fold and *in vitro* studies showed that AGP inhibited the invasion process by blocking parasite-erythrocyte interaction. This study is designed as a descriptive study to look into the possibility of

glycoproteins being expressed and playing a role in *P. falciparum* infection. Results of this study also showed that the 48 kDa glycoprotein was only seen in infected cells from B and O but not A and AB blood groups, thus suggesting that different ABO blood groups may have different glycoproteins involved with parasite invasion. The functional role of the 99 kDa glycoprotein in infected cells seen in this study needs to be determined. Further studies should therefore be performed to characterize these glycoproteins and to identify the carbohydrate and protein components in order to elucidate their important characteristics and their possible roles in the immunopathogenesis of severe and complicated falciparum malaria. It is also hoped that future research in this area could provide a means to identify glycoproteins as potential targets for chemotherapy, as immunodiagnostic markers and as epitopes for vaccine development in view of the fact that malaria continues to be worldwide problem with widespread resistance to existing chemotherapy regimes.

Acknowledgements

We thank Dr Alistair Renwick for his contributions in the design of the study. The work was supported by an internal research grant from the International Medical University, Kuala Lumpur.

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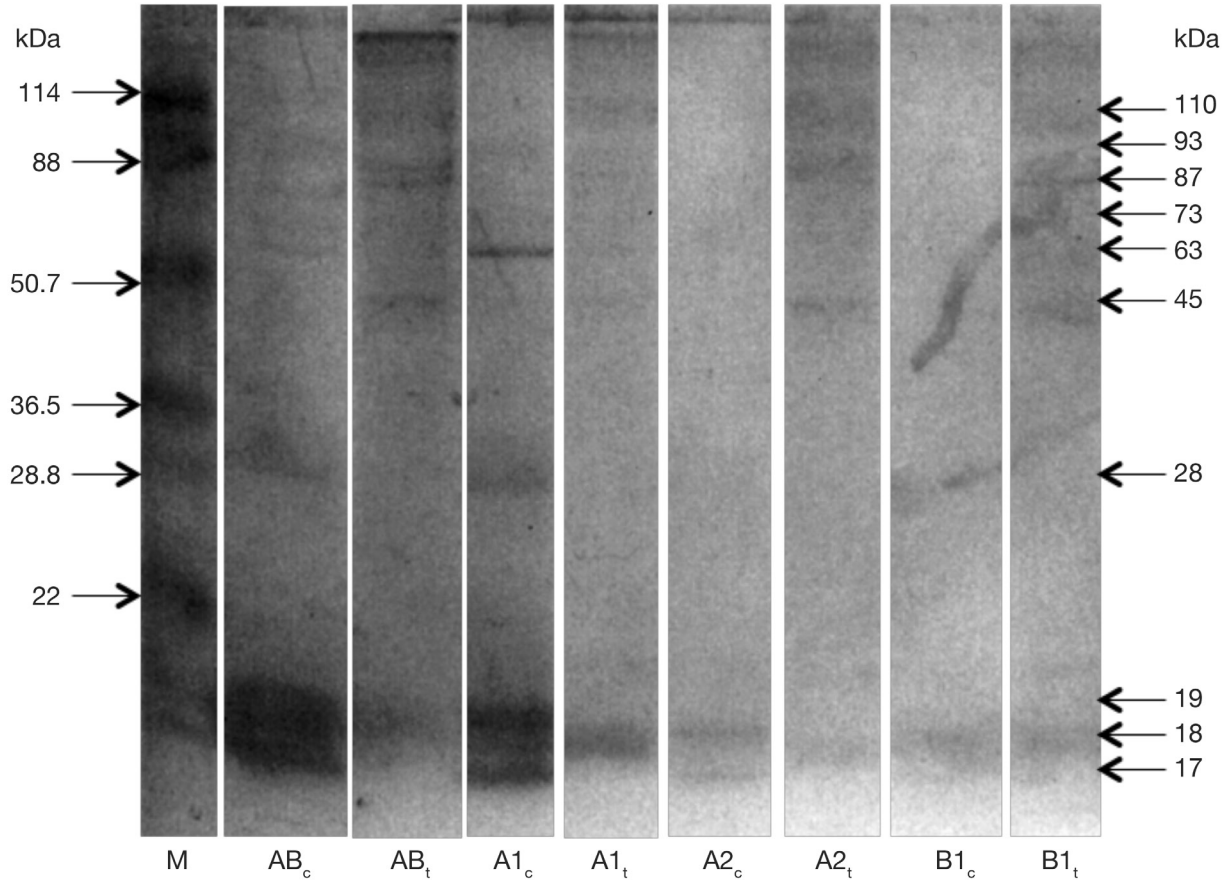

Figure 1. SDS-PAGE gel showing protein profiles of uninfected and *Plasmodium falciparum* infected red blood cells from subjects AB, A1, A2 and B1. Lanes M: protein markers, AB_c: uninfected red blood cells from subject AB, AB_t: *P. falciparum* infected red blood cells from subject AB, $A1_c$: uninfected red blood cells from subject A1, $A1_t$: *P. falciparum* infected red blood cells from subject A1, $A1_c$: uninfected red blood cells from subject A2, $A2_t$: *P. falciparum* infected red blood cells from subject A2, $B1_c$: uninfected red blood cells from subject B1, $B1_t$: *P. falciparum* infected red blood cells from subject B1.

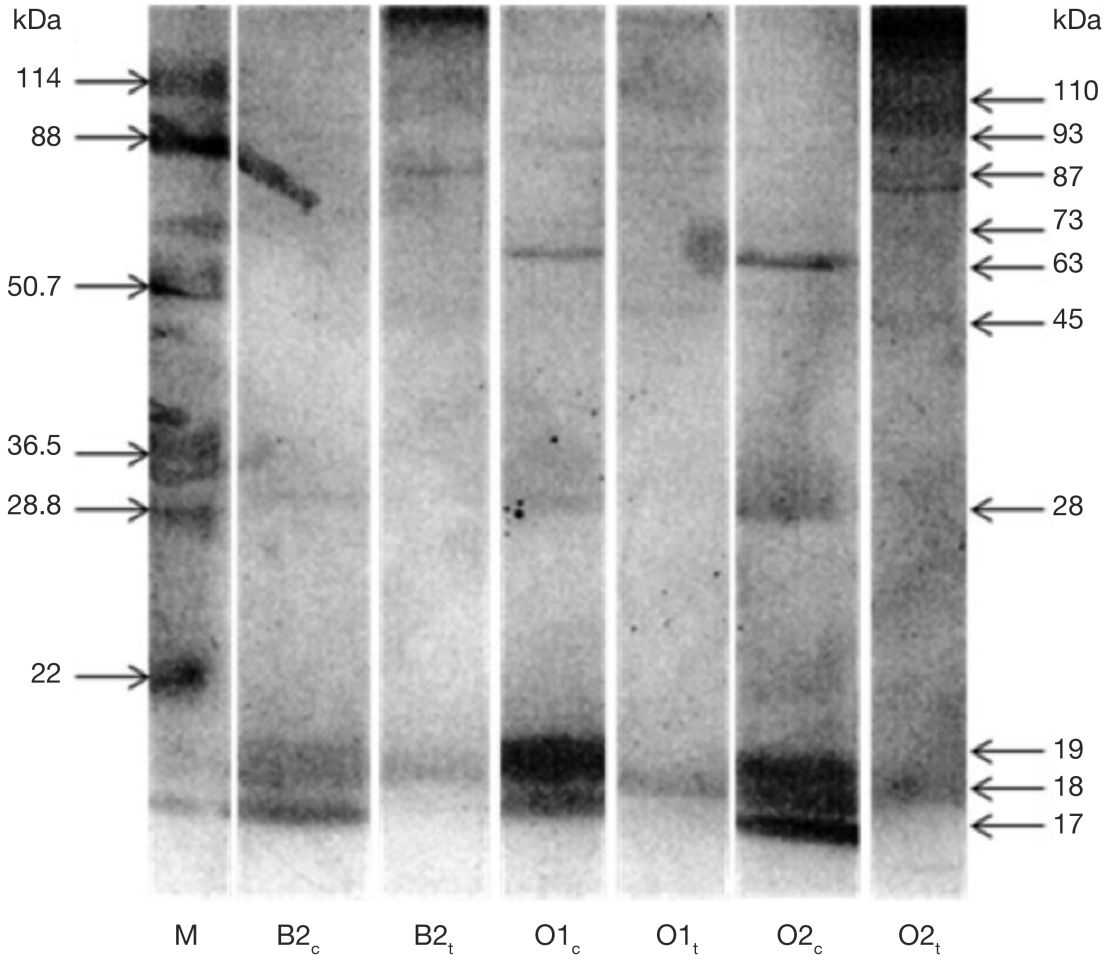

Figure 2. SDS-PAGE gel showing protein profiles of uninfected and *Plasmodium falciparum* infected red blood cells from subjects B2, O1 and O2. Lanes M: protein markers, $B2_c$: uninfected red blood cells from subject B2, $B2_t$: *P. falciparum* infected red blood cells from subject B2, $O1_c$: uninfected red blood cells from subject O1, $O1_t$: *P. falciparum* infected red blood cells from subject O1, $O2_c$: uninfected red blood cells from subject O2, $O2_t$: *P. falciparum* infected red blood cells from subject O2.

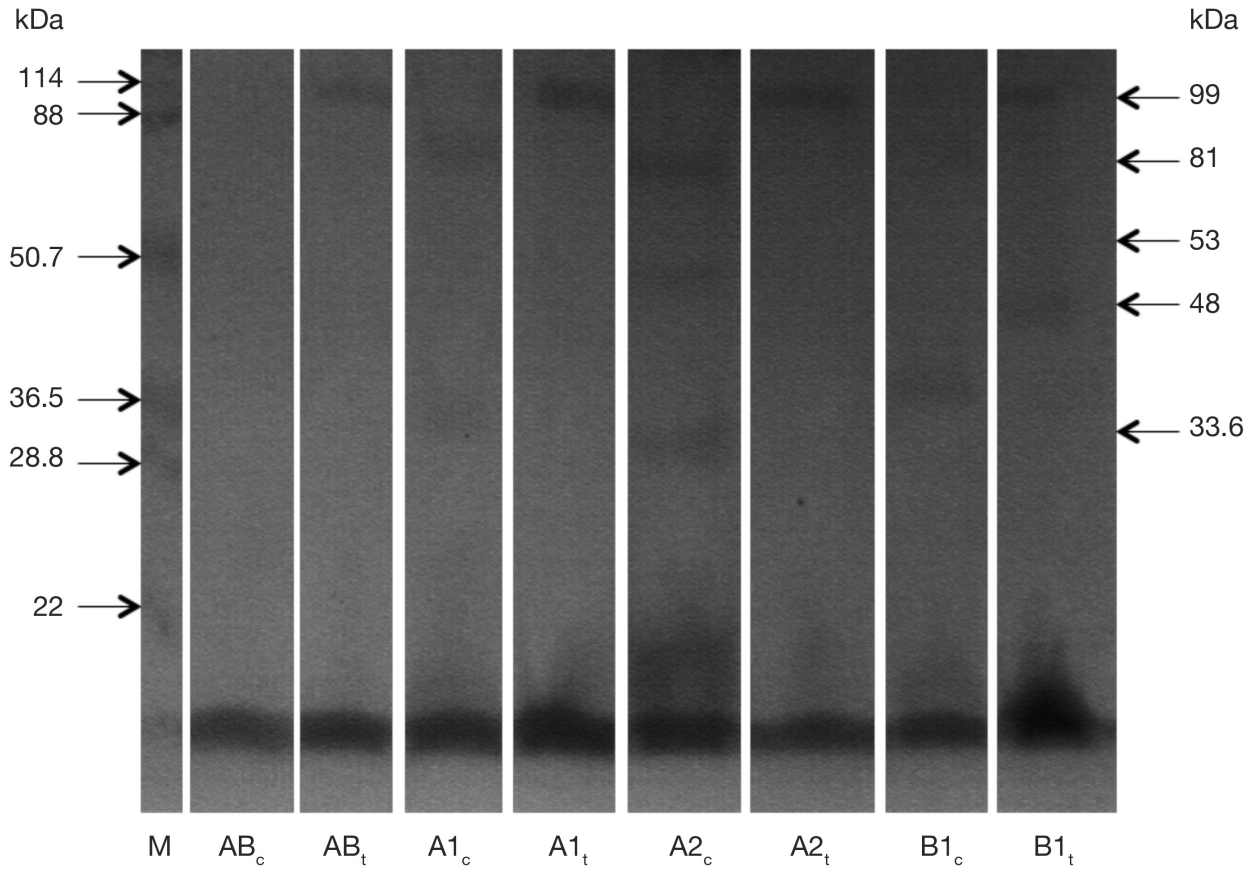

Figure 3. SDS-PAGE gel showing glycoprotein profiles of uninfected and *Plasmodium falciparum* infected red blood cells from subjects AB, A1, A2 and B1. Lanes M: protein markers, AB_c: uninfected red blood cells from subject AB, AB_t: *P. falciparum* infected red blood cells from subject AB, $A1_c$: uninfected red blood cells from subject A1, $A1_t$: *P. falciparum* infected red blood cells from subject A1, $A1_c$: uninfected red blood cells from subject A2, $A2_t$: *P. falciparum* infected red blood cellsfrom subject A2, $B1_c$: uninfected red blood cells from subject B1, $B1_t$: *P. falciparum* infected red blood cells from subject B1.

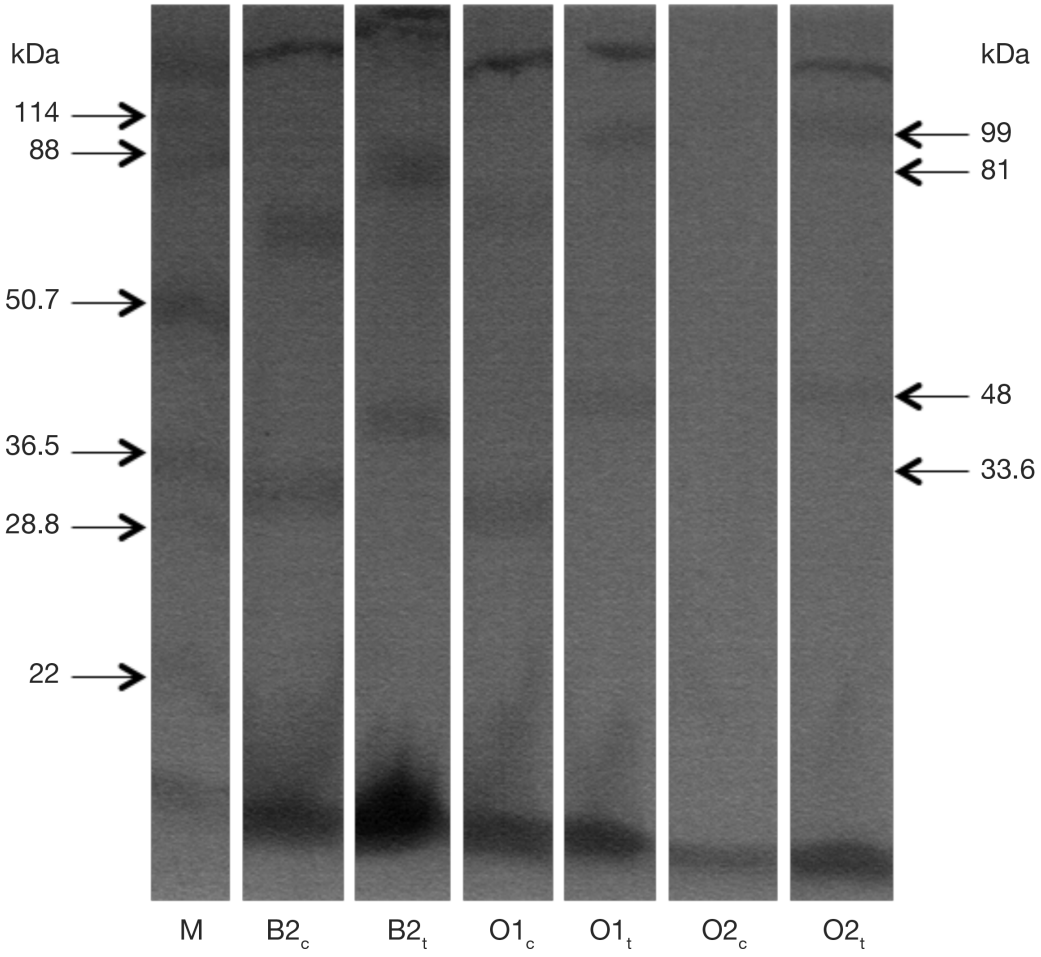

Figure 4. SDS-PAGE gel showing glycoprotein profiles of uninfected and *Plasmodium falciparum* infected red blood cells from subjects B2, O1 and O2. Lanes M: protein markers, $B2_c$: uninfected red blood cells from subject B2, $B2_t$: *P. falciparum* infected red blood cells from subject B2, $O1_c$: uninfected red blood cells from subject O1, $O1_t$: *P. falciparum* infected red blood cells from subject O1, $O2_c$: uninfected red blood cells from subject O2, $O2_t$: *P. falciparum* infected red blood cells from subject O2.