DETERMINATION OF IMMUNOREACTIVITY OF *PLASMODIUM FALCIPARUM* ANTIGENS, SERUM DILUTIONS AND BIOMATERIALS

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ABSTRACT

Immunoreactivity properties of serum dilutions and *Plasmodium falciparum* malaria antigens were measured and compared by ELISA technique using different ELISA plates to evaluate the role of antigens and serum dilutions for optimum binding. Also effort has been made to see the effect of reaction surface and material i.e. ELISA plates for binding capacity. Serological properties were estimated by ELISA methods for detection of malaria and determination of immunological characteristics. Three Pf antigens (PfAg) i.e. ring infected erythrocyte surface antigen: AR-1 (RESA), histidine-rich protein 2 antigen (HRP-2) and glycopospholipid antigen (grown and developed Pf antigen from PSJ-M strain): GPL1 have been used for serological testing of human blood samples by Enzyme Link Immunosorbant Assay (ELISA). 1:100, 1:1000 and 1:10000 dilutions of Pf positive and negative serum (50 samples in each group) and 1:1000 dilution of Pf antigens were used to measure immunoreactive properties by ELISA method. Result of PfAg-serum immunoreactivity study showed that GPL1 has the highest degree of immuno binding reactivity compared to other Pf antigens. HRP-2 and RESA antigens showed no significant difference to each other. Study also found that Costar and Fastec ELISA plates have a better Ag-Ab binding capability compared to Immulon and Falcon plates at all dilutions of serum. Serum dilution of 1:100 showed best binding and reactivity with Pf antigens followed by 1:1000 and 1:10000 showed lowest reactivity.

KEY WORDS

Malaria, Serum dilutions, GPL1, HRP-2, AR-1 antigens, ELISA plates

INTRODUCTION

Plasmodium falciparum malaria is a most common infectious disease of Southeast Asia and causes profound impact on socio-economical life of these countries. Several methods have been made to eradicate malaria from these most prone countries, but due to dynamic nature of protozoan parasite all attempts to control this disease failed and disease endemicity is gradually increasing. Transmission of malaria can be controlled mainly by three methods (i) vector eradication (ii) parasite control (iii) early detection and treatment. There are several methods available for the detection of *P. falciparum* (1-6). Most common method for diagnosis of malaria is microscopic and has been found very reliable and accurate but method requires trained manpower for examination of blood

film (7,8). Other Common methods of detection are immunoreactions dependent techniques, which requires antigen and antibody reaction for detection (enzyme link immunosorbent assay (ELISA), radio immunoassay (RIA) and Laser light Immunoassay (LIA) etc.). Out of all immunodiagnostic methods ELISA method is commonly used for detection of malaria due to its simplistic approach and mass detection capability. Immunoreactivity of ELISA method is dependent on antigen and antibody binding on plastic surface of plates (9). Other malaria immunological methods are based on covalent binding of antigen or antibody to polystyrene beads (10), agarose beads, cellulose (11), nylon material (12) and porous glass (13-15). Several studies have shown that good immunoreaction and sensitivity of ELISA depend on many factors eg. temperature, pH, antigen antigenicity and specificity and type of ELISA plates (3). It has been observed that ELISA plate's material properties play an important role in binding of antigen and antibody and their detection. Also studies have shown that antigenic properties play important role for better serodiagnosis of the disease (16). In this study attempts have been made to investigate the role of ELISA plates and Pf antigens immunoreactivity.

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MATERIALS AND METHODS

Collection of blood samples for testing

(i) Negative control blood collection: The enzyme linked immunosorbent assay (ELISA) was carried out with blood of healthy human subjects. For this human subjects aged between 20-40 years of both sexes were selected after careful medical examinations, those having no history of malaria were used as negative control. Blood samples from negative control subjects were collected in pathological laboratory of I.I.T Hospital. 3 ml blood was aspirated aseptically by 5 ml disposable syringe from left arm antecubital vein. All necessary precautions were observed for aspiration of blood. Collected blood sample was gently transferred into already autoclaved micro plastic tube and name, sex and age label of subject was put on outer surface of plastic tube. Collected blood samples were immediately centrifuged at 5000 rpm for 10 minutes in a refrigerated centrifuge to prevent haemolysis of blood. After centrifugation, serum was removed with the help of sterilized micropipettes and stored at -20°C in deep freezer. Before ELISA testing serum was diluted to desired concentration and ELISA test was performed on different plates with three *Plasmodium falciparum* antigens.

(ii) Malaria positive blood sample collection: Microscopically 50 *Plasmodium falciparum* positive blood samples were collected from endemic area of village Solana. Blood was collected in same manner as described in above mention procedure. Serum samples were stored for ELISA study at -20°C till use.

Pf antigens used in this study:

(i) Ring infected erythrocyte surface antigen (RESA-AR-1) was gifted from Malaria Research Centre, New Delhi (ii) Glycophospholipid antigen (GPL1) was developed by Mya et al. (17) (iii) HRP-2 antigen was gifted by D.N. Rao, AIIMS, New Delhi were used for comparison study of antigen antibody reactivity with different ELISA plates and anti-human horse radish peroxidase (HRPO) conjugate was purchased from Genei, Bangalore, Karnataka.

Isolation and purification of GPL antigen:

Parasite culturing procedure: *Plasmodium falciparum* (PSJ-M strain) obtained from Shahjahanpur, India was grown, adapted and maintained in RPMI 1640 medium for culture study according to the procedure of Trager and Jensen (18). Development and multiplication of the culture were followed by microscopic evaluation. When parasitaemia reached to 5-10% growth then culture supernatant was used for separation study. A control study of culture medium without parasite was performed in similar conditions.

Separation of culture supernatant: Fresh one liter of

culture supernatant was used and centrifuged two times to remove erythrocyte membrane and merozoites. After this, it was dialysis in distilled water for 12 hours and lyophilized three times to make it 20 ml in volume. Lyophilization yields 2 gm of dried material and 1 gm of this dried material was mixed with 380 ml of extraction mixture of Chloroform, methanol and 0.8% KCl (10:20:8). In this extraction solution 76 ml of water and chloroform (1:1) was put to make it in emulsion form. Then it was centrifuged at 500 rpm for 10 min at 15°C to obtain 3 layers i.e. top aqueous, middle emulsified and bottom chloroform layer. All the 3 layers were separated. In middle cloudy layer an equal volume of methanol was added to makes it completely clear solution. All the chloroform extracts were pooled together and subjected to Folch washing (19), caused separation of water and water-saturated chloroform. The water-saturated chloroform portion is called as 'Chloroform component'. It was then washed with water for three times to obtain milky white chloroform extract. At the end it was dried in Buchi rotary evaporator and a sticky residue was obtained in the glass surface of Buchi tube. So obtained sticky material was dissolved in chloroform and transferred to a glass bottle. Later on, it was dried in vacuum desiccator and 60.4 mg sticky product was obtained and parasitized culture supernatant sticky product is designated as GPL1 and non-parasitized product is called as GPL2.

Chemical study of sticky product: Components of sticky product of culture supernatant was chemically analyzed by thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC) and gas liquid chromatography (GLC). Procedure used for chemical analysis of carbohydrate, protein, lipids and phosphate has been described earlier (17).

Procurement of different ELISA plates:

(i) Costar ELISA plates: Costar Corporation, Cambridge, Mass., USA (ii) Immulon ELISA plates: Dytex Laboratories Inc. Winooski, VT, USA (iii) Falcon ELISA plates: BD Biosciences Discovery Labware, Becton, Dickinson and company USA (iv) Fastec ELISA plates: Fujirebio Products, Fujirebio; Inc., Fujizoki Pharmaceutical Co. Ltd. Japan were purchase commercially from suppliers

Preparation of different dilutions of serum samples: 1:100, 1:1000 and 1:10000 dilutions of serum samples were freshly prepared by serial dilution method with phosphate buffer saline (PBS) pH 7.2 and such diluted samples were used for ELISA study.

ELISA study with three serum dilutions, three Pf antigens and four different plates:

ELISA procedure: ELISA procedure was done according to Roy et al., (20). In each ELISA study one

antigen and three different serum dilutions of negative control with three sets of four types of ELISA plates were performed. Same method was used for testing of positive serum blood samples. In similar way other two antigens were also tested with same positive and negative blood samples. Each ELISA reaction plate was coated with 1:1000 dilution of 50 μ l of GPL1, AR-1 and HRP-2 antigens and kept at 4°C for overnight. After twelve hours plates were taken out and kept into incubator at 37°C in humid condition for one hour. Plates were then washed with PBS-Tween20 (0.15M pH 7.2) three times, each for 5 minutes. Antigen coated plates were blocked by adding 100 μ l of 1% BSA in PBS in each well of the plates. Plates were then kept at 37°C in incubator for one hour. After one-hour incubation plates were removed and washed with PBS-Tween20 three times, each for 5 minutes. 50 μ l of serum samples were added to each well of the plates in duplicate. Plates were further incubated at 37°C for one hour. The plates were then again washed thrice with PBS-Tween20. In each well 50 μ l of 1:1000 dilution of freshly prepared anti-human horse radish peroxidase (HRPO) conjugate was added and incubated for 1 hour at 37°C. Plates were then again washed with PBS-Tween20 buffer and in each well of the plates 50 μ l of freshly prepared Ortho-phenyldiamine (OPD) was added. Plates were kept at room temperature (25-30°C) in dark condition for about 10 minutes until color develops. The reaction was stopped by adding 25 μ l of 8.0 N sulfuric acid in each well of the plate. The absorbance of each well of the plate was measured with Diagnostic Pasteur LP300 ELISA reader at 492nm.

Statistical analysis of data: Data obtained from above study has been statistically analysed by paired student 't' test method. Mean, standard deviation and cut of values were calculated for sensitivity measurements.

RESULTS

Negative serum study: Normal subject serum study showed that 1:100 dilution of serum has the maximum binding reactivity with all the three different Pf antigens (AR-1, GPL1 and HRP-2) than other two dilutions (Table 1). Studies of ELISA plates binding and reaction properties found that Fastec plate showed the highest reaction capacity i.e. 0.60 ± 0.14 with AR-1 antigen, but, costar plate gave 0.48 ± 0.06 , whereas Immulon has 0.38 ± 0.07 and which was much higher than Falcon plate. Other antigens GPL1 and HRP-2 showed low reactivity with negative serum in all plates i.e. 0.20-0.24 and 0.20-0.25 at 1:100 dilutions in all the three plates respectively. In all ELISA plates 1:1000 and 1:10000 dilution of serum showed very low antigen and antibody reactivity with negative serum with all the three tested antigens.

Positive serum study: Table 1 shows that 1:100 dilution of positive serum develops a very high degree of

immuno reactivity with GPL1, HRP-2 and AR-1 antigens with Costar and Fastec plates, but Immulon plate showed lower reactivity and Falcon plate showed lowest degree of immuno reactivities. 1:1000 dilution of positive serum showed one fold less reactivity with GPL1, HRP-2 and AR-1 antigens compared to 1:100 dilution. In Costar plate, 1:1000 positive serum showed i.e. 0.58 ± 0.04 for GPL1, 0.52 ± 0.04 for HRP-2 and 0.45 ± 0.03 for AR-1. Fastec and Immulon plates showed nearly identical immunoreactivity in 1:1000 serum dilutions with three antigens i.e. 0.54 ± 0.06 and 0.52 ± 0.06 for GPL1, 0.52 ± 0.07 and 0.53 ± 0.07 for HRP-2 and 0.46 ± 0.07 and 0.42 ± 0.05 for AR-1. Falcon plate showed lowest reactivity amongst all antigens with 1:1000 serum dilutions. All antigens showed very low reactivity with 1:10000 dilution of positive serum compared to 1:100 and 1:1000 dilutions i.e. three-fold lower than 1:100 and one-fold lower than 1:1000 dilution in all plates. Costar, Fastec and Immulon plates showed similar results but falcon shows slightly low absorbance value with 1:10000 dilution of positive serum.

DISCUSSION

Sensitivity properties of three antigens and effect of serum dilutions on reactivity have been studied for selection of best serum dilution. Also attempts have been made to correlate the role of ELISA plates for antigen and antibody reaction/ binding and better detection of malaria. Study found that 1:100 dilution of serum showed maximum reactivity compared to other dilutions in similar conditions. 1:100 positive serum dilution showed a five-fold increase in reactivity against 1:100 dilution of negative serum, ten-fold increase has been found with 1:1000 dilution and 100 fold increase was seen with 1:10000 dilution of negative control serum with all the three antigens. Earlier studies also reported that high sensitivity and reactivity with 1:100 diluted serum for detection of antibody in malaria infected sera (21-23). Recent optimal sensitivity assay study of serum of Drulie et al., (24) found that 10^4 and 10^5 serum dilutions showed lowest positivity for detection of disease compared to 1:100 and 1: 200 serum dilutions.

Our study found that 1:100 serum dilutions showed maximum immuno binding and detection capacity with four different ELISA plates compared to 1:1000 and 1:10000 dilutions. We have done a sensitivity study of different ELISA plates for optimum binding of antigen and antibody. ELISA plate's study found no significant difference in binding and sensitivity properties of antigen and antibody reaction with Costar, Fastec, Immulon and Falcon ELISA plates. When compared with similar negative serum dilutions 1, 10 and 100 folds increase have been observed with 1:100, 1:1000 and 1:10000 positive serum dilutions respectively with GPL1 antigen in Costar plate but HRP-2 and AR-1 did

Table 1: Mean and $\pm$ SD of ELISA absorbance values of antigens and antibody reactivity with different dilutions of negative and positive serum in different ELISA plates

Dilution	Antigen	Plates (50 Negative serum)				Plates (50 <i>Plasmodium falciparum</i> positive serum)			
		Costar	Falcon	Immulon	Fastec	Costar	Falcon	Immulon	Fastec
1:100	AR-1	0.48 $\pm$ 0.06	0.20 $\pm$ 0.04	0.38 $\pm$ 0.07	0.60 $\pm$ 0.14	0.87 $\pm$ 0.08	0.49 $\pm$ 0.07	0.76 $\pm$ 0.06	0.81 $\pm$ 0.08
	GPL1	0.24 $\pm$ 0.04	0.20 $\pm$ 0.04	0.22 $\pm$ 0.05	0.23 $\pm$ 0.04	0.97 $\pm$ 0.05	0.60 $\pm$ 0.06	0.89 $\pm$ 0.07	0.94 $\pm$ 0.05
	HRP-2	0.25 $\pm$ 0.05	0.20 $\pm$ 0.03	0.21 $\pm$ 0.03	0.24 $\pm$ 0.04	0.95 $\pm$ 0.06	0.59 $\pm$ 0.07	0.87 $\pm$ 0.07	0.91 $\pm$ 0.06
1:1000	AR-1	0.15 $\pm$ 0.04	0.14 $\pm$ 0.04	0.10 $\pm$ 0.03	0.22 $\pm$ 0.05	0.45 $\pm$ 0.05	0.27 $\pm$ 0.02	0.42 $\pm$ 0.05	0.46 $\pm$ 0.07
	GPL1	0.14 $\pm$ 0.03	0.14 $\pm$ 0.03	0.11 $\pm$ 0.03	0.14 $\pm$ 0.03	0.58 $\pm$ 0.04	0.39 $\pm$ 0.04	0.52 $\pm$ 0.06	0.54 $\pm$ 0.06
	HRP-2	0.15 $\pm$ 0.04	0.15 $\pm$ 0.04	0.11 $\pm$ 0.03	0.16 $\pm$ 0.04	0.52 $\pm$ 0.04	0.39 $\pm$ 0.05	0.53 $\pm$ 0.07	0.52 $\pm$ 0.07
1:10000	AR-1	0.11 $\pm$ 0.04	0.06 $\pm$ 0.03	0.03 $\pm$ 0.02	0.04 $\pm$ 0.02	0.18 $\pm$ 0.05	0.11 $\pm$ 0.03	0.15 $\pm$ 0.03	0.14 $\pm$ 0.02
	GPL1	0.05 $\pm$ 0.02	0.04 $\pm$ 0.01	0.04 $\pm$ 0.02	0.06 $\pm$ 0.02	0.28 $\pm$ 0.03	0.16 $\pm$ 0.03	0.23 $\pm$ 0.04	0.26 $\pm$ 0.04
	HRP-2	0.08 $\pm$ 0.03	0.05 $\pm$ 0.01	0.04 $\pm$ 0.02	0.06 $\pm$ 0.02	0.25 $\pm$ 0.04	0.12 $\pm$ 0.02	0.23 $\pm$ 0.06	0.22 $\pm$ 0.05

not show much difference in 1:100 and 1:1000 dilutions. In Fastec plate the comparative reactivity study of positive and negative serum with GPL1 antigen showed four-fold increase in 1:100, six-fold in 1:1000 and 100 fold with 1:10000, however HRP-2 and AR-1 antigen showed small significant difference when compared with respective negative serum dilutions. Immulon plate positivity study found four-fold high with 1:100, nine-fold high with 1:1000 and over 100 fold higher with 1:10000 dilutions of negative serum whereas HRP-2 and AR-1 did not show much difference in 1:100, 1:1000 and 1:10000 dilutions. In Falcon plate a 2.5 fold increase has been found with 1:100 serum dilution, four-fold increase has been observed with 1:1000 and fifty-fold increase has been found with 1:10000 dilution of negative serum but HRP-2 and AR-1 showed some differences in sensitivity bindings (Table 1).

Comparative sensitivity study of negative serum with different plates showed highest reactivity with Fastec plate than Costar, Immulon and Falcon plates in 100 and 1000 dilutions. No significant differences have been found between Costar, Fastec and Immulon plates in positive serum dilution study, but Falcon plate study showed significantly lower sensitivity with 1:100, 1:1000 and 1:10000 dilutions. Our comparative studies found that 1:100 serum dilution is the best for detection of disease but 1:1000 dilution can be used for antigen antibody reaction.

ACKNOWLEDGEMENTS

We thank to Dr. Subba Rao, Director Malaria Research Centre, Delhi for permission and necessary facilities for studies. Authors are also thankful to Dr. Shukla Biswas, Mr. Surander Kumar, Mrs. Kamala Negi and Mr. Ajit Singh for technical helps.

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