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Diagnosis of *Plasmodium falciparum* infection from two different endemic areas by two immunological methods using glycophospholipid antigen

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Previously described isolated Glycophospholipid (GPL) antigen's diagnostic sensitivity and specificity properties were tested on malarial and non malarial blood samples from two different malaria endemic areas in Myanmar and India. The control area is a non-endemic area, Delhi, India. Standard Enzyme-Link Immunosorbent Assay (ELISA) and modified Laser Immunoassay (LIA) methods were used. The crude parasite rates were found to be 28.75% in Assam, India and 32.5% in Taikkyi, Myanmar respectively. *P. falciparum* was predominantly high in both forested-hilly areas. Immunoreactivity and specificity of GPL antigen were compared with those of another previously reported RESA derived synthetic peptide antigen (R1). The diagnostic sensitivity properties of GPL antigen were found cent percent sensitive for *P. falciparum* cases in both areas by LIA method but ELISA with R1 antigen showed a slightly lower level of sensitivity than GPL antigen. The specificity studies of GPL and R1 antigens tested on malarial and non malarial diseases sera were found to be very specific for *P. falciparum* as they did not react with other non malarial diseases sera. LIA method with the application of GPL coated latex beads can be an excellent diagnostic tool for *Plasmodium falciparum* infection.

INTRODUCTION

Traditional diagnosis of malaria by microscopic examination of blood smears is an excellent and efficient method, but is highly dependent on human skill and diligence. This human factor has probably been the cause of underreporting of malaria worldwide. As an alternative, sensitive immunoassays like ELISA, radio immunoassay (RIA), Indirect immuno-fluorescent antibody test (IFA) and Opti-Mal tests have been developed and tried for mass application [1,2,3]. These diagnostic methods are quite specific and sensitive but their procedures are very lengthy and expensive. Agglutination assay is the simplest and cheapest immunoassay but has not been employed because of its low sensitivity as the antigen antibody complexes are often microscopic and not visible to the naked eye. Laser immunoassay (LIA) based upon laser light scattering can detect microscopic agglu-tinates and can raise the sensitivity of agglutination assay enormously [4,5]. Recently, development of data analysis by CONTIN method in dynamic light scattering [6] led us to develop LIA for malaria [7], which is as sensitive as ELISA but much simpler in principle and practice. Here we report specific diagnosis of *Plasmodium falciparum* infection by LIA using a new glycophospholipid antigen

isolated from *Plasmodium falciparum* culture supernatant. We demonstrate here that the GPL antigen is very specific and can identify positive infection in clinically diagnosed patients both from India and Myanmar.

MATERIALS AND METHODS

Antigens

The synthetic peptide antigen R1 [8] was gifted from Cambridge Bioresearch Co. UK., and the GPL antigen was isolated from *Plasmodium falciparum* culture supernatant. Isolation of GPL antigen is described briefly as follows:

A single *Plasmodium falciparum* isolate, PSJM, from Sahajahanpur, U.P. India was adapted to continuous culture as described by Trager and Jensen [9]. One litre of healthy culture having parasitemia above 5 percent was selected. Spent culture supernatant was collected after removal of parasites. Supernatant was concentrated, then dialysed against water and lyophilized as a powder. The powder is extracted with chloroform and the chloroform extract yielded a residue, which was characterized as a glycophospholipid having xylose, mannose, galactose and glucose in its sugar moieties. Details of isolation and characterization will be reported elsewhere [10]. To test the parasite origin and immunoreactivity, it was tested against human sera of slide positive cases of *Plasmodium falciparum, Plasmodium vivax* as well as sera of healthy individuals with no history of malaria, as negative control.

Background of study area

Control group

Thirty blood samples from infants (aged between 2 months to 2 years with no history of malaria) were collected from Delhi hospitals by finger prick method on 3mm thick whatman filter paper. Thick and thin blood smears on glass slides were also prepared for microscopic examination to confirm the absence of malarial parasites and served as negative controls.

Test group

Assam: It is a northeastern state of India. The area is hilly, covered with dense forest, and is highly endemic for malaria, predominantly *Plasmodium falciparum* malaria. Population is mostly tribal dependent on forest. Extensive use of bed nets apparently has reduced man-mosquito contacts.

Myanmar: Yeasikan village, Taikkyi Township is in a hilly area situated by the site of the Gyophyu Dam. Malaria transmission is perennial because of mosquito breeding in the water body of Dam. Villagers are mostly dependent on forest products and negligent about malarial drugs and other protection measures.

Collection of test blood samples

Thick and thin blood smears on glass slides were prepared from blood samples of fifty each of Assam and Myanmar human subjects of fever cases and were examined microscopically after Giemsa staining for positive, negative and species identification of parasites. After having written informed consent from all the subjects, finger prick blood was collected from microscopically *P. falciparum* positive cases on filter paper discs, which were stored at -20º C until used for serological examination.

Collection of non malarial blood samples

Ten samples each of six common non-malarial sera (HIV, TB, Asthma, Filaria, Pregnancy, Normal) were obtained from Safdarjung Hospital and AIIMS, New Delhi for specificity study. This study was approved at Malaria Research Center under ICMR, Government of India.

ELISA and LIA

Methods of Enzyme link immunosorbent assay (ELISA) and Laser light immuno-

assay (LIA) using R1 antigen have already been described [8,7]. ELISA results were read on ELISA reader at 492nm wavelength from Organon Teknica, Belgium. LIA was done on a homemade laser scattering set up. Agglutinations of antigen coated polystyrene beads (90nm nano particles) with antibody present in subject serum is monitored by laser light scattering. The agglutinates can be detected microscopically.

Statistical analysis of data

Data obtained from the study were statistically analysed by paired student 't' test method. Mean, standard deviation and cut-off values (Control mean +2SD) were calculated for sensitivity measurement.

against control blood samples by ELISA and Laser immunoassay (LIA) methods. Out of thirty finger prick control negative blood samples collected on filter discs, all samples were tested to determine the cut-off value (control mean +2SD) for determination of malaria positive and negative cases. The cut-off values of GPL and R1 antigens are as follows i.e. 106.07 and 108.5, which were obtained from LIA method. Cut-off values for ELISA method were determined and found to be 0.2416 for GPL and 0.2642 for R1 antigens.

Test group

Microscopic examination

One hundred (Fifty each from Assam and Myanmar) finger prick blood samples were

Table 1. Diagnosis of Assam and Myanmar blood samples for malaria by LIA , ELISA and Microscopy

Category	Microscopic diagnosis		Laser Light Immunoassay				ELISA			
	Thick blood film exam:		GPL-Antigen		R-1 Antigen		GPL-Antigen		R-1 Antigen	
	A	B	A	B	A	B	A	B	A	B
P. falciparum	23	26	23 100%	26 100%	21 91.30%	24 92.31%	22 65.65%	25 96.15%	21 91.30%	23 88.46%
P. vivax	6	4	---	--	5 83.33%	3 75%	---	-	3 50%	2 50%
Negative	21	20	21	20	21	20	21	20	21	20
False negative			6	4	3	3	7	5	5	5
Total positive	29	30	23	26	26	27	22	25	24	25
Total negative	21	20	27	24	24	23	28	25	26	25
Control negative (0-2 yrs)	30		30		30		30		30	
Mean ±SD			(98.36±3.8532)		(98.72±4.89)		(0.1954±0.0231)		(0.1524±0.0559)	
Cut-off value (Control Mean+2SD)	----		106.07		108.5		0.2416		0.2642	
P value			P<0.001		P<0.001		P<0.001		P<0.001	

RESULTS AND DISCUSSION

Results of LIA and ELISA methods evaluated by GPL and R1 antigens are shown in Table 1.

Control group

Initially immunoreactivity properties of GPL and R1 antigens were measured

used for detection of positive and negative cases of malaria in this study. Microscopic examination of thick and thin Giemsa stained slides showed *Plasmodium falciparum* positive in 49 samples and only 10 blood films showed presence of *Plasmodium vivax*. Remaining 41 blood samples were microscopically negative for malaria.

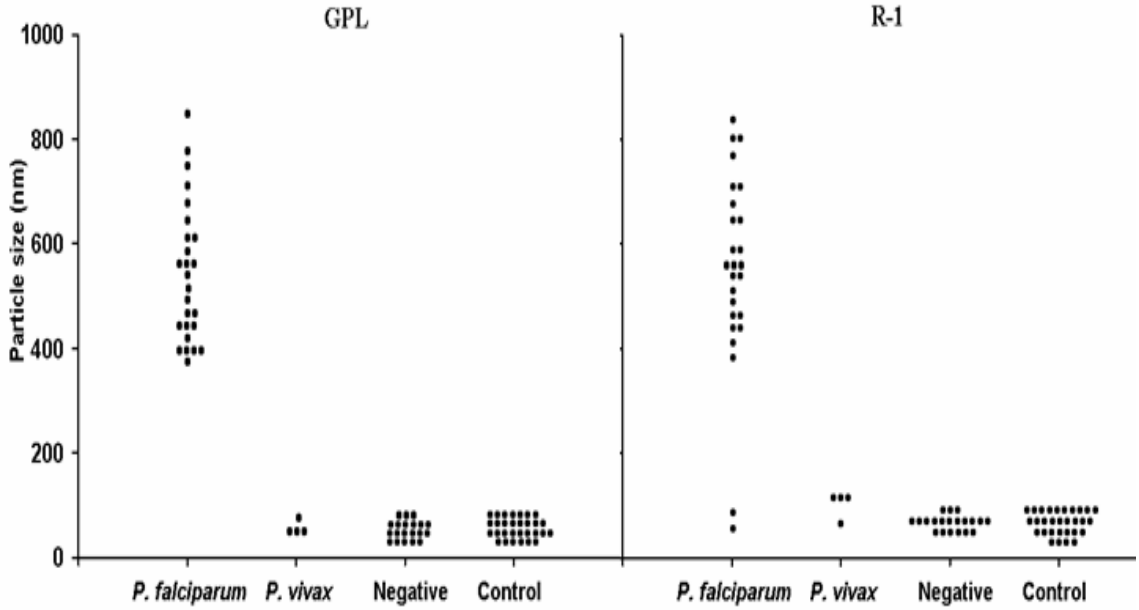

Fig. 1. Estimation of anti-GPL and anti-R1 antibodies by LIA method, samples from Myanmar (Cut-off value-GPL=106.7nm, R1=108.5nm)

LIA method

Detection of malaria in test group by LIA method showed that GPL antigen can detect 49 blood samples of *Plasmodium falciparum*, but R1 antigen could detect only 45 cases of *Plasmodium falciparum* and 8 cases of *Plasmodium vivax* malaria.

ELISA method

Test group blood samples assayed by ELISA method using GPL antigen showed 47 samples *Plasmodium falciparum* positive, however, R1 antigen could detect 44 *Plasmodium falciparum* and 5 *Plasmodium vivax* positive cases.

The results showed that anti GPL titres for *Plasmodium falciparum* cases were about eight to nine folds higher than of *Plasmodium vivax*, negative and control sera ($P<0.001$) but *Plasmodium vivax* titres showed no significance difference between negative and control sera by LIA method. When anti-R1 titres were studied by LIA method, *Plasmodium falciparum* and *Plasmodium vivax* cases were about eight folds ($P<0.001$) and 1.5 folds higher ($P<0.05$) than negative and control sera respectively. The results of ELISA showed that anti GPL titres for *Plasmodium falciparum* cases were about four to five fold higher than *Plasmodium vivax*, negative and control sera but anti R1 titres for *Plasmodium falciparum* and *Plasmodium vivax* cases were about four to five fold ($P<0.001$) and 1.5 fold higher than that of negative and control sera ($P<0.05$).

Such a distinctive difference in results prompted us to find out a potential immunodiagnostic method with higher sensitivity for *Plasmodium falciparum* more systematically by using two different antigens, R1 and GPL in parallel, tested in two different methods, ELISA and LIA. Such a comparative study between ELISA and LIA using R1 as antigen has already been published [7]. We showed that LIA has actually little edge over ELISA in terms of sensitivity and cost.

In this report a new glycophospholipid antigen isolated from *Plasmodium falciparum* culture supernatant was subjected to a similar comparative study and R1 was also included parallely for comparison. Fig.1 gives the LIA results and Fig. 2 the ELISA results obtained with sera

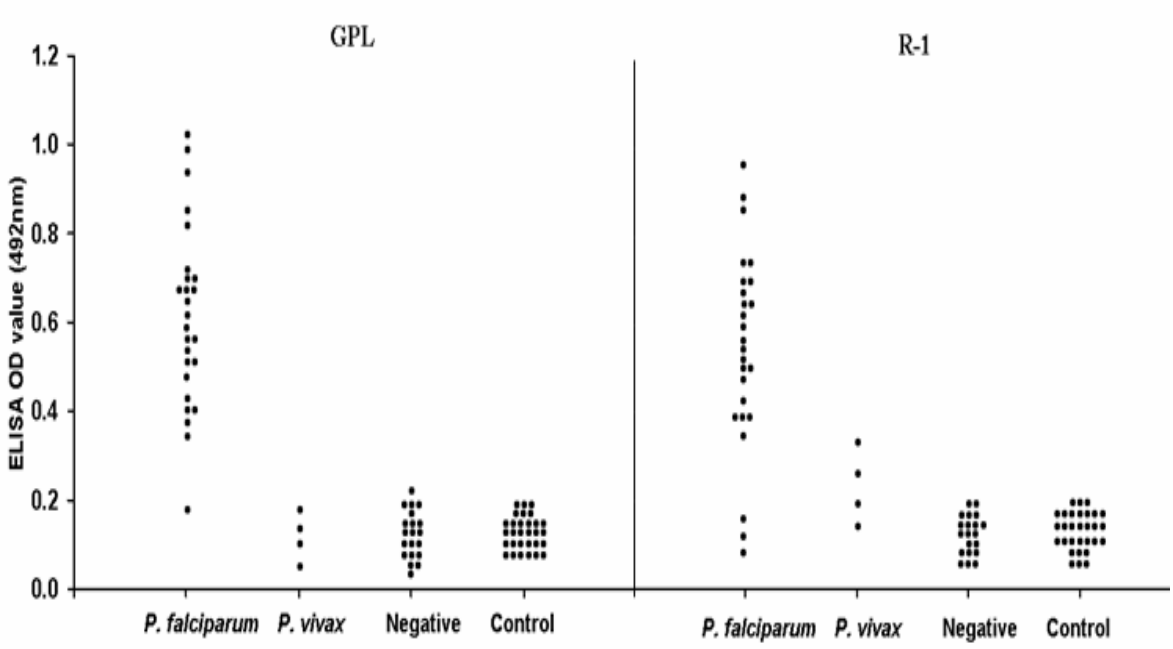

Fig. 2. Antibody titers against GPL and R1 - peptide in the same samples from Myanmar by ELISA (Cut-off value GPL=0.216, R1=0.2642)

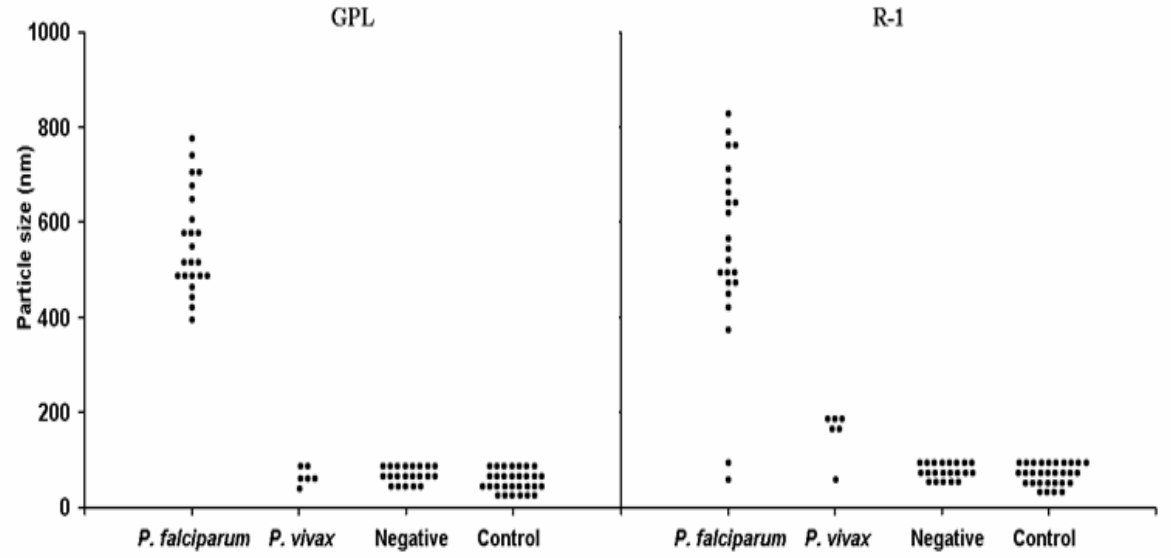

Fig. 3. Estimation of anti-GPL and anti-R1 antibodies by LIA from Assam, India samples (Cut-off value-GPL=106.7nm, R1=108.5nm)

from Myanmar. The ELISA data with R1 antigen are similar as that reported earlier [7]. A large spread in the titre with more overlap between the *Plasmodium vivax* sera and the control sera is evident. The data with GPL antigen are more discriminatory between *Plasmodium falciparum, Plasmodium vivax* and negative cases. Figure 1 shows the results of LIA with the same samples, GPL cannot distinguish between the *Plasmodium vivax* and negative controls, but anti-GPL titres in *Plasmodium falciparum* sera are much higher than the titres in the *Plasmodium vivax* and negative control sera. This indicates that GPL is of *Plasmodium falciparum* origin and is specific. The results with blood samples from Assam, India are given in the Figure 3 and 4. Assam blood samples results showed similar trend as it was observed in Myanmar test group. These results further prove our point of contention that GPL antigen has

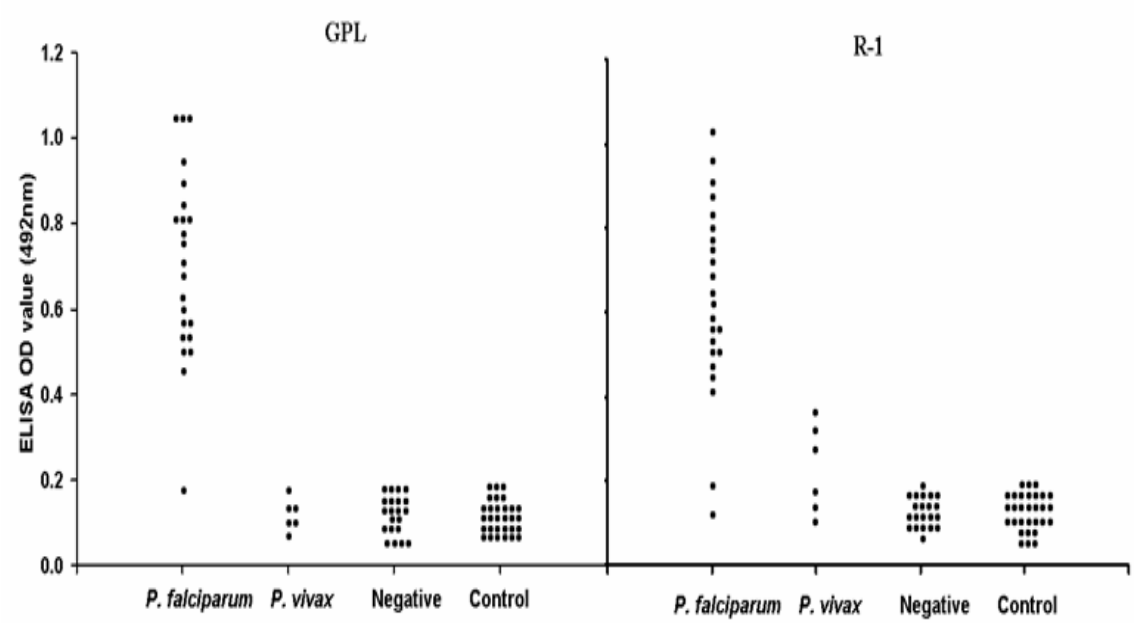

Fig. 4. Antibody titers against GPL and R1 - peptide in the same Assam samples by ELISA (Cut-off value GPL=0.216, R1=0.2642)

better immunodiagnosing reactivity than R1 antigen for *Plasmodium falciparum* malaria as determined by LIA which can be again observed in Figure 3.

We tested the specificity of GPL and R1 antigens also. The results of LIA and ELISA using GPL coated latex beads and non-malarial sera showed no reactivity. The coated beads in absence of sera showed an average diameter of 85-95nm only. No agglutination was shown upon addition of any of the non-malarial sera, which suggests that GPL is non-cross reacting with antigens of other pathogens tested here.

High specificity and discriminatory capacity of GPL antigen for *Plasmodium falciparum* are highly suggestive of its parasite origin. Glycophospholipids synthesis by *Plasmodium falciparum* has been identified [11]. The chemical structure of glycophosphatidylinositol (GPI) of intra-erythrocytic *Plasmodium falciparum* has now been determined including the core glycan [12]. It has been reported that adults who have resistance to clinical malaria contain high level of anti-GPI antibodies whereas, individuals not exposed to the malarial parasite completely lack anti GPI antibodies. It may be noted that Perlman and colleagues reported a blood stage *Plasmodium falciparum* antigen using an invasion inhibitory monoclonal antibody (MabA52A6) obtained from clinically immune donor [13]. This antigen, though not fully characterized chemically, appeared to be a phosphoglycolipid sensitive to phospholipase and is present in the lipid extracts of *Plasmodium falciparum* infected erythrocytes. It was reported that the antigen is mainly synthesized late in schizogony and is also shed from the infected erythrocytes. Amount of GPL obtained by us from 1-liter culture supernatant is very little (10µg) and it is plausible that this may also be sheded by the infected erythrocytes. The specificity and immunoreactivity of the GPL observed here are not surprising if it contains GPL identified by Mabs earlier. Further, our GPL is well conserved among different isolates. We, for the first time, report on this aspect and demonstrate the excellent diagnostic potential of GPL antigen for detect and demonstrate the excellent diagnostic potential of GPL antigen for detection of *Plasmodium falciparum* infection.

The remarkable specificity and sensitivity of GPL in LIA for *Plasmodium falciparum* is demonstrated cent percent correlation with the slide examination results (Table 1). The assay is based on antibody detection, it

cannot be recommended for clinical diagnosis but it can be used as an excellent epidemiological tool. Surveillance and control of malaria require evaluation of Annual parasite index (API), Slide positive rate (SPR) or Slide *Plasmodium falciparum* positive rate (SPFR), which are often under reported due to human tardiness and errors. Also evaluations of these parameters are highly laborious and time intensive. In such situation, GPL/LIA assay described here can be useful for supplementing or cross checking SPR data or determining *Plasmodium falciparum* transmission dynamics in endemic areas.

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