Seroepidemiological study of *Plasmodium falciparum* antigens for detection of malaria

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Attempt has been made to develop a simple and cost-effective diagnostic method for the detection of malaria in field conditions. A new type of *Plasmodium falciparum* antigen (PSJ-M strain) from *in vitro* culture supernatant has been isolated. Purification and chemical analysis of the antigen showed that it is a glycoprophospholipid (GPL1), which contains mannose, xylose, glucose and galactose (3:3:1.5:1.5) in sugar moiety but its structure is devoid of inositol sugar and amino acids. Sensitivity and specificity properties of GPL1, when compared with existing *P. falciparum* antigens by Laser light scattering immunoassay (LIA) and Enzyme linked immunosorbent assay (ELISA), showed that the antigen has a very high sensitivity for detection of *P. falciparum* malaria antibodies; also specificity percentage was found to be 98%. The serological properties of GPL1 antigen have also been evaluated in endemic and non-endemic areas of different regions of the country. A simple, economical and handy malaria detection immunosensor (MDI) has been designed and developed for field areas. Laboratory and field trials for detection of *P. falciparum* malaria by MDI showed highly encouraging and good results. Data obtained by MDI from field areas, when compared in laboratory by LIA and ELISA techniques, showed that MDI method had better diagnostic ability and efficacy for detection of *P. falciparum* malaria (94-98%). Results of the study also suggest that GPL1 antigen and MDI can be used for detection and prevention of *P. falciparum* malaria.

Keywords: antigen, ELISA, field study, glycophospholipid, LIA, MDI, sensitivity, specificity

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Introduction

Malaria is a major public health problem of tropical and subtropical countries. It is one of the most wide spread diseases occurring in the world and intimately connected with socioeconomic conditions of developing countries. Malaria has a high morbidity rate and is responsible for more deaths per year than any other transmitted disease. Nearly 40% of the world’s population is living in malaria prone areas and each year 300-500 million clinical cases of malaria are reported throughout the world. *Plasmodium falciparum* has been found to cause very high morbidity and mortality rate compared to other human malaria. *P. falciparum* malaria is transmitted by female *Anopheles* mosquitoes in human and develops serious and severe pathological symptoms, i.e. cerebral malaria, that may become fatal if not diagnosed and treated well in time. Several attempts have been made in the past to control and eradicate *P. falciparum* malaria but no proper remedial and preventive measures have been found. Therefore, a serious and effective effort is required to control it. Recent studies showed that *P. falciparum* malaria could be controlled if it is detected and diagnosed in early stages of development. Several novel, non-microscopic diagnostic methods for the detection of malaria have been developed in the recent past. In general, however, these diagnostic method are not found suitable in malarialogic areas. Recent studies showed that antigen antibody capturing properties play a very important role in immunological evaluation of malaria. Several *P. falciparum* antigens have been developed and their serological properties evaluated. The detection of parasite antigen has been considered as better alternative for the diagnosis of malaria, but most of the antigen detection tests developed so far showed variable sensitivity and specificity. None of the immunologival methods is found suitable for routine use due to false sensitivity and specificity properties of antigen and cannot be used in field conditions because of expensive equipments and reagents. Thus, there is a need to develop a good, cost effective, handy and reliable diagnostic method for field areas to control *P. falciparum* malaria from poor and developing countries. In this investigation, efforts have been
made to develop a detection method based on antigen isolated from local strain of *P. falciparum* parasite. The seroantigenic properties of isolated antigen have been evaluated and compared with other known antigens in laboratory and field conditions by different diagnostic methods. Also, an attempt has been made to develop an economical, simple, sensitive and portable immunosensor device for the detection of malaria in field conditions.

**Materials and Methods**

**Parasite Culturing Procedure**
*Plasmodium falciparum* (PSJ-M) strain obtained from Shahjahanpur, India was grown, adapted and maintained in RPMI 1640 medium for culture studies according to the procedure of Trager and Jensen\(^1\). Development and multiplication of the culture were followed by microscopic examination. When the parasite 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 litre of culture supernatant was centrifuged two times to remove erythrocyte membranes and merozoites. After this it was dialyzed in distilled water for 12 hrs and lyophilized three times to make it 20 ml in volume, which yielded 2 g of dried material. 1 g of the dried material was mixed with 380 ml of extraction mixture of chloroform, methanol and 0.8% KCl water (10:20:8). In this extraction solution, 76 ml of water and chloroform (1:1) was put to make it in emulsion form. It was subsequently centrifuged at 500 rpm for 10 min at 15°C to obtain three layers: top aqueous, middle emulsified and bottom chloroform. All the 3 layers were separated and to the middle cloudy layer an equal volume of methanol was added to make it completely clear solution. All the chloroform extracts were pooled together and subjected to Folsch washing\(^1\) to separate water and water-saturated chloroform. The water saturated chloroform portion called as ‘Chloroform component’ was then washed with water three times to obtain milky white chloroform extract. At the end, it was dried in Buche rotary evaporator and a sticky residue was obtained on the glass surface of Buche tube. This 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. The parasitized culture supernatant (sticky product) was designated as GPL1 and non-parasitized product as GPL2.

**Chemical Study of Sticky Product**
Components of the sticky product were chemically analyzed by thin layer chromatography (TLC), high performance thin layer chromatography (HPLC) and gas liquid chromatography (GLC). Chemical analysis of carbohydrates, proteins, lipids and phosphates was done using the procedure of Mya et al\(^1\).

**Standardization and Quantification of GPL Antigen**
GPL 1 and GPL 2 antigens were standardized and qualified by ELISA under identical conditions with different serum dilutions. Concentrations of Pf antigens were made as follows: 1mg/ml of Pf antigen (w/v) was prepared in PBS (pH 7.2) and used as stock solution for further dilutions. 1 μl of stock solution was diluted in 1 ml of bicarbonate loading buffer (1μg/ml). 50 μl of this was put into the wells of ELISA plates. Five each serum samples of *P. vivax* positive, *P. falciparum* positive and negative control were tested at 1:100, 1:200, 1:400, 1:800, 1:1000 and 1:10000 dilutions.

**Testing Groups**

**Microscopic Study of Control and Malaria Positive Groups**
Thick and thin blood smears were prepared of control and malaria positive subjects and Giemsa stained slide were examined under oil immersion (×100) by bright field microscope. Groupings of subjects were made after firm findings and results obtained by microscopy with the help of an experienced pathologist at MRC, Delhi.

**Sensitivity Study**
Fifty human subjects of *P. falciparum* positive and negative control blood samples were used.

**Specificity Study**
Ten samples each of non-malarial serums of polio, HIV, tuberculosis, pregnancy, asthma, hepatitis-B, leprosy, filarasis, Leishmaniasis and healthy persons were used.

**Field Area Study**
One hundred human subjects from two regions; endemic (50 from Haldwani Uttar Pradesh) and non-endemic (50 from Surat, Gujarat), were used for testing infection by MDI. Blood smear examination was done by microscopy before testing with MDI. Patients from malaria incidence areas were selected.
by clinical diagnosis of symptoms of fever, etc. in local hospitals with the help of doctors. 50 normal, healthy human volunteers from non-endemic area of Surat, Gujarat who had no history of malaria and diagnosed negative in microscopic tests were used as control for malaria detection by MDI in fields. Samples of patients and healthy human subjects were collected by aseptic finger prick method on filter paper for MDI study in field for *P. falciparum* malaria detection. The filter paper blood samples of patients and normal subjects obtained from field areas were brought to laboratory for ELISA and LIA study for checking and confirming the results of MDI. Four *P. falciparum* antigens (GPL1, RESA, Pf, MRC and HRP-2) were studied under field conditions.

*P. falciparum* Antigens Used for Serological Studies

(i) GPL 1: Isolated glycosphospholipid antigen from PSJ-M strain
(ii) RESA: Ring stage erythrocyte surface antigens derived from *P. falciparum* 155
(iii) Pf: Synthetic *P. falciparum* antigen, a crude preparation of *P. falciparum* parasite, obtained after sonication and enrichment of parasite
(iv) LSAR: Synthetic liver stage parasite antigen
(v) HRP-2: Histidine rich protein 2 antigen
(vi) CSP-60: Circumsporozoite laboratory synthesized peptide
(vii) EENV4: Erythrocyte surface membrane parasite antigen originated from RESA *P. falciparum* 155
(viii) SC-5: Non parasitized synthetic antigen.

Serological Study of Antigens by Different Methods

**ELISA**

ELISA testing was done following the procedure of Roy *et al.* Costar ELISA plates (Denmark) were used for immunoreactions and O.D. values were read by ELISA reader (Diagnostic Pasteur LP 300, France) at 492 nm. Sigma (USA) conjugates and other chemicals were used.

**LIA**

Polystyrene beads (1%) (Sigma, USA) were used for antigen coating. Later on specific antibodies were mixed for reaction in glass capillaries for microagglutination. Scattering of light was measured by He-Ne LASER system (Aerotech, Pittsburg, USA) at 632.8 nm wavelength. Photocurrent values were measured by Brookhaven Instrument (model 9000 AT digital correlator, New York, USA).

**MDI**

A simple, portable and cost effective photometric detection immunosensor device for the detection of *P. falciparum* malaria antibodies in human blood has been developed by authors. In this light of wavelength between 630-680 nm was used for scattering of latex bead agglutination in the media and output of light was measured by highly sensitive photo-detecting cells which in turn converts light energy into signal digital display. MDI device consists of following components:

(i) **Capillary Tube holding System.** It is made up of black plastic tube (A) (5 cm long×5 mm internal diam). The tube is fixed on a solid plastic stand (1.5×1.5×1.0 cm). The base of the plastic stand is fixed in cuboids (12×9×5 cm) of hard plastic sheet of thickness 4 mm. The tube ‘A’ has a hole of 5 mm diam bored through a height of 2.5 cm from bottom surface. Two black tubes B and C of length of 5 mm each are fixed to the holes of tube A on opposite sided for transmission of light through it. The two tubes B and C are attached in turn with the light source and the photo detector system respectively. The upper end of the capillary holder tube was projecting out through a hole of diameter 9 mm on the top surface of the box.

(ii) **Laser Light System.** A LASER diode (LASER Product, class II, USA) of 630-680 nm wavelength of maximum output of <3 mw for light emitting source was used. Laser light passes through a lens system to develop very powerful and intense light system. The light diode required three cadmium batteries 3 CPS (LR44 1.5 V) i.e. 4.5 V (connected in series).

(iii) **Photo Detector Diode.** It is a piezoelectric quartz crystal p-n junction diode (electron-hole concentration) with resistor (56 K) in series and operated on 9 V batteries. It was fixed on the tube ‘C’ to receive directly the scattered light transmitted through the sample in the capillary. The diode was in turn connected with a digital display device for measuring the voltage in mV range (0.01-2000 mV).

(iv) **Digital Display Device.** It is an indigenously fabricated and developed device (ESCORP India company Ltd, Delhi). It is an analogue to digital converter, which changes the electric current into digital signals and operates on 9 V dry cell batteries (Sensitivity: 0.1 mV).

Statistical Analysis of Data

Experimental data obtained from laboratory and field trials have been analyzed statistically and SD and significance values were calculated by paired student ‘t’ test and p value was determined. Seropositivity calculated in terms of cut off value was
decided as mean value of control (healthy) sera plus 2SD (control mean+2SD).

Results

Serological properties of *P. falciparum* antigen have been evaluated to characterize sensitive and specific nature of antigen for simple and accurate diagnosis of malaria. Eight antigens including GPL1 have been used for comparative studies.

Chemical Composition of GPL Antigen

GPL1 component is composed of glycolipid and showed presence of four different kinds of sugars in its carbohydrates moieties. Area measurement study showed a ratio of 3.3:1.5:1.5 for xylose and mannose, glucose and galactose. It was found that GPL1 is totally free from protein part and amino sugars.

Standardization of GPL1 and GPL2 Antigens

It was found that 1:100 dilution of serum and 1:1000 dilution of antigen showed best binding results. GPL1 antigen showed a good correlation with *P. vivax*, *P. falciparum* and control serum. GPL 2 showed no difference amongst three different sera with different dilutions.

Laboratory Study of Sensitivity and Specificity of Antigens

Sensitivity Study

In ELISA, LIA and MDI testings, antigen GPL1 showed the highest detection power of malaria (88, 96 and 92% positivity, respectively) as compared to other antigens (Fig. 1; Table 1). The other malarial antigens showed 65-88% positivity. However, a very low immunoactivity (10%) was seen with non-malarial antigen (SC-5).

Specificity Study

GPL1 showed a very high immunoreactivity with only *P. falciparum* serum (Fig. 2; Table 2). On the other hand, the antigen showed no cross reactivity with non-malarial serum used in the study. However, other malarial antigens showed 10-20% cross reactivity with non-malarial serum.

Evaluation of MDI Detection Capability in Field Areas

MDI showed 94% detection capability with GPL1 antigen, whereas RESA, *P. falciparum* (sonicated) and HRP-2 antigens showed between 86-92% diagnosing power in endemic area (Haldwani). MDI did not show positivity with control serum. Cut off values of antigens have been calculated and found to be 91.73 for GPL1, 93.10 for RESA, 92.45 for *P. falciparum* and 92.57 for HRP-2 antigens.

Discussion

Antigen and antibody reactions and assay are commonly used for early detection of diseases. For this purpose, malarial antigens from different stages of parasitic cycle have been isolated and purified. A Antigen from culture supernatant of *P. falciparum* (PSJ-M) strain has been isolated, purified and chemically characterized\(^1^9\). The antigen is found to be a glycoprophospholipid with no inositol sugar (GPL1) in its carbohydrate moiety. It is also a non-protein based molecule. Earlier, Gerold *et al*\(^1^1\) discovered a new

<table>
<thead>
<tr>
<th>Antigens</th>
<th>ELISA (OD)</th>
<th>LIA (nm)</th>
<th>MDI (mV)</th>
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<tbody>
<tr>
<td>GPL1</td>
<td>88%</td>
<td>96%</td>
<td>94%</td>
</tr>
<tr>
<td>RESA</td>
<td>66%</td>
<td>78%</td>
<td>80%</td>
</tr>
<tr>
<td>Pf</td>
<td>70%</td>
<td>88%</td>
<td>86%</td>
</tr>
<tr>
<td>LSAR</td>
<td>68%</td>
<td>78%</td>
<td>80%</td>
</tr>
<tr>
<td>HRP-2</td>
<td>74%</td>
<td>86%</td>
<td>84%</td>
</tr>
<tr>
<td>CSP-60</td>
<td>70%</td>
<td>74%</td>
<td>72%</td>
</tr>
<tr>
<td>EENV4</td>
<td>66%</td>
<td>66%</td>
<td>74%</td>
</tr>
<tr>
<td>SC-5</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
</tr>
</tbody>
</table>

Fig. 1—Sensitivity study of *P. falciparum* antigens by ELISA, LIA and MDI methods with *P. falciparum* serum
Fig. 2 — Specificity of GPL1 with malarial and non-malarial antigens by ELISA, LIA and MDI

Table 2 — Specificity study of GPL1 with malarial and non-malarial infections determined by ELISA, LIA and MDI

<table>
<thead>
<tr>
<th>Disease</th>
<th>ELISA</th>
<th>LIA</th>
<th>MDI</th>
</tr>
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<tbody>
<tr>
<td>P. falciparum</td>
<td>0.94 ± 0.07</td>
<td>844.2 ± 77.98</td>
<td>254.9 ± 61.65</td>
</tr>
<tr>
<td>P. vivax</td>
<td>0.11 ± 0.03</td>
<td>90.02 ± 2.04</td>
<td>65.1 ± 1.91</td>
</tr>
<tr>
<td>Polio</td>
<td>0.11 ± 0.03</td>
<td>89.3 ± 3.86</td>
<td>64.3 ± 2.58</td>
</tr>
<tr>
<td>HIV</td>
<td>0.10 ± 0.03</td>
<td>89 ± 2.35</td>
<td>64.2 ± 3.99</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>0.11 ± 0.03</td>
<td>91.4 ± 2.17</td>
<td>64.9 ± 2.13</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>0.10 ± 0.02</td>
<td>90.0 ± 2.96</td>
<td>65.0 ± 2.27</td>
</tr>
<tr>
<td>Asthma</td>
<td>0.11 ± 0.03</td>
<td>89 ± 3.97</td>
<td>89.0 ± 3.97</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>0.10 ± 0.03</td>
<td>89.8 ± 2.57</td>
<td>64.6 ± 2.23</td>
</tr>
<tr>
<td>Leprosy</td>
<td>0.12 ± 0.04</td>
<td>89.3 ± 6.16</td>
<td>65.5 ± 2.91</td>
</tr>
<tr>
<td>Filariasis</td>
<td>0.11 ± 0.04</td>
<td>90.5 ± 2.79</td>
<td>64.4 ± 2.32</td>
</tr>
<tr>
<td>Leishmaniasis</td>
<td>0.11 ± 0.04</td>
<td>88.7 ± 3.59</td>
<td>63.5 ± 2.37</td>
</tr>
<tr>
<td>Healthy</td>
<td>0.12 ± 0.04</td>
<td>90.5 ± 3.41</td>
<td>63.9 ± 2.92</td>
</tr>
<tr>
<td>Cut off value</td>
<td>0.2032</td>
<td>98.33</td>
<td>69.74</td>
</tr>
</tbody>
</table>

In the present study, seroantigenic property of GPL1 has been evaluated and it was found that 1:1000 dilutions of antigen and 1:1000 dilution of serum produced best agglutination results. Further, disease detection capability with GPL 1 antigen has been found very high, i.e. 96% by MDI (p< 0.0001), whereas ELISA immunoassay showed only 88% (P<0.0001) detection ability. Sensitivity study of six malarial antigens showed seropositivity between 65-88%, whereas non-malarial antigen, SC-5 showed a very low level of antigenic activity in all the three methods.

Malaria detection rate by MDI was found to be 94 to 97%, which is very close to the LIA results and certainly above the detection ability of ELISA for the diagnosis of malaria. Further, MDI is very suitable for field conditions and on comparison with other methods showed better detection ability. GPL1 antigen, isolated from culture supernatant of PSJ-M strain of P. falciparum, has shown better sensitivity and specificity properties than other antigens. Further, GPL1 showed no cross reaction with all non-malarial diseases and P. vivax positive serum but seven other P. falciparum antigen showed 10-30% cross reactivity with P. vivax positive and non-malarial diseases sera. Roitt reported that accurate immunodiagnosis is dependent upon strong and high specific nature of antigen. The specific detection power of GPL1 may be due to its strong binding affinity with the P. falciparum antibodies. Van Oss and Walker have shown that Ag-Ab reaction depends upon antigen electrostatic property and several other factors. GPL1 showed high reactivity and binding capacity in P. falciparum sera than P. vivax and negative control sera, indicating that GPL1 is specific to P. falciparum. Earlier studies also suggest that glycolipospholipid plays an important role in toxicity versus immunogenecity activities, and this class of antigens can be used for several clinical problems. GPL1 antigen developed and isolated from P. falciparum culture supernatant (PSJ-M strain) showed a high level of detection and
diagnosing capability under field and laboratory conditions.

Acknowledgement

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References