

MONOCLONAL ANTIBODY BASED ELISA: AN EFFECTIVE DIAGNOSTIC TOOL FOR THE DIAGNOSIS OF FALCIPARUM MALARIA

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) system based on the monoclonal antibodies (F2W22C1) originated from Thai strains of *P.falciparum* in mice models and polyclonal antibodies raised against Nepali strains of *P.falciparum* in rabbit models was developed for the detection of local Nepali strain of *P.falciparum* antigens in red cell lysates. The monoclonal-polyclonal antibody based indirect ELISA developed for the detection of *P.falciparum* antigens was specific since it was positive only with *P.falciparum* infected erythrocytes and negative when blood from forty healthy individuals collected from the malaria non-endemic areas and forty *P.vivax* infected erythrocytes were tested. When the test was applied to microscopically confirmed 154 *falciparum* infected blood samples collected from Dhanusha district, Nepal; the assay detected only 138 out of 154 *P.falciparum* samples indicating the sensitivity of the test to be 89.6%. When the assay was used to detect forty samples from the patients of unknown origin of fever other than the malaria collected from the malaria endemic areas, all forty samples were negative with the assay system. A significant correlation was observed ($r = 0.872$; $p = 0.013$) in between the parasitemia and the O.D. values obtained from the MAb-PAb based indirect ELISA. The test developed using monoclonal antibodies raised against Thai *P. falciparum* isolates and the polyclonal antibodies raised against native Nepali strains of *P.falciparum* offered high degrees of sensitivity and specificity. However, the test requires further evaluation with higher number of samples; requires further improvement in sensitivity; before commercial use of the test in patient care.

Key Words: *ELISA, monoclonal antibodies, polyclonal anti-bodies, P. falciparum.*

INTRODUCTION

Malaria is still a major tropical health problem of the world. More than two hundred million cases of malaria worldwide, with over one million deaths, mainly in children occur annually.¹ The majority of deaths due to malaria are reported among young children in Africa, especially in remote rural areas with poor access to health services. The most severe form of the disease is cerebral malaria caused by *P.falciparum*, and is re-

sponsible for the bulk of death in the disease.²

Despite several years of attempted eradication, malaria remains a major public health problem in Nepal predominantly in southern tropical areas. More than 70% of the total population and 64 districts out of 75 districts are at malaria risk in Nepal.³ Out of five development regions of the country, the central development region has been found to be the highest number of malaria cases.

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Normally, the microscopic examination for malaria parasite in peripheral blood smear is very popular and convenient for diagnosis. The sensitivity of this method is high: an experienced microscopist can detect in a 3-minute examination as little as one-five parasite per 10^6 RBCs.⁴ However, because of time limitation and eye fatigueness, after many hours of microscopic examination, may cause inaccuracy. In order to address the above-mentioned problems, techniques-with higher sensitivity, rapidity and cheaper alternative methods have to be sought which can replace the microscopic examination. Demonstration of malaria parasite antigens has become increasingly popular in the diagnosis of malaria infections because they are more closely related to the current illness than antibodies detection which show the previous or recent past infection. A serological method, an enzyme-linked immunosorbent assay (ELISA) was selected in this research study with an objective to offer the aforementioned attractive advantages with the use of affordable apparatus. Moreover, a large number of samples could be tested at the same time. In view of this prospective, the study aimed to develop the indirect ELISA using monoclonal antibodies produced against Thai isolates of *P.falciparum* and polyclonal antibodies produced against Nepali strains of *P.falciparum*. The MAb-PAb based indirect ELISA was applied for the detection of *P.falciparum* antigens in the blood samples collected from the people who have been infected in three health posts of malaria endemic Dhanusha district of Nepal.

MATERIALS AND METHODS

The field study was conducted from July 2001 to July 2002 in Dhanusha district, Janakpur Zone (Godar, Dhalkebar and Lalghad Health Posts) of Nepal.

Informed consent and ethical clearance

A written informed consent was obtained from the patient or from the family member. Those who did not give permission to give blood were not included in this study and there was no discrepancy of sexes on blood sample collection.

Patients with falciparum malaria

One hundred and fifty four blood samples aged between 1-73 years from microscopically confirmed cases of *P.falciparum* positive patients were collected.

Patients with P.vivax malaria

Forty blood samples from microscopically confirmed cases of *P.vivax* were collected from the aforementioned study sites for the specificity of the test.

Healthy individuals from non-endemic areas

Forty blood samples from forty healthy persons were taken re-

siding in Kathmandu where malaria is non-endemic. Those persons without any history of malaria and denied traveling to any malaria endemic areas at least for two years, hence, they were unlikely to have been exposed to malaria during the time of study.

Healthy individuals from endemic areas

Forty blood samples were taken from forty healthy persons residing in malaria endemic areas of Dhanusha district who have been living in their native areas for at least two years.

Patients of unknown origin of fever other than malaria

Forty blood samples from forty individuals with fever of unknown origin more than two weeks were included in this category. Their blood samples were microscopically confirmed negative for malaria by conventional thick and thin blood smear methods.

Blood collection

Two to three milliliters of blood samples were collected from patients with *falciparum* malaria, *vivax* malaria and all control groups separately in a screw capped tubes containing 10 μ l of 10% EDTA per ml of blood. Thick and thin blood films were prepared by the usual method described elsewhere and were stained with Giemsa's staining method and the numbers of infected erythrocytes were counted in light microscope. The buffy coat and plasma portions were removed by centrifuging at 1000 rpm and the remaining packed red cells were aliquoted immediately from the field sites and stored at -70 °C until use.

Preparation of red blood cell lysates

The previously aliquoted and stored patients packed red blood cells from all groups were treated with 9 volumes of 0.01 M Phosphate Buffered Saline (PBS), pH 7.4 containing 0.05% Tween 20 and 0.5% Nonidet P-40 (PBST-NP40) for 10 minutes at room temperature on the testing day.

Preparation of anti-P.falciparum polyclonal antibodies (PAb)

A healthy male rabbit was immunized intramuscularly with three doses of 0.5 ml of approximately 1×10^8 of *P.falciparum* parasitized red cells previously concentrated by Percoll gradient centrifugation with equal volume of Freund's Complete Adjuvant (Difco Laboratories, Michigan, USA) initially and subsequently with two boosts of the same amount of *P.falciparum* parasitized red cells in Freund's Incomplete Adjuvants (Difco Laboratories, Michigan, USA) at of three weeks intervals using the method described elsewhere.⁵ The blood samples were taken from the ear veins of the immunized rabbit before the first immunizing dose and after each booster dose. The serum was separated aseptically by centrifugation at

1000 rpm at room temperature for 10 min and was stored at -20°C. The immune rabbit sera were inactivated at 56°C for 30 minutes and the globulin fractions of the rabbit sera were precipitated by saturated ammonium sulphate as described by Voller and Bidwell⁶ and the concentrations were measured by using Spectronic Unicam Genesystem machine at 280 nm wavelength using PBS pH 7.4 as a blank.

Preparation of anti-P.falciparum monoclonal antibodies

Murine hybridoma namely F2W22C1 secreting antibodies against Thai strains of *P.falciparum* was kindly provided by Prof. Srisin Khusmith, of Mahidol University, Thailand. The hybridoma was produced by fusion of Sp 2/0 myeloma cells with spleen cells from BALB/c mice immunized with the concentrated *P.falciparum* antigens.⁷

Large-scale production of monoclonal antibodies

The monoclonal (F2W22C1) was thawed out by putting the cell ampoules at 37°C water bath immediately after being taken from liquid nitrogen tank. The cells were washed in 10 ml of RPMI IV medium, pelleted by centrifugation and suspended in RPMI 1640 medium. Thereafter, the cells were pipetted into 50 ml tissue culture flask (Costar) and placed in humidified 5% CO₂ incubator at 37°C. The cultures were allowed to grow to exponential phase before passage. About 80-90% of the cell populations were removed before going to the next cycle. The supernatants of the cultures were harvested when the colour of the medium changed to yellow and were then centrifuged at 1000 rpm for 10 minutes at room temperature to remove the cells and pooled.

Concentration of monoclonal antibodies by ammonium sulphate precipitation

The globulin fraction of culture supernatants of monoclonal collected from the antibody producing hybrids were precipitated by ammonium sulphate as described elsewhere by Voller and Bidwell.⁶ The concentrations were measured by using Spectronic Unicam Genesystem spectrophotometer at 280 nm wavelength.

Optimization of the conditions of indirect ELISA

The optimum concentration of monoclonal antibody for plate coating, temperature, working dilution of conjugate and incubation time were initially optimized using checker board titration method before testing. Once optimized the conditions and reagents, these were strictly followed in all the subsequent assay systems.

Development of MAb-PAb indirect ELISA

An indirect ELISA method was developed as described previously with some modifications.⁵ Wells of 96 well flat bottom

micro-ELISA plates (Costar, USA) were each coated with 100 µl of 10 µg/ml F2W22C1 MAb, followed by incubation at 37°C for 2 hours and further at 4°C overnight. The unbound MAb was washed 3 times with Phosphate Buffered Saline (PBS) Tween-20 (PBST 0.05%) and the non-reactive sites were saturated blocked with 200 µl of PBST-2.5% milk 1 hours at room temperature. The plates were thoroughly washed with PBST to which 100 µl of test and control erythrocyte lysates were added to each well followed by incubation for 2 hours at 37°C. After washing, 100 µl of 10 µg/ml of anti-*P.falciparum* PAb was added and incubated for 1 hour at 37°C. The plates were washed again with PBST and then 100 µl of goat anti-rabbit IgG labeled with HRP conjugate (Dako Company, Denmark, 1:2000) was added to each wells and incubated at 37°C for another 1 hour. After washing, the enzyme substrate solution Orthophenylenediamine dihydrochloride (OPD) (Dako Company, Denmark) in citrate buffer at pH 5.0 was added and the reaction was stopped with 50 µl of 0.5 M H₂SO₄ after 30 minutes at room temperature and the O.D. was measured by ELISA reader (Titertek Multiskan MCC/340) at 492 nm.

Data analysis

SPSS 10.0 for Windows was used for the statistical analysis of data. Pearson's Correlation was applied for the estimation of correlation in between the number of parasites/10⁶ erythrocytes and the O.D. values obtained from the MAb-PAb based indirect ELISA for the detection of *P.falciparum* antigens. The cut-off value for the developed ELISA was defined as mean antigen concentration +2 standard deviations (SD) in forty healthy individuals collected from the malaria endemic areas.

RESULTS

Sensitivity and the specificity of MAb-PAb indirect ELISA

When the MAb-PAb indirect ELISA using monoclonal antibodies raised against Thai strains of *P. falciparum* and the polyclonal antibodies raised against local Nepali strains of *P. falciparum* was applied on the blood samples from 40 healthy individuals from malaria non-endemic and 40 healthy individuals from malaria endemic areas, the results showed that the mean O.D. values among healthy individual from endemic areas was 0.078, 0.009 of 1 S.D. and 0.018 of 2 S.D. which was higher than that among healthy individuals from malaria non-endemic areas of mean O.D. values of 0.056, and 0.007, 0.014 of 1 S.D. and 2 S.D values, respectively. Therefore, it was decided to use the mean O.D. among the individuals from endemic areas to establish the cut-off level. The accuracy of the ELISA reader (Titertek Multiskan MCC/340) is 0.05 and the accuracy of the micro ELISA plate (Costar, USA) recommended by the manufacturer is 0.005. In order to determine the precise cut-off level, one should include these error values as well. Therefore, the

test sample was considered positive if its O.D. was equal to or above the O.D. of 0.151 (the mean O.D. plus 2 S.D. plus the error values obtained from the ELISA reader and micro ELISA plate). This cut-off level was used to determine the sensitivity of MAb-PAb indirect ELISA in the clinical samples collected from malaria endemic areas. The specificity of the assay was also determined when tested with forty *P.vivax* blood lysates with the specificity of 100% since all forty *vivax* blood samples were found negative.

Reproducibility of MAb-PAb indirect ELISA

The precision of the ELISA reader given by the manufacturer is 2.5%, difference in absorbance (O.D.) between duplicate samples lower or equal to 2.5% can be considered as the same reading. The reproducibility of the optical density values of 154 *P.falciparum* parasites by the assay system determined in the duplicate testing was within 2.5% hence all the samples were considered as the same reading.

Application of MAb-PAb based indirect ELISA to *P.falciparum* antigen detection

The established cut-off levels were used to determine the sensitivity and specificity of MAb-PAb based indirect ELISA when

all 154 samples of *P.falciparum* infected blood were allowed to react with the anti-*P.falciparum* MAb and anti-*P.falciparum* PAb based indirect ELISA. Only 138 *P.falciparum* infected blood were positive while the assay could not detect 16 out of 154 samples and forty healthy individuals collected from the malaria non-endemic areas of Nepal. The sensitivity of the assay was 89.6% for the detection of *P.falciparum* antigens.

When the *P.vivax* infected erythrocytes from forty cases collected from the malaria endemic same areas where the 154 test samples were collected and subsequently tested, all the O.D. values were below the cut-off value. Therefore, it was considered that the MAb-PAb based indirect ELISA for the detection of *P.falciparum* antigens was 100% specific. In addition, no false positives or false negatives were observed. When the assay was used to detect the blood samples from forty patients of unknown origin of fever - other than the malaria, confirmed negative by microscopically, collected from the same malaria endemic enrolled areas, all forty samples were found to be negative with the assay system supporting that the specificity was 100% for the detection of *P.falciparum* antigens as all the O.D. values were obtained below the established cut-off values (Figure 1).

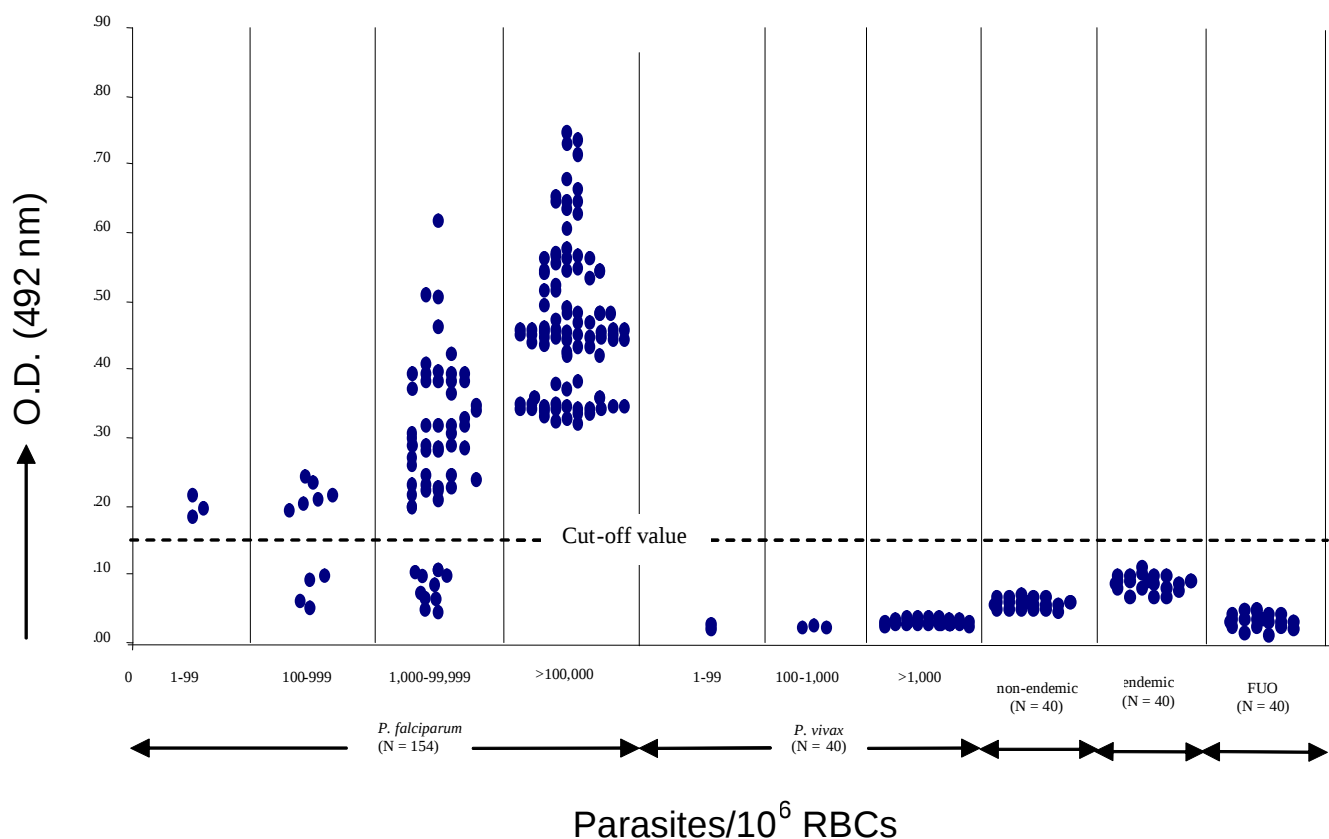


Fig.1 : Scatter diagram of O.D. values from the MAb-PAb based indirect ELISA for the detection of 154 *P.falciparum* samples. 16 out of 154 samples were negative as the O.D. of these samples were below the established cut-off value of 0.151. Forty healthy individuals were from malaria endemic and 40 from non-endemic healthy individuals and 40 *P.vivax* infected individuals showing all negative with the assay system. Likewise, 40 samples of fever of unknown origin were also negative as the O.D. values of all samples were below the established cut-off value.

Correlation between parasitemia and O.D. of MAb-PAb based indirect ELISA

A significant correlation was observed ($r = 0.872$; $p = 0.013$) in between the number of *P.falciparum* parasites/ 10^6 erythrocytes and the O.D. values of MAb-PAb based indirect ELISA.

DISCUSSION

Thick blood film examination is still the best technique worldwide but the method has its own limitations when the parasitemia is low 10-20 parasites/ μ l of blood.⁸ By using this conventional technique, an experienced microscopist can detect as few as 1-5 parasites/ 10^6 erythrocytes.⁹ Furthermore, this technique is sensitive and specific but has the disadvantages of being time consuming and requires skilled personnel to ensure the reliable results.⁶ Some recent molecular diagnostic methods like DNA probe, PCR etc. have been developed which require sophisticated equipments and are not practical in remote rural field settings.

The application of monoclonal antibody in immunodiagnosis confers many advantages, including decreased false positive reactions and cross-reactivity, increased reproducibility, and standardization of testing.¹⁰ ELISA techniques offers the attractive benefits of simplicity, use of inexpensive reagents and instruments and a large number of samples can be tested at the same time and the results can also be read visually by naked eyes. ELISA found to be more sensitive than parasitological examination for malaria diagnosis.¹¹ Newly developed assay by the authors was found to be 100% specific in detecting the *P.falciparum* antigens however the sensitivity was only 89.6%. The reduced sensitivity could be due to the antigenic diversity of the *P.falciparum* blood stage antigens and the binding capacity of the parasite antigens. If the epitope of the antigen is altered due to any reason, it could affect the ability of binding thereby reducing the sensitivity of the assay. However, the reproducibility of the assay was 100%. The obtained data suggested that the monoclonal antibody based indirect enzyme immunoassay by using monoclonal antibody produced against native Thai strains in mice model and the polyclonal antibody produced against the local Nepali strains of *P.falciparum* in rabbit model in Nepal is specific and sensitive to apply even in the rural settings of Nepal.

CONCLUSION

The results in our study indicated that the MAb-PAb based indirect ELISA system developed indigenously offer high degree further evaluate with more blood samples from the people who have been living in such malaria endemic areas. The suc-

cessful ELISA technique could be applied in addition to the routine parasitological examination by microscopy in Nepal.

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