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Use of in-house test system for investigating prevalence of *Plasmodium falciparum* antisporozoite antibodies in a malaria endemic area

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Malaria ranks as first priority disease in Myanmar. For effective control of malaria, the situation of malaria transmission in the area must be known as much as possible. Of several indicators, antisporozoite antibody is considered as a useful indicator for assessing the transmission and could be used in planning the progress of vector control programmes. We developed an in-house Indirect Enzyme Immunosorbent Assay (EIA) system using synthetic peptide $NANP_3$ as the solid phase and peroxidase labeled anti-human IgG (Rabbit) as the conjugate. Checkerboard titration was carried out and the dilutions determined. The antisporozoite antibody levels of 384 subjects from Tarchileik (mean age 34.71 ± 19.2yrs) were determined. The mean antibody levels ranged from 0.08µg to 21.9µg. No significant difference in the anti-sporozoite antibody positive rate was found between males and females (29.2% *vs* 37.6%). A positive correlation ($r = 0.32$) with age ($p<0.001$) was found and highest antisporozoite antibody positive rate was found in 20-40 years age group followed by 40-60 years age group. No association was found with history of malaria. The developed EIA could be used to assess the degree of malaria transmission in a locality.

INTRODUCTION

The detection of antibodies to sporozoites in the sera of individuals living in malaria endemic areas is of epidemiological relevance, since antibody levels should correlate with the intensity of transmission of the disease. In persons living in areas with endemic malaria, prevalence and levels of sporozoite antibodies have been shown to correlate with the entomologic inoculation rate at the same time for the same area. The immunodominant epitope of the *Plasmodium falciparum* CS protein consists of highly conserved tandem repeats of amino acid (Asn-Ala-Asn-Pro=NANP) [1].

An EIA using a chemically synthesized $NANP_3$ peptide has become available [2]. It has been possible to screen large scale samples in shorter interval than needed for salivary gland dissection, and also with greater sensitivity and specificity results. Circumsporozoite (CS) antibodies indicating the occurrence of malaria infection but not necessarily development of disease, have been shown to be reliable indicators of transmission in area with disease [3,4,5]. The results obtained from the in-house EIA test will be applicable in assessment and monitoring of malaria transmission in a locality as well as in planning the control measures of malaria [5,6,7].

MATERIALS AND METHODS

The laboratory-based experimental study was conducted in the Nuclear Medicine Research Division as mentioned below. This producer as reported by M.Knappik was modified for local situation [8]. One-hundred microlitre of $NANP_3$ (1µg/ml~10µg/ml) in PBS was added to polystyrene microtitre plate (NUNC, Germany), incubated at 37˚C for (1~5 hr) and following 4˚C overnight and washing with PBS+0.1% Tween 20. Remaining protein binding sites were blocked by different concentrations of 0.5 ~10% BSA fraction V in 0.15 M PBS pH 7.2 with addition of Tween 20 for varying incubation time from 10 minutes to 2 hours. Following extensive washing with PBS-Tween 20, different sporozoite antibody standard concentrations (0.625 to 5 µg/ml) in PBS or Normal Human Serum plus PBS were incubated for 1 to 2 hours at 37˚C and then washed. Normal Human Serum was used as a negative control. Addition of 100 µl of peroxidase conjugate rabbit anti-human IgG (1:1000~1:10000) in PBS for 1 hour was followed by washing and incubation with substrate solution (40 mg of orthopheny-lenediamide dissolved in phosphate-citrate buffer, pH 5.0) for 30 minutes. The reaction was stopped by adding 2.0 M H_2SO_4. The results were read at 492 nm micro plate reader (Thermal Labsystem Multiskan EX).

Preparation of standard curve

Standard curve was developed by testing circumsporozoite antibody concentrations ranging from 0.625 to 5.0µg/ml.

Study population

All 384 subjects in this investigation were recruited in February 2006 from Tarchileik Township. Serum specimens were stored at -40˚C. The $NANP_3$ EIA with pre coated EIA plates (NUNC, Germany) was used to perform the method developed in our lab. All specimens were screened at 250-fold dilution in duplicate wells. Two negative reference control sera and positive reference control sera confirmed by Immunoradio-metric assay method were included on each plate as quality controls. Results were obtained by use of a micro plate reader (Thermal Labsystem Multiskan EX) with a wavelength capability of 492 nm. A calibration curve was derived by plotting the absorbance values of the calculation sera on linear paper versus defined unit values (µg/ml). The antibody levels of the specimen were determined by interpolation of the absorbance mean of each serum on the calibration curve. Statistical analysis was done using Minitab 14.1 software packages.

RESULTS

The optimal concentration of circumsporo-zoite antigen $NANP_3$ 100 µl used for coating the plate is observed to be 2.5µg/ml.Optimal blocking with 5%BSA-PBS was achieved after 1 hour incubation at 37˚C.

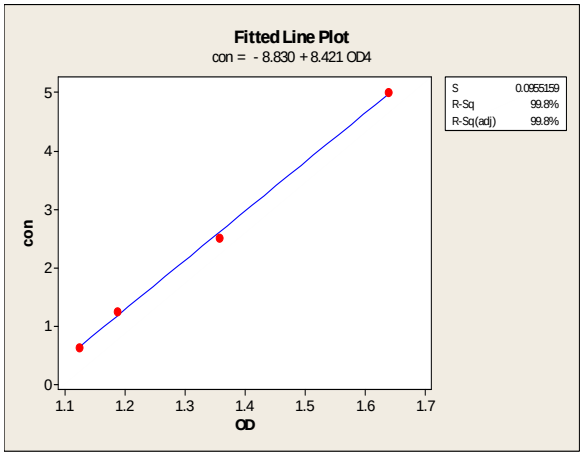

Fig.1. Regression Analysis: con versus OD

The regression equation is
con = - 8.830 + 8.421 OD4
S = 0.0955159 R-Sq = 99.8% R-Sq(adj) = 99.8%
Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	11.2122	11.2122	1228.97	0.001
Error	2	0.0182	0.0091		
Total	3	11.2305			

Fitted Line: con versus OD

The test showed the sensitivity level of detecting circumsporozoite antibody 0.625 µg/ml. The optimal time for incu-bation with 1:1000 peroxidase labeled

antibody conjugate was achieved after 1 hour. The standard curve was obtained as shown in Fig 1.

Table 1. Detection of circumsporozoite (CS) antibody of *P. falciparum* among the subjects in Tarchileik.

Sex	No. of subjects	No. of CS antibody positive (%)	No. of CS antibody negative (%)
Male	113	33 (29.20)	80 (70.80)
Female	271	102 (37.64)	169 (62.36)
Total no. of samples tested	384	135 (35.2)	249 (64.8)

Of the 384 investigated subjects, 113(33.3%) were males and 271(66.7%) were females. The average age was 34.71±19.2 years. By use of calibration sera provided with the test, the cut-off value for measurement of CS antibodies was defined as 7.38 µg/ml. Serum specimens from 135 (35.2%) of the 384 subjects were positive and 249 (64.8%) were negative. No significant difference in the antisporozoite antibody positive rate was found between males and females (Table 1). A positive correlation (r=0.32) with age (p<0.001) was observed and the antisporozoite antibody positive rate was highest in 20-40 years age group followed by 40-60 years age group (Fig. 2).

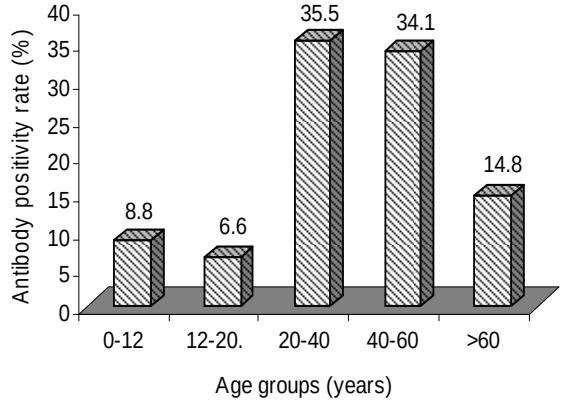

Fig. 2. Age specific circumsporozoite seropositivity rates in Tachileik

DISCUSSION

After performing trial and error assays of EIA system, by using different incubation times, concentrations of $NANP_3$, and conjugate dilution, a reliable and applicable in-house test system of EIA for detecting *Plasmodium falciparum* antisporozoite antibodies was able to develop. Circumsporozoite antigen $NANP_3$ in the concentration of 2.5 µg/ml was needed to coat the polystyrene microtitre plate. Conjugate incubation time for 1 hour with 1:1000 peroxidase labeled antibody conjugate (rabbit anti-human IgG) was necessary to produce a sensitivity level of detecting 0.625 µg/ml circumsporozoite antibody in the human serum. According to the results obtained in the study, it was evident that there is a considerable transmission of malaria in Tarchileik. 35.2% (135) of the study population was recently exposed to malaria infection, in which 29.2% (33) males and 37.64% (102) females were observed to have recent or previous infection. Although statistically not significant, the females were found to have higher infection rates than those of males. The mean antibody levels ranged from 0.08 µg to 21.9 µg. There was no significant difference in antisporozoite antibody positivity rate between males and females. But a positive correlation (r=0.32) with age was found and antisporozoite antibody positive rate was observed to be highest (35.56%) in 20-40 years age group and second highest (34.07%) in 40-60 years age group. It indicated that 20-40 years age groups got highest exposure to malaria infection. 40-60 years age group had second highest exposure to malaria infection. Being 20 - 40 years age group is the most active and most mobile population in that area, the more chance to expose to the malaria infection so that the higher antibody positivity was observed. The second most active population of 40–60 years age group was also evidently found to have the second highest antibody positivity rate. Therefore it can be concluded that the age specific anti-sporozoite antibody positivity rate can be a good indicator for assessing the malaria transmission potential among the population. On analysis, there was no association

observed between a antisporozoite antibody level and history of clinical malaria in this study. In conclusion, the EIA system developed in-house in Nuclear Medicine Research Division could be used as an adjunct in assessment of malaria transmission in a locality where other malariometric diagnoses are not possible.

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