A double antibody sandwich ELISA for the diagnosis of vivax malaria: a tool for further research

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Abstract

A diagnostic assay for *Plasmodium vivax* based on a double antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) was established to detect the merozoite surface protein 1 (PvMSP1) in wild isolates. This assay was based on the recombinant protein p19, a C-terminal processing product of PvMSP1. Of the two anti-*P. vivax* monoclonal antibodies (MAbs) selected, A21 was used as the capture antibody while horse radish peroxidase labelled A8 served as the probing second antibody. Optimized conditions established for p19 based DAS ELISA with the exception of a lower concentration of Tween-20 in buffers were suitable to screen lysed whole blood of malaria patients. This assay had a specificity of 100% for *P. vivax* and all the isolates of *P. falciparum* tested negative. Of the *P. vivax*-infected blood samples, only those containing both compact and schizont stages were diagnosed positive. The rest of the isolates tested negative either due to stage specificity of this assay or to the antigenic diversity of PvMSP1 in wild isolates. This test demonstrated a sensitivity of 27.58% and an accuracy of 63.15%. The positive and negative predicted values of this ELISA were 100% and 57.14%, respectively. Therefore, the *P. vivax* specific DAS ELISA developed and tested in the present study is not sufficiently sensitive to be used as a diagnostic tool for vivax malaria. Nevertheless, a baseline was established for development of diagnostic ELISA for future use with a more appropriate antigen.

Key words: Malaria, *Plasmodium vivax*, diagnosis, double antibody sandwich ELISA, immuno-chromatographic test (ICT).

Introduction

The key feature of the global malaria control strategy is the specific, early and rapid diagnosis of malaria both at village and at district levels. As such, major efforts are being made to develop quick diagnostic methods (1).

Diagnosis of malaria infection has been setback by serious problems both in clinics and in the field. In rural health centers lacking facilities for examination of blood films, samples that are collected have to be transported and examined under a central laboratory. If information is required by health authorities for the control of a potential out break, such delays are unacceptable (2). Also, the conventional diagnosis of malaria by microscopic examination of parasites on a Giemsa stained blood smear is encountered with problems such as lengthy preparation time, being labour intensive and requiring considerable expertise for its interpretation at a lower level of parasitaemia. Hence, many other types of diagnostic tests categorised under immunodiagnosis and nucleic acid probing have been developed (3).

With the aid of novel technology a rapid and specific malaria diagnostic manual test, immunochromatographic test (ICT), has been invented. The principle of ICT is based on double antibody sandwich ELISA that detects parasite antigen captured by an immobilized antibody and probed with a labelled second antibody (2).

Currently there are four ICT's commercially available for malaria diagnosis. ParaSight-F test

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detects *Plasmodium falciparum* (L) Welch, based on the histidine rich protein 2 (PfHRP-2) in haemolysed blood (4). ICT Malaria Pf Test™ detects circulating PfHRP-2 in whole blood (5). OptiMAL test differentiates between live falciparum and vivax malaria parasites based on the antigenic differences between the isoforms of the enzyme, parasite lactate dehydrogenase, within 10 minutes (6). As opposed to the above three dip-stick methods the AMRAD ICT is available in the card form. This test is directed against PfHRP-2 but detects vivax malaria using a second non-specified antigen common to both *P. vivax and P. falciparum* (manufacturer's information, AMRAD Operations Pvt Ltd., Australia). These are rapid manual tests which require only a small volume of blood, can be applied and read by people with limited training and without any special equipment (2).

Half of the malaria infections recorded globally are due to *P. vivax* (1). In Sri Lanka, *P. vivax* accounts for 60%-80% of all reported malaria infections (7). Therefore, the requirement for a specific, rapid manual test for the diagnosis of vivax malaria is imperative. It is apparent that no specific ICT is yet available for the diagnosis of *P. vivox* malaria. The objective of this study was the development of a *P. vivax* diagnostic, double antibody sandwich (DAS) ELISA using two anti-*P. vivax* monoclonal antibodies (Mabs) directed against the merozoite surface protein 1 of *P. vivax* (PvMSP-1). The long-term objective was to seek the possibility of extrapolating this double antibody sandwich ELISA to an ICT to screen wild isolates for early manual diagnosis of malaria caused by *P. vivax*.

Material and Methods

The *P. vivax* diagnostic DAS ELISA was based on a recombinant protein, p19, derived from a C-terminal processing product of the PvMSP1 produced in the baculovirus expression system (8). Monoclonal (Mabs), A8 and A21 recognising two epitopes on p19 were selected for developing the DAS ELISA. These two Mabs were chosen from a panel of thirty hybridomas established against asexual erythrocytic stages of *Plasmodium vivax* obtained from wild isolates (9). Following voluntary informed consent, 1ml of blood drawn by venipuncture was collected into 10 mM ethylene diamine tetra acetic acid (EDTA) from 37 malaria patients, 29 infected with *P. vivax* and 8 with *P. falciparum*. One ml of blood from each of 20 donors who have had no previous history of malaria served as controls. These samples were stored at -70°C until further use.

Ascites fluids of Mabs A8 and A21 produced in BALB/c mice were purified initially by ammonium sulphate precipitation (10) and further purified by column chromatography using Protein G Sepharose 4 Fast flow according to manufacturer's instructions (Pharmacia Biotech, Sweden). Purified A8 antibodies were concentrated using dialysis membrane (Wako Pure Chemical Industries, Japan) against sucrose (Fluka Bio Chemika, Switzerland) to reach a final protein concentration of 8mg/mL and conjugated with horseradish peroxidase enzyme (Sigma Chemical Co., USA) (10).

The optimum concentrations of reagents were selected by checker board titrations for the DAS ELISA. ELISA plates (Immulon 2 HB, Dynex, USA) were coated with 100 μL per well with purified MAb A21 at 4 μg/mL diluted in phosphate buffer, pH 7.4, and incubated overnight at 4°C in a humid chamber. The contents of the plates were aspirated and each well was blocked with 200 μL of blocking buffer [5% non fat milk (Anchor, New Zealand dairy board, New Zealand) in phosphate buffered saline (PBS)]. Plates were incubated for 1 hr at 37°C. Flicked the contents and washed the plates four times with washing buffer (0.1% Tween-20 in PBS). Added the recombinant protein, p19, at 150 μg/mL in dilution buffer (5% non-fat milk in washing buffer) and incubated plates for 1 hr at 37°C in a humid chamber. Washed the plates eight times and 100 μL of HRP labelled MAb A8 at 72μg/mL was dispensed into each well and plates were incubated for 2hr at 37°C. The plates were washed 8 times and 200 μL of substrate

(2,2-azino-bis (3 ethylbenz-thiazoline)-6-sulphonic acid) was pipetted into each well at a concentration of 0.55 mg/mL in substrate buffer (Citrate-phosphate buffer, pH 4.2) with 0.04% H_2O_2. The colour reaction was allowed to proceed for 30 min at 37°C and read the O.D. values at 405 nm using an EL_X 800 ELISA reader (Biotek, UK). Each treatment was performed in triplicate.

Wild isolates of *P. vivax* and *P. falciparum* were screened by this DAS ELISA as described above with the exception of using a lower concentration of Tween-20 in washing buffer (0.005%) to suit this assay system (11). One mL of wild isolate collected as described above were freeze thawed (11), and titrated neat and at dilutions of 1:1, 1:3, 1:7 in dilution buffer. Eight of vivax malaria infested blood samples were selected on a random basis and tested as above and the 1:1 dilution was selected as the best dilution of isolates to be tested in this assay.

For each plate tested, p19 at a concentration of 150 μg/mL and lysates of normal human blood (A and B types) at a dilution of 1:1 in dilution buffer were used as standard positive and disease negative controls, respectively. Each lysate was tested neat and at 1:1 in dilution buffer, in triplicate.

The double antibody sandwich ELISA was validated by calculating the specificity, sensitivity, accuracy, and the positive and negative predicted values (expressed as percentages) using the following formulae (12, 13):

Sensitivity = (No. of true positives)/ (No. of true positives) + (No. of false negatives)

Specificity = (No. of true negatives)/ (No. of true negatives) + (No. of false positives)

Accuracy = (No. of true negatives) + (No. of true positives) / [(No. of true negatives) + (No. of true positives) + (No. of false negatives) + (No. of false positives)]

Positive Predicted Value = (No. of true positives)/ (No. of true positives) +(No. of false positives)

Negative Predicted Value = (No. of true negatives)/ (No. of true negatives) + (No. of false negatives)

Results

As the O.D. values of the 20 disease negative controls by the DAS ELISA were normally distributed, the cut-off value for this test was calculated to be the mean O.D. value plus two standard deviations of the negative controls. Mean O.D. values obtained for test samples falling over and above this cut-off level were expressed as positive responses.

A total of 37 malaria positive isolates collected from 4th July, 1998 to 18th February, 1999, were screened by the DAS ELISA. Of these, 29 were infected with *P. vivax* and the rest (8) with *P. falciparum*. The parasitaemia of these ranged from 0.01% to 1% (Table 1). All the 8 *P. falciparum* isolates, 7 consisting of ring stages and 1 with gametocytes, screened negative by this assay (Table 1).

Of the 29 *P. vivax* isolates only the eight isolates that contained both compact (late trophozoite) stages and schizonts among other blood stages, tested positive by this assay (Table 1). Among the 21 isolates that responded negative to this test, 14 consisted entirely of late ring and amoeboidal (early trophozoites) stages. Of the remaining 7 isolates, one (MR 4335) was treated with chloroquine and another (MR 4249) contained late rings and gametocytes (Table 1). The rest of the *P. vivax* infected test samples (5 isolates) commonly carried compact stages among other immature blood stages (Table 1).

For the purpose of validating this assay, considering all the isolates of *P. vivax* (n=29) the number of true positives were 8 while the rest (n=21) was categorized as false negatives. The number of true negatives was 28 in all that included both the

Table 1

Description of wild isolates of *P. vivax* & *P. falciparum* and their reactivity on double antibody sandwich ELISA

Date of collection	Isolate number	Species	P%[a]	Mean O.D. value (405nm)[b]	Stage composition of isolates
04/07/98	MR4205	Pv[c]	1	0.051	Lr[d], Am[e], Co[F]
04/07/98	MR4206	Pv	0.18	0.037	Am, Co
11/07/98	MR4207	Pv	0.16	0.249[g]	Am, Co, Sc[h]
30/07/98	MR4219	Pv	0.06	0.032	Lr, Am
15/09/98	MR4234	Pv	0.2	0.016	Lr, Am
14/10/98	MR4249	Pv	0.03	0.021	Lr, Ga[i]
15/10/98	MR4250	Pv	0.3	0.112[g]	Am Co, Sc
15/10/98	MR4251	Pv	0.33	0.026	Lr, Am
20/10/98	MR4255	Pv	0.02	0.032	Lr, Am Co
21/10/98	MR4256	Pv	0.23	0.069	Lr, Am, Co
30/10/98	MR4259	Pv	0.07	0.048	Lr, Am
12/11/98	MR4265	Pv	0.2	0.030	Am, Co
26/11/98	MR4274	Pv	0.06	0.028	Lr, Am
07/12/98	MR4276	Pv	0.19	0.062	Lr, Am
15/12/98	MR4283	Pv	0.13	0.188[g]	Am, Co, Sc
21/12/98	MR4285	Pv	0.02	0.038	Lr, Am
04/01/99	MR4298	Pv	0.08	0.076	Lr, Am
18/01/99	MR4302	Pv	0.07	0.078	Lr, Am
20/01/99	MR4306	Pv	0.2	0.069	Lr, Am
02/02/99	MR4321	Pv	0.02	0.109[g]	Am, Co, Sc
03/02/99	MR4322	Pv	0.2	0.142[g]	Am, Co, Sc
05/02/99	MR4325	Pv	0.01	0.126[g]	Am, Co, Sc
08/02/99	MR4327	Pv	0.05	0.072	Lr, Co, Ga
09/02/99	MR4329	Pv	0.01	0.073	Lr, Am
09/02/99	MR4330	Pv	0.3	0.070	Lr, Am
10/02/99	MR4332	Pv	0.4	0.09	Lr, Am
12/02/99	MR4334	Pv	0.13	0.136[g]	Co, Sc, Ga
13/02/99	MR4335	Pv	0.7	0.06	Co, Sc, (CQt)[j]
18/02/99	MR4336	Pv	0.02	0.208[g]	Am, Co, Sc
11/09/98	MR4233	Pf[k]	0.01	0.040	Ga
16/10/98	MR4253	Pf	0.08	0.050	Rings
10/12/98	MR4280	Pf	0.04	0.096	Rings
14/12/98	MR4282	Pf	0.2	0.085	Rings
22/12/98	MR4286	Pf	0.07	0.085	Rings
23/12/98	MR4288	Pf	0.07	0.058	Rings
31/12/98	MR4295	Pf	0.2	0.076	Rings
09/02/99	MR4328	Pf	0.04	0.075	Rings

[a] parasitaemia (% of *Plasmodium* - infected RBC)
[b] mean of triplicate O.D. values obtained from double antibody sandwich ELISA
[c] *Plasmodium vivax*
[d] late rings
[e] amoeboidal stage
[f] compact stage
[g] isolates positive by the DAS ELISA (mean O.D. value of test samples that are above the cut-off value of 0.108)
[h] schizonts
[i] gametocytes
[j] chloroquine treated
[k] *Plasmodium falciparum*

8 *P. falciparum* isolates and the 20 normal, disease negative blood samples. This diagnostic DAS ELISA showed 100% specificity for *P. vivax*. Considering all 57 blood samples tested (both disease positive and disease negative), the sensitivity, accuracy and the positive and negative predicted values of the assay were calculated to be 27.58%, 63.15%, 100% and 57.14%, respectively.

As the sample size was small, 95% confidence limits were obtained for the above estimates by calculation of simultaneous confidence limits (13). The 95% confidence limits for this determination of sensitivity was 11.38% to 43.78%, for accuracy 50.63% to 75.67% and for the negative predicted value was 43.42% to 70.86%. However, only point estimates could be given for sensitivity and the positive predicted value for this assay as both estimates were calculated to be 100%.

Discussion

Specific, early and rapid diagnosis of malaria followed by treatment is currently considered to be the most suitable, effective management method of this disease (1). The immunochromatographic test (ICT) provides an ideal tool for such diagnosis. However, an ICT specific for *P. vivax* malaria is commercially yet not available. The objective of this study, therefore, was to develop a double monoclonal antibody sandwich ELISA specific for *P. vivax* with the long-term objective of seeking the possibility of extrapolating such an assay to establish an ICT for vivax malaria.

ICTs are based on double monoclonal antibody sandwich ELISA which is found to be superior to polyclonal antibody - monoclonal antibody (Pab-Mab) sandwich ELISA as the former shows a good correlation with the level of parasitaemia in malaria infections (3). MAb A21 was selected as the capture antibody in developing the DAS ELISA mainly due to its lack of polymorphism as tested with 50 wild *P. vivax* isolates and its reactivity with almost all blood stages, i.e. amoeboid, compact and schizont (merozoite) stages (9). As this MAb cross reacted with the blood stages of *P. falciparum* (9), by using a second, falciparum-specific labelled MAb for probing, the assay being suitable for detecting both vivax and faiciparum malaria was also a possibility.

It has been well documented that the MSP1 of *P. falciparum* (PfMSP1) is first synthesized by compact stages (mature trophozoites) and is accumulated rapidly during schizogony (14, 15). It is retained by the merozoite during erythrocyte invasion, and is still present on the early ring stage of the next intra-erythrocytic developmental cycle (15, 16). In this context, establishing a double antibody sandwich ELISA to detect PvMSP1 to be used as a diagnostic tool for vivax malaria was justified. With the exception of the late ring and amoeboid stages all other asexual erythrocytic stages would carry either the MSP1 or its processing products. When compared with *P. falciparum* that represent only ring and gametocyte stages in the peripheral blood, all asexual erythrocytic stages of *P. vivax* are found in the circulating blood. Also, multiple stages of the erythrocytic cycle are usually present in a single *P. vivax* isolate at a given time. Therefore, the probability of detecting *P. vivax* based on p19 seemed quite high. Another positive attribute of using p19 for this purpose was the conserved nature of the analogous *P. falciparum* molecule (17).

The double antibody sandwich ELISA developed with MAbs A21, A8 and p19 recombinant proteins appeared to be *P. vivax* specific. Only p19 reacted positively in this system where as the corresponding *P. falciparum* and *P. cynomolgy* recombinant proteins yielded negative results (results not shown).

All the 8 *P. falciparum* isolates tested negative by this DAS ELISA. This was to be expected in the light of the non-recognition of the analogous *P. falciparum* recombinant protein by this assay. Of the 29 *P. vivax*- infected blood samples tested, only 8 screened positive by the DAS ELISA (Table 1). All these isolates were composed of both compact and schizont stages in addition to amoeboidal stages (Table 1). Considering the composition of the blood stages of the 21 *P. vivax* test samples scored negative by this assay, 14 isolates were composed entirely of late ring and amoeboidal

(early trophozoite) stages (Table 1). A plausible explanation for these results may be that this antigen is lacking in the late ring and the amoeboidal (early trophozoite) stages, which is confirmed by these results. Of the 2 isolates MR 4249 and MR 4327 that scored negative by this DAS ELISA, the former contained only late ring and gamete stages while the latter, in addition to these two parasite stages, also consisted of compact stages. Three other isolates that were negative by this assay, all consisted of compact and amoeboidal stages and in addition two of these isolates also contained late rings.

Of the 14 samples that carried among other stages, the compact stage, the 8 isolates that scored positive by this assay also contained shizonts. Of the rest, five isolates, which were lacking in schizonts were not sensitive to this DAS ELISA. The isolate specificity of the labelled second antibody A8 was 94% as compared to 100% of that of the capture antibody MAb A21 (9). Hence, it is possible that these 5 isolates comprising only of compact stages (lacking schizonts) were not recognized by the probing antibody A8 due to antigenic diversity of the epitope recognized by A8. The question of non-reactivity of the compact stages (in the absence of schizonts) with A8 does not arise as reactivity of A8 with compact (late trophozoite) stage of *P. vivax* isolates was previously established by the indirect immunofluorescence assay (9). The remaining isolate out of these 14, MR 4335, that screened negative by this assay did consist both of compact and schizont stages but at the time of bleeding the patient was already treated with 8 tablets of chloroquine. Hence, the non-reactivity of this isolate by this assay was somewhat predictable.

In the light of the above analysis, it is apparent that this DAS ELISA using MAbs A21 and A8 though 100% specific for *P. vivax*, is stage specific for compact and schizont (and merozoite) stages. Considering all isolates tested the sensitivity of the test was calculated to be only 27.58%. It is essential that a larger sample size of both *P. vivax* and *P. falciparum* be screened by this assay to obtain reasonably narrow 95% confidence limits for test parameters such as sensitivity, accuracy and negative predicted values.

Considering the ICTs based on double antibody sandwich ELISA available for rapid diagnosis of malaria, the ParaSight-F test and the ICT Malaria PfTest™ both which detect the *P. falciparum* HRP-2 antigen showed sensitivities of 100% and 92.4% but the specificities were 88% and 88.8%, respectively (5, 18). On the other hand, the OptiMal test that detects both *P. vivax* and *P. falciparum* demonstrate sensitivities of 94% and 88%, respectively, for the two species with specificities of 100% and 99%, respectively (6). Therefore, it is evident that the 100% *P. vivax* specific DAS ELISA developed and tested in the present study at 27.58% sensitivity for *P. vivax* blood stages is clearly not adequately sensitive to be used as a diagnostic assay for vivax malaria.

Nevertheless, the repeatability which is the other important attribute of a diagnostic test, of the DAS ELISA established in the present study was confirmed by producing comparable results for isolates MR4207 and MR 4250 when repeated on 3 different occasions (data not shown).

Although the set objective of this study, i.e. establishing a double antibody sandwich ELISA for detecting *P. vivax* in wild isolates was achieved, the low sensitivity of the test does not permit extrapolating this assay to develop an immunochromatographic test. However, a base line has been laid for the development of a double antibody sandwich ELISA to screen wild isolates of *P. vivax*, which may be immensely beneficial for future research.

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