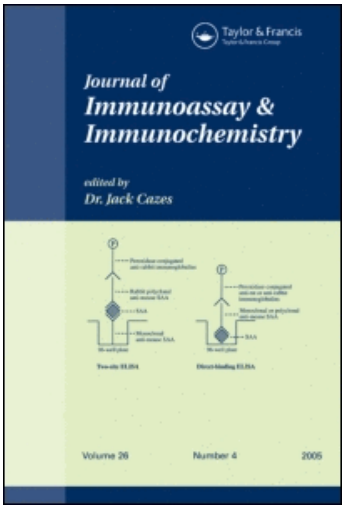


## Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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**To cite this Article** Bracho, Carmen and Pérez, Hilda A.(1996) 'Sensitive Detection of Plasmodium vivax in Blood by a Cell-Elisa Using a Monoclonal Antibody', Journal of Immunoassay and Immunochemistry, 17: 2, 155 — 174
**To link to this Article: DOI:** 10.1080/01971529608005786
**URL:** http://dx.doi.org/10.1080/01971529608005786

JOURNAL OF IMMUNOASSAY, 17(2), 155-174 (1996)

# SENSITIVE DETECTION OF Plasmodium vivax IN BLOOD BY A CELL-ELISA USING A MONOCLONAL ANTIBODY

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## ABSTRACT

An IgM monoclonal antibody (7C5B71) which reacted with the blood stages of Plasmodium vivax, but not with those of Plasmodium falciparum was used in a cell-ELISA to detect parasites in samples of peripheral blood. Blood thin smears were probed with monoclonal antibody 7C5B71 and then reacted with a peroxidase conjugate of the appropriate specificity and the insoluble chromogen amino-ethyl-carbazole. Infected cells which appeared dark red coloured were rapidly identified under a light microscope using a low magnification. The conventional microscopic examination of thin films coloured with Giemsa was used as reference test. Under laboratory conditions the test showed a positive result in samples with a level of parasitaemia of $\leq$ 500 parasites/$\mu$l of blood. In a preliminary field trial the test showed 100 % specificity for the diagnosis of P. vivax malaria.
(KEY WORDS: Plasmodium vivax, diagnosis, cell-ELISA, monoclonal antibodies)

## INTRODUCTION

In Latin America, malaria morbidity is largely related to Plasmodium vivax. Accordingly, of 983,536

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cases officially registered in 1993 by the 21 countries with evidence of malaria transmission, 695,898 (71%) were due to P. vivax (1). Prevalence of this parasite being even higher in some regions of South and Central America. For example, in 1993, countries of the Andean region (Bolivia, Colombia, Ecuador, Peru and Venezuela), reported 311,472 cases of malaria from which 228,612 (73%) were caused by P. vivax. A similar situation occurred in Mexico and Centro America where prevalence of P. vivax malaria reached 98.7% (1). Countries in the area have a limited response capacity in regard to the diagnosis of malaria which is provided mainly through the passive case finding (1). Hence, as one part of the attack of the disease, improved diagnostic methods are sought. In the latter years important efforts to improve malaria diagnosis have been done. Consequently, new diagnosis methods based on monoclonal antibodies (2,3), nucleic acid probes (4,5) or special staining with fluorescent dyes (6,7) have been developed. Large-scale field work is still required to establish the usefulness of the most promising developments, but a common denominator has been to endeavor the diagnosis of Plasmodium falciparum malaria. In this work we have approached the specific diagnosis of P.vivax infections by means of a cell-ELISA using a monoclonal antibody

(mAb) which reacts with a parasite antigen present in the erythrocyte infected with P.vivax but not in those infected with P.falciparum. The test seems of potential value to the specific and sensitive detection of P. vivax malaria.

## MATERIALS AND METHODS

### Monoclonal Antibodies

IgM mAb 7C5B71 which produces a bright immunofluorescent signal with erythrocytes infected with P. vivax and which does not react with blood stages of P. falciparum, was selected for this study. This mAb was produced in our laboratory by immunization of BALB/c mice with human erythrocytes infected with P. vivax. Briefly, Percoll concentrated (8) blood parasites containing asexual and sexual stages were washed with Dulbecco's phosphate buffered saline, 0.15 M, pH 7.2 (PBS) and adjusted to $1.2x10^7$ erythrocytes/ml from which 75% were parasitized erythrocytes. Late trophozoites, schizonts and gametocytes were in the proportion of 7:5:1, respectively. BALB/c mice received an intrasplenic immunization (9) consisting of $4x10^5$ parasitized erythrocytes. Twelve days later mice were given a boosting dose of $8x10^5$ parasites administered by the intraperitoneal route. Fifteen days after the

first immunization the spleen was separated and hybridomas produced by fusing immune splenocytes with the NS-1-Ag4-1 myeloma cell line in the presence of poly-ethylene-glycol (Sigma Chemical Co., St Louis, MO., USA, SIGMA) using a standard protocol (10). Culture supernatants were screened for reactivity with blood stages of P. vivax by indirect immunofluorescence assays (IFA) on acetone fixed Percoll concentrated parasites (11,12). Briefly, aliquots of 5 µl containing $1x10^5$ parasitized erythrocytes were spotted onto circles of teflon coated slides (Eric Scientific, Portsmouth, NH., USA), air dried, fixed with chilled acetone and kept at -20ºC until use. Fixed smears of uninfected erythrocytes served as reference negative controls. To assess reactivity of monoclonal colony supernates 10 µl of a dilution (1:4) of the corresponding supernate was applied onto the cell monolayer and incubated for 30 min at 37°C. Following a washing step, 10 µl of a 1:100 dilution of conjugated fluorescein isothiocyanate (FITC) (Amersham International, Amersham, UK) sheep IgG anti mouse Igs was added for another 30 min at 37°C. The slides were mounted in 70% glycerol in PBS and examined at x400 or x1000 with a fluorescent microscope (Zeiss, Standard Model, Oberkoche, West Germany). To investigate the cross reactivity of monoclonal antibodies (mAbs)

with P. falciparum slides sensitized with acetone fixed blood stage parasites of P. falciparum were prepared from in vitro continuous cultures of the Palo Alto FCM29 strain. The latter was a kind gift of Dr Jacques Le Bras from the "Institut de Medicine et d'Epidemiologie Tropicales, Hopital Bichat-Claude Bernard, Paris, France". One of the mAbs resulting from this fusion was the clone 7C5B71. It reacted with the erythrocytic stages of P. vivax but failed to show reactivity with ring, trophozoite and schizont blood stages of P. falciparum. It was cloned twice by limiting dilution and screened again for specific anti-P. vivax reactivity. Additional slides sensitized with acetone fixed parasites were prepared from short-term in vitro cultures of two wild Venezuelan isolates of P. falciparum and from continuous cultures of the Colombian FCB-1 strain of P. falciparum. These studies further confirmed that mAb 7C5B71 which strongly reacted with the erythrocytic stages of P. vivax failed to bind to the blood stages of P. falciparum. Immunofluorescence assays on acetone fixed parasites counterstained with propidium iodide (SIGMA) (13) showed fluorescence immunostaining of asexual (including rings) and sexual blood stages of P. vivax. The hybridome was cryopreserved in liquid nitrogen. Ascitic fluids were produced in BALB/c mice primed with pristane

(2, 6, 10, 14-tetramethylpentadecane, SIGMA). Class and Sub-class determinations of the hybridomas produced were carried out using a mouse Typer Isotyping Panel from Bio-Rad Laboratories (Hercules CA., USA). MAb 7C5B71 was of the IgM class. It was partially purified from ascitic fluids by precipitation with 50% ammonium sulphate and protein concentration determined by a standard method (14).



## Collection of Blood Samples for Antigen Detection

Patients were submitted to one finger-stick with a standard lancet, from which 40 to 50 $\mu$l of blood were drawn into a single capillary tube. Blood sample in the capillary tube was poured into an eppendorf tube containing 1 ml of PBS, centrifuged (200 x g/3 min) and washed once with PBS. Washed cells were fixed with a solution containing 4% (w/v) paraformaldehyde (SIGMA) and 0.1% glutaraldehyde (EM grade, Polysciences, Inc, USA) (PAF/G) in PBS for 10 min at room temperature, the fixing solution was diluted eight fold with PBS and cells were kept at 4°C until use.

## Cell-ELISA for the Detection of Plasmodium vivax in Blood Samples

The assay was a modification of the immunocytochemical peroxidase test previously described

for the assay of anti-P.vivax antibodies (11). Briefly, 10 µl of PAF/G fixed cells containing 2.5-5 x $10^4$ erythrocytes/µl was introduced into the well of a multiwell slide (Eric-Scientific) and allowed to dry. Cells were dipped into 3% $H_2O_2$ (Merck, Darmstadt, Germany) at room temperature for 5 min to inactivate endogenous peroxidase, washed again with PBS and 10 µl of an appropriate dilution (50 µg/ml) of mAb 7C5B71 applied to the wells. Following incubation for 30 min at 37°C, the slides were washed and a dilution of horseradish peroxidase conjugated goat IgG anti-mouse Igs (Nordic Immunological Laboratories, Turnhout, Belgium) was added to each well for another 30 min at 37°C. The slides were then washed again and placed in a substrate solution containing 0.002 M 2,3 amino 9-ethyl-carbazole, AEC (SIGMA), 5% (v/v) N,N dimethyl-formamide (SIGMA) and 0.02% $H_2O_2$ in 0.1 M acetate buffer pH 5.3 and held at room temperature for 30 min. The slides were rinsed with PBS and mounted in 70% (v/v) glycerol in PBS for microscopic examination (400X). For permanent records cells were mounted in MOWIOL (SIGMA).



## RESULTS

### Immunocytochemical Staining

The immunocytochemical reaction produced by mAb 7C5B71 on erythrocytes infected with P. vivax is shown

in Figure 1. Parasitized cells exhibited immunostaining of the entire surface of the parasite as well a generalized staining of the host cell. Immunostaining was particularly strong in mature schizonts and gametes. Uninfected cells were unreacted.

Sensitivity of the mAb- cell-ELISA to Detect P. vivax Infected Blood

To evaluate the sensitivity of the cell-ELISA for the detection of parasites in infected blood, 0.5 ml of heparinized human blood infected with P. vivax were centrifuged to remove the plasma, washed once with PBS and adjusted to 25% (v/v) in PBS. The cell suspension of parasitized erythrocytes was then diluted with a 25% (v/v) cell suspension of washed uninfected blood cells, to obtain different levels of parasitaemias. Parasitaemia was determined in a thin blood film stained with Giemsa by counting the mean number of parasitized erythrocytes in relation to 10,000 erythrocytes. Erythrocytic cells were counted using standard procedures and this number was used in the calculation of the number of parasites/$\mu$l of the cell suspension. Cells were fixed with PAF/G and used to prepare cell monolayers on microscope slides. Parasitized erythrocytes probed with mAb 7C5B71 using the cell-

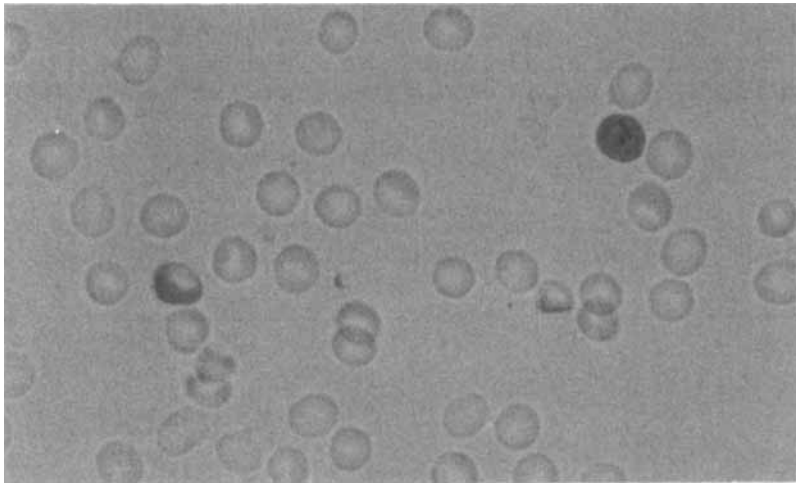

FIGURE 1. The cell -ELISA for the detection of erythrocytes infected with Plasmodium vivax using specific mAb 7C5B71. A positive reaction with PAF/G fixed parasites present in a blood thin smear of a patient. Non-infected cells are unreacted. Magnification X800.



ELISA displayed an intense positive reactivity. Table 1 illustrates the results obtained with individual dilutions of blood of five patients (samples A-E) with P. vivax malaria. Initial parasitaemias ranged between 2,400 to 17,500 parasites/$\mu$l. Positive results were obtained when parasitaemias were diluted up to 300 parasites/$\mu$l of blood. Next experiments were then addressed to evaluate the sensitivity and effectiveness of the cell-ELISA to detect P. vivax infection in the field.

TABLE 1

The Sensitivity of the Cell-ELISA for the Detection of Plasmodium vivax in Blood Using Specific mAb 7C5B71

| Sample | Parasitaemia (parasites/$\mu$l) and the results of the cell-ELISA | | | | | |
|---|---|---|---|---|---|---|
| **A** | 12,500 (+) | 2,500 (+) | 500 (+) | 250 (+) | 25 (-) | 2 (-) |
| **B** | 17,500 (+) | 3,500 (+) | 700 (+) | 140 (+) | 14 (-) | 1 (-) |
| **C** | 2,400 (+) | 1,200 (+) | 600 (+) | 300 (+) | 150 (-) | 75 (-) |
| **D** | 6,000 (+) | 3,000 (+) | 1,500 (+) | 750 (+) | 375 (-) | 188 (-) |
| **E** | 4,000 (+) | 2,000 (+) | 1,000 (+) | 500 (+) | 250 (-) | 125 (-) |



Evaluation of the mAb-cell-ELISA for the Diagnosis of P. vivax in Field Collected Blood Samples

Blood specimens were obtained from miners attending the local malaria clinic in Tumeremo, Bolivar State. Samples gathered in capillary tubes were fixed with PAF/G and stored at room temperature (28-30°C), transported to the laboratory at IVIC and kept at 4°C until use. In parallel, microscopic examination of Giemsa stained thick blood films was done by two skilled microscopist, diagnosed patients received the

appropriate anti-malarial drug treatment. Records of the results of the thick blood film examination were coded to carry out a blind scrutiny of the samples by means of the cell-ELISA.

To screen samples for P. vivax parasites fixed cells were spotted on multi-well slides and probed with mAb 7C5B71. For diagnosis of active infection the test reaches 100% specificity. Positive cells binding mAb 7C5B71 were detected in all parasitized patients. Discordant results were limited to blood specimens with P. falciparum parasitaemias. For estimation of parasitaemia in Giemsa stained thin blood films 300 fields were examined and parasites were counted. Three hundred fields were estimated to contain 0.02 $\mu$l of blood. Table 2 compares the level of circulating parasitaemias, species infecting Plasmodium as determined by the Giemsa staining method and the results of The cell-ELISA in a group of 77 patients.



## DISCUSSION

The application of mAbs as antigen detecting probes for the diagnosis of malaria is a relatively recent development. Specific and sensitive detection of P. falciparum malaria has been reported (15) by using a mAb reacting with P. falciparum Histidine-Rich-Protein

TABLE 2

Detection of Plasmodium vivax in Blood Samples Collected in the Field*, Using Specific mAb 7C5B71

| Infecting parasite** | N° of specimens | Parasites /μl of blood | Cell-ELISA (Total positive) |
|---|---|---|---|
| P. vivax | 11 | 2,900-70,000 | 11 |
| P. falciparum | 8 | 500-11,000 | 0 |
| None | 58 | - | 0 |

* Blood collected in capillary tubes and fixed with PAF/G

** Infecting parasite and level of parasitaemia determined on thin blood smears stained with Giemsa



2 (16) a conserved protein actively secreted from the blood stages of P. falciparum (17). However, no such reagents are yet available for the specific diagnosis of the other three human malaria parasites, P. vivax, Plasmodium malariae and Plasmodium ovale. In the present study, IgM mAb 7C5B71 which reacts specifically with the blood stages of P. vivax but not with those of P. falciparum, showed its potential value for the specific diagnosis of P. vivax malaria. Accordingly, our results have demonstrated that binding of mAb 7C5B71 to the

surface membrane of the P. vivax infected erythrocyte allowed its identification by using a cell-ELISA.

In a preliminary field trial the cell-ELISA showed 100% specificity for the diagnosis of P. vivax malaria and its sensitivity was ≤ 500 parasites/μl of blood. For standard microscopic diagnosis of malaria (examination of 100 fields at 500X -600X), the numerical threshold at which malaria parasites can be detected by an experienced microscopist in blood films is about 100 parasites/μl for thin films. For thick films the threshold is lower, about 10-20 parasites/μl (18). However, a high proportion of parasites are lost during staining of thick films (19) and in the practice there are difficulties to interpret thick films when the level of parasitaemia is below 500 parasites/μl (20). It seems therefore, that the detection sensitivity of the cell-ELISA reported in this study compares favorably with classic microscopic diagnosis of malaria with the further advantage that time needed to ascertain infected erythrocytes was considerably reduced. Infected erythrocytes which appeared dark-red stained in the bright field of the microscope, were easily identified. At low magnification (400X) less than 1 min was usually enough to find the first parasite in samples with parasitaemias ≤ 500 parasites/μl of blood. Other authors

have described immune-detection of P. vivax malaria by using cross-reacting P. falciparum mAbs (21) which facilitated adequate sensitivity (93%) but failed to establish the identity of the infecting Plasmodium species. Recently, we have developed an immunofluorescent antibody test for the specific detection of P. vivax malaria based on a mAb (2C6111) interacting with a 32 KDa antigen present in the cytoplasm but not on the surface of the erythrocyte infected with the mature blood stages (12). Therefore, probing of infected cells with mAb 2C6111 requires permeation of infected cell and those infected with the early asexual stages usually give a negative result. These difficulties were overcome by using mAb 7C5B71 in this study. It reacts with all the asexual stages including young trophozoites and its target antigen is exposed on the surface plasma membrane of the infected erythrocyte.

Other studies of our laboratory on the characterization of P. vivax antigen reacting with mAb 7C5B71 have shown immune recognition of air-dried parasitized erythrocytes (asexual and sexual stages). But in acetone fixed parasitized cells spots of immunostaining were seen close to the parasite body as well as in the cytoplasm of the infected erythrocyte. Immunostaining increased as parasite developed from

ring to schizont, fluorescent signal being particularly strong in the mature asexual stages and gametes. This immunofluorescence pattern of reaction was consistently observed in a total of 27 wild isolates of P. vivax collected in several malaria transmission areas of Venezuela in the States of Amazonas, Apure, Bolivar and Sucre and screened with mAb 7C5B71. Taken together these observations suggest that target antigen of mAb 7C5B71 is related to a conserved secretory protein trafficking from the parasite to the erythrocyte membrane/cytoskeleton. (Pérez, Bracho and De la Rosa, submitted).



The data herein discussed excluded the reactivity of mAb 7C5B71 with the blood stages of P. falciparum but that with P. malariae and P. ovale remains to be established. The latter has been recorded chiefly from tropical Africa and sporadically from the west Pacific region and from southern China, Burma and south-east Asia (18). Plasmodium malariae represents less than 0.05% of the malaria cases officially reported in Latin America (1). However, the specificity of mAb 7C5B71 as concerned to P. ovale and P. malariae merits further investigation. On the other hand, cross reactivity of mAb 7C5B71 with other blood borne parasites such as Trypanosoma cruzi, Leishmania donovani and Toxoplasma

gondii seems unlikely since IFA on acetone fixed tachyzoites of T. gondii, epimastigotes of T. cruzi or amastigotes of L. donovani gave negative results (data not shown). This observation is of interest because Chagas disease and visceral Leishmaniasis are prevalent in many parts of Latin America where transmission of P. vivax malaria is known to occur. Moreover, evidence of antigenic cross-reactivity between Plasmodia and the above mentioned parasites have been given (22-25).

We believe that detection of malaria infected erythrocytes using Plasmodium species specific mAbs can be expected to be a popular choice for the detection of malaria in the near future. Such developments will give strong support to the evaluation of the impact of new control measures, including vaccines, at the population level in endemic areas. Studies in progress in our laboratory are addressed to incorporate mAbs to the specific diagnosis of P. vivax malaria using different formats according to specific needs for either massive or individual diagnosis.



## ACKNOWLEDGMENTS

This research received financial support from Panamerican Health Organization (Grant PAHO DRS/RG VEN 86/126) Corporación Andina de Fomento, (CAF, Contract

438/89) and the Venezuelan Council of Scientific Research (CONICIT Grant RP-IV-130032-7). C. Bracho received a scholarship from BID/CONICIT. Thanks are due to Dr. Jose A. Anselmi from the "Division de Malariología y Saneamiento Ambiental, DMSA", Maracay, for continuous support and to Dr. Alejandro Caraballo from DMSA (Zone III, Tumeremo, Edo Bolivar) for logistic help with the collection of blood samples in the field. The FCB-1 strain of P. falciparum was kindly provided to our laboratory by Dr E.Sanchez (Universidad de Carabobo, Maracay-Edo Aragua). It was originally obtained from the laboratory of Dr S.Herrera (Universidad del Valle, Colombia).



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