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HUMAN MALARIA. STANDARDIZATION OF THE ENZYMEIMMUNOASSAY (ELISA) TO DETECT SPOROZOITES IN MOSQUITOES

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The availability of monoclonal antibodies specific to the major surface coat antigen of the sporozoites of a number of different malaria species (Cochrane et al., 1982; Nardin, 1982) has enabled the development of immunoradiometric assay (IRMA) for detecting sporozoites in infected mosquitoes (Zavala et al., 1982). This assay offers several advantages upon parasitological methods since it can be performed on freshly caught or dead dry mosquitoes and it permits the identification of the species of *Plasmodium*. This procedure has already being successfully applied in field conditions in the Gambia, West Africa (Collins et al., 1984). However, the need of radioisotopes to perform the assay constitute a handicap in most of the laboratories in areas where the malaria is endemic. An alternative method to detect antigen-antibody reaction with high sensitivity and specificity has being the immunoenzimatic procedure (Voller & Draper, 1982; WHO-MAL., 1985). Since this method do not require special laboratory conditions, we decide to define the sensitivity, specificity and reprodutibility parameters utilizing the immunoenzimatic assay to detect infected mosquitoes with *P. falciparum* and *P. vivax* sporozoites. Our ultimate purpose will be the application of present methodology in field conditions to determine the rate of mosquitoes infecied with different sporozoites species in the States of Pará, Northern Brazil.

MATERIAL AND METHODS

Monoclonal antibodies – Ascitic fluids containing monoclonal antibodies (MoAb) specific against *P. falciparum*, *P. vivax*, *P. knowlesi* and *P. brasilianum* circumsporozoite protein, were prepared in the Laboratory of the Department of Medical and Molecular Parasitology, New York University School of Medicine and the immunoglobulin fraction were purified in our Laboratory. The ascitic fluid were precipitated in 1.56M, amonium sulphate at 4°C. After centrifugation and dialysis the antibodies were purified by molecular sieving on Sephadex G-200 column. The peak that correspond to the MoAb were concentrated and dialyzed. After determination of protein content of the purified MoAb by Biuret method, they were stored at -70°C.

Preparation of immunoenzymatic conjugate – Purified MoAb were conjugate to peroxidase (Horseradish Peroxidase, type VI, SIGMA Chem. Co.) according to the method of Nakane & Kawaoi (1974) with few modifications. Briefly, peroxidase was oxidized with 0.1M $NaIO_4$ for twenty minutes at room temperature and dialyzed against 1mM acetate buffer, pH 4.4 overnight. After dialysis, two parts of MoAb by weight, were put in contact with one part of oxidized peroxidase, in alkaline pH, buffer V. The final product was dialyzed against PBS at 4°C, overnight. The conjugate was purified by gel filtration chromotography using Sephadex G-100 and characterized by radial immunodifusion and protein determination. The linkage protein-peroxidase was stabilized with $NaBH_4$ (4mg/ml) for 2 hours at 4°C.

Immunoenzyme assay – ELISA – 1. Sensitization of plastic plates: Wells of polyvinyl microtiter plates were incubated with 50µl of MoAb anti-*P. vivax* or anti-*P. falciparum* at a concentration of 10µg/ml in buffer V, overnight at 4°C. After incubation, the plates were washed with buffer III, and kept at 4°C until used. Before to start the assay the plates were incubated with buffer IV, for 1 hour at 37°C to prevent eventual non specific reaction. 2. Titration of enzymatic conjugate: In order to determine the titer of the conjugates and to evaluate the sensitivity of the ELISA, a block titration system were employed. In each well, of the pre-coated plate, a solution containing decreasing number of *P. vivax* and *P. falciparum* sporozoites, were added in a

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volume of 30μl in buffer II. After incubation for 1 hour at 37°C and three washing cycles with buffer III, dilutions of the enzymatic conjugate in buffer IV was added (dilutions 1/500-1/1000 – 1/2000 – 1/4000 – 1/6000 – 1/8000). After another incubation and washing cycle as described above, the enzymatic reaction was developed. For this, a chromogen solution in buffer VI, was added in each well. After 15 minutes at room temperature the reaction was stopped with 30μl of 1M HCl and the color intensity was evaluated in a minireader apparatus (Dynatech Products). The ELISA specificity were availabled with heterologous systems.

Buffers utilized in all steps of ELISA procedures:

Buffer I: PBS + 1% BSA + 0,5% NP40 + proteases inhibitors (Antipain 25μg/ml; Leupeptin 25μg/ml and Aprotinin 1.7U/ml). Used to crash the mosquitoes.

Buffer II: PBS + 1% BSA + 0,16% NP40.

Buffer III: PBS + 0,05% Tween 20.

Buffer IV: PBS + 1% BSA + 10% normal human serum.

Buffer V: 0,06M sodium carbonate-bicarbonate pH 9.6.

Buffer VI: Ortofenilenodiamine (OPD) – 3.7mM H_2O_2 3.5mM +

Solution A: 11,9g of Na_2HPO_4 in 1000ml of distilled water.

Solution B: 70g citric acid in 1000ml of distilled water.

To prepare this buffer add A solution to B solution for pH 5.0 25ml of prepared buffer + 10μg of 30% H_2O_2 + 10mg of OPD.

RESULTS

Specificity – As shown in Table I *P. falciparum* sporozoites gave strong reactions when tested with *P. falciparum* monoclonal antibody used in the solid phase and conjugated to peroxidase. The same results were observed for *P. vivax* sporozoites (Table II).

When heterologous systems were employed C_1, C_2, C_3, C_4, C_5 and C_6 a very light reaction were observed when the conjugate were diluted 1:500. The block titration systems were used to determine the titer of the conjugates. For *P. falciparum* conjugate the chosen dilution was 1:1000 and 1:5000 for *P. vivax* conjugate.

TABLE I

Standard curve to determine the sensitivity and specificity of immunoenzyme test to detect *P. falciparum* sporozoites, at different titers of the conjugate.

No. of *P. falciparum* Sporozoites	Absorbance (490 nm) Conjugate dilutions		
	1:500	1:1000	1:2000
650	2.45	1.94	1.70
325	1.50	1.35	1.30
160	1.10	1.85	0.60
80	0.80	0.66	0.36
40	0.45	0.32	0.21
20	0.28	0.16	0.11
10	0.20	0.10	0.08
5	0.15	0.07	0.00
C_1	0.01	0.00	0.00
C_2	0.02	0.00	0.00
C_3	0.04	0.00	0.00

*As controls of the reaction we used plates coated with *P. vivax* (C_1); *P. knowlesi* (C_2) and *P. brasilianum* (C_3) MoAb.

Sensitivity – The two site ELISA was able to detect about 5-10 sporozoites of *P. falciparum* and *P. vivax* in 30μl of the solution as showed in Tables I and II. The ELISA seems to be more sensible than IRMA to detect sporozoites in mosquitoes, because IRMA was able to detected as few as 50 sporozoites, as reported by Zavala et al. (1982).

Reprodutibility – The ELISA were tested in duplicate and in different days and the results were the same in all assays.

TABLE II

Standard curve to determine the sensitivity and specificity of immunoenzyme test to detect *P. vivax* sporozoites, at different titers of the conjugate.

No. of *P. vivax* Sporozoites	Absorbance (490 nm) Conjugate dilutions			
	1:2000	1:4000	1:6000	1:8000
400	2.25	1.85	1.66	1.85
200	1.70	1.40	0.81	0.35
100	1.30	0.70	0.48	0.30
50	0.80	0.50	0.28	0.18
25	0.60	0.30	0.17	0.10
12	0.29	0.17	0.13	0.05
6	0.10	0.09	0.09	0.00
3	0.09	0.07	0.05	0.00
C_4	0.01	0.00	0.00	0.00
C_5	0.00	0.00	0.00	0.00
C_6	0.02	0.00	0.00	0.00

*As controls of the reaction we used plates coated with *P. falciparum* (C_4); *P. knowlesi* (C_5) and *P. brasilianum* (C_6) MoAb.

DISCUSSION

The very low sporozoite rate, less than 0.1% found in endemic areas (Warren et al., 1975), and the impossibility of descriminating sporozoite of different species of malaria parasites on a morphological basis are the disadvantages of dissection as an investigational tool in malaria transmission surveys.

The use of monoclonal antibodies specific to defined chemical groups of the major surface coat antigen of sporozoites in the IRMA and ELISA were employed by Burkot et al. (1984) and Zavala et al. (1982). The use of ELISA, avoid the disadvantages of the IRMA related with the short lived radioactive reagents and the need of a gamma counter. The two-site ELISA was able to detect about 5-10 sporozoites in 30μl of solution. This sensitivity is sufficient to detected infected mosquitoes in endemic areas since 90% of them have sporozoites densities exceeding 500 parasites (Pringle, 1966). No eross reactions were observed when heterologous systems were employed. The standardized ELISA for detection of *P. vivax* and *P. falciparum* sporozoites was employed in field conditions in the State of Pará, Northern Brazil (Arruda et al., 1985).

CONCLUSIONS

When compared with parasitological procedures, the enzymeimmunoassay showed to be superior in: I) **Specificity** – the method is specie-specific, no cross reactions were observed among different plasmodia; II) **Sensitivity** – the procedure was enough sensible to detect about 5-10 sporozoite in 30μl of the mosquito extract; III) **Stability** – the reagents employed in this procedure are stable, for about two years; IV) **Processing mosquitoes** – the method may be used for dried or freshly caught mosquitoes and V) A great number of mosquitoes may be processed in a few hours.

REFERENCES

ARRUDA, M.; CARVALHO, M.B.; NUSSENZWEIG, R.S.; MARACIC, M.; FERREIRA, A.W. & COCHRANE, A.H., 1986. Potential vectors of malaria in Northern Brazil. *Am. J. Trop. Med. Hyg.*, *35* :873-881.

BURKOT, T.R.; ZAVALA, F.; GWADZ, R.W.; COLLINS, F.H.; NUSSENZWEIG, R.S. & ROBERTS, D.R., 1984. Identifications of malaria-infected mosquitoes by a two-site enzyme linked immunosorbent assay. *Ann. J. Trop. Med. & Hyg., 33* :227-231.

COCHRANE, A.; SANTORO, F.; NUSSENZWEIG, V.; GWADZ, R.W. & NUSSENZWEIG, R.S., 1982. Monoclonal antibodies identify the protective antigens of sporozoites of *Plasmodium knowlesii. Proc. Natl. Acad. Sci., 79* :5651-5655.

COLLINS, F.H.; ZAVALA, F.; GRAVES, P.M.; COCHRANE, A.H.; GWADZ, R.W.; AKOK, J. & NUSSENZWEIG, R., 1984. First field trial of an immunoradiometric assay for the detection of malaria sporozoites in mosquitoes. *Am. J. Trop. Med. & Hyg., 33* :538-543.

NAKANE, P.K. & KAWAOI, A., 1974. Peroxidase labelled antibody: a new method of conjugation. *J. Histochem. Cytochem., 22* :1084-1091.

NARDIM, E.H., 1982. Circumsporozoites (CS) proteins of human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax. J. Exp. Med., 156* :20-30.

PRINGLE, G., 1966. A quantitative study of naturally-acquired malaria infections in *Anopheles gambiae* and *Anopheles funestus* in a highly malarious area of East Africa. *Trans. R. Soc. Trop. Med. Hyg., 60* :626-632.

VOLLER, R. & DRAPER, C.C., 1982. Immunodiagnosis and seroepidemiology of malaria. *Brit. Med. Bul., 38* :173-178.

WARREN, M.; MASON, J. & HOBBS, J., 1975. Natural infections of *Anopheles albimanus* in a small malaria focus. *Am. J. Trop. Med. Hyg., 24* :545-546.

WHO-MAL. Immunodiagnosis in Malaria, 1985. 1018 Report on an informal consultation held in Geneva on 20-22 June, 1984.

ZAVALA, F.; GWADZ, R.W.; COLLINS, F.H.; NUSSENZWEIG, R. & NUSSENZWEIG, V., 1982. Monoclonal antibodies to circumsporozoites proteins identify the species of malaria parasite in infected mosquitoes. *Nature, 229* :737-738.