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CLONING AND CHARACTERIZATION OF *PLASMODIUM FALCIPARUM* STRAINS

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The Evandro Chagas Institute Malaria Programme has paid special attention to the characterization of strains of P. falciparum *from the Amazon region of Brazil. In these studies, clones were prepared by the serial dilution method described by Rosario (1981). This is the first time that* P. falciparum *has been successfully cloned in Brazil. Two methods were used to characterize* P. falciparum *clones and strains: 1) Isoenzyme analysis of six enzymes: GPI, PEP, LDH, GDH, ADA and PGD, performed on cellulose acetate by the method originally described by Carter & McGregor (1973); 2) drug sensitivity to chloroquine, mefloquine, quinine and amodiaquine using the technique described by Rieckmann et al. (1978).*

Three P. falciparum *strains (IEC-132/83, IEC-145/83 and IEC-51/84) collected in Rio Jari (Amapá), Tucuruí and Marabá (Pará) were cloned. Considerable intraspecific variation was observed amongst 12 clones obtained from these strains by the two methods named above.*

Strain characterization of *Plasmodium falciparum* parasites was initiated at the Instituto Evandro Chagas in 1981. The main objective was to study the diversity of these parasite populations by collecting as many samples as possible from the Amazon region in Brazil which would be submitted to *in vitro* culturing in the laboratory and analyzed to their response to drugs *in vitro* (chloroquine, quinine, mefloquine and amodiaquine), and enzyme typed using acetate cellulose electrophoresis for GPI (glucose phosphate isomerase), ADA (adenosine deaminase), PEP (peptidase), LDH (lactate dehydrogenase), GDH (NADP-dependent glutamate dehydrogenase) and PGD (phospho gluconate dehydrogenase). Since *in vitro* culturing of malaria parasites may exert selective pressure on some parasites, thus establishing a parasite population different from the original isolate we believed that strain characterization should be carried out in the very first weeks after successful adaptation of the parasites to our culturing system. Cloning became a late development in our research project and was carried out whenever enzyme typing showed presence of a mixed population of parasites in a sample.

A total of 365 samples have been characterized showing major variation among the isolates studied and clones obtained from three of these isolates have also shown diversity.

MATERIALS AND METHODS

All the techniques used have already been thoroughly described (Carter & McGregor, 1973; Sanderson & Walliker, 1981; Thaithong, 1983; Couto, Rosario & Walliker, 1983). *In vitro* culturing was based on the reports by Trager & Jensen (1976). Heparinized syringes were used to colect samples from regions distant from the laboratory, and the infected blood was immediately transferred into 15ml tubes with complete RPMI 1640 (5% bicarbonate and 10% A serum). This dilution had the advantage of providing the parasites with some nutrients and also diluting any possible drugs that the patients may have taken prior to blood collection. Only patients exhibiting parasitemias of 2% or higher were selected for blood collection and an accurate questionnaire was submitted orally to each patient. Due to the development of the Amazon region in Brazil these patients were seldom located in one locality for a long period of time, so we believe that not all samples necessarily represented the parasite population of a certain area. However, throughout our questionnaire we tried to locate the origin of the infection. In Fig. 1 a description is shown of the areas where collection of samples was carried out.

Stock solutions obtained from WHO/Geneva were used for drug assays with freshly prepared microplates. Previous experience with predosaged plates, stored for long periods of time suggested that false resistance results were obtained, due perhaps to absorption of the drug by the plate material or to break down of the drug under storage conditions. The assay was based on Rieckmann's *in vitro* micro assay (1978) and has been fully described by Vasconcellos & Rosario (1983). We used cellulose acetate electrophoresis for enzyme typing as described by Couto, Rosario

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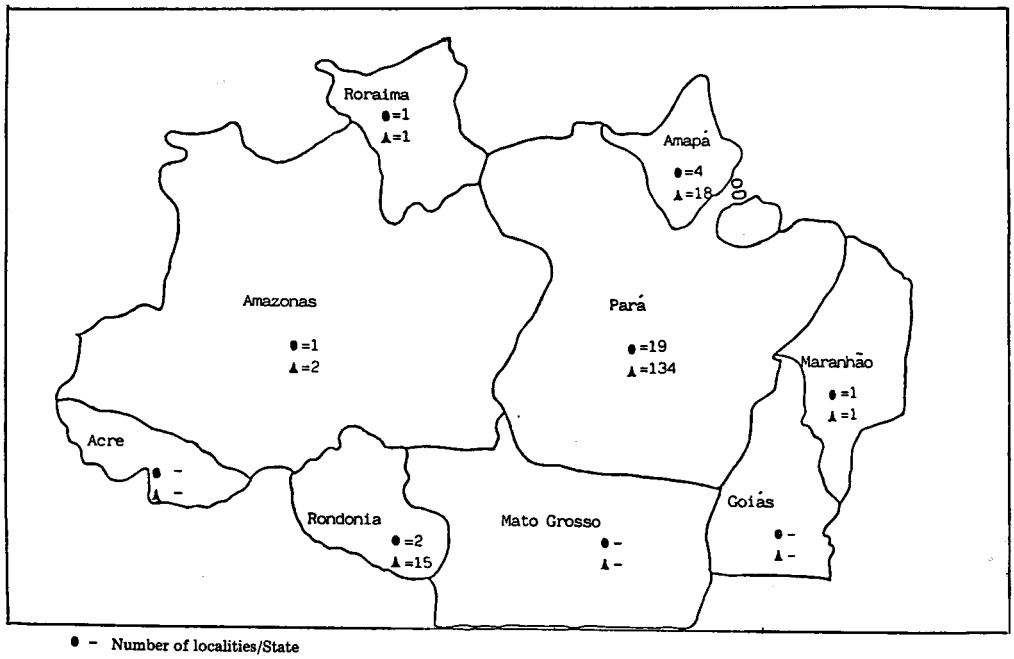

● - Number of localities/State

▲ - Number of strains characterized.

Fig. 1: Brazilian Amazon Region: collection of samples.

& Walliker (1983). Cultures exhibiting a parasitemia of 5% or higher and with predominance of schizonts were spun down and the pellet mixed with two volumes of a 0.15% saponin solution in RPMI 1640, maintained in a water bath at 37°C for 20', washed twice with RPMI 1640 (5% bicarbonate, no serum), and after discharging the supernatant, the pellet was kept at -20°C until electrophoresis was carried out. Prior to use, we added 1-2 drops of distilled water to the parasite pellet, froze and thawed it 2-3 times in order to break the parasite membranes and release the enzymes that were to be analyzed. Running conditions have been described elsewhere (Couto, Rosario & Walliker, 1983).

Isolate IEC 132/83 was collected from a patient from Rio Jari, State of Amapá, on 31 October 1983. The sample was frozen on 2 November 1983, thawed 1 August 1984, and enzyme typed and cloned on 28 August 1984. Isolate IEC 145/83 was collected from a patient from Tucuruí, State of Pará on 8 November 1983. The sample was frozen on 21 November 1983, thawed on 20 February 1985, and enzyme typed and cloned on 5 March 1985. Isolate IEC 51/84 was collected from a patient from Marabá, State of Pará, on 28 May 1984. The sample was frozen on 11 June 1984, thawed on 14 January 1986 and enzyme typed and cloned on 14 February 1986.

We used the method previously described by Rosario (1981) for cloning with modifications according to personal communication by Sodsri Thaithong (Chulalongkorn University, Bangkok, Thailand). All three isolates exhibited both GPI-1 and GPI-2 type of parasites with cellulose acetate electrophoresis. An estimated 1 parasite/0.1ml in a 2% hematocrit culture media was introduced into the wells of a flat bottomed titerplate. The culture media was changed every 48hr. Every fifth day, we added fresh red blood cells (1% haematocrit) to the wells in order to dilute these microcultures. On the twenty first day, thin smears were made and stained to observe for growth. The ratio between the number of positive wells and the total number of inoculated wells was compared to the Poisson distribution in order to establish the probability of the positive ones being pure clones.

RESULTS

In Table I, results obtained with enzyme typing of 152 samples of *Plasmodium falciparum* is shown. There was a predominance of GPI-2 type of parasites (55.3%) against GPI-1 (38.2%) and only 6.5% of these samples contained a mixture of both types. For ADA we detected also a predominance of ADA-2 type (64.9%) against ADA-1 (30.6%) and only 4.5% of the 137 samples

TABLE I

Enzyme typing of *P. falciparum*

Enzyme Type		
GPI	1	58 (38.2%)
	2	84 (55.3%)
	1/2	10 (6.5%)
N. Samples		152
ADA	1	42 (30.6%)
	2	89 (64.9%)
	1/2	06 (4.5%)
N. Samples		137
PEP	1	114 (89.7%)
	2	11 (8.6%)
	1/2	2 (1.7%)
N. Samples		127
LDH	1	102 (100%)
	2	0 (0%)
	1/2	0 (0%)
N. Samples		102

analyzed showed a mixed population. For PEP we detected 89.7% of parasites of type PEP-1, 8.6% of type PEP-2 and 1.7% of these 127 samples contained a mixed population of types 1 and 2. For LDH, GDH and PGD only parasites of type 1 were found.

Table II shows a summary of results on drug assays carried out since 1983. *In vitro* a wide resistance to chloroquine, diversity in the response to quinine and amodiaquine and susceptibility to mefloquine is observed.

The three isolates selected for cloning exhibited high resistance *in vitro* to chloroquine and quinine, and sensitivity to mefloquine; only isolate IEC 51/84 showed sensitivity to amodiaquine and all three were mixtures for GPI 1/2. In Table III, results on clone characterization are shown. From isolate IEC 132/83, IEC 145/83 and IEC 51/84 clones were established and immediatly analyzed for enzyme types and drug response. From all the isolates only clones of type GPI-2 were obtained, GPI-1 type of parasites not having been detected in our cloning experiments. Clones with MIC values lower or even higher than the isolate MIC value, were obtained; drug assays showed that clones differed among themselves confirming presence of mixed populations of parasites in each isolate.

TABLE II

Drug – Susceptibility of *P. falciparum* – *In vitro* Assays

Drugs	MIC*	1983	1984	1985	Total N. Samples Inhibited
Chloroquine Diph.					
(X 10^{-8}M)	1.0	0	0	0	0
	2.0	0	0	0	0
	4.0	1	0	1	2
	6.0	11	8	5	24
	8.0	6	12	8	26
	16.0	12	16	15	43
	32.0	19	21	15	55
N. of Samples		49	57	44	150
Mefloquine chl.	0.5	30	6	9	45
(X 10^{-8}M)	1.0	11	26	22	59
	2.0	8	14	4	26
	4.0	0	7	4	11
	6.0	0	1	2	3
	8.0	0	1	3	4
	16.0	0	0	0	0
N. of Samples		49	55	44	148
Amodiaquine	0.25	1	0	3	4
(X 10^{-8}M)	0.5	0	4	5	9
	1.0	3	8	12	23
	2.0	1	14	8	23
	4.0	4	20	6	30
	8.0	7	6	8	21
	16.0	2	5	2	9
N. of Samples		18	57	44	119
Quinine Sulph.	3.9	0	0	0	0
(X 10^{-8}M)	7.8	0	0	2	2
	15.6	1	11	9	21
	31.25	9	24	16	49
	62.5	6	17	10	33
	125.0	2	1	6	9
	250.0	0	4	1	5
N. of Samples		18	57	44	119

*Minimum inhibitory concentration of drug which kills parasites.

TABLE III

Clone Characterization: Enzyme Typing and Drug – Susceptibility Assays

	Enzyme Types			Minimum Inhibitory Concentration (MIC)			
	GPI	ADA	PEP	Chlq. ($x10^{-8}M$)	Mef. ($x10^{-8}M$)	Amod. ($x10^{-8}M$)	Quin. ($x10^{-8}M$)
IEC-132/83*	1 e 2	2	1	16	1	8	62.5
Clone C	2	2	1	8	0.5	0.5	31.25
Clone E	2	2	1	16	2	2	62.5
Clone F	2	2	1	32	2	4	62.5
IEC-145/83*	1 e 2	2	1	32	4	8	125
Clone 12	2	2	1	32	6	8	125
Clone 13	–	2	1	32	8	16	250
Clone 22	2	2	1	32	8	8	62.5
Clone 27	2	2	1	32	1	8	62.5
Clone 30	2	2	1	32	8	8	62.5
IEC-51/84*	1 e 2	1	–	32.0	2.0	4.0	62.5
Clone 3	2	1	–	16.0	1.0	0.5	31.25
Clone 11	2	1	–	8.0	2.0	4.0	62.5
Clone 12	2	1	–	6.0	0.5	2.0	31.25
Clone 14	2	1	–	8.0	2.0	8.0	62.5

*Isolate
–Not tested
Clq – Chloroquine diphosphate
Mef – Mefloquine chloridrate
Amod – Amodiaquine
Q – Quinine sulphate

DISCUSSION

We have not observed the appearance of new types of enzyme types when compared to the ones previously described in Africa or South East Asia (Walliker, 1982). However, in relation to mixed populations we did find a very small number of samples with GPI 1/2 type of parasites growing simultaneously in Brazil (6.5%) compared to Asia (21.5%) and Africa (26.1%), (Walliker, 1982); for the first time enzyme typing of *Plasmodium falciparum* was carried out in Brazil and it showed similarity to the parasite populations from other countries. Recently Sala-Neto & Tosta (1986) have shown the presence of parasites of type GDH-2, in samples collected in Rondônia; such a result disagrees with our description of samples either from the same locality or from other areas in Brazil, (Couto, Rosario & Walliker, 1983). Such differences require further study; namely the exchange of material and the evaluation of techniques by both laboratories using the same controls is necessary.

Thoughout this presentation we have not described in detail the origin of each isolate. However, we would like to mention that we have observed four samples from Itaituba simultaneously resistant to chloroquine, quinine and mefloquine, in our *in vitro* assays. Assuming that drug resistance is a stable genetic marker capable of recombining with other markers, the continuous use of these drugs might select and disseminate parasites with multiple resistance into other localities.

Table III shows results of the analysis of clones obtained by the limited dilution technique.

From IEC 132/84, three clones were obtained (C, E and F). All clones were of the same enzyme type (GPI-2, ADA-2, PEP-2) but varied in their drug response *in vitro* and when compared with the original isolate.

From IEC 145/83, five clones were obtained; they were all of the same enzyme type (GPI-2, ADA-2, PEP-1) and equally resistant to chloroquine (MIC = 32×10^{-8} M). It is interesting that, with mefloquine, clones 13, 22 and 30 showed a MIC value of 8×10^{-8} M, clone 12 a MIC value of 6×10^{-8} M and clone 27 a MIC value of 1×10^{-8} M; all different from the original isolate whose MIC value was 4×10^{-8} M. From isolate IEC 51/84 we obtained four clones (all of type GPI-2, ADA-1) with different patterns of drug response. Clone 14, for example, was more resistant to amodiaquine than the original isolate. Several explanations for this result can be suggested though these require further studying: a) any variation found in the uncloned population should be represented in the clones since cloning is carried out under no drug pressure, so there is no selection of resistant parasites over sensitive ones; b) it has been shown previously (Rosario, 1981)

that growth patterns of cloned populations differ and such variation in growth may affect the final MIC value for each clone; c) a high MIC obtained from an assay with the uncloned isolate might represent a false description of the composition of this isolate since it shows only the result from growth of the most resistant parasites within the population, the most sensitive clones not being allowed to express themselves in this mixed population of different drug susceptibility patterns; d) such data confirms that descriptions of *in vivo* drug response such as RI, RII and RIII do not represent an accurate description of a parasite population within a certain patient. Namely, a patient with mixed parasites of different drug susceptibility to, for example, mefloquine, most of them being sensitive and only very few highly resistant to the drug, will take longer to exhibit these after treatment with mefloquine since the few surviving parasites will take longer to appear in the blood stream; whereas a patient with a *homogeneous* population of parasites, though *less resistant* to mefloquine might show up earlier giving the false impression of a highly resistant population.

The isolate IEC 51/84 was also cloned by micromanipulation in Chapel Hill, N.C. (Drs. Oduola, A. and Rosario, V.) and surprisingly all seven clones were of type GPI-1 and only one clone of type GPI-2 was obtained; factors such as laboratory culturing conditions or growth rate *in vitro* may have an effect on the final outcome of a specific cloning experiment.

It is important also to associate drug resistance patterns in clones with gametocyte production and mosquito infectivity (Burkot, Williams & Schneider, 1984) so that one can study transmission of drug-resistance.

From this preliminary work we have showed that in the Amazon region (Brazil) it was feasible to carry out these techniques and one hopes to establish new lines of research on strain characterization using more developed techniques for further understanding of the subject.

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