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Electrophoretic Variations of Glucose Phosphate Isomerase (EC. 5.3.1.9) of Clones and Isolates of *Plasmodium falciparum* from Malaysia

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Abstract

Six clones were obtained from each *Plasmodium falciparum* isolate, Gombak A (Malaysian), Gombak C (Malaysian), ST 9 (Malaysian), ST 12 (Malaysian), ST 85 (Malaysian), ST 148 (Malaysian), Gambian (African) and TGR (Thailand) using limiting dilution method. These clones were characterized by electrophoretic isoenzyme analysis of GPI (glucose phosphate isomerase) (EC. 5.3.1.9). Results showed that these were pure clones as they were monovariant with regards to GPI unlike their parent isolates which were divariant.

Key words: *Plasmodium falciparum*; isolates; clones; glucose phosphate isomerase; electrophoretic variations.

Introduction

Isolates of *Plasmodium falciparum* taken on a single occasion from infected hosts are known to contain several types of parasites; for example, early work by Carter and McGregor (1973) revealed that variations within a single isolate of *P. falciparum* occurred when the West African isolate exhibited more than one electrophoretic form of one or more enzymes. Rosario (1981) showed that individual clones could be identified from a single isolate containing a heterogeneous population with regards to the enzyme pattern, by using limiting dilution method. Further studies of parasites collected from various laboratories have shown that isolates contained a mixture of enzymes (Carter and Voller, 1975; Carter and Walliker, 1977; Sanderson *et al.*, 1981; Thaithong *et al.*, 1981; Thaithong *et al.*, 1989; Creasey *et al.*, 1990) and they were used as genetic markers to identify clones from a heterogenous population of isolates (Thaithong, 1983; Thaithong *et al.*, 1984; Graves *et al.*, 1984; Webster *et al.*, 1985; Odoula *et al.*, 1988). However, none of the electrophoresis isoenzyme analysis was performed on the Malaysian isolates.

In this present paper, we summarize an isoenzyme analysis of GPI (glucose phosphate isomerase) (EC. 5.3.1.9) on a total of 48 clones of which 36 clones were obtained from Malaysian *P. falciparum* isolates and the rest from Africa and Thailand, using the limiting dilution method (Rosario, 1981).

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Materials and Methods

Cultivation of malaria parasites.

Blood samples infected only with *P. falciparum* were used in this study. Gombak A, Gombak C, ST 9, ST 12, ST 85 and ST 148 isolates were collected from nomadic aborigines who were treated at the Gombak District Hospital, Ulu Langat District, Selangor, Malaysia. Gambian and TGR isolates were isolated from malaria patients in West Africa and Thailand, respectively, and were brought to Institute for Medical Research, Kuala Lumpur, Malaysia by Professor CR Brockelman, Mahidol University, Bangkok, Thailand. These samples were then cultured *in vitro* at the Filaria and Malaria Division, Institute for Medical Research, Kuala Lumpur and School of Pharmaceutical Sciences, University of Science Malaysia, Minden, Penang, Malaysia using the candle-jar method (Trager and Jensen, 1976).

The infected blood suspensions were dispensed asceptically into 35 mm petri dishes (Linbro Flow

Laboratories, USA) and complete culture medium containing RPMI 1640 powdered medium (GIBCO Laboratories, USA), 25 mM HEPES (Calbiochem-Behring, USA), 0.2% $NaHCO_3$, 10% O^+ human serum (heat-inactivated at 56°C for 30 minutes) and 40 μg/ml gentamycin (Rotex Medica, Germany) were added to give a final concentration of 10% hematocrit. The cultures were incubated at 37°C and washed with culture medium daily. Thin blood films were prepared from each petri dish and stained with 10% Giemsa (BDH, England) for 30 minutes. Cultured parasites were later cryopreserved in liquid nitrogen. When required, these three isolates were thawed out.

Preparation of parasite clones.

After thawing, the isolates were maintained in continuous culture for a month and parasites at rapid growing phase were cloned by limiting dilution method (Rosario, 1981). Diluted samples of cultured parasites, estimated to contain 0.5 to 1 parasite per 0.1 ml at a 2% hematocrit were dispensed in a 96-well microtiter plate and further incubated. Supernatant was discarded every 2 days and replaced with fresh medium. After 4 days post-cloning, supernatant and half of the parasitized erythrocytes were discarded from each well, and fresh medium and erythrocytes were added to each well. This procedure was repeated for 21 days.

Giemsa-stained thin blood smears were prepared from each well at 15 to 21 days. Parasite positive microcultures were transferred to larger wells and eventually to petri dishes to yield adequate materials for isoenzyme assay.

Preparation of malaria parasites for electrophoresis.

The isoenzyme GPI for 48 clones and their parent isolates was studied. Cultures with 5% parasitemia were harvested and centrifuged at 1,500 g for 10 minutes. Supernatant was discarded and infected erythrocytes were washed with normal saline thoroughly. Parasites were freed from their host erythrocytes by incubating with 0.15% saponin (Sigma Chemical, USA) at 37°C for 20 minutes and centrifuged at 3,000 g for 10 minutes. Parasites were washed in RPMI 1640 and centrifuged at 3,000 g for 10 minutes and further lysed using the 1% Triton X-100 (Biorad, USA) in 1 mM EDTA 10 mM Tris-HCl (Sigma Chemical, USA) buffered at pH 7.4. Lysates were then immediately loaded onto filter paper strips and then inserted into the starch gel.

Isoenzyme assay.

Variant forms of GPI in each isolate and clone were identified by starch gel electrophoresis at 3–5°C at 240 V for 4 hours in 0.1 M Tris-HCl at pH 8.0. Substrate for GPI was 10 mg/ml fructose-6-phosphate (Sigma Chemical, USA) whilst the exogenous linking enzyme was 140 units/ml Gd (glucose-6-phosphate dehydrogenase) (EC. 1.1.1.49) (Sigma Chemical, USA) and cofactor was 5 mg/ml NADP (Sigma Chemical, USA). The band positions of the isoenzymes were visualized after electrophoresis by incubating these reagents at 37°C with 2% agar (Difco, USA) solutions containing 5 mg/ml PMS (phenazine methosulphate, Sigma Chemical, USA) and 5 mg/ml MTT (methyl thiazolyl tetrazolium, Sigma Chemical, USA), the latter being an acceptor for dehydrogenase reactions. The addition of agar facilitated handling of the gels and sharpened the enzyme bands.

Classification of the GPI variants.

The isoenzyme forms of GPI were classified following Sanderson *et al.* (1981). Relative mobilities of GPI isoenzyme obtained from *P. falciparum* isolates and clones were characterized as a ratio of:

$$\frac{\text{migration magnitude of GPI isolate or clone from baseline to anode } (R_{fa})}{\text{migration magnitude of GPI erythrocyte (host) from baseline to cathode } (R_{fc})}$$

Results and Discussion

GPI of Gombak A, Gombak C, ST 9, ST 12, ST 85, ST 148, Gambian and TGR isolates and their clones were characterized relative to that of the host. GPI enzyme from host migrated to the cathode and exhibited 2 bands identified as the slow and the fast forms which are consistent to previously reported ones (Sanderson *et al.*, 1981). The values of the relative mobilities of the GPI isolates and clones were expressed with respect to these two bands.

Table 1 GPI isoenzyme variants for *Plasmodium falciparum* isolates and clones

isolate/clone	*R_{fa}	+Relative mobilities of GPI-1	GPI-2
GOMBAK A ISOLATE	0.12, 0.06	2.67, 1.71	1.33, 0.86
A/C1 clone	0.12	2.67, 1.71	–
A/D3 clone	0.12	2.67, 1.71	–
A/D5 clone	0.06	–	1.33, 0.86
A/G4 clone	0.12	2.67, 1.71	–
A/H2 clone	0.06	–	1.33, 0.86
A/H7 clone	0.12	2.67, 1.71	–
GOMBAK C ISOLATE	0.12, 0.06	2.67, 1.71	1.33, 0.86
C/A2 clone	0.12	2.67, 1.71	–
C/B4 clone	0.12	2.67, 1.71	–
C/B7 clone	0.12	2.67, 1.71	–
C/C4 clone	0.06	–	1.33, 0.86
C/C8 clone	0.12	2.67, 1.71	–
C/C10 clone	0.12	2.67, 1.71	–
ST 9 ISOLATE	0.12, 0.06	2.67, 1.71	1.33, 0.86
ST 9/A4 clone	0.06	–	1.33, 0.86
ST 9/A7 clone	0.12	2.67, 1.71	–
ST 9/B5 clone	0.12	2.67, 1.71	–
ST 9/D8 clone	0.12	2.67, 1.71	–
ST 9/D9 clone	0.06	–	1.33, 0.86
ST 9/D10 clone	0.06	–	1.33, 0.86
ST 12 ISOLATE	0.12, 0.06	2.67, 1.71	1.33, 0.86
ST 12/A3 clone	0.06	–	1.33, 0.86
ST 12/A4 clone	0.06	–	1.33, 0.86
ST 12/D5 clone	0.06	–	1.33, 0.86
ST 12/D7 clone	0.06	–	1.33, 0.86
ST 12/E8 clone	0.06	–	1.33, 0.86
ST 12/F8 clone	0.12	2.67, 1.71	–
ST 85 ISOLATE	0.12, 0.06	2.67, 1.71	1.33, 0.86
ST 85/A2 clone	0.12	2.67, 1.71	–
ST 85/A5 clone	0.06	–	1.33, 0.86
ST 85/B3 clone	0.06	–	1.33, 0.86
ST 85/B4 clone	0.12	2.67, 1.71	–
ST 85/D3 clone	0.12	2.67, 1.71	–
ST 85/D7 clone	0.12	2.67, 1.71	–
ST 148 ISOLATE	0.12, 0.06	2.67, 1.71	1.33, 0.86
ST 148/A4 clone	0.06	–	1.33, 0.86
ST 148/A5 clone	0.06	–	1.33, 0.86
ST 148/A6 clone	0.12	2.67, 1.71	–
ST 148/A7 clone	0.12	2.67, 1.71	–
ST 148/F7 clone	0.06	–	1.33, 0.86
ST 148/F8 clone	0.06	–	1.33, 0.86
GAMBIAN ISOLATE	0.125, 0.06	2.78, 1.79	1.33, 0.86
Gm/A1 clone	0.125	2.78, 1.79	–
Gm/B2 clone	0.125	2.78, 1.79	–
Gm/C5 clone	0.06	–	1.33, 0.86

Gm/C6 clone	0.125	2.78, 1.79	–
Gm/H5 clone	0.125	2.78, 1.79	–
Gm/H7 clone	0.125	2.78, 1.79	–
TGR ISOLATE	0.12, 0.06	2.67, 1.71	1.33, 0.86
TGR/A2 clone	0.06	–	1.33, 0.86
TGR/B4 clone	0.06	–	1.33, 0.86
TGR/B7 clone	0.06	–	1.33, 0.86
TGR/C4 clone	0.12	2.67, 1.71	–
TGR/C7 clone	0.06	–	1.33, 0.86
TGR/H2 clone	0.06	–	1.33, 0.86

$$*R_{fa} = \frac{\text{distance moved by GPI variants of } P.\ falciparum \text{ from baseline to the anode}}{\text{distance moved by bromophenol blue from baseline to the anode}}$$

+Relative mobilities are expressed as a ratio R_{fa} to R_{fc} for slow and fast GPI variants of host, whose values are 0.045 and 0.07, respectively.

$$R_{fc} = \frac{\text{distance moved by slow or fast form of GPI host from baseline to the cathode}}{\text{distance moved by bromophenol blue from baseline to the anode}}$$

Table 1 shows the GPI isoenzyme variants for *P. falciparum* isolates and their respective clones. The GPI variants of eight isolates migrated anodally and contained two bands identified as GPI-1 and GPI-2 but did not possess a third band, GPI-3, which was found in the *P. falciparum* parasites isolated from patients in Prachinburi, Thailand (Thaithong *et al.*, 1989) and Zimbabwe, Africa (Creasey *et al.*, 1990).

GPI-1 and GPI-2 of Gombak A, Gombak C, ST 9, ST 12, ST 85 and ST 148 isolates were characterized as having relative mobilities of 2.67, 1.71 and 1.33, 0.86 respectively. The relative mobilities of GPI-1 and GPI-2 of Gambian isolate were 2.78, 1.79 and 1.33, 0.86 whereas values of 2.67, 1.71 and 1.33, 0.86 were found for TGR isolate. The slight difference occured in relative mobilities of GPI-1 for Gambian isolates and clones compared to Malaysian and Thailand isolates and clones were attributed to the slight difference in the R_{fa} which were 0.125 and 0.12 respectively.

On the other hand, clones prepared from isolates exhibited monovariant GPI. From the 6 local isolates, 19 clones out of 36 clones contained GPI-1 with relative mobilities of 2.67, 1.71 whilst the rest, possessed GPI-2 with relative mobilities of 1.33, 0.86. For Gambian isolate, only Gm/C5 clone exhibited GPI-2 with relative mobilities of 1.33, 0.86 whilst the rest, five clones were GPI-1 with values of 2.78, 1.79. In contrast, for TGR isolate, only one clone, TGR/C4 possessed GPI-1 with relative mobilities of 2.67, 1.71 whilst the rest, five clones were GPI-2 with relative mobilities of 1.33, 0.86. This study confirms earlier works showing that isolates obtained from a naturally infected host on a single occasion are genetically heterogeneous with respect at isoenzyme pattern of GPI.

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