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Electrophoretic variations of peptidase E (PEPE) in characterizing clones and isolates of *Plasmodium falciparum* from different geographical areas

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Abstract. Six clones were obtained from each *Plasmodium falciparum* (Welch, 1897) isolate from different geographical areas, Gombak A (Malaysian), Gombak C (Malaysian), ST 9 (Malaysian), ST 12 (Malaysian), ST 85 (Malaysian), ST 148 (Malaysian), Gambian (African) and TGR (Thailand) isolates using the limiting dilution method (Rosario 1981). Forty-eight clones were obtained and were characterized by an electrophoresis isoenzyme analysis of PEPE (Peptidase E) (EC. 3.4.11 or 13). Results showed that they were pure clones as they were monovariant with regards to this enzyme unlike their parent isolates which were divariant.

Natural isolates of *Plasmodium falciparum* (Welch, 1897) taken on a single occasion from naturally infected hosts have shown to contain several types of parasites. For example, the earliest work 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 (Carter and Mcgregor 1973). 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 the limiting dilution method.

Further surveys of parasites collected from various laboratories showed that isolates contained a mixture of enzymes (Carter and Voller 1975, Carter and Walliker 1977, Sanderson et al. 1981, Thaithong et al. 1981, 1989, Creasey et al. 1990) and they were also used as genetic markers to identify clones from a heterogenous population of isolates (Thaithong 1983, Graves et al. 1984, Thaithong et al. 1984, Webster et al. 1985, Oduola et al. 1988). However, none of the electrophoresis isoenzyme on peptidase E (PEPE) (EC. 3.4.11 or 13) was performed on the Malaysian isolates and clones.

Hence the present investigation was taken up to determine the electrophoresis variation of PEPE on a total of 48 clones, 36 of which were obtained from Malaysian *P. falciparum* isolates whilst the rest were from Africa and Thailand using the limiting dilution method (Rosario 1981).

MATERIALS AND METHODS

Eight blood samples infected only with *Plasmodium falciparum* were used in this study. Gombak A, Gombak C, ST 9, ST 12, ST 85 and ST 148 isolates were obtained from Malaysian malaria patients who were treated at the Gombak District Hospital, Selangor, Malaysia. Non-Malaysian isolates, Gambian and TGR isolates, were obtained from malaria patients in West Africa and Thailand, respectively. These were brought to the Institute for Medical Research, Kuala Lumpur, Malaysia by Professor C.R Brockelman, Mahidol University, Bangkok, Thailand. These eight isolates were then cultured *in vitro* following the method of Trager and Jensen (1976, 1977) at the Filaria and Malaria Division, Institute for Medical Research, Kuala Lumpur, and the School of Pharmaceutical Sciences, University of Science Malaysia, Minden, Penang, Malaysia.

The infected blood suspensions were dispensed aseptically 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 every 24 hours. Thin blood film slides were also prepared from each petri dish and stained with 10% Giemsa (BDH, England) for 30 minutes to monitor the % parasitaemia. Only healthy growing parasites were cloned by limiting dilution method (Rosario 1981). Diluted samples of cultured parasites,

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Table 1. PEPE isoenzyme variants for *Plasmodium falciparum* isolates and clones.

isolate/clone	*R_{fa}	*Relative mobilities of PEPE-1	PEPE-2
GOMBAK A ISOLATE	**0.06, 0.10**	**0.33, 0.50**	**0.20, 0.30**
A/C1 clone	0.10	0.33, 0.50	–
A/D3 clone	0.10	0.33, 0.50	–
A/D5 clone	0.06	–	0.20, 0.30
A/G4 clone	0.06	–	0.20, 0.30
A/H2 clone	0.10	0.33, 0.50	–
A/H7 clone	0.10	0.33, 0.50	–
GOMBAK C ISOLATE	**0.06, 0.10**	**0.33, 0.50**	**0.20, 0.30**
C/A2 clone	0.10	0.33, 0.50	–
C/B4 clone	0.10	0.33, 0.50	–
C/B7 clone	0.10	0.33, 0.50	–
C/C4 clone	0.06	–	0.20, 0.30
C/C8 clone	0.06	–	0.20, 0.30
C/C10 clone	0.10	0.33, 0.50	–
ST 9 ISOLATE	**0.06, 0.10**	**0.33, 0.50**	**0.20, 0.30**
ST 9/A4 clone	0.06	–	0.20, 0.30
ST 9/A7 clone	0.06	–	0.20, 0.30
ST 9/B5 clone	0.10	0.33, 0.50	–
ST 9/D8 clone	0.10	0.33, 0.50	–
ST 9/D9 clone	0.06	–	0.20, 0.30
ST 9/D10 clone	0.06	–	0.20, 0.30
ST 12 ISOLATE	**0.06, 0.10**	**0.33, 0.50**	**0.20, 0.30**
ST 12/A3 clone	0.06	–	0.20, 0.30
ST 12/A4 clone	0.06	–	0.20, 0.30
ST 12/D5 clone	0.10	0.33, 0.50	–
ST 12/D7 clone	0.06	–	0.20, 0.30
ST 12/E8 clone	0.10	0.33, 0.50	–
ST 12/F8 clone	0.10	0.33, 0.50	–
ST 85 ISOLATE	**0.06, 0.10**	**0.33, 0.50**	**0.20, 0.30**
ST 85/A2 clone	0.06	–	0.20, 0.30
ST 85/A5 clone	0.10	0.33, 0.50	–
ST 85/B3 clone	0.10	0.33, 0.50	–
ST 85/B4 clone	0.06	–	0.20, 0.30
ST 85/D3 clone	0.06	–	0.20, 0.30
ST 85/D7 clone	0.06	–	0.20, 0.30
ST 148 ISOLATE	**0.06, 0.10**	**0.33, 0.50**	**0.20, 0.30**
ST 148/A4 clone	0.06	–	0.20, 0.30
ST 148/A5 clone	0.06	–	0.20, 0.30
ST 148/A6 clone	0.06	–	0.20, 0.30
ST 148/A7 clone	0.10	0.33, 0.50	–
ST 148/F7 clone	0.10	0.33, 0.50	–
ST 148/F8 clone	0.10	0.33, 0.50	–
GAMBIAN ISOLATE	**0.06, 0.10**	**0.33, 0.50**	**0.20, 0.30**
Gm/A1 clone	0.06	–	0.20, 0.30
Gm/B2 clone	0.06	–	0.20, 0.30
Gm/C5 clone	0.06	–	0.20, 0.30
Gm/C6 clone	0.06	–	0.20, 0.30
Gm/H5 clone	0.10	0.33, 0.50	–
Gm/H7 clone	0.10	0.33, 0.50	–
TGR ISOLATE	**0.06, 0.10**	**0.33, 0.50**	**0.20, 0.30**
TGR/A2 clone	0.06	–	0.20, 0.30
TGR/B4 clone	0.06	–	0.20, 0.30
TGR/B7 clone	0.10	0.33, 0.50	–
TGR/C4 clone	0.06	–	0.20, 0.30
TGR/C7 clone	0.06	–	0.20, 0.30
TGR/H2 clone	0.10	0.33, 0.50	–

estimated to contain 0.5 to 1 parasite per 0.1 ml of sample at a 2% hematocrit were dispensed in a 96-well microtitre plate and later incubated at 37°C in a candle-jar. Supernatant was discarded every 2 days and replaced with fresh medium but the erythrocytes still remained at the bottom of the wells. Supernatant and half of the parasitized erythrocytes were discarded from each well after 4 days post cloning. This was followed by the addition of fresh medium and erythrocytes to each well in the microtitre plate and this was repeated for 21 days. Giemsa-stained thin blood smears were prepared from each well from 15 to 21 days and only positive microcultures were transferred to Petri dishes to yield adequate materials for isoenzyme assay. Negative wells were rechecked on one or more occasions and microcultures without any evidence of parasite by 21st day after isolation were discarded. The clones were named according to the positions in the 96-well microtitre plate.

The isoenzyme PEPE 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 thoroughly with normal saline. Parasites were freed from their host erythrocytes by incubating with 0.15% saponin (Sigma Chemical, USA) at 37°C for 20 minutes and subsequently, concentrated by centrifuging at 3,000 g for 10 minutes. The supernatant layer was discarded leaving a grey brown pellet of packed cells. Parasites were resuspended in RPMI 1640 and centrifuged at 3,000 g for 10 minutes. Supernatant was discarded and parasites were further lysed using the 1% Triton X-100 (Biorad, USA) in 1 mM EDTA (ethylenediaminetetraacetic acid) 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. Bromophenol blue, a tracker dye, was used in gel electrophoresis to confirm the status of the system.

The experimental protocol for isoenzyme assay of PEPE was precisely the same as previously reported (Sanderson et al. 1981). Relative mobilities of PEPE isoenzyme obtained from *P. falciparum* isolates and clones were characterized as a ratio of migration magnitude of PEPE isolate or clone from baseline to anodes / migration magnitude of PEPE erythrocyte (host) from baseline to anode.

RESULTS AND DISCUSSION

Generally, PEPE of these eight isolates and their respective clones were characterized relative to that of the host. PEPE from host migrated to the anode and exhibited two bands identified as the slow and fast forms which were consistent to those previously described (Sanderson et al. 1981). The values of the relative mobilities of the PEPE isolates and clones were expressed with respect to these two bands.

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

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

R_{fan} – slow PEPE variant of host = 0.2; R_{fan} – fast PEPE variant of host = 0.3; *Relative mobilities = $\frac{*R_{fa}}{R_{fan}} = \frac{*R_{fa}}{0.2}; \frac{*R_{fa}}{0.3}$

Table 1 shows PEPE isoenzyme variants for *Plasmodium. falciparum* isolates and their respective clones. The PEPE variants of eight isolates migrated anodally and contained two bands identified as PEPE-1 and PEPE-2, but did not possess a third band, PEPE-3, despite this being found in parasites obtained from Tanzanian and Congolese patients in Africa (Sanderson et al. 1981).

PEPE-1 and PEPE-2 of Gombak A, Gombak C, ST9, ST12, ST85, ST148, Gambian and TGR isolates were characterized as having relative mobilities of 0.33, 0.50 and 0.20, 0.30 respectively. However, clones prepared from these isolates exhibited only monovariant, either PEPE-1 or PEPE-2 with regards to PEPE.

From the 6 local isolates (Gombak A, Gombak C, ST9, ST12, ST85, ST148), only 18 out of 36 clones, A/C1, A/D3, A/H2, A/H7, C/A2, C/B4, C/B7, C/C10, ST 9/B5, ST 9/D8, ST 12/D5, ST 12/E8, ST 12/F8, ST 85/A5, ST 85/B3, ST 148/A7, ST 148/F7 and ST 148/F8 clones possessed PEPE-1 with relative mobilities of 0.33 and 0.50, whilst the rest, A/D5, A/G4, C/C4, C/C8, ST 9/A4, ST 9/A7, ST 9/D9, ST 9/D10, ST 12/A3, ST 12/A4, ST 12/D7, ST 85/A2, ST 85/B4, ST 85/D3, ST 85/D7, ST 148/A4, ST 148/A5 and ST 148/A6 possessed PEPE-2 with relative mobilities of 0.20, 0.30.

For the Gambian isolate, only two clones, Gm/H5 and GFor m/H7 exhibited PEPE-1 with relative mobilities of 0.33 and 0.50, whilst the rest, Gm/A1, Gm/B2, Gm/C5 and Gm/C6 clones exhibited PEPE-2 with relative mobilities of 0.20 and 0.30. Similarly, only two TGR clones, TGR/B7 and TGR/H2, showed PEPE-1 with relative mobilities of 0.33 and 0.50, whilst the rest, TGR/A2, TGR/B4, TGR/C4 and TGR/C7, showed PEPE-2 with relative mobilities of 0.20 and 0.30. Gambian and TGR isolates were incorporated into the present study mainly to serve as references of isoenzyme analysis of PEPE compared to those of the local isolates.

In general, this study shows that PEPE-3 did not exist in any of the local, West African or Thailand isolates despite that the third variant was found in African patients (Sanderson et al. 1981). Beside this, it further confirms that isolates obtained from a naturally infected host on a single occasion are genetically heterogenous with respect to isoenzyme patterns of PEPE.

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