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# Enzyme typing of *Plasmodium falciparum* from African and some other Old World countries

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## Summary

Freeze-dried and freshly cultured isolates of *Plasmodium falciparum* from different countries have been examined for variation in six enzymes, GPI, PGD, LDH, GDH, ADA and PEPE, using starch gel electrophoresis. Variant forms of each enzyme occur. For most enzymes, similar forms occur in isolates from different parts of the world, although differences in frequencies of each form are found. LDH-2 has been found only in African isolates. PEPE variants vary markedly in their frequency between East and West Africa, although further work is needed to confirm this finding.

## Introduction

Enzyme electrophoresis has proved to be a valuable technique for identifying genetic differences between micro-organisms. In malaria the method has been applied most extensively to the parasites infecting rodents (CARTER, 1978). This work has shown that each of the four species of these parasites can be differentiated by their enzymes, and that distinct sub-species occur in different regions of Africa. Some enzyme work was carried out on freeze-dried samples of *Plasmodium falciparum* from The Gambia (CARTER & MCGREGOR, 1973) and Tanzania (CARTER & VOLLER, 1975) which suggested that in these two countries the organisms were genetically similar. This work was limited, however, because only four enzymes had proved suitable for electrophoresis work with malaria, and because it was difficult to obtain sufficient quantitites of parasite material. In this and the following paper we report the results of a more extensive survey of enzyme variation in *P. falciparum* using more enzymes than were previously available and examining isolates from a larger number of countries by making use of *in vitro* cultures of parasites.

## Material and Methods

*Sources and preparation of parasite samples*

The parasite material was of two types, (a) freeze-dried blood or placental material and (b) fresh parasites from *in vitro* cultures.

(a) *Freeze-dried samples*: These were obtained from The Gambia, Tanzania and Congo (Brazzaville). Gambian isolates were obtained by Dr. R. Carter in 1973, and were of venous blood from 54 infected patients, which was cultured for 24 hours to produce trophozoites and schizonts. Tanzanian isolates, collected by Dr. Carter in 1975, were of placental blood from eight patients. Isolates from The Congo, collected by Dr. J-F. Molez in 1979, were of placental blood from six patients. All samples were freeze-dried in their countries of origin before being sent to Edinburgh. They were stored under vacuum at −20°C until required for electrophoresis.

Gambian and Tanzanian isolates have been examined previously for variation in the enzymes GPI, PGD and LDH (CARTER & MCGREGOR, 1973; CARTER & VOLLER, 1975), and those from The Gambia additionally for GDH (CARTER & WALLIKER, 1977).

(b) *Cultured parasites*: 15 cultured parasite lines from ten countries have been kindly made available to us by workers in other laboratories (see Table II). Cultures of each line were sent to Edinburgh, either fresh or deep-frozen in liquid nitrogen.

**Table I—Distribution of enzyme types in freeze-dried samples of *P. falciparum* from three African countries. The figure in each column represent the number of isolates exhibiting the enzyme forms shown**

| | GPI | | | PGD | | | LDH | | | GDH | | | ADA | | | PEPE | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Origin | 1 | 2 | 1+2 | 1 | 2 | 3 | 1 | 2 | 1+2 | 1 | 2 | 1+3 | 1 | 2 | 1+2 | 1 | 2 | 3 |
| Gambia | 23 | 6 | 25 | 46 | 2 | 1 | 38 | 3 | 13 | 51 | 0 | 1 | 49 | 2 | 2 | 52 | 0 | 0 |
| Tanzania | 4 | 1 | 3 | 5 | 0 | 1 | 6 | 0 | 2 | 5 | 2 | 0 | 1 | 1 | 6 | 0 | 6 | 2 |
| Congo | 3 | 3 | 2 | 4 | 0 | 0 | 6 | 2 | 0 | 8 | 0 | 0 | 6 | 1 | 1 | 6 | 0 | 1 |

**Table II—Distribution of enzyme types in 15 cultured isolates of *P. falciparum* + =presence of enzyme form. − = absence of enzyme form**

| Origin and Isolate No. | GPI | | | PGD | | | LDH | | | GDH | | | ADA | | | PEPE | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 1+2 | 1 | 2 | 3 | 1 | 2 | 1+2 | 1 | 2 | 1+3 | 1 | 2 | 1+2 | 1 | 2 | 3 |
| Gambia FCR-3 | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| Gambia R-FCR-3 | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| Gambia FCR-4 | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| Gambia G1 | − | + | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| Gambia M17 | − | − | + | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| Senegal M13 | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| Ghana NF7 | − | + | − | + | − | − | + | − | − | + | − | − | + | − | − | − | + | − |
| Zaire Z90 | − | + | − | + | − | − | + | − | − | + | − | − | + | − | − | −? | + | − |
| Tanzania NF56 | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| E. Africa M19 | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | −? | + | − |
| Viet Nam FCR-1 | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| China M21 | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| Indonesia NF58 | − | + | − | + | − | − | + | − | − | + | − | − | + | − | − | − | + | − |
| Netherlands NF36 | − | − | + | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| Netherlands NF54 | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |

Cultures FCR-1, FCR-3, RFCR-3 (chloroquine-resistant line derived from FCR-3) and FCR-4 were sent by Dr. W. Trager, Rockefeller University, New York, USA. Cultures G1 and Z90 were supplied by Dr. R. Carter, National Institutes of Health, Bethesda, USA. Cultures M13, M17, M19 and M21 were supplied by Dr. L. Perrin, University of Geneva, Switzerland. Cultures NF7, NF36, NF54, NF56 and NF58 were sent by J. H. E. Th. Meuwissen, Nijmegen, The Netherlands. NF7, NF56 and NF58 were isolated from patients in the Netherlands who had recently returned from tropical countries. NF36 and NF54 however were isolated from patients in the Netherlands who had never visited tropical countries.

On arrival they were established in continuous culture using the petri-dish method of TRAGER & JENSEN (1976).

For enzyme work parasites were freed from their host cells by saponin lysis and concentrated by centrifugation. Cultures exhibiting a parasitaemia of at least 5% were harvested from three petri-dishes (60 × 15 mm) and centrifuged at 500 *g* for 10 min. The supernatant was discarded and the packed red cells incubated with 0·15% saponin in complete RPMI 1640 medium (1·5 volumes saponin solution : 1 volume packed cells) at 37°C for 20 min. The material was then resuspended in ten volumes of complete RPMI medium and, after thorough mixing, centrifuged at 3,000*g* for 10 min. The supernatant and red cell ghost layer were discarded, leaving a grey-brown pellet of packed parasites. Although some host-cell material remained, principally red cell membranes as well as unlysed red cells, this procedure was normally effective in removing nearly all host enzyme activity. The parasite material, which was sufficient for about ten electrophoreses, was either freeze-thawed and used immediately for enzyme work or stored in sealed glass capillaries under liquid nitrogen until required.

*Enzyme electrophoresis*

Six enzymes were studied using starch gel electrophoresis.

(i) Glucose phosphate isomerase (GPI) (EC 5.3.1 9).
(ii) 6-phosphogluconate dehydrogenase (PGD) (EC 1.1.1.44).
(iii) Lactate dehydrogenase (LDH) (EC 1.1.1.27).
(iv) NADP-dependent glutamate dehydrogenase (GDH) (EC 1.4.1.4).
(v) Adenosine deaminase (ADA) (EC 3.5.4.4).
(vi) Peptidase E (PEPE) (EC 3.4.11 or 13).

The preparation of starch gels and conditions of electrophoresis for GPI, LDH and GDH were carried out basically as described by CARTER (1978) using his methods (a) for GPI and LDH. PGD and ADA were studied using the methods of HARRIS & HOPKINSON (1976). For PGD the samples were placed in slots approximately half way up the gel rather than near the cathodal end. This was because an inactive area migrated up the gel after some hours of electrophoresis and sometimes overtook the parasite enzyme if samples were inserted too far back. When PGD was developing, frequent checks were made while the bands were developing because host superoxide dismutase which has a similar mobility to PGD-1, sometimes destained bands which were beginning to form.

PEPE was examined as in HARRIS & HOPKINSON (1976) with the following modifications. The electrode buffer for this enzyme was 0·225M Tris/0·22M sodium dihydrogen orthophosphate, pH 8·0. The gel buffer was a one in 20 dilution of the electrode buffer. Electrophoresis was carried out at 5 v/cm for 14 hours. The enzyme assay solution consisted of 25 ml 0·02M citrate phosphate buffer pH 5·5; 20 mg of the peptide substrate (either phenylalanyl-glycyl-phenylalanyl--glycine or leucine nitroanilide); 6 mg snake venom amino-acid oxidase (Sigma Chemical Co. Ltd.

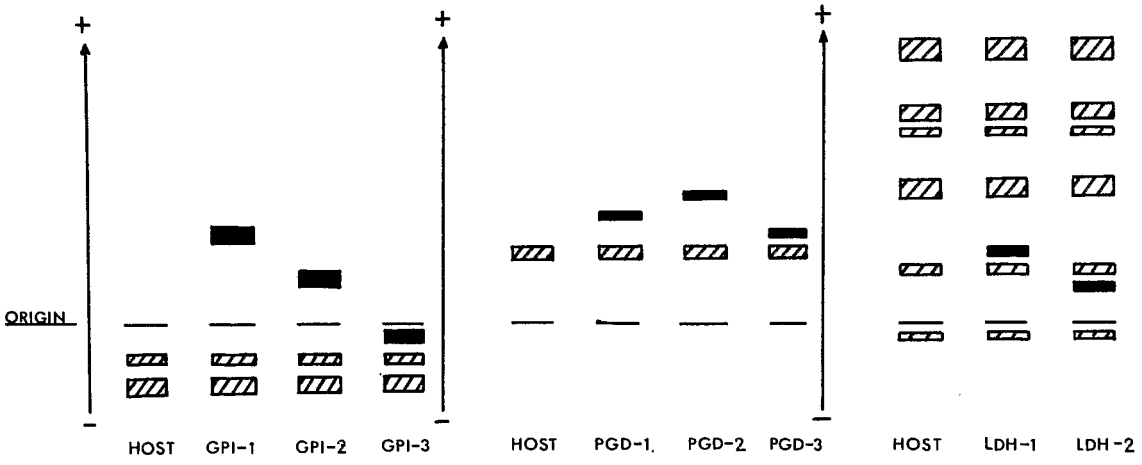


Fig. 1. Electrophoretic forms of GPI, PGD and LDH in *P. falciparum*.

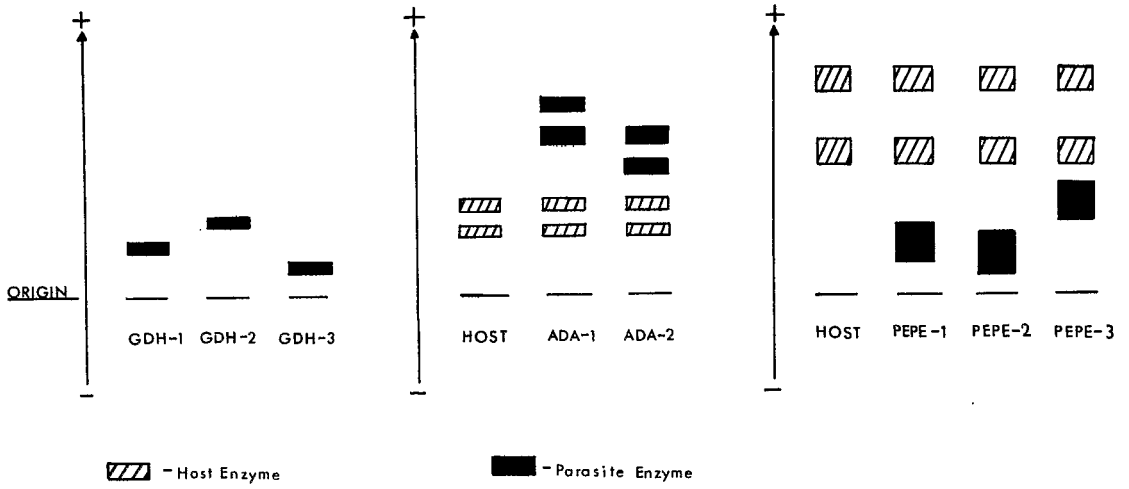


Fig. 2. Electrophoretic forms of GDH, ADA and PEPE in *P. falciparum*.

product number V-6875); 4 mg peroxidase (80 units/mg); and 20 mg manganese chloride. This solution was warmed to 45°C, mixed with 25ml 2% agar also at 45°C and poured on to the cut surface of the gel. After the agar had solidified a piece of Whatman No.1 filter paper, soaked in a solution of 3-amino-9-ethyl carbazole (12·5 mg amino ethyl carbazole in 2 ml ethanol) was laid on the surface. PEPE activity was indicated by gradual develop-

starch gel electrophoresis described, parasite enzymes could be clearly distinguished from those of the host. In the case of GDH, no host enzyme occurs.

Two forms of GPI and LDH and three of PGD were found, as described previously by CARTER & MCGREGOR (1973). A third form of GPI, GPI-3, has so far only been detected in one isolate of *P. falciparum* from Thailand (see following

The Gambia, Tanzania and Congo. The total numbers shown for each enzyme vary because a number of isolates failed to give bands for certain enzymes. Table II shows the enzyme forms of 15 cultured isolates of *P. falciparum* from a variety of Old World countries.

## Discussion

In this work we have made use of both freeze-dried and fresh cultured parasites for enzyme electrophoresis. We have found that freshly prepared cultured parasites, either put directly on to gels or after storage under liquid nitrogen showed much better enzyme activity than freeze-dried material stored under vacuum at −20°C. GPI especially suffers after several years of freeze-dried storage, the bands of activity becoming less discernible. The freeze-dried material used in this work also suffered from the disadvantage that no further living samples of the isolates were available. It was thus impossible to check whether rare variant forms of some enzymes were due to contamination by other malaria species or even due to other organisms.

In the earlier work on enzyme typing of *P. falciparum,* CARTER & VOLLER (1975) found the same electrophoretic forms of GPI, PGD and LDH among isolates from The Gambia and Tanzania. Furthermore, the frequencies of the different forms of the most variable enzymes GPI and LDH were similar in each country, indicating that the parasites were genetically similar in each region.

The present work extends the range of enzymes available to include GDH, ADA and PEPE. Of these GDH has previously been used extensively in studies of rodent malaria, but only to a limited extent with *P. falciparum* (CARTER & WALLIKER, 1977). In the present studies a single form of GDH (GDH-1) was found in almost all isolates. A second form (GDH-2) occurred in two Tanzanian samples and a third form (GDH-3) in one Gambian sample.

Two electrophoretically distinct forms of ADA were found. The more common form, ADA-1, occurred in 51 out of 53 freeze-dried Gambian isolates showing activity for this enzyme, seven out of eight Tanzanian and seven out of eight Congolese isolates. ADA-1 was the only form of this enzyme detected in the 15 cultured samples. ADA-2 occurred most commonly in Tanzania, one of the eight isolates showing this form exclusively, whereas six showed ADA-1 and ADA-2. In the Gambia only four isolates showed ADA-2, either alone or in combination with ADA-1, and in the Congo, two isolates possessed ADA-2.

The parasite bands of PEPE were large, diffuse and slow to develop. As shown in Fig. 2, three forms have been recognized, denoted PEPE-1, PEPE-2 and PEPE-3. Of these PEPE-1 and PEPE-2 possess only a slight difference in mobility with considerable overlap between the bands. By lowering the concentration of parasite material inserted into the gel, the edges of the bands contract towards the centre. Thus unless the parasite concentration of each sample is similar it is difficult to distinguish these two forms. For this enzyme, therefore, it is important to examine each sample several times using different concentrations and carefully selected controls before definite identification of PEPE-1 and PEPE-2 can be made. While most samples examined could be classified with some confidence, the PEPE type of a few isolates must be regarded as provisional at present (see Table II).

The distribution of the three forms of PEPE appears to show some regional variation. Among the freeze-dried samples, Gambian isolates possessed only PEPE-1, Tanzanian only PEPE-2 and PEPE-3 and Congolese only PEPE-1 and PEPE-3. A cultured isolate from a patient in the Netherlands, recently returned from Tanzania (NF 56), possessed PEPE-1 as did most other cultured samples. PEPE-2 occurred in isolates from Ghana (NF7) and Indonesia (NF58) and was provisionally identified in those from Zaire (Z90) and East Africa (M19).

With regard to the forms of GPI, PGD and LDH originally described by CARTER & MCGREGOR (1973), the present results confirm the frequencies of the variant forms in The Gambia and Tanzania. GPI-1 and GPI-2 are also found among the various cultured isolates from other countries, as well as in freeze-dried samples from Congo. Parasites exhibiting the form of LDH denoted LDH-2 have been found only in freeze-dried isolates from The Gambia, Tanzania and Congo. No cultured samples from Africa or any other country show this form. The rare forms of PGD, PGD-2 and PGD-3 have been found so far only in The Gambia.

In contrast to rodent malaria species, isolates of *P. falciparum* from different regions of the world appear to possess remarkably similar enzyme forms. There is, however, a difference in frequency of the enzyme variants in different regions. LDH-2, for example, occurs in The Gambia, Tanzania and Congo, but so far has not been detected in isolates from other countries. The different PEPE variants also vary markedly in their frequency, particularly between East and West Africa. This observation needs to be confirmed by further work, particularly in view of the similarities between PEPE-1 and PEPE-2 as revealed by our system of electrophoresis. We also need to examine more isolates from other parts of the world, especially from South America, to help complete the world wide picture of enzyme variation in *P. falciparum.*

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