

POLYMERASE CHAIN REACTION TO DETERMINE FREQUENCY OF PYRIMETHAMINE (DARAPRIM) - RESISTANT *PLASMODIUM FALCIPARUM* IN VOLTA AND GREATER ACCRA REGIONS OF GHANA

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Abstract

Treatment of malaria is being hampered by the emergence of drug resistant strains of malaria parasites. In this study, mutation specific polymerase chain reaction assays using 3'-mismatched oligonucleotide primers which annealed to the wild or mutated parasites' gene encoding dihydrofolate reductase-thymidylate synthase (DHFR-TS) were used to survey *P. falciparum* strains in two southern regions of Ghana (Volta and Greater Accra regions). Mutations were identified directly from blood samples obtained from patients attending out patient departments in hospitals. A DNA amplification product of 337 base pairs was obtained when reaction products were analysed by electrophoresis. Of 162 smear positive samples collected, 13 (8.0%) contained the Asn-108 codon AAC that confers pyrimethamine resistance, 139 (85.8%) samples contained only the wild-type Ser-108 codon AGC. Out of the 13 pyrimethamine resistant cases, 10 were found in samples obtained from the Volta Region while the rest (3) were from Korle-Bu Teaching Hospital in the Greater Accra Region. No PCR product was found in 10 (6.2%) of 162 samples. After second (nested) PCR, only one sample showed amplified product. Contamination was negligible. PCR amplification of the DHFR-TS could be used to determine frequency of pyrimethamine resistant *P. falciparum* strains in malaria blood.

Résumé

MAK-MENSAH, E. E., LARBI, J. A. & GYANG, F. N.: Réaction en chaîne par polymérase pour déterminer la fréquence de pyriméthamine (daraprim) - Plasmodium falciparum résistant dans les régions de Volta et de Greater Accra du Ghana. Le traitement du paludisme est en cours d'être gêné par l'émergence de souches de parasites de la malaria résistantes au médicament. Dans cette étude les essais de réaction en chaîne par polymérase (RCP) de mutation spécifique utilisant les primers oligonucléotide de 3'-mal assortis qui recusaient le gène de parasite muté ou naturel le synthase thymidylate-dihydrofolate réductase (ST-DHFR) encodant étaient utilisés pour enquêter sur les souches de *P. falciparum* dans deux régions au sud du Ghana (les régions de Volta et de Greater Accra). Les mutations étaient identifiées directement dans les prélèvements de sang obtenus de malades qui visitent les hôpitaux pour les services de consultation externe. Un produit d'amplification d'ADN de 337 paire de base était obtenu quand les produits de réactions étaient analysés par électrophorèse. De 162 frottis positifs prélevés, 13 (8.0%) contenait Asn-108 Codon ACC qui confère la résistance pyriméthamine, 139 (85.8%) prélèvements contenait seulement le type-sauvage Ser-108 Codon AGC.

Introduction

Pyrimethamine is a dihydrofolate reductase (DHFR) inhibitor and, hence, an antifolate antimalarial (Zolg, Chen & Plitt, 1990). Pyrimethamine, capable of clearing the pre-erythrocytic stages of the malaria parasite, is used for malaria prophylaxis. Recent observations indicate that strains of the parasites

resistant to combinations of sulfones or sulfonamides and DHFR inhibitors (Fansidar) have appeared in southeast Asia, South America (Cortese *et al.*, 2002), Africa (Roper *et al.*, 2003) and Kumasi, Ghana (Marks *et al.*, 2005). Resistance of *P. falciparum* to pyrimethamine alone has not been reported in southern Ghana. Documentation of this

resistance is, therefore, necessary.

Resistance of *P. falciparum* to pyrimethamine could result from a number of mechanisms including point mutation of DHFR-TS gene (Hapuarachchi *et al.*, 2006; Gregson & Plowe, 2005; Gyang, Peterson & Wellems, 1992), amplification or increased production of DHFR enzyme (Thaithong *et al.*, 2001) and decreased activity of structurally altered DHFR enzyme (Walter, 1986). The resistance may result from drug pressure or extensive use of any antimalarial (Gregson & Plowe, 2005), subcurative doses and/or self-medication (Neequaye, 1989). Resistance may also arise from gross misuse and abuse of drugs, partly due to lack of control and over prescription (Riekmann, 1990), and partly due to use of pyrimethamine for malaria prophylaxis (Marks *et al.*, 2005).

Some methods of determining point mutations, such as restriction fragment length polymorphism analysis, cloning and sequencing, or hybridization with sequence-specific oligonucleotides and microscopy are time consuming and labour intensive (Peterson *et al.*, 1991). Polymerase chain reaction (PCR) offers a quick, easy and sensitive way of detecting low levels of parasite DNA (Sethabutr *et al.*, 1992). PCR is rapid and efficient for detection of point mutations (Newton *et al.*, 1989; Gyang, Peterson & Wellems, 1992).

The point mutation is in the nucleotide sequence resulting in a change from Ser to Asn in the amino acid in position 108 of DHFR-TS gene. The specific codon sequence of this mutation has not been found to vary among resistant parasites (Gyang, Paterson & Wellems, 1992). Resistant parasites could therefore be detected by using mutation specific primers with 3'- mismatches in a PCR assay (Gyang, Paterson & Wellems, 1992).

The study seeks to determine frequency of resistant *P. falciparum* to pyrimethamine and pyrimethamine-containing drugs in southern Ghana so as to obtain additional information about the effectiveness, or otherwise, of these drugs. This will help clinicians to prescribe alternative but

equally appropriate drug combinations in order to provide more effective prevention and control of *falciparum* infection.

Experimental

Patients who showed signs of fever, 72 h of illness, temperature $\geq 38.6^{\circ}\text{C}$ and parasitaemias (+1 to +4) were randomly selected. Parasitized blood samples (1 ml) were drawn into sterile tubes with anticoagulant acid citrate dextrose (ACD) by venipuncture from the patients. Informed consent was obtained before blood extraction. Non-parasitized blood samples were also collected. Samples were then stored at -20°C . All samples were collected upon permission from the Senior Medical Officers in charge of the hospitals.

Blood processing

Frozen blood sample was thawed at 37°C , and 200 μl portion was dispensed into sterile 12-ml centrifuge plastic tubes with caps. Ten millilitres ice cold 5 mM sodium phosphate buffer pH 8 (SP8) was added, vortexed for 30 s to suspend the cells and centrifuged at 6000 g for 10 min at 4°C . The supernatant was discarded and the process (washing) repeated 3–5 times until the pellet became clear. Final supernatant was discarded and the pellet resuspended in 200 μl sterilized double distilled water (SDDW), boiled for 10 min, and centrifuged at 6000 g for 10 min. The supernatant containing the DNA was removed into a sterile eppendorf tube and stored at -20°C (Foley, Ranford Cartwright & Babiker, 1992).

PCR and electrophoresis

About 2–10 μl of DNA preparations from each blood samples were amplified by PCR using mutation specific oligonucleotide primers DIA 3 5'-GAA-TGC-TTT-CCC-AGC-3', and DIA 12 5'-GGA-AAT-GCT-CTT-TCC-CAT-T-3', each with a common primer SP1 5'-ATG-ATG-GAA-CAA-GTC-TGC-GAT-3'. The primers (Promega) were designed from the *P. falciparum* DHFR-TS gene sequence (Bzik *et al.*, 1987) adjusted for the point mutation (Peterson

et al., 1991).

PCR method for detection of the point mutation at position 108 of DHFR (Ser-108 →Asn-108) is described previously (Zolg, Chen & Plitt, 1990 and Peterson *et al.*, 1991). DNA templates (8-10 µg) and 1.5 units *Taq* DNA polymerase (Promega) were mixed with buffer (Zolg, Chen & Plitt, 1990) in 50 µl total volume. About 50 µl mineral oil was layered on the PCR mix in 1.5 ml sterile eppendorf tubes to prevent evaporation. The eppendorf tubes were inserted into the heat block holes of the thermocycler (Technique PH2-Dri Block[®]) containing drops (1 in each hole) of mineral oil to ensure thermal contact. Forty cycles of amplification were employed, each consisting of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and primer extension at 74 °C for 45 s. DNA templates from parasite clones 3D7 (pyrimethamine-sensitive; pyr^s) and HB3 (pyrimethamine-resistant; pyr^r) were used as controls, and also to optimize the PCR assays (Peterson, Millions & Willems, 1990).

Second or nested PCR reactions were carried out by adding 2 µl of the 50 µl PCR product (as templates) to 48 µl PCR reaction mix containing PCR buffer, primers, dNTPs, *Taq* DNA polymerase and SDDW and amplified for 32 cycles at the same temperatures. Twenty micro litre PCR products were mixed with 2.5 µl gel-loading buffer (0.25 g bromophenolblue, 4 g sucrose and 10 ml SDDW), carefully loaded into the slots of 1%/2% ethidium bromide containing agarose nusieve composite gel and electrophoresis done using 0.5× TBE buffer. The first slot was loaded with DNA molecular weight marker (Sigma). Photographs of the gels were taken after the electrophoresis.

Results

The primer DIA 3 specifically amplified the DHFR sequence containing the wild-type Ser-108 (AGC) codon while DIA 12 was specific for the mutant Asn-108 (AAC) codon (Zolg, Chen & Plitt, 1990). These primers gave 337 base pair products (Fig. 1 and 2) as obtained by Peterson *et al.*, 1991. A single base exchange in position 323 of the DHFR coding region (Zolg, Chen & Plitt, 1990) changed

Ser-108 in pyr^s (3D7) to Asn-108 in pyr^r (HB3).

Discussion

PCR detection of parasites in the blood samples was 93.8 per cent (152 out of 162 samples) compared to microscope detection. PCR failed to detect parasites in 10 malaria blood samples out of a total of 162 samples (Table 1) giving a percentage failure of 6.2 per cent (10 out of 162 samples). This observation is consistent with the reports that PCR specimens are occasionally negative (5-7%) in samples in which organisms were found microscopically (Sethabutr *et al.*, 1992). Some samples may have threonine rather than serine or asparagine at position 108 in the DHFR-TS protein (Peterson, Millions & Willems, 1990) to which the primers used in the study could not anneal perfectly and could avoid detection. Of the 152 samples, 139 samples contained pyr^s while 13 had pyr^r (Table 1). The frequency of Asn-108 mutation (pyr^r) in the samples (Table 1) was 8 per cent (13 out of 152 samples).

Higher percentage of pyrimethamine-resistance 6.2 per cent (10 out of 162 samples) in the Volta Region than 1.8 per cent (3 out of 162 samples) in the Greater Accra Region (Table 1) may be due to self-medication and inaccurate dosage by patients (Neequaye, 1989) or drug pressure (Gregson & Plowe, 2005) through the use of pyrimethamine combinations or alone for malaria prophylaxis (Marks *et al.*, 2005; Riekman, 1990). These explanations may account for the different percentages of pyrimethamine-resistance seen in all communities: Sogakofe 10 per cent, Dzodze 25 per cent and Aflao 16.6 per cent (Table 1).

Point mutations were detected by PCR assays that employed the primers DIA 3 and DIA 12 against a common primer SP 1. Fig. 1 and 2 are representative of what was obtained when the two pair of primers were used for each of the samples, followed by agarose gel electrophoresis. Primer pair DIA 3/SP 1 produced amplified products with KB1 and KB2 (Fig. 1, lanes 3 and 4) while no product was seen in Fig. 1, lanes 6 and 7, with

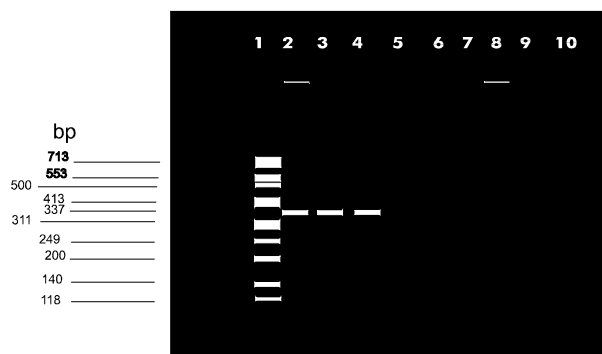


Fig. 1. PCR products of *P. falciparum* isolates and DIA3/SPI primer pair specific for Ser-108 on gel.

Legend: Lane 1 contained marker (ϕ X 174 RFI DNA digested with Hinf I). Lanes 2-7 (positive control), KB1, KB2, HB3 (negative control), KB3 and KB4 respectively, lane 8, HB3 (template control - no primer) and lane 9 (no template), lane 10 had no PCR product.

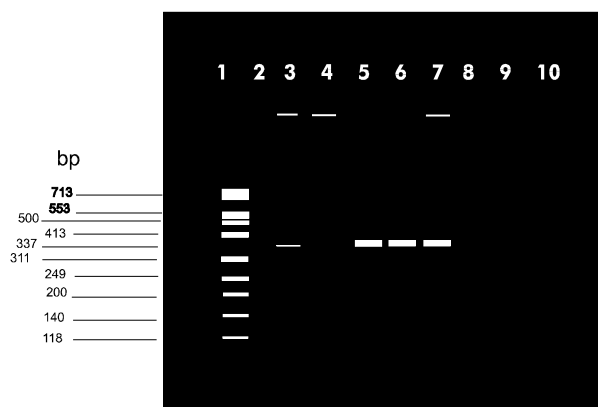


Fig. 2. PCR products of *P. falciparum* isolates and DIA12/SPI primer pair specific for Asn-108 on gel.

Legend: Lane 1 contained marker (ϕ X 174 RFI DNA digested with Hinf I). Lanes 2-7 contained DNA from 3D7 (negative control), KB1, KB2, HB3 (positive control), KB4 respectively. Lane 8 contained water control (no template). Lanes 9 and 10 had no PCR product.

KB3 and KB4 as templates. KB1 and KB2, hence, contained Ser-108 codons AGC which were complementary at their 3' ends to the diagnostic primer DIA 3 used. KB3 and KB4 may not contain Ser-108 codon to perfectly match with DIA 3 for PCR amplification.

No PCR product was seen in Fig. 1, lanes 8 and 9 because lane 8 was loaded with a PCR reaction product of 3D7 as template with no primers (template control) while lane 9 (water control) lacked template. These reactions were used to eliminate mispriming and contamination of PCR

TABLE 1
Assessment of sensitivity of *P. falciparum* to pyrimethamine

Communities	Samples	pyr ^s	Sensitivity (%)	pyr ^r	Resistance (%)	PNS
KB	59	56	94.9	3	5.1	0
L	6	6	100.0	0	0.0	0
K	11	6	54.5	0	0.0	5
D	4	1	25.0	1	25.0	2
A	20	18	90.0	2	10.0	0
B	16	12	75.0	2	12.5	2
S	40	36	90.0	4	10.0	0
AF	6	4	66.6	1	16.6	1
Total	162	139		13		10

pyr^s%=Pyrimethamine-sensitive *P. falciparum* isolates.

pyr^r%=Pyrimethamine-resistant *P. falciparum* isolates.

PNS=PCR negative samples. KB, L, K, D represent Korle-Bu Teaching Hospital, University Hospital, Legon, Keta Government Hospital and St Anthony's Hospital, Dzodze, respectively, while A stands for E. P. Church Hospital, Adidome, B for Catholic Hospital, Bator, and S and AF represent Sogakofe Health Centre, Comboni Clinic and New Hope Clinic, Aflao, respectively.

TABLE 2
Nested PCR

Communities	PNS	PPS
K	5	1
D	2	0
B	2	0
AF	1	0

Nested PCR reactions were performed with DNA templates which did not produce amplified products in the first PCR reactions. PPS-PCR positive samples. Only one DNA template (sample) produced PCR product after the second amplification. This shows that contamination of the PCR assays is negligible.

assays with DNA from sources other than from the *P. falciparum* isolates. High molecular weight fragment seen in Fig. 1, lane 8 is a parasite DNA since Fig.1, lane 2 containing 3D7 DNA as template also gave similar fragment size.

Fig. 2, lane 5 contained PCR product of HB3 with DIA 12/SP1 primer pair specific for Asn-108

codon AAC (positive control). Since KB3 and KB4 also gave amplified products with DIA 12/SP 1, it can be concluded that their template DNAs contained Asn-108 (Fig. 2, lanes 6 and 7). No band was seen in Fig. 2, lane 4 loaded with KB2 DNA - DIA 12/SPI PCR reaction product. It might be that KB2 lacked Asn-108 codon.

A faint band is seen in Fig. 2, lane 3 loaded with PCR product of KB1 and DIA 12/SP I. The same KB1 gave amplified product with DIA 3/SP 1 (Fig.1, lane 3). It may be probable that pyr^s and pyr^r isolates were present in the KB1 sample. This double infection supports the reports that mixture of parasites with different drug responses

occur commonly in natural infections (Webster *et al.*, 1985). High molecular weight fragments seen in Fig. 2, lanes 3, 4 and 7 may be parasite DNAs.

Conclusion

Asn-108 mutation in *P. falciparum* DHFR-TS gene which confers parasites' resistance to pyrimethamine occurs in Volta and Greater Accra regions of Ghana at a frequency of 8 per cent.

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