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PROGRESS REPORT

DIAGNOSIS OF DRUG RESISTANT PLASMODIUM FALCIPARUM BY DNA PROBES

Project No. C8-126

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Diagnosis of Drug Resistant Plasmodium falciparum by DNA Probes

Objective

Correlate drug resistance with DNA markers. Two markers were investigated: a) Pattern of methylation b) RFLP sites. These markers were followed by using two probes: BS 11, a repetitive sequence (kindly provided by Dr. J. S. Scaife, 1) and pHBE 6.1, a clone containing part of the DHFR-TS gene and a 2.3 Kbp fragment localized upstream to the gene (kindly provided by Dr. T. E. Wellems).

Results

The two isoschizomers Msp I and Hpa II, recognizing the sequence CCGG were used to investigate the state of cytosine methylation. While Msp I is indifferent to the state of methylation of the internal cytosine in the recognition sequence, Hpa II will not cut this sequence when the internal cytosine residue is methylated. The results of a comparative Msp I/Hpa II restriction analysis is presented in Fig. 1. As can be seen, (panels A and B), Msp I and Hpa II yield identical patterns. However, it can also be

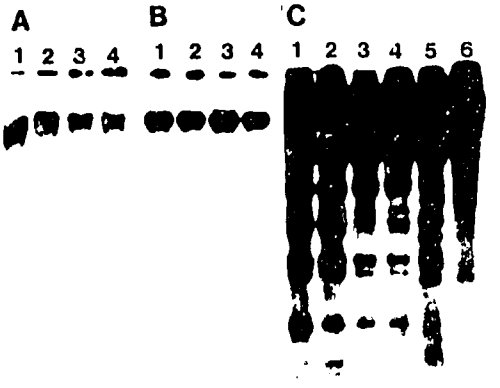


Fig. 1: Msp I/Hpa II analysis: DNA (3ug) was digested with Msp I (lanes 1,3,5) and Hpa II (lanes 2,4,6) and analysed by Southern blotting, using BS 11 (A,C) and pHBE 6.1 (B) as probes. In panel C, DNA was cut also with Hind III. DNA used was from strains FCR-3 (lanes 1,2), ItG-2G1 (lanes 3,4) and D6 (lanes 5,6).

observed that these enzymes cut the DNA very scarcely, yielding high molecular weight fragments which are poorly separated on the gel, and thus not allowing a definite conclusion. In order to overcome this problem, analysis of the state of cytosine methylation of regions recognized by the probe BS 11 was determined on DNA cut simultaneously with Msp I (or Hpa II) and Hind III. As can be seen in Fig. 1 (panel C), the hybridization patterns obtained by the two enzymes are identical, indicating the lack of methylated cytosine in CCGG sites at this region.

Additional restriction enzymes which can detect methylated bases were used. Sau3A I, Mbo I and Dpn I are isoschizomers recognizing the sequence GATC. Dpn I will cut this sequence only if the adenosine residue is methylated, while Mbo I will cut it only if it is not methylated. Sau3A I activity is indifferent to the state of methylation of the adenosine residue. In addition, Sau3A I and Mbo I can serve to detect methylation of the terminal cytosine residue in the sequence GATC, as the former will not be able to cut this site if this residue is methylated while the latter will not be affected. Analysis of this type is presented in Fig. 2 for the repeated sequence and in Fig. 4 for the DHFR-TS gene.

Fig. 2: Sau3A I/Mbo I/Dpn I analysis of repetitive sequences. DNA (3 ug) was digested with Sau3A I (lane 1), Mbo I (lane 2) and Dpn I (lane 3) and analysed by Southern blotting using BS 11 as a probe. DNA analysed were from a=FCR-3; b=ItG-2G1; c=D6 and d=3A-B4 P. falciparum strains. The first three strains are pyrimethamine sensitive. The fourth (3A-B4) is a resistant strain.

Fig. 2 shows identical Sau3A I and Mbo I restriction patterns (lanes 1 vs 2) for each of the four different strains tested, indicating that these regions do not contain neither methylated cytosines nor methylated adenines. The fact that no digestion is observed by DpnI (lane 3), support the notion about the lack of adenine methylation.

A similar approach was undertaken for the analysis of the DHFR-TS gene and its neighbouring regions. As the sequence of this gene is known, the size of some of the fragments obtained after restriction is known (see Fig. 3).

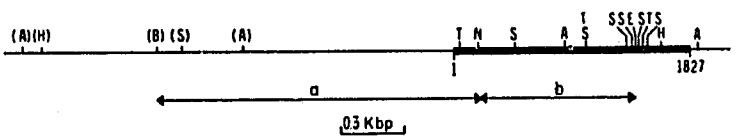
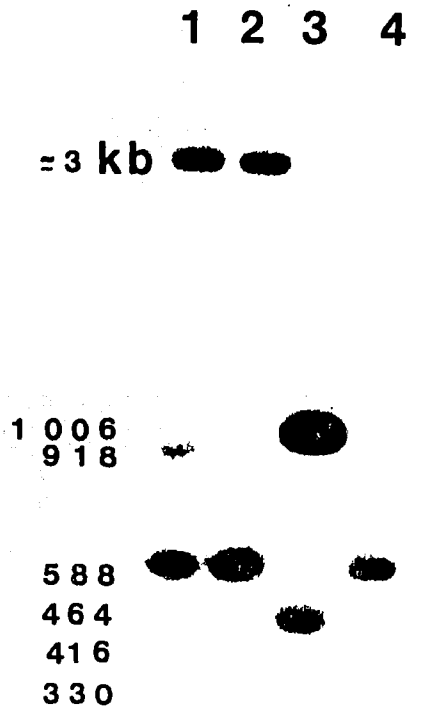


Fig. 3: Restriction map of P. falciparum DHFR-TS gene and its neighbouring regions. Heavy line presents the coding region and the numbers below indicate the first (1) and last (1827) nucleotide of the gene. Restriction sites in this region were determined according to the known sequence of the gene (2,3). Restriction sites in the region upstream to the 5' end of the gene were determined in this study. The probe used for this study is indicated below the restriction map. The pHBE 6.1 probe was cleaved by Nco I producing a Bcl I-Nco I fragment (a) of approx. 2500 bp and a 1279 bp Nco I-Eco RI fragment (b). Symbols of restriction enzymes (site of cleavage): A=Acc I (811, 1905, -1700,-3450), B=Bcl I (-2300); H=Hha I (1621,-3300); N=Nco I (139); S=Sau3A I (442, 1030, 1360, 1396, 1441, 1509, -2150); T=Taq I (26, 1032, 1496).

The results of such an experiment are shown in Fig. 4. As evident from both panels of the figure, all bands appearing in the Mbo I digests appear also in the Sau3A I digests and DpnI does not cut the DNA. These results indicate that this region also does not contain methylated adenine residues. However, the Sau3A I digest in panel A (lane 1) contains a 918 fragment which does not appear in the Mbo I digest. Prolonged incubation times, excess of enzymes and kinetic studies indicate that this fragment is

Fig. 4: Sau3A I/Mbo I/Dpn I analysis of DHFR-TS gene and neighbouring regions. See legend to Fig. 2. A and B denote hybridization with probe b and a respectively.

not the result of a decreased activity of the enzyme toward a certain GATC site in this region. Thus, it seems that this fragment may be the result of the inability of Sau3A I to cut a site which contains a methylated cytosine. As the exact positions of the GATC sites in the gene are known, the most probable site whose methylation can produce a fragment similar in size to the one observed in the experiment, is the site at position 1030. As part of this site comprises part of an adjacent Taq I site (-GATCGA-), the treatment of a Sau3A I digest with Taq I should abolish this band. As can be seen in Fig. 5, this is the case. The 918 bp fragment is cut by Taq I, yielding the expected fragments (lane 4). It should be noted that this site is only partially methylated as the 588 bp and 330 bp fragments (lane 1 in Fig. 4A and Fig. 5) are the result of this site being cut. Hha I activity is also sensitive to the presence of a methylated cytosine in its recognition site. One such site is found in the DHFR-TS gene (at position 1621). This site is flanked from both sides by two Acc I sites 1094 bp apart (at positions 811 and 1905). If the Hha site is methylated, the 1094 bp fragment should be observed also in a mixed digestion of DNA with these two enzymes. As Fig. 6A indicates, this is not the case.



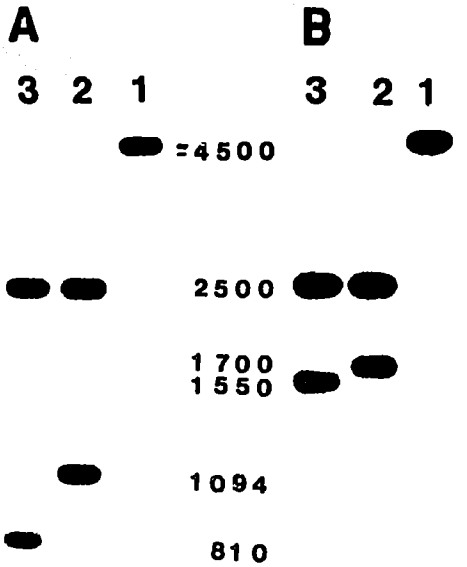


Fig. 5 (left): Localization of methylated GATC site. FCR-3 (3 ug) was cut with Sau3A I (lane 1), Mbo I (lane 2), Taq I (lane 3) and Sau3A I + Taq I (lane 4). Probe b (see Fig. 3) was used for hybridization.

Fig. 6 (right): Hha I analysis of DHFR-TS genes and neighbouring regions. FCR-3 DNA (3 ug) was digested with Hha I (lane 1), Acc I (lane 2) and Acc I + Hha I (lane 3). A=probe b; B=probe a.

This fragment (lane 2) is cut by Hha I yielding the expected 810 bp fragment (lane 3). (The remaining 284 bp fragment cannot be detected by the probe). A 1700 bp fragment detected by probe a in a Acc I digest (Fig. 6B, lane 2), was further cut by Hha I (lane 3), indicating that this site is also not methylated.

In conclusion, no methylated adenine residues were found in the genome, while cytosine methylation was observed. However, the pattern of this methylation is identical in both sensitive and resistant lines (Fig. 4 A, a,b,c,vs d). Thus, at this stage, this parameter cannot be used to distinguish sensitive from resistant strains.

In this study, a difference, not related to methylation, between sensitive and resistant strains was however observed. In Fig. 4 (A,B), a fragment, detected by both a and b probes, is smaller in the resistant strain (d, marked with an arrow), than the equivalent fragment in the sensitive strains (a,b,c).

At this stage, it is not known whether this is a result of the existance of a polymorphic site or a deletion, and whether or not this change is related to the Hinf I polymorphic site described by Paterson et al (4) in the pyrimethamine resistant strain, HB3, which is closely related to the resistant strain used in this study.

In order to find out whether this change can be used for distinguishing sensitive from resistant strains, more strains have to be analysed. This will be done during the forthcoming year on DNA isolated from different isolates collected in the past year in Papua New Guinea by the principal co-investigator, Dr. Michael Alpers.

I intend to visit the Madang laboratory in Papua New Guinea in February. During my stay there, I will be involved in the isolation of DNA from different isolates collected for this project. In addition, staff members of the Institute will be trained in the techniques used in this project.

In Papua New Guinea, in addition to collecting isolates and determining their response to antimalarial drugs, plans for the new facility at Maprik were drawn up and are now almost at completion.

The results described above were submitted for publication to Mol. Biochem. Parasitol.

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1st PROGRESS REPORT

DIAGNOSIS OF DRUG RESISTANT PLASMODIUM FALCIPARUM BY DNA PROBES

Project No. C80126

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DIAGNOSIS OF DRUG RESISTANT PLASMODIUM FALCIPARUM BY DNA PROBES

Following the signing of the contract by both parties, the following steps were initiated:

a. Culturing of several different isolates of P. falciparum. All of these isolates have been cultured in vitro for several years, and some of them show different levels of drug sensitivity.

b. Isolation of DNA from these isolates.

c. Evaluation of the organization of a highly repeated DNA sequence in the genome of isolates.

d. In vitro induction of drug resistance in sensitive isolates.

e. Arrangement for collection of isolates in Papua-New Guinea, in vitro culturing of these isolates and determining their drug sensitivity.

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