Attempted Isolation of the Gene Encoding the 21 Kd *Plasmodium berghei* Ookinete Transmission Blocking Antigen from *Plasmodium Yoelli* and *Plasmodium vivax*

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The 21kD ookinete antigen of Plasmodium berghei *(Pbs 21) has been shown to elicit an effective and long lasting transmission blocking immune response in mice. Having cloned and sequenced this antigen (Paton et al. 1993) the sequence was compared to the genes of the same family previously identified in* P. falciparum, P. gallinaceum *(Kaslow et al. 1989) and* P. reichenowi *(Lal et al. 1990). Four conserved areas were identified in this comparison, to which degenerate oligonucleotides were designed. PCR amplification and screening of genomic libraries was then carried out using these oligonucleotides. The* P. yoelii *gene was successfully cloned and a number of novel* P. vivax *genes identified but the* P. vivax *homologue of Pbs21 remains elusive.*

Key words: *Plasmodium vivax* - *Plasmodium yoelii* - transmission blocking - ookinete

A number of studies have shown that antibodies to proteins expressed on the surface of the sexual stages of the malarial parasite can completely block transmission from the vertebrate host to the mosquito vector (Carter et al. 1979, Mendis et al. 1987). Of these the most effective, so far isolated, are a family of proteins expressed on late zygotes and ookinetes with molecular weights of between 21-28kD these proteins are characterised by the presence of four complete or partial domains similar to the 6 cysteine domain found in EGF (Kaslow et al. 1988). Homologues have been identified in *P. falciparum* (Pfs25; Kaslow et al. 1989), *P. gallinaceum* (Pgs25; Kaslow et al. 1989)) (Pgs 28; Duffy et al. 1993), *P. reichenowi* (Prs25; Lal et al. 1990) and *P. berghei* (Pbs21; Paton et al. 1993). Cross hybridisation of the Pbs21 gene to pulsed field gel blots of other rodent malarial species has also been reported (Paton et al. 1993) suggesting that this gene is highly conserved within the rodent malaria.

Both Pfs25 (Vermulen et al. 1985) and Pbs21 (Tirawanchi et al. 1991) have been shown be capable of eliciting a long lasting immune response capable of a 100% blockade in transmission. Premawansa et al. (1990), reported that monoclonal antibodies raised against *P. vivax* gametes recognised proteins in the range of 20-24kD which were capable of blocking transmission. It is therefore reasonable to expect that one of these proteins might be a *P. vivax* homologue of Pbs21. The identification of such a transmission blocking antigen from *P. vivax* could be of prime importance for its control (Kaslow et al. 1989). A study by Waters et al. (1991), on the evolution of malaria suggested that the *Plasmodium* genus has evolved three major subgroups. These are the avian malaria's to which *P. falciparum* has been linked; secondly the simian malaria's *P. cynomolgi* and *P. fragile* to which *P. vivax* appears most closely related and finally the rodent malaria's which appear quite distant from the rest of the genus (Waters et al. 1991). The isolation of the rodent species early in the evolution of the genus has been suggested as a possible reason for their extreme divergence (Garnham 1966). If regions of any gene are conserved between two such highly divergent species such as *P. berghei* and *P. falciparum* then it is plausible to expect that such regions might be conserved throughout the genus. If such is the case then Polymerase Chain Reaction (PCR) amplification using oligonucleotides designed against the *P. berghei* sequence might therefore permit a rapid cloning of the homologous gene from the entire genus. The PCR amplification of genes is an extremely useful technique (Saiki et

	REGION ONE	REGION TWO	REGION THREE
Pbs21	TTTTTTATCCAACTTGC	GAGTAATCATTACGAATG	GTGGTGAATAATCTATATG
Pfs 25	C - - - - C - - T - - - - - - AG	- - - - CG - - - - - TG - - - - -	- - - - A - - T - T - - - C - A - - -
Pgs 25	- - - - - C - - T - - - - - - - T	- - - - - - - - - - - - TT - - - - -	- - - - C - - T - TC - - - - A - - -
Prs 25	C - - - - C - - T - - - - - - AG	- - - - GGC - - - - TG - - - - -	- - - - A - - T - T - - - C - A - - -
P. yoelii	- - - - - - - - - - - - - - - - - -	- - C - - G - - - - - TA - - - - -	- - - - - - - - - - - - G - - - - - - -
Pgs 28	- - - - - - - - T - - - A - - - -	- - - C - - - - - - ATT - - G - -	- - - C - - - - - - - - G - - - G - - -

	REGION FOUR	REGION FIVE	OLIGO 585 REVERSE
Pbs21	TGCTCATGTGATATAGG	CTAAATGTTTATTGAAATG	CCAAACCCCAAGCACCAGG
Pfs 25	- - - - - - - - TA - - - - - - -	- - - - - - - - C - C - - - A - - - - -	GT - - - GATGG - TTTAT - AT
Pgs 25	- - - - - - - - TTT - - - - - -	- - G - - - - - AC - - - A - - - G -	GT - - GGATGGTTACA - TTT
Prs 25	- - - - - - - - TA - - - - - - -	- C - - - - - C - C - - - A - - - - -	GT - - - GATGG - TT - - T - AT
P. yoelii	- - T - - - - - - - - - - - - - -	- C - - - - - - GC - - - - - - - - -	Primer from Pbs 21
Pgs 28	- - T - - - - - - A - - - - T - -	- - - - - - - - AC - C - AG - - - -	AATTCTGGGTCTGGGTCT -

Fig.1: this figure illustrates the regions of Pbs 21 (Paton et al. 1993), and its homologues Pfs 25 (Kaslow et al. 1989); Pgs 25 (Kaslow et al. 1989) and Prs 25 (Lal et al. 1991) to which oligonucleotides were designed. Dashes indicate were a base is the same as that found in the Pbs 21 sequence. Following a comparison of the first four sequences degenarative primers were designed to regions 2,3,4 and 5. Non degenarative primers to regions 1 and 6 were also used in this study. The bottom two sequences, Pgs 28 (Duffy et al. 1993) and the *P. yoelii* homologue (present study), have been isolated subsequently.

al. 1988) and one which overcomes many of the problems normally associated with the isolation of *P. vivax* genes. Problems often exacerbated by the lack of material, due either to the inability to culture *P. vivax in vitro* or due to the low parasitemias which occur in natural infections (Sharma et al. 1991).

MATERIALS AND METHODS

PARASITES

The *P. vivax* DNA used for the PCR amplification was collected from patients during a visit to Dr Rodriguez laboratory in Tapachula (Chiapas, Mexico). Blood was collected and passed down a CF-11 (Whatman) column and the DNA extracted according to Sambrook et al. (1989). The DNA used for the library construction was a kind gift from Dr M Hollingdale.

P. yoelii DNA was obtained following passage in mice. Infected blood with a parasitemia of approximatly 30% was withdrawn in heparin and the DNA extracted as above.

OLIGONUCLEOTIDES

Following a comparison of the gene sequences of Pfs25, Pgs25, Prs25 and Pbs21 genes five regions were identified in which the sequences appeared to be conserved (Fig. 1). Non degenerate primers were designed to the 5' and 3' ends of Pbs21 as well as degenerate primers to four of the conserved regions, these were as follows.

5' oligonucleotides
Region One
(1)TATTTTTATCCAACTTGCA

Region Two
(2)GAGT(AG)(AG)TCATT(AT)(CTG)GAATG

Region Three
(3)GTGG(ATC)GA(TA)T(TA)(TC)TC(TC)A(AT)ATG

3' oligonucleotides
Region Four
(4) CCTATA(AT)(CAT)ACATGAGCA

Region five
(5)CA(TC)TT(TC)AAT(GA)(TA)(AG)CATT(TC)(AG)G

oligo 585 reverse
CCTGGTGCTTGGGGTTTGA

PCR AMPLIFICATION

PCR amplification was carried out using standard techniques (Saiki et al. 1988) and Taq DNA polymerase (Perkin Elmer). Any PCR products obtained were run on low melting point agarose gels and the bands cut out and purified. The Products were then blunt ended and EcoR1 adapters added prior to sub cloning into PUC 18 (Sambrooke et al, 1989). Double strand sequencing was carried out using the Sequenase protocol and reagents (United States Biochemical Co). All sequences were analysed using Mac Vector software (version 4.1; Eastman Chemical Company) and by comparison to the GenBank database (NCBI).

SOUTHERN BLOTTING

Southern blotting was carried out according to standard protocols (Sambrooke et al. 1989). A 743bp fragment of Pbs21 was used as a probe. After hybridisation according to Ausubel et al. (1989), the filters were washed to a stringency of 0.1 XSSC (1M sodium chloride, 0.1M sodium citrate, pH 7.0)/ 0.5% SDS at 37°C for 30 minutes. Auto radiography was carried out at -70°C overnight.

LIBRARY SCREENING

A genomic library of partial- RSA digested *P. vivax* was constructed in λgt11 according to the usual protocols (Sambrooke et al. 1989). This library was plated using Y 1088 cells to give semi-confluent plaques and filters lifts taken using Nylon filters (Amersham Hybond N). These filters were denatured and neutralised (Ausubel et al. 1989) and the DNA fixed by baking at 80° C for two hours prior to hybridisation. Hybridisation was carried out using a formamide based buffer at 37° C for 16hr (Sambrooke et al. 1989) and using 25ng of probe. Washing was carried out using 6 x SSC/ 0.1% SDS for 15 min at room temperature and 15 mins at 37°C. Auto radiography was carried out at -70°C for up to 2 weeks in order to identify positive colonies. The library was first screened with a 500 bp PCR fragment of the *P. vivax* CSP gene (Arnot et al. 1985) to ascertain the number of *P. vivax* genome equivalents it represented. The filters were then stripped (Sambrooke et al. 1989) and reprobed with the oligonucleotides listed above. Positive plaques were PCR amplified and sub cloned into PUC 18 for sequencing as described above.

RESULTS

All the oligonucleotides listed above when used in various combinations amplified the expected bands from *P. berghei* DNA by PCR amplification. Southern blotting however, revealed that the bands amplified using primer 5 with both *P. berghei* and *P. yoelii* DNA did not hybridise with the Pbs21 probe therefore these bands were non-specific. The largest specific band amplified from *P. yoelii* DNA and recognised by the Pbs21 probe proved to be a 500bp product produced using primers 1 and 6 . When this band was isolated and the product cloned and sequenced it was found to have 85% homology with Pbs21. PCR amplification of *P. vivax* DNA using the degenerate primers failed to amplify any product despite using a wide range of annealing temperatures and extension times. The only combination of oligonucleotides which gave any *P. vivax* product after PCR amplification was oligonucleotide 1 and 585 reverse. When these were used a 500bp product was amplified, following Southern transfer this band did not cross hybridise to the Pbs21 probe but as we have shown that the Pbs21 probe does not cross hybridise to *P. vivax* DNA this is not surprising. This PCR product was cloned and sequenced but was found to have no homology with Pbs21. An alternative approach which involved the screening of a novel partial RSA digested genomic library of *P. vivax* in λgt11 was also tried. This library was first probed with a 500bp PCR product of part of the *P. vivax* CSP and 6 positive clones were identified. This indicated that the library was representative of a sufficient number of genome equivalents to make it worth while screening. The library was then screened using the oligonucleotides listed above. An initial screen using individually labelled oligonucleotides followed by auto radiography for 48hr proved negative, consequently all the oligonucleotides were labelled and hybridised together and the auto radiography extended for 2 weeks. This resulted in the identification of 10 positive clones with insert sizes varying from 1-4 Kb. A comparison of these clone revealed four of them to be identical. A comparison of all 10 clones against the Genbank database found no homology with any other transmission blocking antigen and as yet we have no real indication as to their function.

DISCUSSION

In this study we have isolated the *P. yoelii* homologue of Pbs21 but not the *P. vivax* equivalent. The degenerate oligonucleotides designed, appeared to be incapable of hybridisation to the homologous *P. vivax* sequence. There is a number of possibilities why this might be. After cloning and sequencing the *P. yoelii* gene some differences were found within the conserved regions used for designing the degenerate oligonucleotides (Fig. 1). A gene coding for another *P. gallinaceum* transmission blocking antigen with a molecular weight of 28kD has also recently been sequenced (Duffy et al. 1993). This gene appears to be strikingly similar to Pgs25 and to contain four EGF like domains. It is now apparent that Pbs21 bears a closer resemblance to this gene than to Pgs25. The detection of two very similar genes within *P. gallinaceum* (Pgs25 and Pgs28) raises the question are two such genes to be found in the other species? Surprisingly the conserved regions previously identified after the comparison of Pgs25 and Pbs21 were mostly retained (Fig. 1). If this is maintained then oligonucleotides to these regions should prove capable of isolating both genes from the other species tested. This study provided no evidence for such an occurrence in either *P. berghei.* or *P. yoelii.* We therefore question whether two genes exisit in these rodent parasites. The differences identified within the conserved regions following the cloning of the new genes if exaggerated in the *P. vivax* homologues it might explain the lack of hybridisation of our oligonucleotides. The DNA of *P. vivax* is known to consist of two components the first which resembles the other primate malaria's and which has a 30% G and C component and the second which resembles most of the genus with an 18% G-C component (McCutchan et al. 1984). It is possible that if a homologous gene to Pbs21 exists in *P. vivax* then its G/C content might be higher than that found in the genes so far cloned. This question might be resolved by cloning the homologue from one of the other primate species with a similar G/C content from which material can be more readily obtained.

Both Pfs25, Pgs28 and Pbs21 gene were originally isolated either by monoclonal antibody recognition of clones from a cDNA library or by using oligonucleotides designed to peptide sequences derived from affinity purified protein. In normal circumstances, this might therefore be considered the method of choice for isolation of a *P. vivax* homologue. Due to the difficulties in obtaining sufficient amounts of *P. vivax*, indicated earlier, neither of these methods have to date proved feasible. By sequencing the homologous gene from further rodent species or from other primate malaria's it is hoped that in future we will be able design fresh oligonucleotides with which the *P. vivax* to homologue may be identified.

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