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Cycle DNA sequencing with [α-^{35}S]dATP demonstrates polymorphism of a surface antigen in malaria parasites from Sri Lankan patients ☆

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Abstract

Structural diversity in a 45 kDa surface antigen on *Plasmodium falciparum* merozoites (termed GYMSSA, MSP-2 or MSA-2) and other candidate molecules for developing a malaria vaccine need to be investigated in parasites obtained directly from patients in different malaria endemic countries. A double-stranded DNA sequencing method suitable for this purpose, and also for studying diversity in genes of other haploid cells, is described. A first round polymerase chain reaction (PCR) on DNA isolated from blood was carried out with a primer containing the GCN4 binding site to amplify and subsequently purify the coding region of the MSA-2 gene on GCN4 coated tubes. A second round PCR with more internal primers incorporating M13 forward and reverse primer sequences was then performed. Cycle sequencing was done with unlabelled M13 primers and [α-^{35}S]dATP by the dideoxynucleotide procedure. Two different allelic forms of MSA-2 were identified in samples of *Plasmodium falciparum* from patients in Sri Lanka.

Keywords: Cycle sequencing; DNA sequence; Genetic diversity; Malaria; Membrane protein; PCR

1. Introduction

Variation in the sequences of enzymes and antigens of the human malaria parasite *Plasmodium falciparum* occur under drug [1] and immune [2] selection pressure. The frequency of different alleles of a 185–200 kDa precursor to the major merozoite surface antigen (MSA-1), a candidate molecule for a synthetic malaria vaccine, in parasites directly isolated from patients is different from those found in in vitro cultured *P. falciparum* [3]. Variant proteins are therefore best studied in parasites isolated directly from patients without adaptation to culture. Strain-specific markers for malaria parasites are also useful in epidemiological studies. Determination of silent mutations, that do not affect protein structure and therefore antigenicity, requires a method for determining parasite DNA sequence from small samples of infected blood. The haploid genome of blood stage *Plasmodium* is an advantage for this purpose.

A 45-kDa glycosylated and myristilated smaller surface antigen (GYMSSA, MSP-2 or MSA-2) on *P. falciparum* merozoites is another candidate for vaccine development since antibodies against this molecule inhibit parasite growth and reinvasion in in vitro cultures [4,5]. Two allelic forms of MSA-2 in in vitro culture lines of *P. falciparum* represented by sequences in the K1 or FC27 isolates [6,7] and 3D7 or Camp isolates [8,9] differing chiefly in a central region containing repetitive sequences, have been identified. Within each allele occur sub-families [9] and there is limited recombination between alleles [10].

The polymerase chain reaction (PCR) can be used to amplify a target double-stranded DNA (ds DNA) sequence [11] which can then be single strand-sequenced after cloning into M13 or after isolating one of the strands [12]. However, this procedure is time-consuming and therefore methods have been developed for directly sequencing ds-DNA by cycle sequencing (or linear PCR) with dye [13] or ^{32}P-labelled primers [14]. We report here on the use of a simple manual PCR-based sequencing technique for ds-DNA utilising unlabelled primers and [^{35}S]dATP to demonstrate polymorphism of MSA-2 in Sri Lanka.

Abbreviations: ds DNA, double-stranded DNA; MSA, merozoite surface antigen; NTP, nucleoside triphosphate

☆ The nucleotide sequence data reported in this paper have been submitted to the EMBL/GenBank Data Libraries under the accession numbers X76087 (P2) and X76298 (D2).

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2. Materials and methods

2.1. Isolation of DNA from infected blood

15 1-ml samples of microscopically confirmed *P. falciparum*-infected blood (parasitaemia 0.06–0.90%) were collected in acid citrate dextrose from hospitals in malaria endemic, Dambulla, Galewala, Kurunagala and Polgahawela areas of Sri Lanka [15]. After removal of plasma, the cells were treated with 7.5 ml of 6 M guanidine hydrochloride in 0.1 M sodium acetate (pH 5.5) and stored at −80°C until use. Five hundred μl of lysed blood cells were centrifuged at $100\,000 \times g$ for 20 min at 4°C. The supernatant was transferred to microfuge tubes containing 20 μl glassmilk (Prep-a-gene, Bio-Rad, Richmond, USA) and the DNA purified and collected in 50 μl water according to the manufacturer's instructions.

2.2. Primers

For the first PCR amplification and purification of amplified DNA by binding to yeast GCN4 protein [16,17], the following primer pairs were used: 5′ GTCAAAATGAAGGTAATTAAAAC 3′(VM 558/1) and 5′ GGATGACTCATATGAATATGGCAAAAGATA (VM 570/1). The GCN4 binding site incorporated into VM 570/1 is underlined. GCN4 is a yeast regulatory protein that has a high affinity for double-stranded DNA containing the palindromic binding sequence. The pair of primers

D2

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1    M K V I K T L S I I N F F I F V T F N I
1   ATGAAGGTAATTAAAACATTGTCTATTATAAATTTCTTTATTTTTGTTACCTTTAATATT
          VM 558/1                    VM/570/3
21   K N E S K Y S N T F I N N A Y N M S I R
61  AAAAATGAAAGTAAATATAGCAACACATTCATAAACAATGCTTATAATATGAGTATAAGG
                                        VM 591/1
41   R S M A N E G S N T T S V G A N A P N A
121 AGAAGTATGGCAAATGAAGGTTCTAATACTACTAGTGTAGGTGCAAATGCTCCAAATGCT

61   D T I A S G S Q S S T N S A S T S T T N
181 GATACTATTGCTAGTGGAAGTCAAAGTAGTACAAATAGTGCAAGTACTAGTACTACTAAT

81   N G E S Q T T T P T A A D T P T A T E S
241 AATGGAGAATCACAAACTACTACTCCTACCGCTGCTGATACCCCTACTGCTACAGAAAGT

101  N S R S P P I T T T E S N S R S P P I T
301 AATTCACGTTCACCACCCATCACTACTACAGAAAGTAATTCACGTTCACCACCCATCACT

121  T T E S N S R S P P I T T T E S S S S G
361 ACTACAGAAAGTAATTCACGTTCACCACCCATCACTACTACAGAAAGTTCAAGTTCTGGC

141  N A P N K T D G K G E E S E K Q N E L N
421 AATGCACCAAATAAAACAGACGGTAAAGGAGAAGAGAGTGAAAAACAAAATGAATTAAAT
                    VM 591/3      R 92/1
161  E S T E E G P K A P Q E P Q T A E N E N
481 GAATCAACTGAAGAAGGACCCAAAGCTCCACAAGAACCTCAAACGGCAGAAAATGAAAAT

181  P A A P E N K G T G Q H G H M H G S R N
541 CCTGCTGCACCAGAGAATAAAGGTACAGGACAACATGGACATATGCATGGTTCTAGAAAT
                                  VM 591/2
201  N H P Q N T S D S Q K E C T D G N K E N
601 AATCATCCACAAAATACTTCTGATAGTCAAAAAGAATGTACCGATGGTAACAAAGAAAAC

221  C G A A P S L L S N S S N I A S I N K F
661 TGTGGAGCAGCACCATCCCTCTTAAGTAACTCTAGTAATATTGCTTCAATAAATAAATTT

241  V V L I S A T L V L S F A I F I *
721 GTTGTTTTAATTTCAGCAACACTTGTTTTATCTTTTGCCATATTCATATAA
          VM 570/2                    VM 570/1
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Fig. 1. Nucleotide and deduced amino acid sequences of MSA-2 from *P. falciparum* isolate D2. The locations of primers used for PCR are underlined. Nucleotides 1–49 and 736–771 are deduced from primer hybridisation and homology to other MSA-2 genes.

P2

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1    M K V I K T L S I I N F F I F V T F N I
1   ATGAAGGTAATTAAAACATTGTCTATTATAAATTTCTTTATTTTTGTTACCTTTAATATT
          VM 558/1                    VM 570/3
21   K N E S K Y S N T F I N N A Y N M S I R
61  AAAAATGAAAGTAAATATAGCAACACATTCATAAACAATGCTTATAATATGAGTATAAGG
                                        VM 591/1
41   R S M A E S N P S T G A G G S A G G S G
121 AGAAGTATGGCAGAAAGTAATCCTTCTACTGGTGCTGGTGGTAGTGCTGGTGGTAGTGGT

61   S A G G S A G G S A G G S A G G S A G S
181 AGTGCTGGTGGTAGTGCTGGTGGTAGTGCTGGTGGTAGTGCTGGTGGTAGTGCTGGTTCT

81   G D G N G A N P G A D A E G S S S T P A
241 GGTGATGGTAATGGTGCTAAXCCTGGTGCAGATGCTGAGGGAAGTTCAAGTACTCCCGCT

101  T T T T T T T T T T T T T T N D A E A S
301 ACTACCACAACTACCACAACTACCACAACTACCACAACTACTAATGATGCAGAAGCATCT

121  T S T S S E N P N H N N A K T N P K G N
361 ACCAGTACCTCTTCAGAAAATCCAAATCATAATAATGCCAAAACAAATCCAAAAGGTAAT
          VM 591/4      R 92/2
141  G G V Q K P N Q A N K E T Q N N S N V Q
421 GGAGGAGTTCAAAAACCAAATCAAGCAAATAAAGAAACTCAAAATAACTCAAATGTTCAA

161  Q D S Q T K S N V P P T Q D A D T K S P
481 CAAGACTCTCAAACTAAATCAAATGTTCCACCCACTCAAGATGCAGACACTAAAAGTCCT

181  T A Q P E Q A E N S A P T A E Q T E S P
541 ACTGCACAACCTGAACAAGCTGAAAATTCTGCTCCAACAGCCGAACAAACTGAATCCCCC

201  E L Q S A P E N K G T G Q H G H M H G S
601 GAATTACAATCTGCACCAGAGAATAAAGGTACAGGACAACATGGACATATGCATGGTTCT
                                  VM 591/2
221  R N N H P Q N T S D S Q K E C T D G N K
661 AGAAATAATCATCCACAAAATACTTCTGATAGTCAAAAAGAATGTACCGATGGTAACAAA

241  E N C G A A T S L L N N S S N I A S I N
721 GAAAACTGTGGAGCAGCAACATCCCTCTTAAATAACTCTAGTAATATTGCTTCAATAAAT

261  K F V V L I S A T L V L S F A I F I *
781 AAATTTGTTGTTTTAATTTCAGCAACACTTGTTTTATCTTTTGCCATATTCATATAA
          VM 570/2                    VM 570/1
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Fig. 2. Nucleotide and deduced amino acid sequences of MSA-2 from *P. falciparum* isolate P2. The locations of primers used for PCR are underlined. Nucleotides 1–49 and 791–837 are deduced from primer hybridisation and homology to other MSA-2 genes.

are based on the conserved 5′ and 3′ ends of the MSA-2 gene (Figs. 1 and 2). For a second PCR amplification, internal primers incorporating M13 forward or reverse primer sequences were utilised. Details of the primers, which are based on nucleotide sequences found in laboratory isolates, are given in Table 1 and their relative locations in the MSA-2 gene shown in Figs. 1 and 2.

The M13 sequences incorporated into the primers were as follows: M13F-5′ TGTAAAACGACGGCCAGT 3′ and M13R-5′ CAGGAAACAGCTATGACC 3′. VM 558/1, 570/1, 570/2, 570/3, 591/1, 591/2, 591/3 and 591/4 were kindly provided by Vicky Marshall and Drs. R.F. Coppel and D. Kemp. R92/1 and R92/2 were synthesised at the Department of Biochemistry, La Trobe University, Melbourne.

Table 1

Primer	Isolate	Primer sequence
VM 570/2	K1/3D7	M13R-GTGTTGCTGAAATTAAAACAA C
VM 570/3	K1/3D7	M13F-ATTTCTTTATTTTTGTTACC
VM 591/1	K1/3D7	M13F-GCTTATAATATGAGTATAAGG
VM 591/2	K1/3D7	M13R-CATATGTCCATGTTGTCCTG
VM 591/3	K1	M13R-CTCTCTTCTCCTTTACCGTC
VM 591/4	3D7	M13R-TTCTGAAGAGGTACTGGTAG
R 92/1	K1	M13F-GACGGTAAAGGAGAAGAGAG
R 92/2	3D7	M13F-CTACCAGTACCTCTTCAGAA

2.3. First PCR amplification

This was carried out essentially as described previously [10]. The PCR mix contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 1% gelatine, 5 pmol primers, 2.5 U of Taq polymerase and 10 μl of DNA from blood. The PCR was carried out for 40 cycles in Perkin Elmer Cetus model 480 thermal cycler with the following program: 94°C 45 s (denaturation), 55°C 60 s (annealing) and 70°C 80 s (primer extension).

2.4. Purification of PCR-1 product on GCN4

Fifty μg/ml of GCN4-glutathione-*S*-transferase (Amrad, Melbourne) was prepared in 0.01 M phosphate-buffered saline, PBS (pH 7.2) and 100-μl aliquots used to coat 0.5 ml microfuge tubes for 2 h at 26°C. The tubes were then washed in PBS and exposed to low wavelength UV to shatter contaminating DNA. Twenty-five μl of PCR-1 product was transferred to the GCN4 coated tubes and allowed to bind for 20 min to 1 h at 26°C. The tubes were then washed in PBS before proceeding with second round PCR amplifications.

2.5. Second PCR amplification

The second PCR reaction was carried out for 40 cycles identically to the first PCR except that the target was bound to the GCN4-coated tubes [17]. Different pairs of internal primers containing universal M13 forward and M13 reverse primer sequences were used for amplification. The amplified DNA was separated from primers by electrophoresis on 1.2% low melting agarose, the band of dsDNA excised, mixed with 3 vols. of Bio-Rad binding buffer and heated to 37–55°C to dissolve the agarose. Twenty-five μl of glass milk was added to bind and purify DNA according to the manufacturer's instructions.

2.6. Cycle sequencing of the PCR-2 product

The sequences of the universal M13 sequencing primers were as follows: M13 forward 5'GTAAAACGAGGCAGT3'; M13 reverse 5'TTCACACAGGAAACAG3' (US Biochemicals, USA). To 1 pmol of the primer in 5 μl water was added 4.5 μl of 10 × Taq sequencing buffer, i.e., 300 mM Tris-HCl, 50 mM $MgCl_2$, 500 mM KCl (pH 9.0), 50–100 fmol of template DNA in 18 μl water and 2.5 μl (2.5 U) of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) to give a final volume of 28 μl. Six μl of this pre-reaction mix was aliquoted into four tubes containing 2 μl of termination mix A (2 mM ddATP and 50 μM each of dATP, dCTP, 7-deaza-dGTP and dTTP), mix C (1 mM ddCTP and 50 μM dNTP), mix G (0.2 mM ddGTP and 50μM dNTP) and mix T (2 mM ddTTP and 50 μm dNTP). To each tube was added 2 μl containing 20 μCi of [α-^{35}S]dATP (Amersham International, UK: spec. act. > 1000 Ci mmol^{-1}). The components were mixed, overlaid with 10 μl mineral oil and subjected to cycle sequenc-

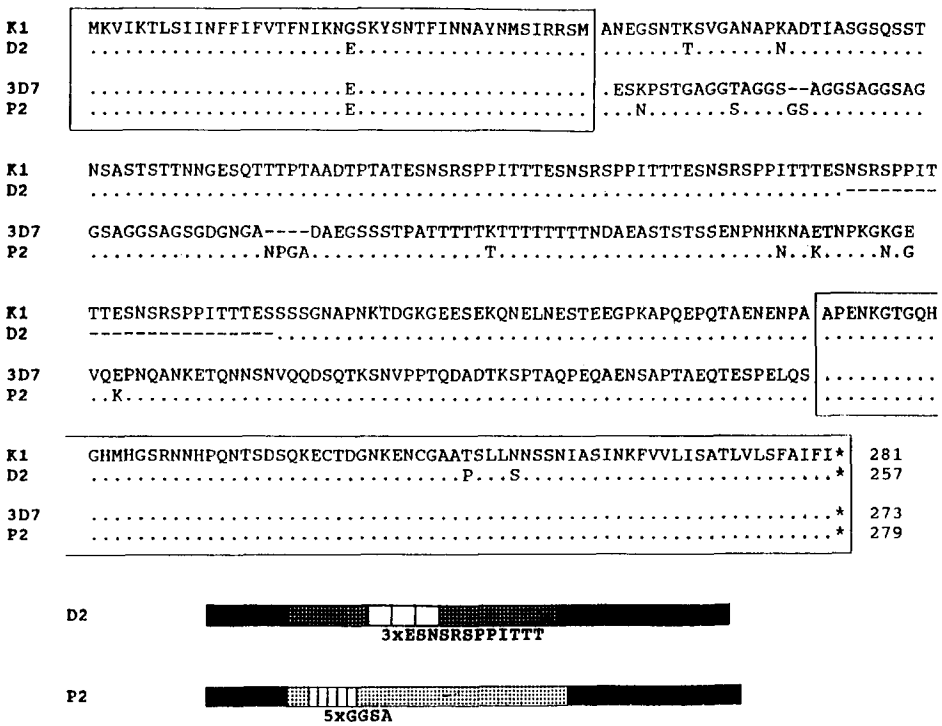

Fig. 3. Amino acid sequences of MSA-2 in the *P. falciparum* isolates D2 and P2 compared with MSA-2 from in vitro cultured isolates K1 and 3D7. The N and C-terminal 'constant' regions are shown boxed. Dashes have been inserted where necessary to optimise alignment of sequences. A diagrammatic representation of the conserved (black), repetitive (striped) and the variable flanking regions of each gene is shown at the bottom.

ing. For M13 forward primer the conditions were: 20 cycles 94°C 30 s, 55°C 30 s, 70°C 60 s and 10 cycles 94°C 30 s, 70°C 60 s. With the M13 reverse primer, the program was: 20 cycles 94°C 30 s, 42°C 30 s and 55°C 1 min and 10 cycles 94°C 30 s and 55°C 60 s. The sequences were determined on both strands for the D2 and P2 genes. At the end of PCR, 5 μl of stop solution (95% formamide, 10mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, pH 8.0) was added. The sequencing reaction mix was then heated to 90°C for 5 min and 3–4 μl aliquots loaded onto standard sequencing gels containing 7 M urea and 5% polyacrylamide. To read the complete DNA sequence, multiple loadings at different intervals were performed. Gels were fixed in 10% methanol, 10% acetic acid, then dried and autoradiographed at −80°C for 1–3 days using intensifying screens.

3. Results

Separation of the PCR amplified products from 15 Sri Lankan patients by agarose gel electrophoresis showed significant size polymorphism in the MSA-2 gene. The largest gene derived from a sample from Polgahawela, termed P2, and one of smaller genes from a Dambulla sample, termed D2 [15], were selected for sequencing. DNA sequences were partially read into the second round PCR primers in all instances. However, nucleotide residues 1–49 and 736–771 in D2 and 1–49 and 791–837 in P2 are assumed to be exactly conserved in samples D2 and P2 as they are in most laboratory isolates sequenced to date (an exception is a C to A transversion in position 794 of the KF1916 isolate, ref. [9]). Hybridisation of the oligonucleotides VM 558/1, 570/1, 570/2 and 570/3 to the genomic DNA of D2 and P2 partially supports this assumption. The sequences of P2 and D2 suggest that in the translated region of MSA-2, D2 contains 771 bp (256 aa, m.wt. 27.0 kDa) and P2, 837 bp (278 aa, m.wt. 28.3 kDa) (Figs. 1 and 2). Comparision of their sequences with known laboratory isolates revealed that D2 and P2 belonged to the FC27/K1 and 3D7/Camp allelic forms respectively (Fig. 3). Despite sequence differences, the overall amino acid compositions of MSA-2 in D2 and P2 were remarkably similar (data not shown).

Most MSA-2 genes characterised to date have conserved N terminal (first 43) and C terminal (last 75) translated sequences (Fig. 3). The conserved blocks enclose small allele specific sequences flanking a repetitive block consisting of either a 32 aa sequence followed by a 12 aa proline and threonine-rich sequence in FC27/K1 or GGSA-like sequences characteristic of the 3D7/Camp allele. Our data show that only the D2 gene differs in the conserved C terminal block at residues 225 and 229. These result in the substitution of P and S for T and N respectively (Fig. 3). The two changes are present in in vitro cultured 7G8 and one of these (S for N) in the in vitro cultured isolates FC27 and KF1916 [9].

In the variable region, the D2 sample contained a single intact 32 aa sequence as in K1, which was followed by three tandemly repeated ESNSRSPPITTT sequences corresponding to nucleotides 295–402. Five of these 12 aa repeats are observed in the K1 isolate [7]. Mutations in the D2 MSA-2 gene result in a T for K change in position 51 and an N for K change in position 59 (Fig. 3). An N for K change in position 51 and an N for K change in position 59 have been observed in FC27 when compared to K1 GYMSSA [7].

Sample P2 did not differ from the consensus sequence for the N and C-terminal conserved blocks. The repetitive region of P2 contained five GGSA repeats. Changes observed in the repetitive region and the flanking sequences in P2 are known to occur in similar positions in other members of the 3D7/Camp family of MSA-2 genes (Fig. 3 and ref. [9])

4. Discussion

The study demonstrates that cycle sequencing or linear PCR may be used in conjunction with appropriate unlabelled oligonucleotide primers and [^{35}S]dATP to sequence PCR amplified dsDNA. The use of [^{35}S]dATP in cycle sequencing is advantageous because it is less hazardous than [^{32}P]dATP and because automated sequencing with dye-labelled primers requires expensive instrumentation. The method can also be used for investigating diversity in genes of other haploid cells, using appropriate oligonucleotide primers.

Taq polymerase has a significantly higher random error rate in copying template DNA (estimated to be 1 per 1421 nucleotides) as shown by cloning PCR amplified DNA into M13 and single strand sequencing by the dideoxynucleotide technique [18]. However, the use of the mass of PCR amplified dsDNA for cycle sequencing eliminates random errors introduced by *Taq* polymerase and yields only the predominant sequence. Multiple linear PCR cycles and polymerisation at 70°C also reduce artefacts. Indeed no variation was seen when samples of independently amplified D2 and P2 DNA were sequenced (data not shown).

The results suggest that MSA-2 in *P. falciparum* obtained directly from patients in Sri Lanka is represented by at least the two major allelic families of MSA-2 described previously in culture lines of the parasite. Additional unpublished observations made by us showed that PCR amplified MSA-2 genes from all 15 samples hybridised in Southern blots to repetitive region-based oligonucleotide probes specific for D2 (ATCACAAACTACTACTC) or P2 (GGTGGTAGTGCTGGTGGTAG). 13 of the 15 samples were D2-like and the remaining two were P2-like by this analysis (unpublished data). While this work was in

progress similar MSA-2 alleles were reported in wild *P. falciparum* isolates from patients in Colombia [19] and Irian Jaya [13]. Our observations are consistent with reports [13,19] that the D2/FC27/K1 allelic family predominates in field isolates. Introns are absent in the MSA-2 gene [6]. An N-terminal signal sequence and a putative C-terminal sequence coding for the attachment of a glycophosphatidyl inositol membrane anchor are present in MSA-2 [6]. Mutations detected in the two sequenced samples D2 and P2 are restricted to (a) change(s) in specific positions in the N and C terminal conserved regions that are seen in other MSA-2 genes (b) variations in the number of repeats and insertion or deletion of a part of the repetitive sequence and, (c) limited substitutions in specific positions in the variable regions flanking the repeats that are also seen in other MSA-2 genes. The limited polymorphism in D2 and P2 as well as the MSA-2 genes sequenced by others probably reflects constraints on the structure and function of MSA-2. However, polymorphism in a membrane protein recognised by growth inhibitory antibodies, particularly in immunodominant repetitive sequences, provides a survival advantage to the parasite. Further studies on MSA-2 diversity in the variable region may be done by using oligonucleotide probes derived from the variable regions for probing Southern blots and then sequencing DNA from any non-reactive samples using the method described here. The present results are supported by the detection of antibodies in up to 59% adults and 38% of 8- to 15-year-old children living in malaria-endemic areas of Sri Lanka, to peptide epitopes in conserved and variable regions of MSA-2 predicted from the elucidated sequences [20]. The conserved N and C terminal sequences or a mixture of the two major variable region sequences of MSA-2 may be a basis for designing synthetic malaria vaccines.

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