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Allelic forms of the knob associated histidine-rich protein gene of *Plasmodium falciparum*

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Abstract **The knob associated histidine-rich protein (KAHRP) gene was cloned and sequenced from two Indian isolates of *Plasmodium falciparum*, Pf3–92 and Pf29–92. These isolates showed major sequence differences in the C-terminal repeat domain of KAHRP. However, the biologically important domains such as spectrin-actin binding region remained highly conserved. The PCR amplification of a variable C-terminal repeat domain from the clinical isolates of *P. falciparum*, from Rajasthan epidemic, showed the presence of multiple alleles of KAHRP gene. The presence of multiple alleles indicates the existence of several *P. falciparum* strains in India. This should be taken into account for future malaria control strategies such as molecular therapy and vaccines.**

Key words: Knob protein; Falciparum malaria; Nucleotide sequence; Allele

1. Introduction

Plasmodium falciparum is responsible for the majority of deaths in malaria. This is because the mature parasite is able to sequester in brain capillaries and thereby causing cerebral malaria. The cerebral capillaries of these patients were found to be packed with infected and uninfected erythrocytes. The electron microscopic studies have shown the attachment of infected erythrocytes to the host endothelium through knobs. Knobs are cup shaped protuberances on the surface of parasitized erythrocytes. They contain several host and parasite derived molecules. The knob mediated cytoadherence phenomenon is through a receptor–ligand type of interaction. The host and parasite molecules involved in this interaction are reviewed elsewhere [1].

The formation of knobs has been found to be associated with the synthesis of knob associated histidine-rich protein (KAHRP) [2]. This protein is synthesized by ring and early trophozoite stages of the parasite [3]. KAHRP is then transported to the red cell membrane where it induces knobs by interacting with several host as well as parasite derived molecules [4]. Earlier, we have reported the cloning and sequencing of the KAHRP gene from a Gambian isolate FCR3 [3,5]. Here, we report the sequence of KAHRP gene from two Indian isolates of *P. falciparum*, indicating that they are different. The PCR data showed the presence of multiple alleles of this gene among Indian isolates, an indication of various *P. falciparum* strains in India.

2. Materials and methods

2.1. Parasite isolates

Different *P. falciparum* isolates, adapted in culture, were obtained from the parasite bank of Malaria Research Centre Delhi. These isolates were from various parts of the country. The clinical samples from Jaisalmer, Rajasthan (North-Western India) epidemic were not adapted in culture. DNA from these samples was isolated directly from the patient's infected blood. DNA from FCR3-Gambia and HB3-Hondura was used as positive control

2.2. Polymerase chain reaction

The sequence of the primers used in PCR is as follows: Fd, 5′-CCC TAA CCA CAG CAT CCTG-3′; Fe, 5′-AAT AAT GGA AAC GGA TCC GGT GAC-3′; Ff, 5′-GTA CTG CAC TAG CTC CTG TAG TTG-3′; Fi, 5′-GAA ACA AAA AAC ACC GCT G-3′. To set the PCR reaction, 100 ng DNA was used. In this reaction mixture, 2 μM Primers, 200 μM nucleotides, 2.5 units of Taq DNA polymerase were used. The DNA was denatured at 95°C for 5 min for the first time and then for 1 min in the subsequent cycles. The primer annealing was carried out at 48°C for the Fd and Fe pair and 45°C for the Ff and Fi pair. The polymerization/DNA synthesis was carried out at 72°C for 1 min. The amplification was carried out for 30 cycles. After these cycles, the product was left for 5 min at 72°C for polymerization. The amplified product was analyzed on 2% agarose gel.

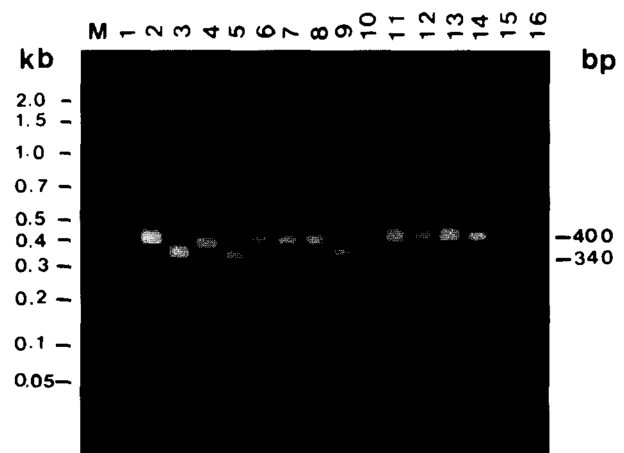

Fig. 1. PCR amplification of KAHRP gene segments from C-terminal domain (Region III) of *P. falciparum* cultured isolates from India. Primer set of Fi and Ff was used. The sequence of the primers are given in the text. Lane M, marker (50 bp–2 kb ladder); Lane 1, no DNA; Lane 2, Pf3–92 from Delhi; Lane 3, FCR3 from Gambia; Lane 4, HB 3 from Hondura; Lane 5, Pf29–92 from Delhi; Lane 6, FDL1 from Delhi; Lane 6, clone 2 from Shankargarh; Lane 7, clone 87 from Assam; Lane 8, clone 89 from Orissa; lane 10, GP-30 from Ghaziabad; Lane 11, FSJA 5 from Shahjahanpur; Lane 12, FSA from Assam; Lane 13, FSJB 6 from Shahajahanpur; Lane 14, Rajasthan; Lane 15, human; Lane 16, *P. vivax*.

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Abbreviations: KAHRP, knob associated histidine-rich protein; PCR, polymerase chain reaction.

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2.3. Cloning the PCR product

The PCR product (~1.8 kb), obtained by using primers Fd and Fe, covers the entire main coding region of KAHRP gene. This fragment was cloned into the PCR-script SK(+) plasmid. Briefly, the PCR product (~1.8 kb band) was purified from agarose gel using the QIAEX kit (Qiagen Inc., Chats Worth, USA). The ends of the eluted fragments were polished before cloning into the pCR script plasmid. The *Taq*I and *Rsa*I fragments of the KAHRP gene from these PCR clones were subcloned into the *Cla*I or *Sma*I site of pGEM 3z or 7z plasmid.

2.4. Nucleotide sequencing

The CTAB-DNA precipitation method was used to isolate DNA for nucleotide sequencing [6]. The nucleotide sequencing was performed on the double stranded DNA using sequenase version 2.0 (sequencing kit

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Pf3-92   C N N G N G S G D S F D F R N K R T L A Q K Q H E H H H H H H H Q H E   35
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NF7      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - H - H

Pf3-92   H Q H Q A P H Q A × × × × H H H H H H G E V N H Q A G Q V H Q Q V H G   70
Pf29-92  - - - - - - - - - × × × × - - - - - - - - - - - - - P - - - - - - - -
FCR-3    - - - - - - - - - × × × × - - - - - - - - - - - - - P - - - - - - - -
NF7      - - - - - - - - - P H Q A - - - - - - - - - - - - - P - - - - - - - -

Pf3-92   Q D Q A H H H H H H H H H Q L Q P Q Q L Q G T V A N P P S N E P V V R   105
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - K
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - K
NF7      - - - - - - - - - - - - - H - H - - - P - - - - - - - - - - - - - - K

Pf3-92   T Q V I R E A R P G G G F K A Y E E K Y E S K H Y K L K E N V V D G K   140
Pf29-92  - - - F - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - F - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NF7      - - - F - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Pf3-92   K D C D E K Y E A A N Y A F S E E C P Y T V N D Y S Q E N G P N I F A   175
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NF-7     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Pf3-92   L R K R F P L G M N D E D E E G K E A L A I K D K L P G G L D E Y Q N   210
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NF-7     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Pf3-92   Q L Y G I C N E T C T T C G P A T I D Y V P A D A P N G Y A Y G G S A   245
Pf29-92  - - - - - - - - - - - - - - - - A - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NF-7     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Pf3-92   H D G S H G N L R G H D N K G S E G Y G Y E A P Y N P G F N G A P G S   280
Pf29-92  - - - - - R - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - G - - - - - - - - - - - - - - - - - - - - - - -
NF-7     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Pf3-92   N G M Q N Y V P P H G A G Y S A P Y G V P H G A A H G S R Y S S F S S   315
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NF-7     - - - - - - - H - W S - - - - - - - - - - - - - - - - - - - - - - - -
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Fig. 2. The KAHRP sequence homology between Indian isolates (Pf3–92 and Pf29–92), Gambian isolate (FCR3) and Ghana isolate (NF7). The common amino acid sequences to Pf 3–92 are shown by a dash (-) and the deletions are shown as cross (x). The repeat domains are underlined. The boxes indicate the homologous region to erythroid skeletal protein 4.1 which binds to host spectrin–actin.

```
Pf3-92   V N K Y G K H G D E K H H S S K K H E G N D G E G E K K K K S K K H K   350
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NF-7     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Pf3-92   D H D G E K K K S K K H K D N E D A E S V K S K K H K S H R C E K K K   385
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - D - - - - -
NF-7     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - D - - - - -

Pf3-92   S K K H K D N E D A E S V K S K K S V K E K G E K H N G K K P C S K K   420
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NF-7     - - - - - - - - - - - - - - - - - V L - K R E K S I M E - N H A A - -

Pf3-92   T N E E N K N K E K T N N L K S D G S K A H E K K E N E T K N T A G E   455
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NF-7     L T K K I K I - K - - - - S - - - - - - - - - - - - - - - - - - - - -

Pf3-92   N K K A D S T S A D N K S T N A A T P G A K D K T Q G G K T D K T G A   490
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - T - - - - - - - - - - - - - - - -
NF-7     - - - V - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Pf3-92   S T N A A T N K G Q C A A E G A T K G A T K E A S T S K E A T K E A S   525
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - S - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - T - - - T - - - - - - - - - - - - - - -
NF-7     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Pf3-92   T S K E G T K E A S T S K E G T K E A S T S K G A T K E A S T T E G A   560
Pf29-92  - - - G A - - - - - - T × × × × × × × × × × × × × × × × × × × × - - -
FCR-3    - - - G A - - - - - - T × × × × × × × × × × × × × × × × × × × × - - -
NF-7     - - - - A - - - - - - - - - A - - - - - - - - - - - - - - - - - - - -

Pf3-92   T K G A S T T A G S T T G A T T G A N A V Q S K D G T A D K N A A N N   595
Pf29-92  - - - - - - - - - - - - E - - - - - - - D - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NF-7     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Pf3-92   G E Q V M S R G Q A Q L Q E A G K K K K K R G C C G Stop                621
Pf29-92  - - - - - - - - - - - - - - - - - - - - - - - - - - -
FCR-3    - - - - - - - - - - - - - - - - - - - - - - - - - - -
NF-7     - - - - - - - - - - - - - - - - - - - - - - - - - - -
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Fig. 2 (*continued*).

from US Biochemicals, Cleveland, USA) according their instructions. M_{13} universal primers were used to sequence the *Taq*I and *Rsa*I clones. The internal primers based on known knob protein gene sequence [5] were also synthesized and used for sequencing.

2.5. EMBL accession numbers

The accession numbers for the nucleotide sequences reported here are X92413 (for Pf3–92) and X92414 (for Pf29–92).

3. Results

The knob-associated histidine-rich protein (KAHRP) of *P. falciparum* has three different domains: N-terminal histidine-rich domain (Region I), central lysine-rich domain (Region II), and C-terminal decapeptide repeats (Region III) as described in [5,7]. The amplification of region III of this gene was carried

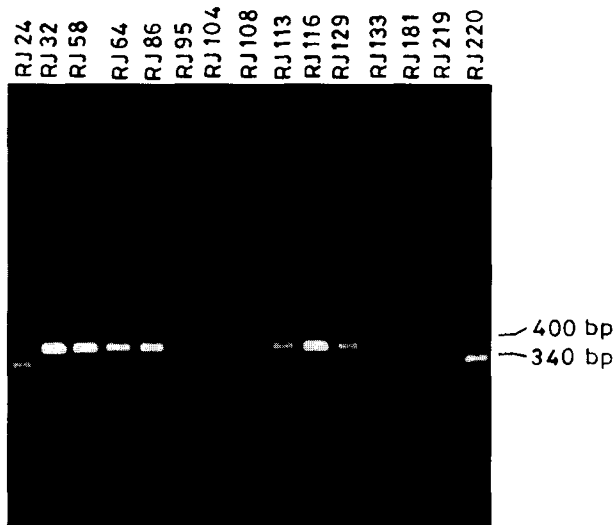

Fig. 3. PCR amplification of the C-terminal repeats domain of KAHRP gene from the patients blood. These patients were suffering with falciparum malaria during the epidemic in Jaisalmer, Rajasthan. Numbers at the top belong to the patient number. The primers were the same as in Fig. 1.

out on the available cultured parasites of *P. falciparum* from India. The results showed the presence of two distinct alleles (Fig. 1). Based on these results we decided to sequence the entire main coding region of both alleles. For this purpose the KAHRP gene from each of the two Indian isolates (Pf3–92 and Pf29–92) was amplified and cloned. Sequencing of 2 to 4 independent clones, for each allele, was carried out to rule out the possibility of PCR mistakes. The deduced amino acid sequence of both Indian isolates (Pf3–92 and Pf29–92) was compared with the available sequences from FCR3 (Gambia) and NF7 (Ghana) as shown in Fig. 2 [5,8]. The histidine-rich domain of Indian isolates was the same as of FCR3 except at position 62 where Proline has been replaced by glycine in Pf3–92 but not in Pf29–92. There were certain point mutations at scattered places in the gene. This has resulted in a single amino acid change at certain places. However, the sequences required for the interaction with host protein spectrin and actin (homologous to erythroid skeletal protein 4.1) were found to be conserved among all the isolates (residue 318 to 330 and 347 to 355 in Fig. 2 and ref. [4]).

Except few minor point mutations, the first two domains were conserved among both the Indian isolates. The major differences were noticed in the C-terminus domain where Pf3–92 contained two extra decapeptides compared to Pf29–92. Therefore, in this region, Pf29–92 showed more homology to FCR3 whereas the Pf3–92 showed homology to NF7. The other major differences observed between NF7 and other isolates (amino acid residue 402 to 434) were due to reading frame change in NF7. The N-terminus histidine-rich domain of Indian isolates [$(His)_7$ — $(His)_6$ — $(His)_9$] was also different from NF7 [$(His)_{11}$ — $(His)_6$ — $(His)_{10}$]. The NF7 also has an extra tetra-peptide Pro-His-Gln-Ala in this region.

The above mentioned observations were further extended to the clinical isolates of *P. falciparum* from Rajasthan epidemic [9]. The PCR results showed some distinct differences among the clinical isolates (Fig. 3). The majority of clinical isolates from this North-Western State epidemic showed the PCR product of the same size as of Pf3–92 (~400 bp). Nevertheless, some individuals showed the presence of both KAHRP gene alleles (RJ 24 and RJ 108 in Fig. 3). We have also observed an intermediate sized band in one of patient (RJ 181) which was similar to HB3 from Hondura. Besides, patients having both alleles, only one patient (RJ 220) had the low molecular weight band (~340 bp), similar to pf29–92 (Fig. 3). So far, we have analyzed 29 clinical isolates from this epidemic and found that 79% (23/29) had a pattern like Pf3–92 (~400 bp), 10% (3/29) had the mixture of Pf3–92 and Pf29–92 and one each showing the banding pattern of Pf29–92 and an intermediate like HB3, respectively. It is interesting to note that the PCR product from cultured isolates (Fig. 1) did not show such mixture or intermediate form (compare Fig. 1 and Fig. 3). The loss of the slow growing strain from the mixture, during the long term parasite culture, cannot be ruled out at this stage. But there was no difference in the PCR profile of two isolates (RJ24 and RJ108) before and after one month of in vitro culture: in both cases two bands were observed (data not shown).

4. Discussion

The observed variations in the KAHRP gene are mainly in the C-terminal peptide repeat domain whereas the other two domains (N-terminal and central) remained almost unaltered. Of these three domains, the central and C-terminal domains have been reported to be immunogenic in human [7]. But only the C-terminal domain showed variations although both have peptide repeats. The noticeable difference between these two domains is the presence of a spectrin–actin binding region within the central domain, which is conserved among all the isolates (Fig. 2). This indicates that the changes in peptide repeats at the C-terminal domain could be allowed by the parasite to evade the host immune system whereas the biologically important domain is still maintained unaltered during evolution. Although it is a difficult question to address as to which isolate is recently evolved. In general, the immunogenecity of the peptides seems to be increased with an increased number of repeats. Therefore, if the parasite is evading the host immune system for its survival (and thus more evolved) then it should contain more repeats. The generation of such variation is possible due to genetic recombination. But the other isolate, having less peptide repeats in the C-terminal domain, being more evolved cannot be ruled out since deletions are more easy to occur. However, to retain the function of evasion of host immune system, for its survival, the parasite may opt for the recombination event.

Recently three labs have independently cloned the gene for a highly variable knob antigen called as *P. falciparum* erythrocyte membrane protein-1 (PFEMP-1) [10–12]. It has been speculated that the C-terminal acidic domain of PFEMP-1 must be interacting with the positively charged KAHRP through salt bridges [11]. It will be interesting to map this interacting domain of KAHRP to explore the possibility of its being conserved among different isolates of *P. falciparum*. The complexity of the PFEMP-1 gene has shown the ability of the parasite to express a large number of variant forms of this protein; each erythrocytic cycle can have a new variant of PFEMP-1. This antigenic variation has been correlated to the evasion of host immune attack by the parasite [13]. Nevertheless, the respective interact-

ing domains of KAHRP and PFEMP-1 must remain conserved.

The antigenic variation and strain polymorphism are major cause of concern for malaria vaccine programmes. Raising of a universal malaria vaccine, therefore, has not been very successful. This warrants the development of alternative strategies such as molecular therapy, using anti-sense RNA or ribozymes. The KAHRP can be the best candidate for this purpose since it is involved in the induction of knobs [2]. The knobby parasites get sequestered in deep vasculature where they are attached to host endothelium and also to normal RBCs to form rosettes and thereby causing pathogenecity [14,15]. Both of these cytoadherent phenomena are shown to be knob mediated [14,16]. The knobless parasites, which were produced during long term cultures in the lab, were found to have lost their virulence and the capacity to synthesize KAHRP [2,3,17–19]. The molecular reagents to cease the knob formation will therefore be useful to treat the falciparum malaria. In this regard we are attempting to develop the ribozyme, based on the conserved KAHRP sequences.

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