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Evidence for negative selection on the gene encoding rhoptry-associated protein 1 (RAP-1) in *Plasmodium* spp.

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ABSTRACT

Assessing how natural selection, negative or positive, operates on genes with low polymorphism is challenging. We investigated the genetic diversity of orthologous genes encoding the rhoptry-associated protein 1 (RAP-1), a low polymorphic protein of malarial parasites that is involved in erythrocyte invasion. We applied evolutionary genetic methods to study the polymorphism in RAP-1 from *Plasmodium falciparum* ($n = 32$) and *Plasmodium vivax* ($n = 6$), the two parasites responsible for most human malaria morbidity and mortality, as well as RAP-1 orthologous in closely related malarial species found in non-human primates (NHPs). Overall, genes encoding RAP-1 are highly conserved in all *Plasmodium* spp. included in this investigation. We found no evidence for natural selection, positive or negative, acting on the gene encoding RAP-1 in *P. falciparum* or *P. vivax*. However, we found evidence that the orthologous genes in non-human primate parasites (*Plasmodium cynomolgi*, *Plasmodium inui*, and *Plasmodium knowlesi*) are under purifying (negative) selection. We discuss the importance of considering negative selection while studying genes encoding proteins with low polymorphism and how selective pressures may differ among orthologous genes in closely related malarial parasites species.

1. Introduction

Worldwide, malaria is one of the most important causes of disease and death in humans, the causal agents are four species of parasitic protozoa belonging to the genus *Plasmodium*. Despite considerable biological differences among human malarial parasites, all of them invade erythrocytes. This process involves the orchestrated action of a variety of proteins, several of them considered prime targets for anti-malarial vaccines (Richards and Beeson, 2009). Given its importance, highly polymorphic proteins involved in this process have been the subject of several evolutionary genetic studies. The overall approach has been to determine whether the polymorphism observed at a specific gene is subject to positive-balancing selection, and if so, it is considered to be involved in the onset of natural acquired immunity (Escalante et al., 2004; Conway, 2007; Tetteh et al., 2009). Less attention, however, has been given to genes encoding proteins that exhibit low polymorphism.

Among these low polymorphic proteins, there are several associated with pear-shaped membrane-bound vesicles located at the apical end of the merozoite called rhoptries (Preiser et al., 2000), such as the rhoptry-associated protein 1 (RAP-1). RAP-1 in *Plasmodium falciparum* (*Pf*RAP-1) shows minimal genetic polymorphism with few amino acid substitutions identified (Ridley et al., 1990a; Howard and Peterson, 1996; Escalante et al., 1998b; Tetteh et al., 2009). Thus, given that the limited polymorphism observed is not under balancing selection, RAP-1 seems to be immunologically irrelevant. However, several lines of evidence indicate that RAP-1 is recognized by the host immune system. Monoclonal antibodies directed against rhoptries-associated proteins *Pf*RAP-1 and *Pf*RAP-2 have inhibited the erythrocyte invasion *in vitro* (Schofield et al., 1986; Harnyuttanakorn et al., 1992; Howard et al., 1998; Jacobson et al., 1998) and immunizations of *Saimiri sciureus* and *Saimiri boliviensis* monkeys with the *Pf*RAP-1 and *Pf*RAP-2 complex demonstrated partial protection against *P. falciparum* (Perrin et al., 1985; Ridley et al., 1990b; Collins et al., 2000). In addition, naturally exposed individuals have shown antibodies against *Pf*RAP-1 conserved linear epitopes and recombinant proteins (Jakobsen et al., 1993, 1997; Stowers et al., 1997; Fonjungo et al., 1998; Moreno et al., 2001; Curtidor et al., 2004). Yet, unlike surface antigens expressed in the merozoite (the parasite stage that invades the erythrocyte), RAP-1 immunogenicity does not translate into the maintenance of observable

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polymorphism. It could be possible that the dynamics of anti-RAP-1 immunity is more complex than previously thought.

Recent investigations have shown that anti-*Pf*RAP-1 immune responses (*falciparum* malaria) may be associated with clinical manifestations of disease such as anemia, a major cause of sickness and death among children with *P. falciparum* malaria in sub-Saharan Africa (Sterkers et al., 2007; Awah et al., 2009) and also one of the clinical manifestations of severe malaria by *Plasmodium vivax*. Specifically, it has been reported that non-protective levels of immunity against *Pf*RAP-1 tagged erythrocytes may trigger their destruction (Sterkers et al., 2007; Awah et al., 2009). If partial anti-RAP-1 immunity could actually be detrimental to the host, then polymorphism could be selected against, leading to low variation at the protein level. Unfortunately, assessing how natural selection, negative or positive, operates on genes with low polymorphism is extremely difficult. As an example, alternatively to negative selection, low polymorphism could be simply the result of demographic processes such as bottlenecks, a phenomenon expected in malarial parasites due to their origins, a result of host switches that lead to recent population expansions (Escalante et al., 2005; Krief et al., 2010). Consequently, there is no evidence so far indicating that negative natural selection could be acting on the limited polymorphism observed in *Pf*RAP-1 (Escalante et al., 1998a,b; Tetteh et al., 2009) or any other ortholog genes in the genus *Plasmodium*.

We explored the mechanisms that shaped the genetic diversity observed in the orthologous genes encoding RAP-1 in several primate malarias, including the two major human malarial parasites: *P. falciparum* and *P. vivax*. While no compelling evidence was found for natural selection, negative or positive, acting on RAP-1 in the two human parasites using these samples, all of the RAP-1 orthologs from non-human primate malarial parasites show patterns consistent with negative selection. Thus far, this study provides the first evidence indicating that negative selection may be acting on a gene encoding a protein involved in the erythrocyte invasion in any *Plasmodium* species. These results suggest that some genes with low diversity may be indeed under negative selection and such a possibility should be carefully explored.

2. Materials and methods

2.1. Parasite strains

All strains and field isolates used in our study were provided by the Centers for Disease Control and Prevention. We have taken an approach to obtain a limited set of sequences from different geographic locations, which increases the probability of sampling the most divergent alleles in order to infer the history and processes involved in the evolution of the observed polymorphism (Kliman and Hey, 1993). In the case of *P. falciparum*, we sequenced the RAP-1 gene in 28 field isolates: nine from India (Delhi collected in 2000, see Escalante et al., 2001), seven from Thailand (hospital based samples from Bangkok, year 2000, see Escalante et al., 2001), four from Kenya (Asembo Bay, 1996–1997), three from Cameroon (Yuonde, Cameroon, 2001), four from Venezuela (Tumeremo, Bolivar State, 2002), and the strain Honduras I from Central America. In addition, we included in our investigation four published sequences of the *Pf*RAP-1 under the following accession numbers: strain FVO from Vietnam (AF205284), FCC1/HN from China (AF206631), FC27 (U20985), strain K1 (M32853), and the RAP-1 from *P. reichenowi* (U20986) in order to compare the RAP-1 sequences that have been reported.

In the case of *P. vivax*, in addition to the Salvador I strain available in the gene bank (XM_001616799), we sequenced RAP-1 in five laboratory isolates (Chesson from New Guinea, Indonesia I, Mauritania I, Sumatra I, and Vietnam II). These sequences were compared with four sequences from different isolates of *P. cynomolgi* (strains Berok, Gombok, PT1, and PT2 all from Malaysia, see Coatney et al., 1971) and five sequences from isolates of *Plasmodium inui* (Leaf Monkey II, Leucosphyrus, OS from Malaysia; Taiwan I and II from Taiwan, see Coatney et al., 1971). We also included in our studies two strains of *P. fieldi* (ABI and N-3 from Malaysia), one sequence from *P. simiovale*, one sequence from *P coatneyi*, one sequence from *P. hylobati* (a gibbon parasite), and five strains of *Plasmodium knowlesi* (H, Hackeri, Malayan strains from Malaysia; the Nuri strain from India, and the Philippine strain; see Coatney et al., 1971). The sequences reported in this investigation are deposited in the GenBank under the accession numbers GQ281604–GQ281655. In order to estimate the RAP-1 phylogeny, we also included the orthologs from two rodent malarial parasites, *P. yoelii* (PY00622) and *P. chabaudi* (PCAS_103190).

2.2. PCR amplification, cloning, and sequencing

Amplification reactions were carried out in a 50 μl volume and included 20 ng/μl of total genomic DNA, 2.5 mM $MgCl_2$, 1× PCR buffer, 1.25 mM of each deoxynucleoside triphosphate, 0.4 mM of each primer, and 0.03 U/μM AmpliTaq® Gold polymerase (Applied Biosystems, Roche-USA). Specific primers were designed in order to amplify the RAP-1 gene by polymerase chain reaction (PCR) in all isolates from *Plasmodium* spp. The primers forward 5′ TAT AAT GAG TTT CTA TTT GGG TAG 3′ and reverse 5′ CCT TCA AGA GAT TAG ATT AAG AAT A 3′ were used to amplify the complete gene in *P. falciparum* isolates. The PCR conditions for amplifying were: a partial denaturation at 94 °C for 1 min and 30 cycles of 1 min at 94 °C, 1 min at 52 °C, and 3 min extension at 72 °C, and a final extension of 10 min was added in the last cycle. In the specific cases of *P. vivax*, *P. cynomolgi*, *P. inui*, *P. fieldi*, *P. fragile*, *P. simiovale*, *P. knowlesi*, and *P. coatneyi*, we used the primers forward 5′ ATG ATA ACK TRC GYA AGT TC 3′ and reverse 5′ CCA ATC KCT TGT AGA GCA AAT 3′ to amplify their RAP-1 genes. We also obtained partial sequences of *P. hylobati* (2235 bp) and *P. fragile* (2223 bp) using primers forward 5′ GCA CTS TAC CAA AAT GTT TCC 3′ and reverse 5′ ATA ATC ATY RCG CAT TTC C 3′. The PCR conditions were: a partial denaturation at 94 °C for 3 min and 35 cycles with 1 min at 94 °C, 1 min at 50–56 °C, and 2 min extension at 72 °C, and a final extension of 10 min was added in the last cycle.

The amplified product was purified, cloned using the pGEM®-T Easy Vector Systems I from Promega (USA), and sequenced. Both strands were sequenced from at least two clones. Two independent alignments were made using ClustalX Version 1.83 with manual editing: one alignment included all *Plasmodium* species, and another for *P. vivax* and related species found in non-human primates from Southeast Asia.

2.3. Evolutionary genetic analyses

We performed phylogenetic analyses on both protein and nucleotide sequences on two alignments: one with all known RAP-1 orthologous from malarial parasites species found in mammals and, separately, *P. vivax* and closely related species found in non-human primates from Southeast Asia. The rationale was that the distances among the major mammalian *Plasmodium* species may reduce the resolution of the phylogeny within the group of species that includes *P. vivax*, given problems aligning highly divergent proteins. The gene phylogenies were first determined by using the Neighbor-Joining method (Saitou and Nei, 1987) with the Tamura–Nei model in the case of nucleotides and the Dayhoff (PAM) models for proteins as implemented in MEGA. The reliability of the nodes in the NJ trees was assessed by bootstrap method with 1000 pseudo-replications (Nei and Kumar, 2000). Additionally, Bayesian phylogenetic analyses were performed as implemented in