Available online at www.sciencedirect.com
ScienceDirect
Diagnostic Microbiology and Infectious Disease 59 (2007) 415–419
DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE
www.elsevier.com/locate/diagmicrobio

Parasitology

A new polymerase chain reaction/restriction fragment length polymorphism protocol for *Plasmodium vivax* circumsporozoite protein genotype (VK210, VK247, and *P. vivax*-like) determination

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Received 20 March 2007; accepted 27 June 2007

Abstract

For the molecular diagnosis of *Plasmodium vivax* variants (VK210, VK247, and *P. vivax*-like) using DNA amplification procedures in the laboratory, the choice of rapid and inexpensive identification products of the 3 different genotypes is an important prerequisite. We report here the standardization of a new polymerase chain reaction/restriction fragment length polymorphism technique to identify the 3 described *P. vivax* circumsporozoite protein (CSP) variants using amplification of the central immunodominant region of the *CSP* gene of this protozoan. The simplicity, specificity, and sensitivity of the system described here is important to determine the prevalence and the distribution of infection with these *P. vivax* genotypes in endemic and nonendemic malaria areas, enabling a better understanding of their phylogeny.

Keywords: *Plasmodium vivax* genotypes; PCR/RFLP; Diagnosis; Circumsporozoite protein; *Plasmodium vivax*-like; VK210; VK247

1. Introduction

Plasmodium vivax is the second most prevalent malaria parasite affecting more than 75 million people each year (Imwong et al., 2005). The circumsporozoite protein (CSP) of the infective sporozoite is the main target for the development of recombinant malaria vaccines (Qari et al., 1993; Gonzales et al., 2001; Herrera et al., 2007). Nevertheless, the data generated require special considerations because of the discovery of sequence variations in the central portion of the *CSP* gene (Gopinath et al., 1994). Rosenberg et al. (1989) described a variant of *P. vivax* in Thailand (VK247), and Qari et al. (1993) reported on the *P. vivax*-like variant in Papua New Guinea, which morphologically resembles the classic form (VK210) and VK247 but has a distinctive repeated portion of the central region of the *CSP* gene. Another important issue generated by the existence of these genotypes is the possibility of differential variant-linked responses to treatment. The first evidence that *P. vivax* develops resistance to chloroquine (CQ) was reported in Papua New Guinea (Rieckmann et al., 1989) and, consequently, studies carried out by Kain et al. (1993) suggested that the response to CQ can vary depending on the *P. vivax* genotypes. In addition, Machado et al. (2003) confirmed a significant correlation between parasite clearance and its 3 genotypes.

By serological and/or molecular approaches, different authors evaluated the global occurrence of these variants.

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0732–8893/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.diagmicrobio.2007.06.019

The proportion of positive sera, specific for the VK210 and VK247 variants, ranged from 28% to 66% in Thailand (Wirtz et al., 1990). Nevertheless, VK247 genotype was identified in 58% of all patients infected with both genotypes (Kain et al., 1992, 1993). In Brazil, all variants were genotyped, but only VK210 was found as a single agent of infection, whereas the other 2 occurred as mixed infections (Machado and Póvoa, 2000; Silva et al., 2006). On the other hand, serological approaches had shown higher levels of positivity for antibodies against the 3 variants in Brazilian endemic and nonendemic areas (Arruda et al., 1996; Oliveira-Ferreira et al., 2004). VK247 variant was mainly found as a single infection in West Africa and the Indian subcontinent. In addition, the majority of the studied individuals had mixed infections with both variants, the predominant and VK210 (Kain et al., 1991; Gonzales et al., 2001). In Southern Mexico, it was observed that all patients were infected with VK210 and most of them also had VK247 (Rodriguez et al., 2000). All variants were detected in field isolates from malarious regions of Papua New Guinea, Indonesia, and Madagascar, although no pure *P. vivax*-like isolate was verified (Qari et al., 1991, 1993).

Kain et al. (1992) developed a genotype-specific polymerase chain reaction (PCR) technique by ^{32}P-end–labeled oligoprobes to detect the VK210 and VK247 variants, whereas Kho et al. in 1999 investigated the polymorphisms of the *CSP* gene in isolates from Korea by the PCR/restriction fragment length polymorphism (RFLP) technique. The first methodology developed to identify *P. vivax* genotypes was PCR/hybridization, which also uses radiolabeled oligoprobes, but the technique is expensive and time-consuming, and also requires an adequate laboratorial structure for elimination of the oligoprobes proper disposal (Qari et al., 1993). Six years ago, Machado and Póvoa (2000) optimized the Glass Fiber Membrane (GFM)/PCR/enzyme-linked immunosorbent assay (ELISA) method; however, it needs much time, as well as uses initiating biotinylated primers and digoxigenin-labeled probes, raising the cost of the procedure. In 2006, a protocol of nested-PCR/RFLP was standardized for the diagnosis of 2 of the 3 genotypes: VK210 and VK247 (Zakeri et al., 2006).

The analysis of RFLPs of PCR products is a fast and simple technique (Trost et al., 2004) normally used in molecular biology laboratories in malaria endemic countries, requiring only basic equipment (Tahar et al., 1998). Here, we report on the standardization of a new PCR/RFLP for the identification of the 3 described *P. vivax CSP* gene variants.

2. Materials and methods

2.1. Samples

For PCR standardization, we used 3 different plasmids (BlueScript, Stratagene, La Jolla, CA), one for the characteristic *CSP* repetitive region of each variant (VK210, VK247, and *P. vivax*-like), and 45 frozen plus 10 fresh blood samples collected in different endemic areas of the Brazilian Amazon region, all with positive results for *P. vivax* thick blood films (TBFs). TBFs were examined by independent experienced microscopists who were unaware of each result as recommended by the World Health Organization. Furthermore, molecular confirmation of *P. vivax* was performed for all samples according to the method described by Kimura et al. (1997). The protocol for this study was reviewed and approved by the Ethics Research Board of the Medicine School in São José do Rio Preto, Brazil.

2.2. Target DNA sequences and design of synthetic oligonucleotides

DNA was extracted from blood samples by the phenol–chloroform method (Pena et al., 1991). To amplify the *CSP* gene, 2 sets of forward and reverse primers were designed based on the conserved central portion of the *CSP* gene. The *CSP* sequences are available in the GenBank database (VK210, accession number 11926; VK247, accession number M69061; *P. vivax*-like, accession number L13724). The sequences were amplified using the following set of primers: PR1 (5′-ACT TTT ATT CGA CTT TGT TGG TC-3′) and PR2 (5′-ATG GAC TCC ATG CAG TGT AAC C-3′). The optimal specificity was achieved using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). A conformational analysis was made to investigate the possibility of secondary structure formations (primer dimer). All oligonucleotide primers were synthesized by the Integrated DNA Technologies (Coralville, IA).

2.3. PCR standardization

Different PCR conditions were tested, varying PCR mixer concentrations, primer annealing, and number of cycles. After optimization, DNA (1.5 μL) was amplified in a total reaction volume of 25 μL consisting of 1× PCR buffer (10 mmol/L Tris–HCl, pH 8.3, 50 mmol/L KCl), 1.5 mmol/L of $MgCl_2$, 1.0 μmol/L of each primer, 200 μmol/L deoxyribonucleotide triphospate (dNTPs), 2.5 U ampli-Taq DNA polymerase, 1% betaine, and water (25 μL). Twenty-five cycles of amplification were performed in a thermocycler (DNA MasterCycler, Eppendorf, Hamburg, Germany) after initial denaturation of DNA at 94 °C for 5 min. Each cycle consisted of a denaturation step at 93 °C for 60 s, an annealing step at 41 °C for 90 s, and an extension step at 72 °C for 2 min, with a final extension at 72 °C for 10 min after the last cycle. The PCR products were analyzed by electrophoresis using 1.5% agarose gels and stained with ethidium bromide.

2.4. Restriction digests of PCR products

The selected enzymes were required to have at least 1 cleavage site in the amplification of each variant, resulting in DNA fragments that are easily visible in polyacrylamide gel. Restriction digests were set up with 10 μL of PCR product and 1 U of the respective enzyme (*Alu*I and *Dpn*I, Promega,