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Genetic characterization of *Plasmodium falciparum* allelic variants infecting mothers at delivery and their children during their first plasmodial infections

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ABSTRACT

Introduction: Infants born to mothers with placental malaria at delivery develop *Plasmodium falciparum* parasitemia earlier than those born to mothers without placental infection. This phenomenon may be explained by the development of immune tolerance due to exposure to *P. falciparum* antigens *in utero*. The hypothesis of this study is that this increased susceptibility might be related to infections by parasites expressing the same blood stage allele's antigens as those to which the infants were exposed *in utero*.

Methods: The comparison of *P. falciparum msp2* (*3D7* and *FC27*) and *glurp* gene polymorphisms of infected mothers at delivery to those of their offspring's infections during infancy was realized and the possible associations of the different polymorphisms with clinical outcomes were assessed. A second approach consisted in the use of a Geographic Information System to determine whether the antigen alleles were homogeneously distributed in the area of study. This was necessary to analyze whether the biological observations were due to high exposure to a particular antigen allelic form in the environment or to high infant permissiveness to the same allelic antigen polymorphism as the placental one.

Results: Infants born to mothers with placental malaria at delivery were more susceptible to infections by parasites carrying the same *glurp* allele as encountered *in utero* compared to distinct alleles, independently of their geographic distribution.

Conclusion: The increased permissiveness of infants to plasmodial infections with shared placental-infant *glurp* alleles sheds light on the role that *P. falciparum* blood stage antigen polymorphisms may play in the first plasmodial infections in infancy.

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1. Introduction

Plasmodium falciparum placental infection is notably associated with low birth weight and consequently with increased infant morbidity and mortality (McGregor, 1984). Epidemiological studies conducted in Cameroon (Le Hesran et al., 1997), Tanzania (Mutabingwa et al., 2005), Gabon (Schwarz et al., 2008), Kenya (Malhotra et al., 2009) and Benin (Le Port et al., 2011) showed that children born to mothers with placental infection at delivery developed a *Plasmodium* parasitemia earlier than those born to mothers without placental infection. The development of immune tolerance by the infant might explain this phenomenon (Le Hesran et al., 1997). The immune tolerance process derives from alterations in immunity as a result of the inflammatory environment. In fact the accumulation of infected erythrocytes in the placenta creates an inflammatory environment that could be associated with lower proliferation of neonate-B lymphocytes (Ismaili et al., 2003), partial maturation of plasmacytoid and myeloid dendritic cells (Fievet et al., 2009; Lutz and Schuler, 2002), and T cell anergy (Malhotra et al., 2009). Furthermore the accumulation of infected

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erythrocytes in the placenta is associated with transplacental transfer of *P. falciparum* antigens (Jakobsen et al., 1998; Malhotra et al., 2006; Redd et al., 1996) – inducing *in utero* exposure. Other studies detected fetal malaria specific immunoglobulin (Ig) E and M – isotypes that do not cross the placenta and are produced by the fetus (Deloron et al., 1997; Desowitz et al., 1993; Rasheed et al., 1995), corroborating the exposure *in utero* and the fetus immune stimulation. Moreover, it has been shown that *in vitro* stimulation of cord blood B lymphocytes from newborns living in malaria endemic areas induces the production of specific IgGs directed to the merozoite surface protein 1 (MSP1) (Dent et al., 2006; King et al., 2002). Malhotra et al. showed that infants exposed to malaria *in utero* acquired and conserved during childhood a tolerant phenotype to the 42 kDa fragment of MSP1, the Apical Membrane Antigen 1 (AMA1) and the F-2 region of the 175 kDa Erythrocyte Binding Antigen (EBA-175) (Malhotra et al., 2009). Overall, these studies suggest an *in utero* exposure of the fetus to soluble parasite antigens associated with an increased susceptibility to *P. falciparum* infections during infancy.

Nevertheless, the numerous polymorphisms of *P. falciparum* blood stage antigens were not taken into account in these studies. The present study investigates if the infant susceptibility to malarial infections increases when infections are characterized by the same blood stage *P. falciparum* polymorphic antigen form as the one identified in placental blood. A comparison of *P. falciparum* blood stage antigen allelic polymorphisms between mother infections at delivery and infections of their offspring during infancy could definitely clarify this question. The antigens MSP2 and GLURP were selected in this study because of (i) their extensive polymorphism that guarantees an accurate antigen molecular characterization and (ii) their ability to cross the placenta and be exposed to the fetus (Jakobsen et al., 1998). The objective of our study was to evaluate the relationship between the antigens found both in placental infections and in malaria episodes of the offspring with regard to (i) infant clinical outcomes: parasitemia, time to first infection, delay between infections and clinical manifestations of malaria (ii) the absolute number of infant infections taking into account the exposure to the allelic forms in the mother-and-child residing area (Coulibaly et al., 2013).

2. Materials and methods

2.1. Sample collection and clinical survey

The study design has been presented in detail elsewhere (Le Port et al., 2011). Briefly, the study was carried out in Benin, in nine villages of the rural area of Tori Bossito, located 40 km North West of Cotonou. Between June 2007 and January 2010, 597 women were included at delivery. Their infants were followed-up monthly until 18 months. At delivery, peripheral and placental blood smears were realized. In addition blood spots were collected onto filter paper. At each systematic visit, a blood smear and blood spots were performed. Monthly thick blood smears (TBS) were read *a posteriori*. Children were visited once a week by a nurse from the field team to control axillary temperature. In case of temperature $\geqslant$37.5 °C or fever suspicion, infants were invited to attend the local dispensary where a clinical examination was realized and both a TBS and a rapid diagnosis test (RDT) were performed. A blood spot was collected as well. In case of positive RDT and/or TBS, infants were treated with artemether lumefantrine (posology was adapted to infant weight). The efficacy of the treatment was supervised every two days until the disappearance of the symptoms and blood smears were collected to ascertain the clearance of circulating parasites. In case of fever between two weekly visits, mothers were invited to bring their children to the dispensary where the same protocol was carried out. All blood spots were conserved at 4 °C with dessicant. Meanwhile the entomologic inoculation rate in the area was approximately of 14 infected bites/person/year (Cottrell et al., 2012; Djenontin et al., 2010). The study was authorized by the institutional ''Ethics Committee of the Faculté des Sciences de la Santé'' (FSS) from the Université d'Abomey-Calavi (UAC) in Benin and by the Comité Consultatif de Déontologie et d'Ethique (CCDE) from the french Institut de Recherche pour le Développement (IRD). The nature of the project was explained in detail to the participants and an informed consent was obtained from women included in the study.

In the present study, the 68 mothers diagnosed by microscopy with *P. falciparum* placenta infection at delivery were selected as well as their offspring (Fig. 1). Parasitemia was defined as the number of *P. falciparum* trophozoites $\times$ 100/number of leucocytes. The counting was performed until either leucocytes or parasites reached 500 (Le Port et al., 2012). The sample collection included the placental blood spot of the 68 mothers and the infants' blood spots collected at each visit (monthly systematic visits and medical consultations in the local dispensary). A molecular analysis using polymerase chain reaction (PCR) was performed for the 46 mother/infant pairs with a malaria infected placenta (Fig. 1). Malaria infection was defined by both a positive placental blood smear and a positive PCR determination of the *P. falciparum 18S* ribosomal gene and a positive PCR determination of *msp2* or *glurp* genes.

2.2. DNA extraction

The Chelex-100 resin method (Biorad, France) was used to extract DNA from every blood spot as described in Plowe et al. (1995). Briefly, filter paper discs of blood were deshemoglobinized overnight at 4 °C in 1 mL of phosphate buffered saline (PBS) containing 0.5% saponin. Chelex-100 resin (6.5%) was added to the suspension and samples boiled for 10 min. After two centrifugation steps at 13,800*g* during 5 and 10 min at 4 °C, supernatants containing DNAs were recovered and conserved at −20 °C.

2.3. P. falciparum PCR detection

A nested PCR was realized to select the positive samples. First primers amplified the *18S* ribosomal gene of *Plasmodium* (Plowe et al., 1995; Snounou et al., 1993). Briefly, amplifications were realized in a final volume of 50 μL containing 7.5 μL of extracted DNA, 250 nM of each primer (rPLU1 and rPLU5 (Singh et al., 1999)), 1.25 U of GoTaq flexi DNA polymerase (Promega, France), 4 mM of $MgCl_2$, Taq PBS buffer 1$\times$ and 200 μM of deoxyribonucleotide triphosphates (dNTPs, Applied Biosystems, France).

DNA was denatured at 94 °C for 4 min. The five first PCR cycles included denaturation (94 °C for 30 s), hybridization (63 °C for 1 min) and extension (72 °C for 1 min); then PCR was performed for 30 cycles following the same protocol. However the hybridization temperature was set at 55 °C. Finally, an additional extension of 4 min was performed.

Thereafter, a nested PCR was performed using 1.5 μL of outer PCR products in a final 20 μL mixture and specific *P. falciparum* species primers named FAL1 and FAL2 (Snounou et al., 1993). The program described above was performed at a hybridization temperature of 63 °C for the first 5 cycles and at 58 °C for the following 30 (cycles).

2.4. Msp2 and glurp genotyping

The polymorphic repetitive block 3 region of *msp2* (Smythe et al., 1990) and the RII region of *glurp* (Borre et al., 1991) were

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