Original article

Association analyses of NCR3 polymorphisms with 
P. falciparum mild malaria

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Received 25 July 2006; accepted 7 November 2006
Available online 5 December 2006

Abstract

Plasmodium falciparum malaria is a major cause of morbidity and mortality in many developing countries especially in sub-Saharan Africa. A susceptibility locus for mild malaria has been mapped to the MHC region, and TNF polymorphisms have been associated with mild malaria. The Natural Cytotoxicity-triggering Receptor 3 (NCR3) gene is located in the peak region of linkage, and is 15 kb distal to TNF. In this study, we considered NCR3 as a candidate gene, and we genotyped ten NCR3 single nucleotide polymorphisms (SNPs). Here, we report evidence of an association between mild malaria and NCR3/C0412G > C polymorphism located within the promoter. Population-based association analysis showed that NCR3/C0412C carriers had more frequent mild malaria attacks than NCR3/C0412GG individuals (P = 0.001). Using the family-based association test (FBAT) program and its phenotype (PBAT) option, we further found that NCR3/C0412C (P = 0.0009) and a haplotype containing NCR3/C0412C (P = 0.008) were significantly associated with increased risk of mild malaria, and that the association was not due to the association of TNF with mild malaria. These observations suggest that there are at least two genes located on the central region of MHC involved in genetic control of human malaria. The association of NCR3 with malaria should provide new insights into the role of Natural Killer cells in this common disease.

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Keywords: Natural Killer Receptor; NKp30; Plasmodium falciparum; Mild malaria; Association; Genetic linkage

1. Introduction

The outcome of malaria infection depends on parasite and host genetic factors. In humans, twin studies and familial clustering of the disease indicate that genetic factors control mild malaria [1,2]. Candidate gene studies have evidenced association of mild malaria with some genes, such as the beta-globin gene (HBB) [3,4]. Recently, Mackinnon et al. only attributed a small proportion of the variation of mild malaria incidence to HbS and α-thalassemia, and pointed out that other genes are involved in malaria resistance [2].

Linkage studies have mapped genes controlling mild malaria on chromosome 6p21–p23 [5,6], with a peak close to the TNF gene. In addition, several TNF polymorphisms have been associated with mild malaria [6,7]. However, the TNF allele frequencies were not high, suggesting that TNF polymorphisms per se unlikely explain linkage of mild malaria to the MHC region. This was further supported by linkage analyses showing that linkage of mild malaria to the MHC region remained significant when taking into account TNF polymorphisms as covariates (Barbier et al., unpublished data). This raised the possibility that other genes close to TNF may be involved. Among the genes located within the central region of MHC, the Natural Cytotoxicity-triggering Receptor 3 (NCR3) gene that encodes the activating NK receptor NKp30 could be considered as a candidate gene. Recently,
it was shown that human NK cells directly interact with *P. falciparum*-infected erythrocytes (iRBC) and that it was necessary for IFNγ production [8,9]. In addition, NK cells were shown to mediate cytolysis of iRBC [10,11]. It is thought that neoantigens expressed on the surface of iRBC interact with NK receptors, and that they provide an activating signal.

We therefore searched for NCR3 polymorphisms that might be associated with mild malaria in an African population living in a *P. falciparum* endemic area. We studied 34 families comprising 193 individuals (53 parents and 140 sibs) with previous evidence of linkage [6].

We investigated the linkage disequilibrium (LD) patterns in the central region of MHC, and we tested family-based association between NCR3 polymorphisms and phenotypes related to mild malaria.

### 2. Materials and methods

#### 2.1. Subjects

The study population consisted of 193 individuals (53 parents and 140 sibs) belonging to 34 families living in Burkina Faso. Linkage of mild malaria to chromosome 6p21–p23 was reported elsewhere in the study population [6]. The mean age of sibs was 12.1 ± 6.2 years (range 1–34 years). The study population and the area of parasite exposure have been described elsewhere [12]. The Medical Authority of Burkina Faso approved the study protocol.

#### 2.2. Clinical data and phenotype determination

Clinical diagnosis and phenotype determination have been described elsewhere [6]. Briefly, according to the WHO, a diagnosis of malaria attack was based on *P. falciparum* parasitemia fever (axillary temperature more than 37.5 °C) and the classical symptoms (headache, aching, vomiting or diarrhea in the children). According to the recommendation of the Centre National de Lutte contre le Paludisme (CNLP) of Burkina Faso, each episode of illness was treated with 25 mg/kg chloroquine during three days or until recovery. Parasitemia was checked at the end of the treatment. In all, 62 of the 193 family members (57 sibs and 5 parents) presented at least one uncomplicated malaria attack during the survey. After treatment, these individuals had neither parasite nor fever. They were considered in the analysis as affected individuals. Sibs from 5 families were unaffected. Eleven, 13, 5, and 1 families contained 1, 2, 3, and 5 affected sibs, respectively. The first phenotype was thus a binary trait (P1).

To take into account the influence of known covariates on the phenotype, we performed logistic regression using the statistical SPSS software (SPSS, Boulogne, France). The explicative variables were treated as continuous variables. The analysis revealed that age and the number of measurements using the statistical SPSS software (SPSS, Boulogne, France). The explicative variables were treated as continuous variables. The analysis revealed that age and the number of measurements had an effect on maximum parasitemia (*P* = 0.0001). The residual of the regression model, which took into account age and the number of measurements, was the third phenotype used in association and linkage analyses. All the sibs were included in linkage and association analyses.

#### 2.3. Genotyping

DNA was extracted from mononuclear cells separated by Ficoll-Hypaque density gradient as described [12]. DNA was preamplified with the Primer Extension Preamplification method [14]. Since exon 1 does not contain known SNP, and since exon 3 contains only a synonymous SNP, we focused our analyses on the promoter, and exons 2 and 4. We checked, nevertheless, that exon 3 did not contain new SNPs in 30 unrelated individuals (data not shown). To identify NCR3 mutations in the promoter region, exon 2 and exon 4, we performed sequencing analysis of three defined PCR products. Three primer pairs were designed with the PRIMER program (NCR3Frag1: 5’-GATGGGTCTGGGTACTGGTG-3’ and 5’-GGATCTGAGCACGTAGGGTC-3; NCR3Frag2: 5’-ATCCTGTCCTCTGGGTGT-3’ and 5’-CTGTACCAGCCTCTAAGCTGA-3; NCR3Frag3: 5’-CTGAACTTCTCCCTTCCCCACC A-3’ and 5’-GGTCCAGCGCTAAAAACCA-3’). PCR amplification was carried out with AmpliTaq (PE Biosystems) in 50 μl reactions, using a Hybaid apparatus. Thermocycling started with a single denaturation step for 5 min at 95 °C, followed by 40 cycles of denaturation for 40 sec at 95 °C, annealing for 30 sec (64 °C for NCR3Frag1 primers, 60 °C for NCR3Frag2 primers, and 64 °C for NCR3Frag3 primers) and extension at 72 °C for 10 sec. One final extension step was added for 10 min at 72 °C. Before starting the sequencing reaction, the PCR products were purified with the Qiagen QIAquick PCR purification kit and quantified by 2% agarose gel electrophoresis. Sequencing reaction was performed with the CEQ 8000 kit and a CEQ 8000 automated fluorescent
scribed (Beckman Coulter). LTA 252 mutation was detected by PCR-RFLP after NcoI digestion of PCR products as described [15] (5'-CCCGTGCTTCGTGCTTTGGACTA-3' and 5'-AGAGCTGTTGGGACATGTCTG-3').

2.4. Allele frequencies, haplotype reconstruction and LD analysis

All genotypes passed a Mendelian check with the program FBAT [16]. Using MERLIN program, we further searched for improbable recombination events from SNP maps to detect genotyping errors [17]. The detectable genotype errors in the sample were less than 0.1%. Allele frequencies were calculated by gene-counting. No deviation from Hardy–Weinberg equilibrium was detected by using a χ² with 1 df.

We generated haplotypes on the basis of family genotypic data with GENEHUNTER program [18]. Pair-wise LD was calculated with GOLD program [19]. We tested LD between pairs of biallelic markers by the r² statistic [19].

2.5. Statistical analyses

Genotype-binary trait (mild malaria attack) correlations were first assessed using contingency-table analysis. This was performed for the two categories (affected versus unaffected) of mild malaria attacks, with a focus on the presence versus the absence of mutation. The χ² test and Fisher’s exact test were used. Since age and hemoglobin genotype are known to influence the development of malaria attacks [4,13], stepwise multivariate logistic analysis that took into account age and hemoglobin genotype was then applied on all variables. We started with all SNPs as covariates, and we eliminated non-significant covariates through the likelihood ratio criterion. The goodness-of-fit of the model was tested by the Hosmer–Lemeshow statistic: a significant test indicated that the model poorly fitted the data. The χ² test, Fisher’s exact test, and logistic regression were performed with the SPSS software. Only terms significant at the 5% level were retained.

Association in the presence of linkage was assessed using family-based association tests (FBATs) [16], which avoid biases due to population stratification, population heterogeneity, or population admixture. PBAT program (the phenotype option of FBAT) was used to identify the most relevant regression model that described the phenotypes as a function of covariates [20]. We performed either 100,000 permutations to calculate empirical P values or data analysis allowing pheno-typic non-normal distribution using FBAT or PBAT programs.

By use of the Bonferroni correction, the significance level of the FBAT statistic was adjusted for the number of FBATs computed (12 tests corresponding to 6 markers and 2 phenotypes analysed), that is, an adjusted significance level of $P = 0.004$. Since the Bonferroni correction procedure does not take into account allelic association, we also used a FBAT multi-marker test estimating the covariance between markers and circumventing multiple testing problems. In this case, the null hypothesis was no linkage or association between any marker and mild malaria. This procedure does not require resolving phase. We also performed haplotype association analysis that requires resolving phase. To tackle multi-testing in haplotype association analysis, we used the multi-allelic procedure computed by FBAT program, and we calculated empirical $P$ values.

3. Results

3.1. Identification of polymorphisms in NCR3

We focused the studies on three polymorphisms within the promoter, five within the exons, and one within the 3' untranslated region (UTR) (Fig. 1). All the variants were previously reported in dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) (Table 1). The NCR3 2859 and NCR3 3571 polymorphisms were the two known non-synonymous mutations reported in the NCR3 gene. We did not detect the NCR3 2859, NCR3 3008 and NCR3 3649 polymorphisms in the study population. The NCR3 −172 polymorphism was identified in one family. The rare allele had a frequency <1%, and the power of association analysis was too small for this polymorphism. Therefore, this polymorphism was excluded from further analyses.

3.2. Population-based association of NCR3 −412 (rs2736191) with mild malaria

To assess the association of polymorphisms with mild malaria, we first conducted statistical calculations for each SNP by χ² tests with 1 df. According to the distribution of NCR3 −412 genotypes GG, GC and CC in affected sibs, 31% of GG sibs, 51% of GC sibs and 57% of CC sibs presented at least one malaria attack (P1) during the study (Table 2). NCR3 −412C carriage was associated with a higher risk of mild malaria attacks ($\gamma^2 = 6.46$, df = 1, $P = 0.011$). No association was detected with the other SNPs. Since age and hemoglobin genotype are known to influence the development of malaria attacks [4,13], we performed logistic regression analysis to re-evaluate the association between NCR3 −412 polymorphism and mild malaria attack. The logistic regression analysis that took into account the influence of age and hemoglobin genotype confirmed this association ($\gamma^2 = 10.7$, df = 1, $P = 0.001$). The result of the Hosmer–Lemeshow test indicated that the model fitted the data. The odds of malaria attack between NCR3 −412GG and NCR3 −412GC/CC was 2.93 (95% confidence interval 1.49–5.76).

3.3. Family-based association of NCR3 −412 with mild malaria

Family-based association tests were performed for each SNP. NCR3 −412 polymorphism demonstrated nominal association, using the standard quantitative FBAT statistic [21], with the binary trait P1 ($P = 0.024$) and the quantitative trait P2 related to mild malaria attacks ($P = 0.00092$) (Table 3). In contrast, the NCR3 −412 polymorphism was not associated with maximum parasitemia (P3), while NCR3 2708T was
negatively associated with P3 \( (P = 0.032) \). NCR3 \(-412C\) was positively associated with P1 and P2. These two associations between the NCR3 \(-412\) polymorphism and the phenotypes P1 and P2 were confirmed with the PBAT program [20], using the GFBAT statistic which is adjusted for environmental correlation within families [22] (Table 3). Nevertheless, the association of the binary trait P1 with the NCR3 \(-412\) polymorphism was not significant when it was corrected for multiple testing. In contrast, the association of the quantitative trait P2 with the NCR3 \(-412\) polymorphism was robust even with a conservative Bonferroni correction of the significance level, and with a multi-marker correction procedure implemented in FBAT.

### 3.4. LD analysis, haplotype analysis, and family-based association model including TNF polymorphisms as covariates: the association of NCR3 \(-412\) with mild malaria was not due to the association of TNF with mild malaria

To test whether the association of NCR3 polymorphisms with traits related to mild malaria could be due to the association of neighbouring polymorphisms with the same traits, we generated haplotypes using NCR3, TNF and LTA genotypes, and we calculated pair-wise LD coefficients (Table 4). TNF 1304 and TNF \(-238\) that were associated with P3 [7] were in LD with NCR3 2708. TNF 267 and TNF \(-244\) that were not associated with mild malaria [7] were in slight LD with NCR3 \(-412\). No TNF variant associated with mild malaria [7] was in LD with NCR3 \(-412\). In particular, NCR3 \(-412\) was not in LD with TNF 1304 and TNF \(-308\). Besides, NCR3 \(-412\) was in strong LD with LTA 252 \( (r^2 = 0.366, P = 0.0001) \), which was not found to be associated with mild malaria (data not shown). The common haplotype characterized by NCR3 \(-412C\) and LTA 252C had a frequency >20%.

The haplotype association results were consistent with our results based on SNP-by-SNP association. An haplotype containing NCR3 2708T was negatively associated with the quantitative trait P3 \( (P = 0.033) \). In addition, we found a positive association of the quantitative trait P2 with the haplotype represented by NCR3 \(-412C\) and LTA 252C \( (P = 0.008) \). Multi-allelic procedure that simultaneously analyzes the association of all the haplotypes with P2 also yielded significant results \( (P = 0.0005) \).

To further dissect the pattern of association between NCR3 polymorphisms and traits related to mild malaria, we utilized conditional power calculation using PBAT [20]. On this basis, we identified the most statistically significant set of covariates in the conditional mean model. According to the PBAT screening procedure, the most relevant set of covariates for the association analysis with NCR3 \(-412\) were TNF 1304 and TNF \(-308\) polymorphisms associated with mild malaria [7], and the LTA 252 polymorphism which was in strong LD with NCR3 \(-412\). When TNF 1304, TNF \(-308\) and LTA 252 were simultaneously included as covariates, we found that the association of NCR3 \(-412\) was strongly associated with mild malaria attack \( (P = 0.00008) \) (Table 3). This association did remain significant after Bonferroni correction for multiple testing. In contrast, when TNF \(-238\) and TNF 1304 were taken into account as covariates, we found that the association of NCR3 2708 with P3 was no longer significant (data not shown).

### Table 1
Overview of the NCR3 polymorphisms genotyped in the African population

<table>
<thead>
<tr>
<th>PCR product</th>
<th>SNP</th>
<th>Mutation</th>
<th>NCBI number</th>
<th>Position in the gene</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCR3Frag1</td>
<td>-412</td>
<td>G &gt; C</td>
<td>rs2776191</td>
<td>Promoter</td>
<td>0.248</td>
</tr>
<tr>
<td></td>
<td>-204</td>
<td>T &gt; C</td>
<td>rs11575836</td>
<td>Promoter</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>-172</td>
<td>G &gt; A</td>
<td>rs11575837</td>
<td>Promoter</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>NCR3Frag2</td>
<td>2708</td>
<td>C &gt; T</td>
<td>rs11575839</td>
<td>Exon 2</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>2859(^\text{c})</td>
<td>G &gt; A</td>
<td>rs11575840</td>
<td>Exon 2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3008</td>
<td>G &gt; A</td>
<td>rs11575841</td>
<td>Intron 2</td>
<td>0</td>
</tr>
<tr>
<td>NCR3Frag3</td>
<td>3571(^\text{c})</td>
<td>G &gt; T</td>
<td>rs3179003</td>
<td>Exon 4</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>3577</td>
<td>G &gt; A</td>
<td>rs3179004</td>
<td>Exon 4</td>
<td>0.062</td>
</tr>
<tr>
<td></td>
<td>3649</td>
<td>A &gt; G</td>
<td>rs3179005</td>
<td>Exon 4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3790</td>
<td>T &gt; C</td>
<td>rs986475</td>
<td>3' UTR</td>
<td>0.024</td>
</tr>
</tbody>
</table>

\(^\text{a}\) NCR3Frag: NCR3 fragment.
\(^\text{b}\) Position relative to transcription start site (+1).
\(^\text{c}\) Wild allele > variant allele. The allele with the higher prevalence was considered the wild one.
\(^\text{d}\) The frequency of the variant allele is shown.
\(^\text{e}\) Non-synonymous mutation.
4. Discussion

We report here the first genetic association of a NK cell receptor with malaria. Our results show that NCR3 –412 polymorphism was associated with the risk of developing mild malaria attack. NCR3 –412 polymorphism was not, however, associated with maximum parasitemia, which was based on the highest parasitemia in each individual during the study. Similarly, we previously reported that TNF –308 polymorphism was associated with mild malaria, and that it was not associated with maximum parasitemia [7]. Conversely, we found that TNF –238 polymorphism was associated with maximum parasitemia, and that it was not associated with mild malaria. Although we also described TNF variants associated with both mild malaria and maximum parasitemia, our data suggest that the mechanisms involved in the control of malarial infection and disease may partly differ. It is conceivable that some variants may affect mild malaria by influencing the cytokine production of effector cells, and that these variants may only weakly influence parasitemia.

NCR3 –412 polymorphism may influence the expression of NKp30 on the surface of NK cells and may alter the response of NK cells to *P. falciparum*-infected erythrocytes. In this way, the GGTCTT sequence containing NCR3 –412 polymorphic site is a RREB1/LZ321 binding motif [23]. This cis-acting element stimulates transcription independent of the direction in which it is inserted. In addition, it is thought to be involved in the Ras-Raf signalling pathway [23], which participates in the regulation of NK cell activation [24]. It should be stressed, however, that the biological role of NKp30 and NCR3 –412 polymorphism remains to be supported by direct functional evidence. We cannot exclude that NCR3 –412 polymorphism may be in LD with a causative polymorphism.

### Table 2
Clinical data of sibs for NCR3 –412 polymorphism

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of unaffected sibs</th>
<th>Number of affected sibs&lt;sup&gt;a&lt;/sup&gt;</th>
<th>All sibs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>53 (68.9%)</td>
<td>24 (31.1%)</td>
<td>77 (100%)</td>
</tr>
<tr>
<td>GC</td>
<td>27 (48.2%)</td>
<td>29 (51.8%)</td>
<td>56 (100%)</td>
</tr>
<tr>
<td>CC</td>
<td>3 (42.8%)</td>
<td>4 (57.2%)</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>All</td>
<td>83 (59.3%)</td>
<td>57 (40.7%)</td>
<td>140 (100%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sibs with at least one malaria attack during the 24 months of the study.

### Table 3
Family-based association tests for NCR3 –412 polymorphism

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>FBAT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GFBAT&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GFBAT with SNP covariates&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaria attack (P1)</td>
<td>0.024</td>
<td>0.027</td>
<td>0.028</td>
</tr>
<tr>
<td>Risk of developing malaria attacks (P2)</td>
<td>0.00092</td>
<td>0.001</td>
<td>0.00008</td>
</tr>
</tbody>
</table>

<sup>a</sup> FBAT is the family-based association test.
<sup>b</sup> GFBAT is the FBAT statistic which is adjusted for environmental correlation within families.
<sup>c</sup> SNPs in covariates: TNF –308, TNF 1304 and LTA 252.

### Table 4
LD coefficients for the locus NCR3-TNF-LTA

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NC3</td>
<td>0.022</td>
<td>0.007</td>
<td>0.020</td>
<td>0.008</td>
<td>0.009</td>
<td>0.008</td>
<td>0.007</td>
</tr>
<tr>
<td>NCR3 –204</td>
<td>0.005</td>
<td>0.005</td>
<td>0.003</td>
<td>0.002</td>
<td>0.006</td>
<td>0.005</td>
<td>0.004</td>
</tr>
<tr>
<td>NCR3 –238</td>
<td>0.023</td>
<td>0.001</td>
<td>0.002</td>
<td>0.001</td>
<td>0.002</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>TNF –308</td>
<td>0.007</td>
<td>0.007</td>
<td>0.009</td>
<td>0.009</td>
<td>0.009</td>
<td>0.009</td>
<td>0.009</td>
</tr>
<tr>
<td>NCR3 –238</td>
<td>0.002</td>
<td>0.002</td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
</tr>
</tbody>
</table>

<sup>*</sup> Significant result, *P* < 0.05. ** Significant result, *P* < 0.0001.
unknown locus may be harboured by the haplotype characterized by NCR3 -412C and LTA252C. However, we showed that the association of NCR3 -412 with mild malaria was not due to LTA 252 or TNF polymorphisms. In parallel, we found that the weak association of NCR3 2708 with maximum parasitemia was due to TNF variants. In all, these data suggest that both NCR3 -412 and TNF variants influence mild malaria. Alternatively, we cannot exclude that undiscovered polymorphism or haplotype defined by several undiscovered polymorphisms in the chromosomal region would be solely responsible for genetic control of mild malaria. This hypothesis implies that the putative undiscovered polymorphisms would be in linkage disequilibrium with both NCR3 -412 polymorphism and TNF polymorphisms associated with mild malaria.

Besides, it is unknown if NKp30 interacts with infected erythrocytes and if it is involved in parasite killing or in IFNγ production by NK cells activated by infected erythrocytes. Alternatively, NKp30 that plays a central role both in the killing of immature dendritic cell (DC) [25] and in the induction of DC maturation [26] may influence the development of innate and adaptive immune responses to P. falciparum-infected erythrocytes. Interestingly, the effect of NKp30 engagement on the maturation of DC is dependent on TNF, the gene of which is close to the NCR3 gene and was also associated with mild malaria [7]. Thus, genetic variation in neighbouring NCR3 and TNF genes may alter both the maturation of DC and the response to the parasite. Under this hypothesis, NKp30 may not directly interact with P. falciparum-infected erythrocytes, but may influence DC maturation, which is also regulated by P. falciparum-infected erythrocytes or their products [27,28].

There is a growing body of evidence for a role of NK cells in human malaria infection [29]. Our results are consistent with this hypothesis, and suggest that genetic variation in NK cell receptors may account both for the heterogeneity of human NK cell reactivity to P. falciparum-infected erythrocytes [30] and for the heterogeneity of human resistance to P. falciparum malaria. Functional analyses and replication studies are, nevertheless, required to validate our findings.

Acknowledgements

We thank all volunteer families of Bobo-Dioulasso (Burkina Faso, West Africa). This work was supported by the French Ministry of Research and Technology (Agence Nationale de la Recherche, Microbiology-Immunology Program). N.F.D. is supported by a studentship from the French Ministry of Research and Technology.

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