8-17-2017

# Genetic Diversity of CD14 Promoter Gene Polymorphism (rs2569190) is Associated With Regulation of Malaria Parasitemia and Susceptibility to Plasmodium falciparum Infection.

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Infectious Diseases: Research and Treatment
Volume 10: 1–6

DOI: 10.1177/1178633617726781
SAGE

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**ABSTRACT:** CD14 is a multifunctional receptor expressed on many cell types and has been shown to mediate immune response resulting in the activation of an inflammatory cascade, with polymorphism of its promoter (rs2569190) found to be associated with susceptibility to several diseases. In malaria infection, the CD14 gene demonstrated a pathogenic profile in regulating experimental cerebral malaria, with reports of elevated levels of soluble CD14 in serum of patients but no definitive conclusion. We present a detailed analysis of genetic diversity of CD14 promoter gene (snp −159 C/T; rs2519190) polymorphism between a malaria-infected group and uninfected controls and its association with clinical parameters of disease. Genomic DNA samples obtained from 106 *Plasmodium falciparum* malaria–infected patients and 277 uninfected controls were elucidated with a polymerase chain reaction-restriction fragment length polymorphism (RFLP) assay. Our results show a significant diversity ($P = 3.32E{-}06$) in the genotypic frequency (3.8% versus 22.4%) of the rs2569190 mutant variant between the malaria-infected group and controls, respectively. The mutant allele had the lowest frequency among the malaria-infected group demonstrating its necessity for infection. Mean parasitemia (parasites/μL of blood) was significantly regulated based on CD14 polymorphic profile (19 855 versus 37 041 versus 49 396 for homozygote mutants, heterozygotes, and homozygote wild type, respectively). Interestingly, we found no association between CD14 genetic variants with fever, age of patients, or anemia. How this affects disease severity between subregional and continental groups deserves further clarification, including extending these studies in a larger group and among severe and asymptomatic patients with malaria.

**KEYWORDS:** CD14, malaria, sub-Saharan Africa, polymorphisms, parasitemia, severity, *Plasmodium falciparum*

**RECEIVED:** May 8, 2017. **ACCEPTED:** July 15, 2017.

**PEER REVIEW:** Three peer reviewers contributed to the peer review report. Reviewers' reports totaled 1271 words, excluding any confidential comments to the academic editor.

**TYPE:** Short Report

**FUNDING:** The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This project was supported by the funding provided by a Research Laboratory Support and Faculty Development Awards, College of Health Sciences and Technology, Rochester Institute of Technology and an American Association of Immunology Undergraduate Faculty Travel Grant to present this work at the annual conference was provided (B.N.T.). Y.L. was supported by China Natural Science Foundation grants #31501002 and 31470070.

**DECLARATION OF CONFLICTING INTERESTS:** The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Introduction

Inflammatory reactions, secondary to pathogenic stimuli, are important for the initiation of necessary immune response for combating the external stimuli when effectively and efficiently deployed. Infection with *Plasmodium falciparum*, the causative agent of malaria, especially common in sub-Saharan Africa,[1,2] is one such pathogen for which the inflammatory cascade would be necessary, considering possible infection severity, especially in nonimmune travelers or children.[3–5] Several innate immunity genes have been implicated, at one time or another, as having significant roles in disease severity, infection outcome, or development of possible sequelae, postinfection.[6] One such gene is CD14, a coreceptor to the toll-like receptor (TLR)-4/MD2 complex on leukocytes,[7–10] and expressed on many cell types. It has been demonstrated to mediate innate immune response due to its specificity for bacterial lipopolysaccharide and consequent pro-inflammatory signaling and initiation of phagocytosis.[11–13] It lends its capacity to T-cell plasticity, driving a $T_H1$ differentiation, especially with infections such as malaria.

In published reports, genetic variations leading to dysfunctional monocyte functions have been implicated in disease susceptibility and outcome, probably clarifying how human populations have adapted to major infectious diseases through the ages.[14] Genetic variants of the CD14 gene (a C-to-T transition, snp −159 C/T) have been shown to be associated with susceptibility to multiple diseases and conditions including allergic rhinitis,[15] functional dyspepsia,[16] inflammatory bowel disease,[17] gram-negative bacteria, and respiratory infections, including brucellosis.[18–21] Its significance in allergic reactions and outcome in patients with asthma have also been documented.[21,22–24] In human, malaria infections particularly, elevated levels of soluble CD14 have been found in patients with

acute infections,[25] concluding that soluble CD14 levels may play a critical role in disease severity. A murine infection model has been the clearest to delineate a specific pathogenic role for CD14 during experimental cerebral malaria infection, showing a protective outcome in *Plasmodium berghei*–infected transgenic CD14 mice.[9]

Although differing outcomes have been reported for the critical role of TLRs alongside CD14, its co-receptor, in mediating disease susceptibility or outcome in malaria infection,[26–29] it is abundantly clear that this promoter gene potentially has a significant role in disease outcome. Would CD14 promoter gene (rs2519190) single-nucleotide polymorphism (SNP) be a mediating factor in who develops severe or acute disease in sub-Saharan Africa? Will the hereditary variants regulate initiation of immune response thereby affecting (positively or negatively) phagocytosis and the inflammatory cascade following infection as well as clinical parameters of disease? Dissecting the essentiality of CD14 promoter gene polymorphism in acute *P falciparum* malaria–infected patients is imperative, necessary to bridge the existing knowledge gap.

Our objective is to investigate any association between CD14-snp159 C/T (rs2569190) gene polymorphism and susceptibility to acute malaria infection or other measures of disease severity, such as parasitemia, fever, and anemia. To do this, we conducted a detailed analysis of genetic diversity of CD14 promoter gene polymorphism in an infected group versus uninfected controls, recruited from sub-Saharan Africa. Genomic DNA samples obtained from 106 *P falciparum* malaria–infected (microscopy and polymerase chain reaction [PCR] confirmed) patients and 227 uninfected controls from southwestern Nigeria were subjected to a PCR-RFLP analysis. We evaluated association with parasitemia, age of patients, fever, and anemia (packed cell volume [PCV]).

## Materials and Methods

### *Subjects and demographics*

A total of 106 patients (age range: 6 months-5 years), who presented with presumptive, acute malaria infection (initial diagnosis positive for *P falciparum* by rapid diagnostic test [RDT]) at St Mary Catholic Hospital, from November 2013 to November 2014, and whose parents/guardians provided informed consent were recruited from Ibadan, south-western Nigeria, to participate in this study. Rapid diagnostic test was performed with the SD Bioline histidine-rich protein 2 (HRP-2) kit (Standard Diagnostics, Gyeonggi-do, Republic of Korea), with parasite species detected according to manufacturers instruction. Patients, recruited from same geographical area, with similar ethnic mix (Yoruba) were examined, and temperature reading, indicative of fever, was recorded. Capillary blood from a finger prick was spotted onto Whatman filter papers (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA), after sterilizing the fingertip with an alcohol swab. Thin and thick blood smears were made for microscopy purposes, whereas anemia was determined by PCV. Genomic DNA was extracted from blood samples with a Qiagen Blood Mini Kit, according to standard protocols (Qiagen Inc., Valencia, CA, USA). DNA elution volume of 100 µL total was stored at −20°C, until further analysis. In addition, 277 genomic DNA samples, from same geographical location and same ethnic mix (RDT and PCR negative for malaria infection), served as uninfected controls. This project was approved by the University of Ibadan/University College Hospital Institutional Review Committee (approval number UI/EC/12/0279) and conducted in accordance with Helsinki declaration of 1975. Fever was determined by measuring the axillary body temperature. Anemia was determined by measuring the PCV, expressed as a percentage, whereas parasite count was evaluated per microliter of blood by counting the maximum number of parasite per 200 leukocytes multiplied by 8000.

### *PCR confirmation for malaria infection*

To confirm microscopy results, PCR assay targeting the 18S ribosomal RNA gene of *Plasmodium* spp, with primers, protocols, and reaction setup, was conducted.[31,32] Polymerase chain reaction was performed on a Prime G thermal cycler (Bibby Scientific, Staffordshire, UK) in a total volume of 25 µL and amplified using 1× PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl, and 1.5 mM of MgCl2), 0.125 mM of dNTPs, 0.25 mM of each primer, and 1 U *Taq* DNA polymerase (New England Biolabs, Hitching, UK). About 5 µL of amplified products was loaded onto 2% agarose gels (SeaKem Agarose, Lonza, Rockland, ME, USA), and band size was determined poststaining with SYBR Green 1 gel stain (Cambrex Biosciences, East Rutherford, NJ, USA).

### *Genotyping for CD14 promoter gene (rs2569190) polymorphism*

To assay for the SNP of the CD14 promoter gene (rs2569190), we used a previously published primer and protocol,[33] with some amendments. We amplified 1 µL of genomic DNA as template, with conditions optimized to 25 µL of final volume, as described.[1] About 5 µL of PCR products was examined on a 2% ethidium bromide–stained agarose gel; positive amplification yielded products of 561 base pairs (bp) and size estimated with a GeneRuler 100bp Plus DNA ladder (Thermo Fisher Scientific Inc, Springfield Township, NJ, USA).

### *RFLP assay*

To determine genotypic and allelic frequencies of the polymorphic variants, we set up a RFLP assay using the FastDigest *BsuRI* enzyme (Thermo Scientific), following manufacturer's guidelines, as previously described.[34] Digested products were analyzed on a 3% agarose gel, and band sizes were determined,

**Table 1.** Genotypic and allelic frequencies of CD14 (snp –159 C/T; rs2519190) promoter gene polymorphisms between patients with *Plasmodium falciparum*–infected malaria and healthy, uninfected controls.

| POLYMORPHISM | GENOTYPE | MALARIA: N = 106 (%) | CONTROLS: N = 277 (%) | ODDS RATIO (95% CI) | *P* VALUE |
|---|---|---|---|---|---|
| CD14 (rs2569190) | C/C | 60 (56.6) | 127 (45.8) | 1.54 (0.96–2.48) | Reference |
| | C/T | 42 (39.6) | 88 (31.8) | 1.41 (0.86–2.30) | .15 |
| | T/T | 4 (3.8) | 62 (22.4) | 0.14 (0.04–0.38) | 3.32E–06 |
| | ALLELE | MALARIA: N = 212 (%) | CONTROLS: N = 554 (%) | ODDS RATIO (95% CI) | *P* VALUE |
| CD14 (rs2569190) | C | 162 (76.4) | 342 (61.7) | 2.00 (1.38–2.94) | Reference |
| | T | 50 (23.6) | 212 (38.3) | 0.50 (0.34–0.72) | .00012 |

Abbreviations: C, cytosine; CD14, cluster of differentiation 14; CI, confidence interval; RDT, rapid diagnostic test; T, thymine.
Control group populations are individuals without malaria parasitemia and healthy control populations are individuals without malaria infection determined by RDT, microscopy, and PCR and recruited from south-western Nigeria. C/T denotes alleles at the CD14 locus. Odds ratio was calculated by Fisher 2-tailed exact test. $P < .05$ was considered significant.

as described.[1] Restriction analysis was conducted by 2 investigators, with 100% concordance. Homozygote wild-type variants (snp –159 C/C; rs2569190) produced bands of 204, 201, and 156 bp, whereas homozygote mutant variants (snp –159 T/T; rs2569190) produced bands of 360 and 201 bp.

### *Statistical analysis*

Tests for deviation from Hardy-Weinberg equilibrium (HWE) were performed, with SNPs rejected based on the recommended threshold of $P < .05$ in control individuals.[35] Differences between malaria and control groups were assessed by odds ratio. Original files were converted to an EH program format to resolve genotypic and allelic frequencies, as previously described.[30]

## Results

We examined the genetic diversity of the CD14 gene promoter (–159 C/T; rs1800829) SNP that has been demonstrated to exhibit significant relevance within and between disease groups and evaluated its association with disease outcome in *P falciparum* malaria–infected patients. To do this, we subjected genomic DNA samples extracted from malaria-infected patients (n = 106) and control group (n = 277) to a PCR-RFLP assay. Genotypic frequencies at each variant were compatible with the HWE ($P > .05$). Our results show that there is a widespread diversity in the genotypic frequency of the CD14 promoter gene between malaria-infected and control populations, with this diversity more pronounced in malaria-infected patients but not among uninfected controls (Table 1). The wild-type variant (*snp* –159 C/C) of the CD14 promoter gene had a higher frequency in patients with malaria compared with controls (Figure 1). In fact, the mutant variant (*snp* –159 T/T) (depicting a nonsynonymous polymorphism and possibly functional significance) was extensively widespread and significantly diverse in its frequency between both groups (3.8% versus 22.4% for malaria-infected and control groups, respectively; $P = 3.32E–06$). In other words, the mutant allele was less

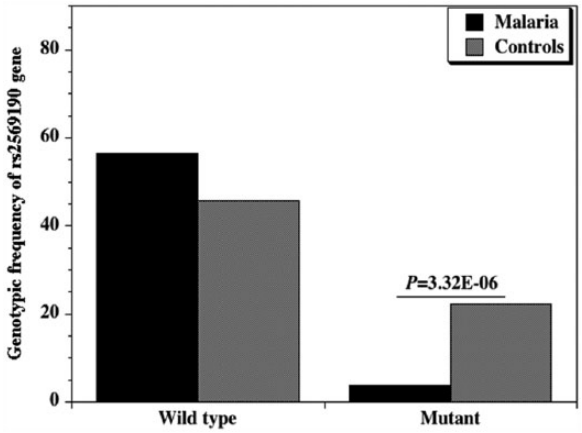


**Figure 1.** Genotypic frequency of CD14 gene promoter polymorphism (snp –159 C/T; rs2569190) between *Plasmodium falciparum* malaria–infected patients and healthy controls. Amplified PCR products were digested with FastDigest *BsuRI* restriction endonuclease (Thermo Scientific, NJ, USA) and expressed on a 3% ethidium bromide–stained agarose gel. Wild-type variant (snp –159 C/C) produced 3 bands (204, 201, 156 bp), whereas mutant variant (snp –159 T/T) produced 2 bands (360 and 201 bp) on digestion. Marker: GeneRuler 100bp Plus DNA ladder (Thermo Scientific). Black bars indicate patients with malaria; gray bars indicate uninfected controls.

common among malaria-infected patients compared with uninfected controls. Examining the allelic frequency of this gene revealed a similar pattern to that for genotypic distribution, with the mutant allele significantly less frequent (Figure 2) than the wild-type variant (23.6% versus 38.3% for malaria-infected and uninfected controls, respectively; $P = .0001$).

To determine whether the CD14 gene promoter genetic diversity results correlate with markers of clinical disease or severity, we evaluated parasite load (parasites/μL of blood), fever (°C), anemia (PCV), and age (months) among malaria-infected patients. We found no statistically significant difference or any association for age (mean age: 31.9 versus 38.7 months; $P = .42$), fever (mean temperature: 38.4°C versus 38.7°C; $P = .70$), or anemia (mean PCV: 29.4% versus 31.7%; $P = .37$) among malaria-infected patients with either the

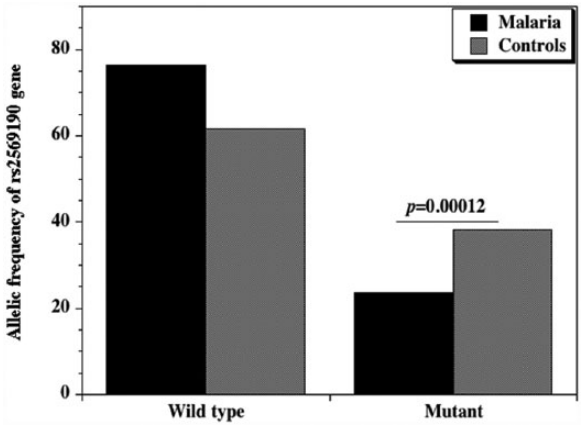


**Figure 2.** Allelic frequency of CD14 gene promoter polymorphism (snp –159 C/T; rs2569190) between *Plasmodium falciparum* malaria–infected patients and healthy controls. Amplified PCR products were digested with FastDigest *BsuRI* restriction endonuclease (Thermo Scientific, NJ, USA) and expressed on a 3% ethidium bromide–stained agarose gel. Black bars indicate patients with malaria; gray bars indicate uninfected controls.

wild-type (*snp* –159 C/C) or mutant (*snp* –159 T/T) variants, respectively. Surprisingly, we found mean parasitemia was mildly significantly regulated ($P = .07$), based on CD14 gene promoter polymorphic profile (19 855 parasites/μL of blood versus 37 041 parasites/μL of blood versus 49 396 parasites/μL of blood for patients with mutant, heterozygote, and wild-type variants, respectively). This implies that among *P falciparum* malaria-infected patients in sub-Saharan Africa, genetic diversity of the CD14 rs2569190 promoter gene is potentially a significant factor regulating parasite load and consequently disease severity (Figure 3).

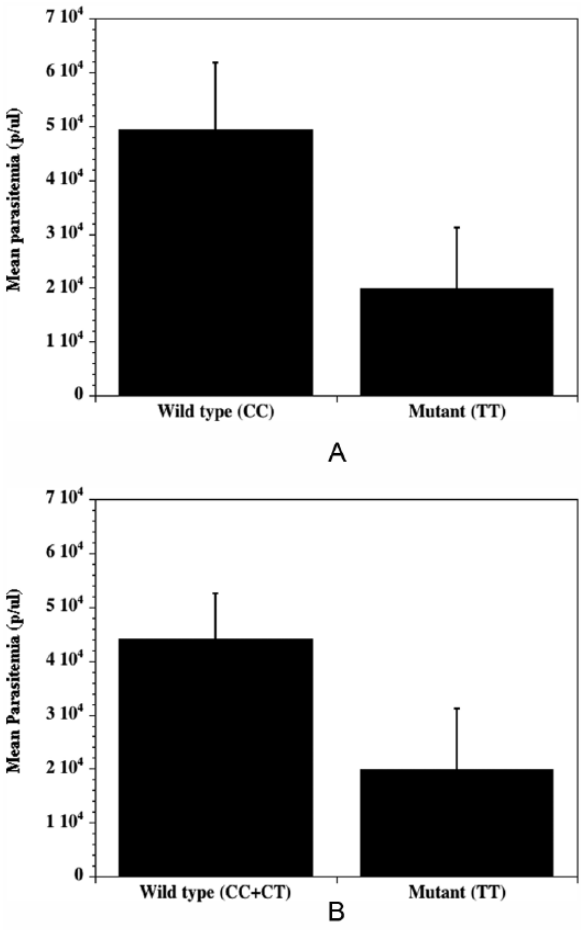


**Figure 3.** Mean parasite distribution between patients with malaria with either (A) wild-type (–159 C/C) or mutant (–159 T/T) variants and (B) wild-type (–159 C/C+C/T) versus mutant (–159 T/T) variants of the CD14 gene promoter polymorphism (snp –159 C/T; rs2569190).

## Discussion

Malaria is still a disease that confounds a lot of researchers globally and the millions who fall sick to this infection constantly, especially considering the possibility of multiple infections in a single year. Despite the intensified efforts toward designing a vaccine or new therapeutic regimen to replenish the stock of available antimalarials, thereby reducing the scourge of disease due to resistant parasites, there is a further need for deconvoluting the various factors contributing to the diversity in disease severity or susceptibility among endemic populations. One of such potential factors is CD14, a leucine-rich repeat surface protein linked to macrophage TLRs that functions as a receptor for bacteria lipopolysaccharide and microbial ligand recognition,[14,18] as well as host response to viral and fungal infections.[36,37] Its polymorphism has been shown to be a major regulator of innate immune response,[10] and its demonstrated expression in the brain is a critical factor in the development of cerebral malaria.[9] Significantly, it has been shown that CD14 genetic factors that affect differential expression of downstream genes would potentially have functional imperative in the immune response to infectious pathogens or immunological diseases.[10] To this end and as part of our ongoing work on immune regulatory patterns contributing to disease diversity and severity, we set out to determine the role, if any, of the genetic diversity of CD14 gene promoter polymorphism (snp –159 C/T; rs2519190) on markers of clinical disease or severity among and between *P falciparum* malaria–infected patients from sub-Saharan Africa. We found that the polymorphism was equally distributed, showing no deviations from HWE ($P > .05$) within malaria-infected and control groups, implying our result is not due to selection pressure, that can be attributed to the disease.

Even though there are a number of publications referencing CD14 and its regulatory or contributory role in several conditions and diseases, including allergy, just a handful is directly related to malaria. A signature publication on murine cerebral malaria showed that deletion of the CD14 gene confers protection against disease and a negative effect on parasite growth in transgenic mice challenged with *P berghei* ANKA[9] and recommended that this observation be elucidated in human infections. Our result among *P falciparum* malaria–infected individuals confirm this finding, whereby the wild-type variant of CD14 gene promoter polymorphism had the highest

statistically significant genotypic and allelic frequencies among patients with malaria when compared with uninfected controls. In addition, the mutant variant was less frequent in malaria-infected patients compared with the higher frequencies in uninfected controls. This observation has significant implications for disease susceptibility among endemic populations and could potentially clarify clinical differences or degrees of disease severity in subcontinental population groups across sub-Saharan Africa. Examining this finding among *Plasmodium vivax* malaria–infected groups within and outside sub-Saharan Africa would be an added benefit of this present report.

Oakley et al also reported on the beneficial role of CD14 in regulating parasitemia among CD14 transgenic and wild-type *P berghei* ANKA–infected mice, with the transgenics showing 2-fold reduction in parasitemia postinfection as well as longer survival rate. This is a very interesting and significant finding superbly confirmed by our result in human infection. We found the highest mean parasitemia among malaria-infected individuals with the CD14 gene promoter wild-type variant and a 2.5-fold reduction (mildly significant) in mean parasitemia among individuals with the mutant variant. This is very important considering the significance of immune regulatory markers in initiating immune response to antigenic stimuli. Despite the fact that Togbe et al[38] saw no conferred protection by the loss of the CD14 gene in murine cerebral malaria, this present finding clarifies possible differences between human and murine infections, and potential benefit of the CD14 gene in human *P falciparum* infection, not seen in animals (multiple backcrosses, differing genetic background, parasite lines/clones), which are completely different from field infections.

In addition, although Wenisch et al[25] reported that they found no relationship between soluble CD14 and markers of disease severity in malaria infection, our present result states otherwise, based on genetic diversity analysis. Our results qualitatively delineate the potential reasons for the results obtained by Wenisch et al. This is an excellent study that should move this field further, especially among malaria-infected individuals from differing ethnic or endemic backgrounds or while examining severity of infection among nonimmune travelers from nonendemic regions. We recommend further studies with larger sample sizes and different population groups across sub-Saharan Africa.

The results in this study further confirm reports that CD14 polymorphism potentiates a $T_H1$ differentiation and subsequent IFN-γ response leading to reduced malaria parasitemia. We believe that a variant CD14 would drive a deficient response leading to an immune cascade that could potentially make disease worse, leading to complications, compared with a normal immune response, in which infection can be adequately managed or controlled. Such complications could potentially include high-circulating immune complex levels,[1] extensive parasite diversity,[39] incomplete clearance of parasites posttreatment and potential for recrudescence,[2] or possibly development of drug-resistant strains. To this end, it is imperative that our results be extended by analyzing pro-inflammatory biomarkers in serum assays from similar patient groups as well as patients with cerebral or severe malaria, in addition to clarifying any contributory role TLR polymorphisms may play in regulating disease markers. In addition, we advocate strengthening awareness and understanding of CD14 regulatory effects on innate immune response, especially with malaria infection in sub-Saharan Africa, and potential to exacerbate disease outcome among infected individuals carrying the CD14 promoter gene mutant variant. We conclude that *P falciparum* malaria severity in sub-Saharan Africa is significantly regulated by CD14 promoter gene (snp –159 C/T; rs2569190) polymorphism, in addition to its importance for clinical disease, markers of severity, and immune response. How this diversity affects clinical presentation or disease severity between subregional or intercontinental population groups deserves further elucidation.

## Acknowledgements

The authors appreciate Maria Gazouli, University of Athens, Greece, and Iman Farid of Rochester Institute of Technology, Rochester, NY, USA, for technical assistance.

## Author Contributions

BNT conceived, designed experiments, and drafted the manuscript. RIF, COF, and OO recruited patients; obtained consent; and collected blood samples. TJS, NA, and BNT performed genotyping and endonuclease digestion. YL, RIF, COF, OO, and BNT conducted statistical analyses. OO and COF contributed to scientific content. All authors read and approved the final version of the manuscript.

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