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Short communication

Plasmodium ovale curtisi and *Plasmodium ovale wallikeri* in Chinese travelers: Prevalence of novel genotypes of circumsporozoite protein in the African continent

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ABSTRACT

Imported malaria due to *Plasmodium ovale curtisi* and *P. ovale wallikeri* infections from African countries has increased recently (2011–2014) in Chinese travelers. We report novel genotypes, their prevalence and the predominant haplotypes of *P. ovale curtisi* and *P. ovale wallikeri* circumsporozoite protein (CSP) from 20 African countries in Chinese travelers. These genotypes should be considered while designing a CSP-based vaccine against *P. ovale* malaria.

1. Introduction

With the implementation of the National Malaria Elimination Program (NEMP, 2010–2020), there has been a considerable reduction in local malaria cases in China (Zhang et al., 2014). However, imported malaria cases mostly originating from Sub-Saharan African countries poses a big threat leading to the rise of malaria cases in China (Cao et al., 2016; Li et al., 2016). A recent study in Jiangsu Province of China, on *P. ovale* imported cases from Sub-Saharan African countries (from 2011 to 2014) reported that 48% cases were due to *P. ovale curtisi* and 52% cases were due to *P. ovale wallikeri* (Cao et al., 2016). Though *P. ovale* infection were suspected to be present along the south-west border of Yunnan provinces in China, only imported cases was observed in the past-60 years. *Plasmodium ovale* is tertian malaria which has two sympatric sub-species *P. ovale curtisi* and *P. ovale wallikeri* (Sutherland et al., 2010) and has so far received little attention in medical research. Due to its low parasitemia, ability to cause relapse in patients, and its morphological resemblance with *P. vivax* makes its diagnosis difficult by microscopy (Collins and Jeffery, 2005). However, previous studies have also reported severe disease and fatal cases due to *P. ovale* (Facer and Rouse, 1991; Lau et al., 2013).

In the context of the development of a successful malaria vaccine, an understanding of antigen polymorphism is very crucial for proper vaccine design, deployment, and evaluation. The circumsporozoite protein (CSP) is the most abundant protein on the sporozoite's surface and has a sequence comprising a central repeat that is flanked by polymorphic N-terminal and C-terminal non-repeat regions. The *P. falciparum*-CSP antigen is the primary component of the RTS,S vaccine (Gordon et al., 1995), which is one of the leading malaria vaccine candidates. It is the only one to have shown moderate, but promising efficacy during Phase II trials in adults in The Gambia and children in Mozambique (Alonso et al., 2004) and allele-specific high vaccine efficacy in children in the age group of 5–17 months old (Neafsey et al., 2015). However, during Phase III trials in 10 African countries, RTS, S vaccine has shown low efficacy (Mahmoudi and Keshavarz, 2017).

Given the high number of imported *P. ovale* infections in China and the lack of genetic studies on *P. ovale curtisi* and *P. ovale wallikeri* vaccine candidates in African countries, we conducted this study to understand the genetic variations in the *csp* and determine the crucial genotypes in clinical isolates of Chinese cases imported from 20 African

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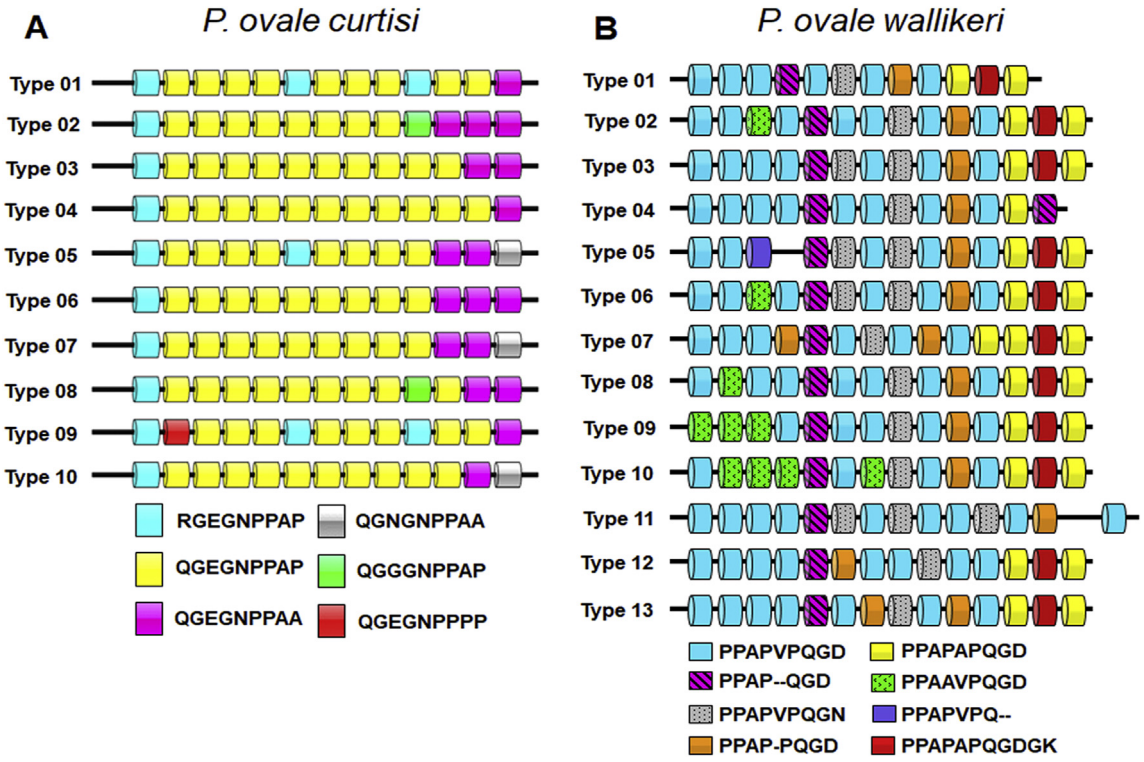

Fig. 1. Schematics of genotypes and repeat motif sequences of *P. ovale curtisi csp* (A) and *P. ovale wallikeri csp* (B) from African isolates.

countries and discuss its importance in relevance to future vaccine design against two *P. ovale* sub-species.

2. Materials and methods

We used previously collected samples from Jiangsu Province, China (n = 198, Table S1) from 2012 to 2016 where imported cases were reported from 20 Sub-Saharan African countries (Cao et al., 2016). All *P. ovale* samples were initially screened using microscopy and real-time PCR to differentiate between the two sub-species using methods from previous report (Cao et al., 2016). A total of 198 blood samples were obtained and details of the real-time PCR results with demographic information, parasitemia are summarized in Table S1. This study was approved by ethical clearance was obtained from the Institutional Review Board of Jiangsu Institute of Parasitic Diseases (IRB00004221), Wuxi, China. Genomic DNA was extracted from the blood samples using Qiagen DNeasy Blood Tissue Kit (QIAGEN, Valencia, CA) according to the manufacturers' protocol. The *poccsp* and *powcsp* genes were then amplified (Bio-Rad MyCycler™) as follows. The full-length *poccsp* gene was PCR amplified using the oligonucleotide primer pair PoC CSP-F 5′ GGCTTGCATATACAATCTTTCATTA 3′ and PoC CSP-R 5′ ACACCAATA TCAGGTGCTATTTATT 3′ primers based on published *P. ovale curtisi* genome sequence GH01_00239700. The primers were designed flanking the *csp* gene such that on amplification and sequencing the full-length is obtained and the expected band size was 1224 bp. The amplification reaction was carried out in the Taq master mix (Vazyme, Nanjing, China) and 1 µL of DNA template with nuclease-free water used to obtain a final volume of 20 µL. The PCR amplification was initiated at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1.5 min and a final extension at 72 °C for 5 min. For the *powcsp* gene, PCR amplification was carried out by semi-nested PCR with the primers PoW CSP-F 5′-CTTGCGCATATAACAATACACATC-3, PoW CSP-F1 5′GTAGGCGGCT GGCTCTACTTTCGA-3′ and PoW CSP-R 5′- ATAACGCGTGCGCATGTA TATAC-3′ which were designed based on published *P. ovale wallikeri* genome sequence POVWA1_023660 and the expected band size was 1008 bp. The amplification reaction was carried out using the Vazyme Master mix as above. The purified PCR amplified fragments were then sent for sequencing to Shanghai Talen-Biological Technology Co. Ltd., China with the primers used for the PCR amplification. Any single nucleotide polymorphism (SNP) that was not observed in two or more samples was only included in the analysis after validity was confirmed by a repeated cycle of amplification and sequencing. Sequences with two calls at particular sites, indicative of mixed genotype infections, were also among those excluded.

The DNA sequences generated were analyzed using SeqMan Software in Lasergene v 7.0. The sequences were aligned and edited by using CLUSTAL-W against the reference strains of *P. ovale curtisi* (GH01_00239700) and *P. ovale wallikeri* (POVWA1_023660) for the CSP gene and then exported to the MEGA 5.6 programme for further alignment and analysis. Sequence diversity (π), the number of polymorphic sites, the number of synonymous and non-synonymous substitutions, haplotype diversity (Hd), the number of haplotypes (h) within the *poccsp* and *powcsp* sequences were determined by DnaSP v5.10 software (Librado and Rozas, 2009). Graphical representation of diversity was calculated with window length 100 bp and step size 25 bp in Dnasp. The rates of synonymous (dS) and non-synonymous (dN) mutations were computed in MEGA5 using the method of Nei and Gojobori and compared by the *Z*-test ($P < .05$) in MEGA5 with the Jukes and Cantor (JC) correction and 1000 bootstrap replications (Tamura et al., 2011). Genealogical relationships between the *csp* haplotypes were constructed using the median-joining method in NETWORK software version 4.6.1.2 (Bandelt et al., 1999). The *poccsp* and *powcsp* sequence information were obtained from 82 and 58 samples, respectively, from 20 African countries (Accession number: MG865292 - MG865373 and MG865374 - MG865431) (Fig. S1).

3. Results and discussion

Alignment and comparison of 82 *poccsp* sequences (1076 bp) revealed that only the central repeat region was polymorphic with 26 SNPs (yielding 15 haplotypes) and the N and C-terminal non-repeat

Table 1
Prevalence of genotypes and repeat motif sequences of *P. ovale curtisi csp* from African isolates.

Country	No. of isolates (%) in each type and repeat motifs								
	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Type 7	Type 8	Type 9
	1[**2**]$_4$**1**[**2**]$_3$**1**[**2**]$_2$[**3**]$_2$[**4**]$_2$	**1**[**2**]$_8$**5**[**3**]$_3$[**4**]$_3$	**1**[**2**]$_{10}$[**3**]$_2$[**4**]$_3$	**1**[**2**]$_{11}$[**3**]$_2$[**4**]$_2$	1[**2**]$_4$1[**2**]$_4$[**3**]$_2$[**4**]$_4$	**1**[**2**]$_9$[**3**]$_3$[**4**]$_3$	**1**[**2**]$_9$[**3**]$_2$[**4**]$_4$	**1**[**2**]$_8$**52**[**3**]$_2$[**4**]$_3$	**16**[**2**]$_3$**1**[**2**]$_3$**1**[**2**]$_2$[**3**]$_2$[**4**]$_2$
Equatorial Guinea	5(6.1)	1(1.2)	10(12.2)	7(8.5)	4(4.8)	4(4.9)	3(3.6)	–	1 (1.2)
Nigeria	6(7.3)	–	2(2.4)	1(1.2)	–	–	1(1.2)	–	–
Angola	1(1.2)	7(8.5)	–	–	–	–	–	–	–
Congo	1(1.2)	3(3.6)	–	–	–	–	–	–	–
Cameroon	4 (4.9)	–	–	–	1(1.2)	–	–	–	–
DR Congo	1(1.2)	4(4.9)	1(1.2)	–	–	–	–	1(1.2)	–
Mozambique	–	1(1.2)	–	–	–	–	–	–	–
Zambia	–	–	–	–	–	–	–	1(1.2)	–
Malawi	–	–	–	–	–	–	–	1(1.2)	–
Niger	–	–	–	–	–	1(1.2)	–	–	–
Liberia	1(1.2)	–	–	–	1(1.2)	–	–	–	–
Chad	–	–	1(1.2)	–	–	–	–	–	–
Guinea	–	–	1(1.2)	–	–	–	–	–	–
South Africa	1(1.2)	–	–	–	–	–	–	–	–
Sierra Leone	1(1.2)	–	–	–	–	–	–	–	–
Gabon	1(1.2)	–	–	–	–	–	–	–	–
Ghana	–	–	–	–	–	–	–	–	1(1.2)
Total	22(26.8)	16(19.5)	15(18.3)	8(9.7)	6 (7.3)	5(6.1)	4(4.8)	3(3.6)	2(2.4)

The nona peptide repeat motifs were named as: **1**, RGEGNPPAP; **2**, QGEGNPPAP; **3**, QGEGNPPAA**; 4**, QGNGNPPAA; **5**, QGGGNPPAP; **6**, QGEGNPPPP.
Additional Type 10: **1**[**2**]$_{10}$**3**[**4**]$_4$; 1(1.2), Nigeria, The number in parenthesis indicates percentages of each type.
-, no data.

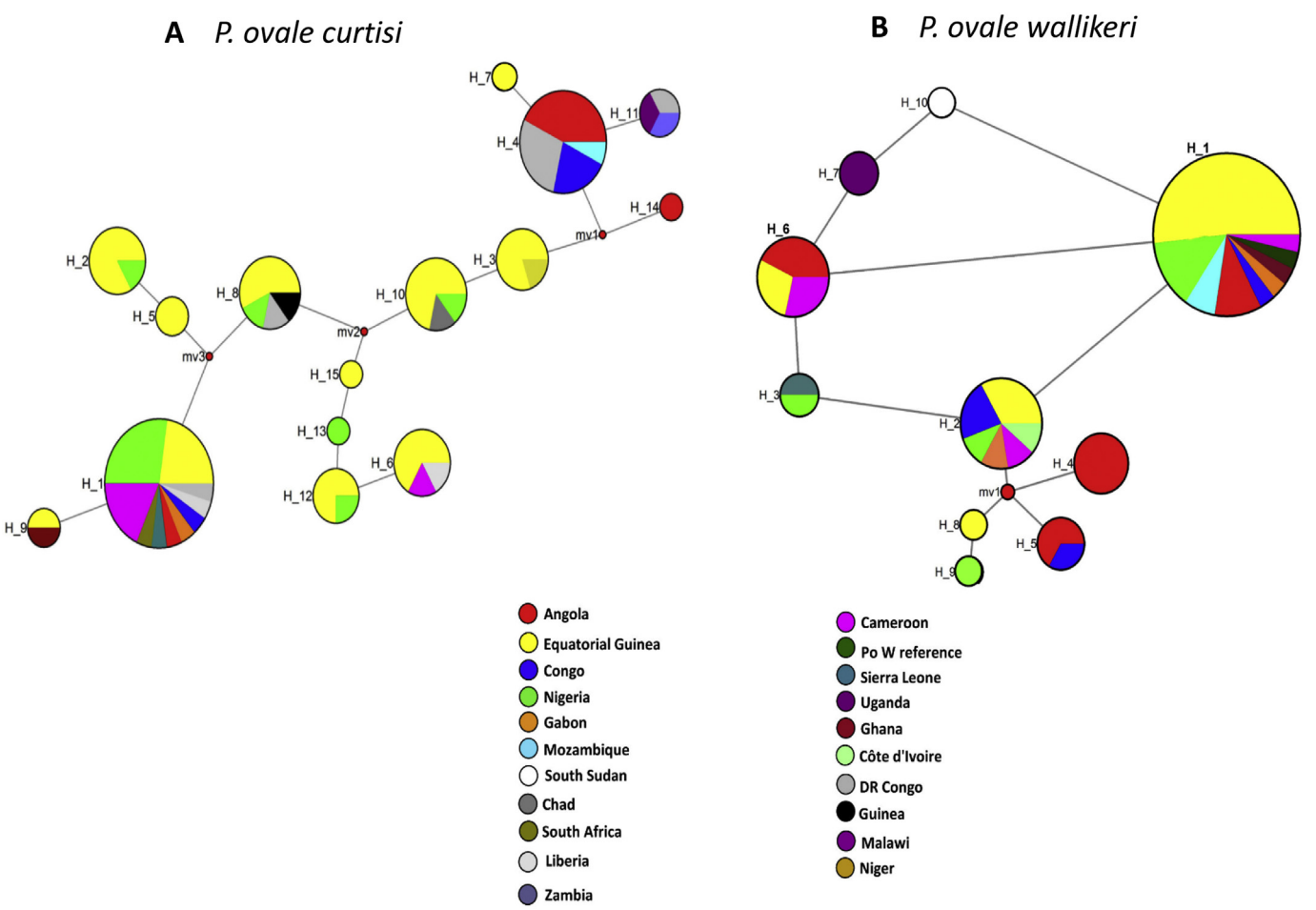

Fig. 2. Haplotypes of *P. ovale curtisi csp* (A) and *P. ovale wallikeri csp* (B) from African isolates.

regions were conserved (Fig. S2). We also observed low nucleotide diversity across the *csp* gene (Table S2) compared to *P. falciparum* (Escalante et al., 2002) and only the central region was found to be diverse ($\pi = 0.02$) and the non-repeat regions were conserved (Fig. S2). There were excess of synonymous substitutions compared to non-synonymous substitutions within the central region which leading to negative selection (Table S2). This conservation was also observed in the C-terminal T-cell epitope regions Th2r; PSEDDIKKYIDKIRNDI and Th3r; GVRVRRKAGASAKKAQELTLSD indicating the absence of host immune selection pressure in *P. ovale curtisi* similar to the observation in *P. malariae* CSP (Tahar et al., 1998) (Fig. S3). Based on the deduced amino acid alignments we noted the following nona-repeat motifs: 1, RGEGNPPAP; 2, QGEGNPPAP; 3, QGEGNPPAA; 4, QGNGNPPAA; 5, QGGGNPPAP and 6, QGEGNPPPP in the central repeat region of *P. ovale curtisi* CSP (Fig. 1A). Among these repeat motifs, three repeat motifs 2, 3 and 4 were tandem repeats and repeat motif 2 was most abundant with 2–11 repeats in a sequence. Unlike *P. falciparum* which has a NANP/NVDP tetrapeptide tandem repeats (Escalante et al., 2002) we found nonapeptide tandem repeats QG(E/N)GNPPA(P/A) in *P. ovale curtisi* which is similar to nonapeptide repeat motifs in *P. vivax* (Hernandez-Martinez et al., 2011). For simplicity, we characterized the central repeat region into 10 genotypes (Type 1 to Type 10) based on the arrangement of these repeat motifs (Table 1). The most abundant genotype among the 18 African countries was Type 1 (26.9%, 22/82) followed by Type 2 (19.5%, 16/82) and Type 3 (18.3%, 15/82) (Table 1). Among these, Type 1 was prevalent in 10 countries with Nigeria (7.3%, 6/82) sharing the highest followed by Equatorial Guinea (6.0%, 5/82) (Table 1).

The median-joining network tree for the *poccsp* (central repeat region) haplotypes from 15 African countries identified two major shared haplotypes [Hap_1: n = 22 (ARREPPEE) and Hap_4: n = 14 (AQQGAAEN)] (Fig. 2A). Hap_1 was shared among 8 countries and Hap_4 was shared among 6 African countries (Fig. 2A). Four minor haplotypes (Hap_8, 12, 6 and 3) were shared among 4 countries. Six unique haplotypes were identified which originated from Angola, Equatorial Guinea, and Nigeria.

Fifty-eight *powcsp* sequences also showed that the N and C-terminal non-repeat regions were conserved and central repeat region (from 274 to 668 nucleotides [nts]) was polymorphic (Fig. S2). We observed size variations at the repeat regions due to 6 nucleotides deletion in some isolates leading to size variations from 884 to 990 nts. In total, there were 31 SNPs across the central repeat region with lower nucleotide diversity ($\pi = 0.01$) compared to *poccsp* yielding 10 haplotypes (Table S2). Similar to the findings with PoC CSP, the C-terminal T-cell epitope regions; Th2r; PSEDDIKKYIDKIRKDI and Th3r; GVRVRRKAGASAKKANELTIND were conserved in all 58 sequences analyzed unlike *P. falciparum* (Zeeshan et al., 2012) and negative section was observed based on the central region. Based on the amino acid alignments we noted the following repeat motifs: 1, PPAPVPQGD; 2, PPAPQGD; 3, PPAPVPQGN; 4, PPAP–QGD; 5, PPAPAPQGD; 6, PPAAVPQGD; 7, PPAPVPQ- and 8, PPAPAPQGDGK in the central repeat region of *P. ovale wallikeri* CSP (Fig. 1B). Among these repeat motifs, 1, 5 and 6 were tandem repeats and repeat motif 1 was most abundant with 2–4 repeats in a sequence. The nona-peptide tandem repeats PPAP(V/A)PQGD in *P. ovale wallikeri* were also similar to repeat motifs in *P. vivax* indicating closer phylogenetic relationship compared to *P. falciparum* as observed earlier (Rutledge et al., 2017) and the existence of extensive polymorphism in the central region (Hernandez-Martinez et al., 2011). We characterized

Table 2
Prevalence of genotypes and repeat motif sequences of *P. ovale wallikeri csp* from African isolates.

Country	No. of isolates (%) in each type and repeat motifs						
	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Type 7
	[1]$_3$213141585	**[1]$_2$612[1]$_2$3141585**	**[1]$_4$2313141585**	**[1]$_4$2[1]$_2$3141[5]$_2$–5**	**[1]$_2$7-2313141585**	**[1]$_2$612313141585**	**[1]$_3$4213141[5]$_2$85**
Equatorial Guinea	14(24.1)	2(3.4)	3(5.1)	–	–	–	1(1.7)
Nigeria	1(1.7)	–	1(1.7)	3(5.1)	–	1(1.7)	–
Angola	3(5.1)	3(5.1)	2(3.4)	–	–	–	3(5.1)
Congo	1(1.7)	–	3(5.1)	–	–	–	–
Cameroon	1(1.7)	2(3.5)	–	–	1(1.7)	–	–
DR Congo	–	–	–	–	–	–	–
Mozambique	–	–	–	2(3.4)	–	–	–
Cote d'ivoire	–	–	1(1.7)	–	–	–	–
South Sudan	–	–	–	–	–	–	–
Uganda	–	–	–	–	–	–	–
Liberia	–	–	–	–	–	–	–
Guinea	–	–	–	–	–	–	–
Sierra Leone	–	–	–	–	–	1(1.7)	–
Gabon	1(1.7)	–	–	–	1(1.7)	–	–
Ghana	1(1.7)	–	–	–	–	–	–
Total	22(37.9)	7(12)	10(17.2)	5(8.6)	2(3.4)	2(3.45)	4(6.9)

The repeat motifs were named as: **1**, PPAPVPQGD; **2**, PPAPQGD; **3**, PPAPVPQGN; **4**, PPAP–QGD**; 5**, PPAPAPQGD; **6**, PPAAVPQGD **7**, PPAPVPQ-; **8**, PPAPAPQGDGK.
Additional genotypes:
Type 8: **16[1]$_2$2[1]$_2$3141585**; 1 (1.7), South Sudan
Type 9: **[6]$_3$12[1]$_2$3141585**; 1 (1.7), Uganda
Type 10: **1[6]$_3$2163141585;** 1 (1.7), Uganda
Type 11: **[1]$_4$2313[1]$_2$314–1**; 1(1.7), Equatorial Guinea
Type 12: **[1]$_4$24[1]$_2$3[1]$_2$585**; 1(1.7), Nigeria
Type 13: **[1]$_4$2143141585**; 1(1.7), Equatorial Guinea
The number in parenthesis indicates percentages of each type.
-, no data.

the central repeat region into 13 genotypes (Type 1 to Type 13) based on the arrangement of these repeat motifs (Table 2). The most abundant genotype among the 20 African countries was Type 1 (37.9%, 22/58) followed by Type 3 (17.2%, 10/58) and Type 2 (12.0%, 7/58) (Table 2). Among these, Type 1 was prevalent in 7 countries with Equatorial Guinea (24.1%, 14/58) sharing the highest followed by Angola (5.2%, 3/58) (Table 2).

The haplotype network tree for *powcsp* (central repeat region) from 17 African countries identified one major haplotype [Hap_1: n = 29 (PPPDPDNDVDAAGNQ)] (Fig. 2B) which was shared among 8 African countries. Minor haplotype [Hap_2: n = 9, (PAPDPDNDVDAAGNQ) was shared between 6 African countries (Fig. 2B). Five unique haplotypes were identified which originated from Angola, Equatorial Guinea, Nigeria and South Sudan.

Our study is the first to report the *csp* genotypes and their heterogeneity within *P. ovale curtisi* and *P. ovale wallikeri* parasites from African countries. One limitation of this study is that we could not generate sequence information from all samples in the study due to sequencing problem and limited sample size from some countries; nevertheless, we could find multiple *csp* genotypes which are prevalent in many African countries. The T cell epitope regions (Th2r/Th3r) for both the species were conserved indicating that only the B cell epitope region within the repeat region were diverse and absence of host immune pressure on the T cell epitope regions as observed for *P. falciparum* (Kumkhaek et al., 2005). Future studies should focus on the immunological basis of these *P. ovale* repeat genotypes with relevance to vaccine design and malaria elimination of imported cases from Sub-Saharan African countries.

Our study highlights the novel *csp* genotypes of *P. ovale curtisi* and *P. ovale wallikeri* among 20 African countries from imported cases in China and it is an important step forward in understanding the parasite variants as well as in the development of a CSP-based vaccine in context to the African continent.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.02.008.

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