Single Nucleotide Polymorphism Analysis of *Pvmdr-1* in *Plasmodium Vivax* Isolated from Military Personnel of Republic of Korea in 2016 and 2017

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Research Article

Keywords: Plasmodium vivax, Multi-drug resistance protein 1, Single nucleotide polymorphism, Chemoprophylaxis

Posted Date: December 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-273974/v2

Abstract

Background: Malaria chemoprophylaxis using chloroquine (CQ) and primaquine (PQ) has been administered to resident soldiers in the 3rd Army of Republic of Korea (ROK) to prevent malaria infection since the year 1997. Due to mass chemoprophylaxis against malaria, concern exists about occurrence of chloroquine resistance (CQR). Herein, we investigated the single nucleotide polymorphisms (SNPs) of the *Plasmodium vivax* multi-drug resistance protein-1 (*pvmdr-1)* gene to monitor the risk of CQR.

Methods: SNPs of the *pvmdr-1* gene were analyzed in 73 soldiers of the 3rd Army of ROK diagnosed with infection by *Plasmodium vivax (P. vivax)*.

Results: Quintuple mutations (G698S, L845F, M908L, T958M, and F1076L) were detected in 73 soldiers. Mutation in the Y541C position was firstly detected in soldiers at a frequency of 1.3% (1/73). In addition, synonymous mutations were detected at positions K44, L493, T529, and E1233. Based on these SNPs, *pvmdr-1* sequences of ROK were classified into 6 haplotypes. The phylogenetic analysis closed to Type of North Korean showed that *P. vivax* malaria of ROK could be a reason of influx from North Korea. In this study, there was no therapeutic resistance (CQ-mediated parasite clearance within 72 hours) for clinical samples that possessed various SNPs of *pvmdr-1*. Various SNPs including a newly identified non-synonymous mutation (Y541C) had been introduced into *P. vivax* malaria-endemic areas in ROK.

Conclusions: Our study showed that synonymous and non-synonymous mutations of *pvmdr-1* were introduced to the malaria chemoprophylaxis-executed regions of ROK from 2016 to 2017. Thus, to prevent the emergence of CQR, continuous surveillance for SNPs of *pvmdr-1* related with CQR in the malaria-endemic regions of ROK is essential.

Introduction

Malaria, life-threatening disease caused by *Plasmodium* parasites, endangers about 40% of the world's population [Price et al., 2007; Guerra et al., 2009]. Depending on recent WHO malaria report, all malaria infection cases have been declined from 238 million cases in 2000 to 229 million cases in 2019. In case of *P. vivax* infected malaria, it was decreased from about 7% in 2000 to 3% in 2019 [WHO, World malaria report 2020]. This worldwide malaria reduction trends is attributed to WHO's preventive policy of eradicating malaria. Nevertheless, annually, more than 300 million people of the world`s population are infected with malaria, and about 500,000 people die from malaria. The re-emergence of *P. vivax* malaria in ROK, which supposed to eradicated, was reported in Paju City of Gyeonggi Province in 1993 [Chai et al., 1994; Yeom et al., 2012]. According to Korea Disease Control and Prevention Agency, since re-emergence occurred in a soldier of the 3rd Army of ROK, the incidence of malaria infections has been steadily increasing. In ROK, *P. vivax* malaria endemic regions are localized near the DMZ (demilitarized zone; the border between ROK and the Democratic People's Republic of Korea, DPRK) [KCDA]. Thus, soldiers and civilians residing in DMZ have been classified as high-risk group of malaria infection. Among the total malaria patients of ROK, military (Soldiers and military veterans) accounted for a large proportion.

Because of this, members of the army of ROK have undergone prophylactic chemotherapy against malaria to prevent patient outbreak since 1997 [Yeom et al., 2005]. The detailed procedure of chemoprophylaxis is as following, 300 mg CQ is weekly administrated to military personnel nearby DMZ from July to October for 15 weeks, and 15 mg PQ is subsequently administrated daily for 2 weeks [WHO, Guidelines for the Treatment of Malaria 3rd. 2015].

CQ is effective to eradicate *P. vivax* in asexual blood stages and gametocytes, and PQ is responsible for killing the hypnozoite form of *P. vivax* parasite in the liver stage. Due to the risk of CQR by massive and long-term use of chemoprophylaxis for prevention of malaria [Lee et al., 2009], the army of ROK has been monitoring drug resistance by analyzing mutations in *pvmdr-1* [Chung et al., 2015]. To date, various CQR cases in several regions including Indonesia, Southeast Asia, India, and Central and South America have been reported [Schuurkamp et al., 1992; Rieckmann et al., 1989; Schwartz et al., 1991; Baird et al., 2004; Ruebush et al., 2003; Soto et al., 2001; Marlar-Than et al., 1995]. There is concern about the influx of malaria resistance from Southeast Asian countries [Feng et al., 2015]. Recent studies have been shown that long-term chemoprophylaxis could induce genetic mutation. For example, polymorphism of *pvcrt-o* and *pvmdr-1* was detected by CQ treatment [Golassa et al., 2015; Anantabotla et al., 2019; Barnadas et al., 2008]. Thus, we hypothesized that genetic changes could occur in malaria chemoprophylaxis-executed malaria-endemic regions of ROK. On the basis of our hypothesis, we examined the relevance between SNPs of *pvmdr-1* and drug-mediated parasite clearance, which military hospitals confirmed CQ-mediated parasite clearance within 72 hours in all malaria samples. Herein, our study was to focus on the surveillance of genetic mutation in *pvmdr-1* (Malaria drug resistance-related gene).

Materials And Methods

Ethics statement and sample preparation

This study was approved by the ethics committee of the Armed Forces Medical Command (Approval No. AFMC-16067-IRB-16-056, September 2016). An approval form was used to obtain written informed consent and permission from each participant for providing a 5 ml blood sample.

Collection of clinical isolates

172 venous blood samples from male patients infected with malaria or malaria suspected patients with fever were collected in three Armed Forces Hospitals (Yangju, Koyang, and Ildong) near the DMZ located in northern Gyeonggi Province and in the northwest region of the ROK from 2016 to 2017. Armed Forces Hospitals performed a rapid diagnostic test (STANDARD DIAGNOSTICS Inc., USA), and blood smear test with microscopy for all participant samples with a history of fever in the 48 hours.

Plasmodium vivax **nested PCR analysis (**18S rRNA **and** pvmdr-1)

Genomic DNA was extracted from 200 ul whole blood using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. For screening *P. vivax* infection, purified DNA

samples from all patients were diagnosed by nested polymerase chain reaction (PCR) targeting *P. vivax 18S rRNA*. 1,100 bp or 120 bp was detected by 2nd nested PCR. For the amplification of DNA templates, using Master cycler nexus X2 thermal cycler (Eppendorf Inc., Germany) and specific primer sets [The first round of PCR primer sets: rPLU5 forward primer (5'-CCT GTT GTT GCC TTA AAC TTC-3') and rPLU6 reverse primer (5'-TTA AAA TTG TTG CAG TTA AAA CG-3'), The second round of PCR primer sets: rVIV1 forward primer (5'-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3') and rVIV2 reverse primer (5'-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3')], Nested PCR was conducted as shown in previous reference [Barnadas et al., 2008]. The first round of PCR was performed under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 5 min. The second PCR was performed under the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for 35 sec, and a final extension at 72°C for 5 min.

As shown in previous report [Barnadas et al., 2008], the *pvmdr-1* gene was amplified using the indicated primer sets of nested PCR (Table 1). The first round of PCR was performed under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 4.5 min, and a final extension at 72°C for 10 min. The second PCR was performed under the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 4 min, and a final extension at 72°C for 10 min. PCR products were analyzed on 1.5% agarose gels using 1 Kb Plus DNA Ladder (Thermo Fisher Scientific) by electrophoresis (Figure 1) and visualized by Fluor Chem FC3 (PROTEIN SIMPLE, USA).

Sequencing of pvmdr-1

After the amplification, sequence analysis was performed by specific sequencing primers as shown in Table 1. Sequencing primers were designed to cover near full-length *pvmdr-1* gene (4,395 bp). Direct sequencing of PCR products was performed by using Big Dye^{TM} Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and the products were resolved on ABI 3730XL Genetic Analyzer (Applied Biosystems, CA, USA).

Phylogenetic analysis

We used nearly full-length sequences (from 104 bp to 4,290 bp) of *pvmdr-1* to perform phylogenetic relationship analysis. The sequences of Sal I strain (GenBank Accession# AY571984) and global parasites in PlasmoDB [Aurrecoechea et al., 2009] were used as reference sequences. *pvmdr-1* sequences of 73 *P. vivax* clinical samples isolated from military personnel of ROK army in 2016 and 2017 were analyzed using BioEdit Sequence Alignment Editor [Hall et al., 1999]. The phylogenetic analysis was constructed by 1,000 bootstrap replications [Pattengale et al., 2010] using the neighbor-joining method [Saitou et al., 1987].

Results

Nested PCR and SNP analyses of the pvmdr-1 gene

73 of 172 samples were identified as *P. vivax* infected specimen through nested PCR analysis targeting *18S rRNA* (Figure 1a). For SNP analysis of *pvmdr-1*, we conducted nested PCR targeting *pvmdr-1* using 73 positive specimens (Figure 1b), which were used to sequence analysis. As shown in Table 2, five non-synonymous mutations (G698S, L845F, M908L, T958M, and F1076L) were detected in all specimens (2016: 20/20, 2017: 53/53). Interestingly, a novel non-synonymous mutation (Y541C) was detected in 1 soldier at a frequency of 1.8% (2017: 1/53). In addition, silent mutations in K44 [2016: 4/20 (20%), 2017: 24/53 (45.2%)], L493 [2016: 5/20 (25%), 2017: 14/53 (26.4%)], T529 [2016: 15/20 (75%), 2017: 30/53 (56.6%)], and E1233 [2016: 8/20 (40%), 2017: 30/53 (56.6%) positions were detected in specimens. Alignment and mapping data of *pvmdr-1* wild-type and mutant-type sequences were provided (Additional File 1; Figure S1).

Genotypic classification of the pvmdr-1 gene

As shown in Table 3, all specimens were clustered into 6 groups from Type 0 to Type 5, and all haplotypes possessed 5 non-synonymous mutations (G698S, L845F, M908L, T958M, and F1076L). We genotypically classified the 6 haplotypes based on *pvmdr-1* sequences from 73 specimens in ROK army from 2016 to 2017 (Figure 3). Type 0 was found in 1 case in 2016, and there was no mutation other than the 5 non-synonymous mutations. For synonymous mutation, K44 mutant was found in Type 1 (2016; 1/20, 2017; 8/53) and Type 2 (2016; 3/20, 2017; 16/53), T529 mutant was found in Type 3 (2016; 5/20, 2017; 14/53), Type 4 (2016; 10/20, 2017; 14/53), and Type 5 (2016; 0/20, 2017 1/53), E1233 mutant was found in Type 2 (2016; 3/20, 2017; 16/53) and Type 3 (2016; 5/20, 2017; 14/53), and L493 was only found in Type 3 (2016; 5/20, 2017; 14/53). Type 5 (2017; 1/53) was nearly the same as Type 4, except it harbored the first identified Y541C mutation. In the years 2016 and 2017, Type 4 (24/73) was abundant and showed a 100% match with the *pvmdr-1* sequence of North Korean.

Phylogenetic analysis of the pvmdr-1 gene

We performed analysis of phylogenetic relationships on the overall *pvmdr-1* sequence and classified them into 6 haplotypes (Table 3). Type 4 sequence was identical to the *MDR* sequence in North Korean (*length* = 0.00001). The neighborhood types of 6 haplotypes were identified as China_NB-16 and Papua New Guinea-PNG58/Thailand_VKBT-106 (Figure 2).

Discussion

P. vivax endemic area is localized in the Gyeonggi province of the ROK near the DMZ. In this study, we analyzed CQ-mediated parasite clearance and *pvmdr-1* SNPs of *P. vivax* using malaria-infected blood specimens in malaria-endemic regions including Goseong, Cheorwon, Yeonchen, Paju, Gimpo, and Hwacheon (Figure 3). Since 1997, the ROK Army has conducted continuous malaria chemoprophylaxis (CQ and PQ) to prevent and reduce transmission of malaria for approximately 100,000 military personnel. Due to the massive chemoprophylaxis efforts, there is a consistent concern regarding CQR. According to various reports [Yeom et al., 2012; Lee et al., 2009], long-term or massive chemoprophylaxis could cause

CQR. Thus, the surveillance system should be needed to estimate the risk of chemoprophylaxis-mediated drug resistance. ROK army also has been interested in analyzing SNPs of drug resistant-related gene along with the implementation of chemoprophylaxis. Thus, we investigated the SNP analysis of *pvmdr-1* and CQ-induced therapeutic clearance of parasite against malaria-infected soldiers near chemoprophylaxis-executed malaria-endemic regions.

CQR has occasionally been observed in malaria endemic regions that follow extensive chemoprophylaxis. Through preventive CQR study, CQR was confirmed in 2 of 484 enrolled patients [Park et al., 2009]. CQR was also studied via the treatment responses of *P. vivax* malaria patients in the ROK monitored during 2003-2007 [Lee et al., 2009]. Until recently, it was reported that *P. vivax* resistance to CQ had emerged in South America and French Guiana [Goncalves et al., 2014; Musset et al., 2019]. In the ROK, to date, most malaria patients in the military have been cured with malaria chemotherapy. However, issues with chemoprophylaxis-mediated resistance have recently been reported [Lee et al., 2009; Chai et al., 2020]. For example, Yeom and colleagues suggested that malaria resistance to prophylactic agents could decrease CQ susceptibility, and they cited that the mass chemoprophylaxis with CQ in the ROK Army could contribute to this issue [Yeom et al., 2012]. Therefore, the relationship between CQ susceptibility and chemoprophylaxis should also be investigated. To date, although CQR cases of *P. vivax* malaria have been reported in many other areas of the world, the drug resistance in malaria-endemic regions of ROK has been reported rarely [Lee et al., 2009]. However, the issue of drug insusceptibility to CQ and rapid transition of *pvmdr-1* SNPs has been raised [Schousboe et al., 2015].

In this study, the Y541C, K44, L493, and T529 mutations centered around quintuple mutations (G698S, L845F, M908L, T958M, and F1076L) in the *pvmdr-1* gene were detected in malaria-infected military personnel of the ROK. Fortunately, there was no CQR in the aforementioned mutations, based on CQ-induced normal clearance of the parasite. Based on the CQ-induced normal clearance of the parasite in malaria-infected military personnel from the ROK, we inferred that quintuple mutations (G698S, L845F, M908L, T958L, and F1076L) could represent a background mutation. However, other reports have showed that SNPs of M908L, T958M, and F1076L were associated with CQR [Chehuan et al., 2013; Li et al., 2020].

As the reports investigating the relationship of CQR and *pvmdr-1* SNP, Suwanarusk *et al.* revealed that the Y976F mutation was linked to CQR based on an increase in the IC_{50} value [Suwanarusk et al., 2008]. In other report, the F1076L mutation was linked to the Y976F SNP as a background mutation [Tacoli et al., 2019]. Our study did not identify any SNPs at the Y976F position and also did not conclude whether Y976F was linked with F1076L. This study showed no CQR with the Y541C mutation (Data not shown). In detail, in the patient with the Y541C phenotypic mutation, normal parasitic clearance to CQ treatment was observed, and parasitic clearance was also confirmed after one month. Thus, we inferred that the Y541C mutation was not related to CQR.

Phylogenetic analysis showed that Type 4 (T529, G698S, L845F, M908L, T958M, and F1076L) was significantly related to the Type identified for North Koreans. In addition, a review by Chai indicated that a

mosquito with blood from a malaria patient in North Korea near the DMZ flew south and infected Korean soldiers [Chai et al., 2020]. Based on this result, we deduced that *P. vivax* malaria was introduced from North Korea, rather than as an influx from foreign countries.

Here, we focused SNPs of *pvmdr-1* and CQ-induced therapeutic clearance of parasite against malaria-infected soldiers near chemoprophylaxis-executed malaria-endemic regions. However, for a more practical resistance analysis and effectiveness of malaria chemoprophylaxis, *in vivo* or *ex vivo* resistance studies via analyzing malaria infection ratio or CQ metabolite under malaria chemoprophylaxis via further research. therefore, we are also planning these studies in cooperation with AFMC and KDCA (Korea Disease Control Agency).

Conclusions

In this study, there was no therapeutic CQR (CQ-mediated parasite clearance within 72 hours) for clinical samples that possessed various SNPs of *pvmdr-1*. Thus, we inferred that quintuple mutation (G698S, L845F, M908L, T958M, and F1076L) was a prevalent background mutation based on CQ-induced normal clearance of the parasite in malaria-infected military personnel of the ROK. Phylogenetic analysis indicated that the malaria type was close to a type seen in North Korean, indicating that *P. vivax* malaria in the ROK could have come from North Korea. Furthermore, consistent monitoring of chemoprophylaxis-linked *pvmdr-1* polymorphisms should be conducted to detect rapidly changed SNP profiling against *P. vivax* malaria in the ROK.

Declarations

Acknowledgements

None.

Author's contributions

Kyung Tae Noh, Qu-Ehn Park, and Wonsig Lee designed the project, Wonsig Lee conducted experiments, Jin-Jong Bong and Kyung Tae Noh analysed the results, Jin-Jong Bong and Kyung Tae Noh wrote the manuscript, all authors reviewed the manuscript, all authors gave final approval of the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the Internal Research Program from the Armed Forces Medical Research Institute of ROK (Fund code #16-internal-1).

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author(s) on reasonable request.

Ethics approval and consent to participate

This study was approved by the ethics committee of the Armed Forces Medical Command (Approval No. AFMC-16067-IRB-16-056, September 2016). An approval form was used to obtain written informed consent and permission from each participant.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Information of primers used to amplify *pvmdr-1* gene

Primers	Sequences (5`→ 3`)
pvmdr-1 **(1st F)**	ATG AAA AAG GAT CAA AGG CAA C
pvmdr-1 **(1st R)**	CTA CTT AGC CAG CTT GAC GTA C
pvmdr-1 **(2nd F)**	TTG AAC AAG AAG GGG ACG TT
pvmdr-1 **(2nd R)**	CTT ATA TAC GCC GTC CTG CAC
Sequencing primer 1	TAC GGA ACG AGA ATC ATC AT
Sequencing primer 2	GAT GGT CAG CAT GAA CAT AC
Sequencing primer 3	TCA GGT GGA CAG AAG CAG AG
Sequencing primer 4	GCG AAC TCG AAT AAG TAC TC
Sequencing primer 5	AAG TCC CTC ATC GAC GTG AG

pvmdr-1 primers set were adapted from Barnadas *et al.,* 2008. Sequencing primers were newly designed for this study.

Table 2. The list and frequency of *pvmdr-1* mutations in ROK army from 2016 to 2017

Mutations in the *pvmdr-1* gene	No.(%) of mutated isolates	
	2016 (N=20)	2017 (N=53)
K44 (AA**G** -132- AA**A**)	4/20 (20%)	24/53 (45.2%)
L493 (**T**TA -1477- **C**TA)	5/20 (25%)	14/53 (26.4%)
T529 (AC**A**-1587-AC**G**)	15/20 (75%)	30/53 (56.6%)
Y541C (TAC-1622-TGC)	0	1/53 (1.8%)
G698S (**G**GC-2092-**A**GC)	20/20 (100%)	53/53 (100%)
L845F (**C**TC-2533-**T**TC)	20/20 (100%)	53/53 (100%)
M908L (**A**TG-2722-**C**TG)	20/20 (100%)	53/53 (100%)
T958M (A**C**G-2873-A**T**G)	20/20 (100%)	53/53 (100%)
F1076L (**T**TT-3226-**C**TT)	20/20 (100%)	53/53 (100%)
E1233 (GA**G**-3699-GA**A**)	8/20 (40%)	30/53 (56.6%)

Quintuple non-synonymous mutations (G689S, L845F, M908L, T958M, and F1076L) and synonymous mutations (K44, L493, T529, and E1233) were detected in specimens. Furthermore, a novel non-synonymous mutation (Y541C) was detected in 1 patient.

Table 3. The haplotypes of the *pvmdr-1* gene in Republic of Korea from 2016 to 2017

Primers	Sequences (5`→ 3`)
pvmdr-1 (1st F)	ATG AAA AAG GAT CAA AGG CAA C
pvmdr-1 (1st R)	CTA CTT AGC CAG CTT GAC GTA C
pvmdr-1 (2nd F)	TTG AAC AAG AAG GGG ACG TT
pvmdr-1 (2nd R)	CTT ATA TAC GCC GTC CTG CAC
Sequencing primer 1	TAC GGA ACG AGA ATC ATC AT
Sequencing primer 2	GAT GGT CAG CAT GAA CAT AC
Sequencing primer 3	TCA GGT GGA CAG AAG CAG AG
Sequencing primer 4	GCG AAC TCG AAT AAG TAC TC
Sequencing primer 5	AAG TCC CTC ATC GAC GTG AG

All haplotypes (Type 0 to Type 5) contained quintuple non-synonymous mutations (G689S, L845F, M908L, T958M, and F1076L, marked in bold). The frequency of non-synonymous (Y541C) and synonymous mutations (K44, L493, T529, and E1233) from 2016 to 2017 was listed.

Figures

Figure 1

Gel electrophoresis of *18S rRNA* and *pvmdr-1* nested PCR

(a) *18S rRNA* nested PCR. Blood samples were collected from malaria patients who agreed to the study. All blood samples collected from 3 military hospitals (Yangju, Koyang, and Ildong), during 2016-2017 were screened using *18S rRNA* nested PCR. Based on the screened result, 73 specimens are identified as positive samples. 1,100 bp or 120 bp was detected by 2nd PCR of *18S rRNA*. (b) Nested PCR targeting *pvmdr-1* amplicons ranged from 104 bp to 4,254 bp were observed in 73 *P. vivax* positive samples using *pvmdr-1* nested PCR. The amplicons were adjusted to sequencing analysis for SNP analysis using listed primers in Table 1.

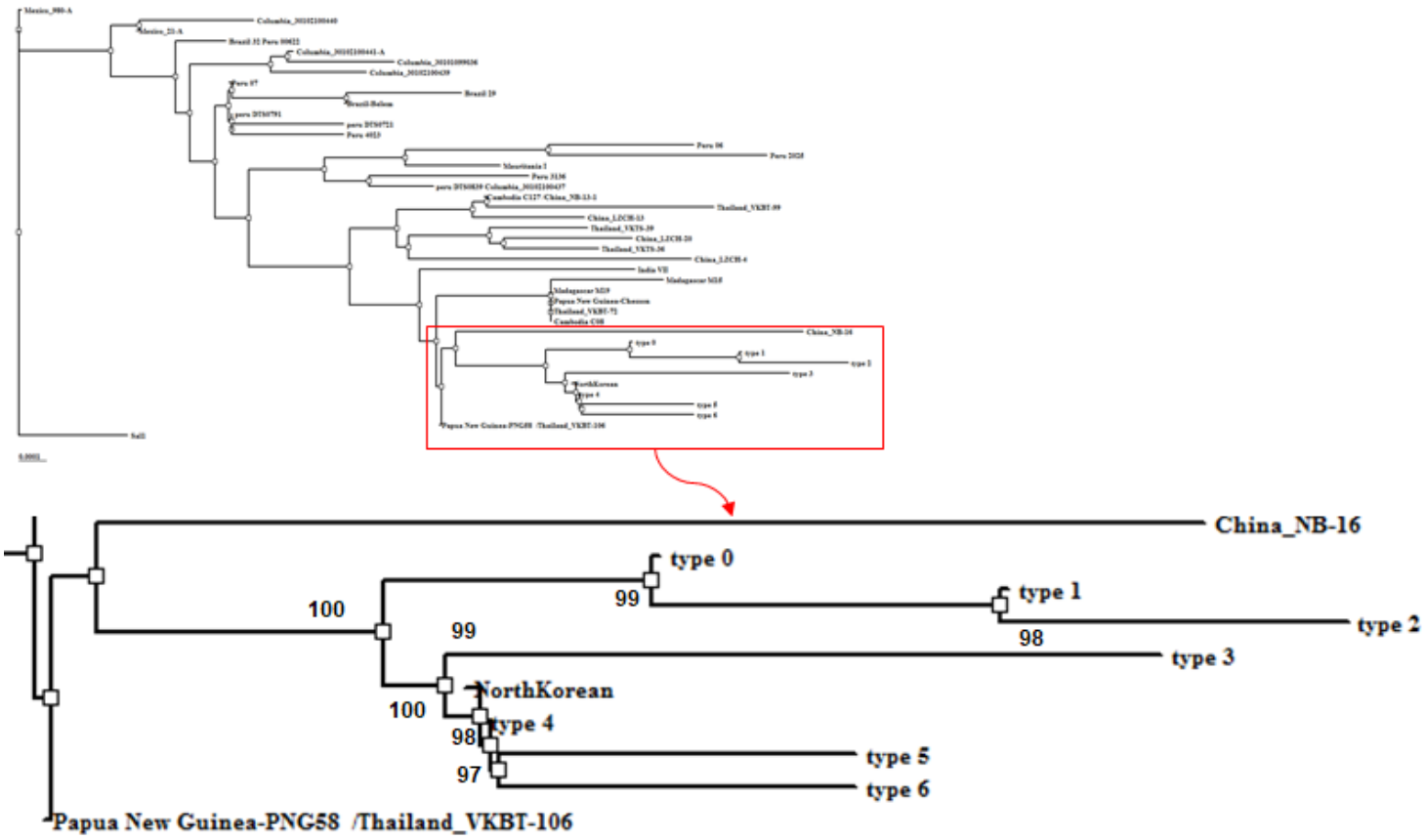

Figure 2

Phylogenetic analysis of 6 classified haplotypes via *pvmdr-1* SNP analysis of 73 *P. vivax* clinical samples isolated from military personnel of ROK army in 2016 and 2017

The sequences of Sal I strain (GenBank Accession# AY571984) and global parasites in PlasmoDB [Aurrecoechea et al., 2009] were used as reference sequences. *pvmdr-1* sequences of 73 P. vivax clinical samples isolated from military personnel of ROK army in 2016 and 2017 were analyzed using BioEdit Sequence Alignment Editor [Hall et al., 1999]. The phylogenetic analysis was constructed by 1,000 bootstrap replications [Pattengale et al., 2010] using the neighbor-joining method [Saitou et al., 1987].

Figure 3

The malaria-endemic region and *pvmdr-1* haplotypes of ROK in 2016 and 2017

The malaria-endemic regions were near the DMZ located in northern Gyeonggi Province and in the northwest region including Goseong, Cheorwon, Yeonchen, Paju, Gimpo, and Hwacheon of the ROK from 2016 to 2017. Six haplotypes were classified from 73 specimens collected in Armed Forces Hospitals (Yangju, Koyang, and Ildong). The red triangle indicated malaria-endemic region. Numeric letters mean 6

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