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Biological Resistance of Hydroxychloroquine for *Plasmodium vivax* Malaria in the Republic of Korea

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Abstract. The Republic of Korea (ROK) Army instituted a vivax malaria chemoprophylaxis program (hydroxychloroquine [HCQ] 400 mg per week) in 1997 that was expanded to nearly 200,000 soldiers by 2007, raising concerns for the emergence of drug-resistant vivax malaria. Therefore, a study of whole blood HCQ concentrations for all malaria patients admitted to four ROK Army hospitals was conducted from June through September 2007. For all 142 vivax malaria patients enrolled, fevers returned to normal by Day 3 post-treatment and all thin blood films were negative for parasites by Day 7. Pre-treatment whole blood concentrations of HCQ for 14 patients were > 100 ng/mL. Eight of the patients were enrolled in the ROK Army chemoprophylaxis program that reported taking HCQ as directed, with the last pill taken ≥ 4 days before diagnosis. Although there was no evidence of clinical resistance, chemoprophylaxis data indicates the biological resistance or tolerance to HCQ in ROK.

INTRODUCTION

Chloroquine (CQ) has been the treatment of choice for vivax malaria for more than 40 years with no alternative chemotherapy comparable in efficacy, safety, and cost.[1,2] Chloroquine-resistant *P. vivax* (CRPV) malaria, reported in Papua New Guinea, India, Burma, Guyana, and Indonesia, poses a serious health threat to millions of people who reside or travel to vivax malaria endemic regions of high incidence and long transmission periods.[3,4] The reemergence of *Plasmodium vivax* in the Republic of Korea (ROK) was reported in 1993 along the demilitarized zone (DMZ) and became endemic in this area, whereas there have been no autochthonous transmission reports of *P. falciparum*, *Plasmodium malariae*, or *Plasmodium ovale* malaria.[5,6] Malaria transmission occurs from late April through October with the first cases presenting in late May/June through early November with sporadic cases occurring from November through May as a result of latent malaria transmitted during the previous malaria season.[7–9] Nearly all transmission has been attributed to exposure in northern Gyeonggi and northwestern Gangwon Provinces near the DMZ that separates the ROK and North Korea and where many ROK Army personnel are stationed.[6,9] Because of its strategic and geographic distribution,[7] relatively large numbers of ROK soldiers contract vivax malaria annually. To address this problem, the ROK Army instituted a chemoprophylaxis program (hydroxychloroquine [HCQ] 400 mg 2 weeks before the onset of the malaria season and weekly thereafter through mid October followed by terminal chemoprophylaxis with primaquine, 15 mg daily/14 days) for personnel assigned to units located in malaria high-risk areas (malaria incidence > 10/100,000) near the DMZ. The program was initiated in 1997 with approximately 16,000 ROK soldiers placed on chemoprophylaxis and has been expanded annually to include nearly 200,000 ROK soldiers by 2007. Although it is suggested that this program contributed to the reduction of malaria cases from 2000 to 2004,[10] it is difficult to assess the effectiveness of this program because the number of malaria cases increased in 2005–2007 when the malaria chemoprophylaxis program was expanded.[8] Although CRPV has not been confirmed since the implementation of the ROK Army chemoprophylaxis program, there is much concern regarding potential breakthroughs as suggested by soldiers developing malaria while taking chemoprophylaxis as directed. Additionally, extensive use of chemoprophylaxis may serve to hasten the development of drug resistance when drug plasma concentrations, caused by non-compliance or other factors, are not high enough to kill circulating parasites. Therefore, confirmation of *P. vivax* HCQ resistance is necessary to address this potential significant health threat in the ROK. In this study, we investigated potential chemotherapy failures and evaluated clinical resistance in ROK Army soldiers that developed malaria during the 2007 malaria season.

MATERIALS AND METHODS

Volunteer patients. All ROK Army soldiers admitted to the Armed Forces Capital Hospital, Armed Forces Yangju Hospital, Armed Forces Ildong Hospital, or Armed Forces Byukjae Hospital with a history of fever within 48 hours and microscopically confirmed malaria from June through September 2007 were prospectively enrolled. These four ROK military hospitals are the primary health care facilities for treatment of malaria for all ROK soldiers assigned to the Seoul Metropolitan Area and Gyeonggi Province where more than one-half of all ROK soldiers in malaria-endemic areas were stationed. The ROK soldiers assigned to units in

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malaria-risk areas remained in the same location throughout the study period and were admitted to one of the four primary health care hospitals when medical providers at local military health clinics suspected malaria. Upon hospital admission from June through September 2007, venous blood was drawn (pre-treatment, Day 0 [D0]), thin blood films prepared, and if positive for vivax malaria parasites, patients were included under an approved human use protocol (Ethics Committee, Armed Forces Medical Command, Seoul, Korea) as part of the malaria surveillance program in Korea. All ROK Army soldiers admitted to one of the four hospitals with malaria were ≥ 19 years of age. Patients were excluded from the study if they were diagnosed and treated for malaria within 30 days at local military health clinics before hospital admission or if they elected not to participate in the study. Additional venous blood samples of vivax malaria patient volunteers were drawn on D2, D3, and D7, post-treatment, following informed consent for each blood draw. All venous blood samples were used for blood film preparation and blood spots dried on filter paper for measuring whole blood levels of total hydroxychloroquine (THCQ). THCQ is comprised of both hydroxychloroquine and its active metabolite, desethylhydroxychloroquine. A questionnaire of patient history, including chemoprophylaxis usage (compliance), was completed by each volunteer on D0 after informed consent.

Patients were given a total of 2,000 mg hydroxychloroquine (HCQ; Korean United Pharmaceutical Inc., Seoul, Republic of Korea); 800 mg at 0 hr after diagnosis, 400 mg at 6 hr, 400 mg at 24 hr, and 400 mg at 48 hr, followed by 15 mg primaquine (Myung In Pharmaceutical, Seoul, Republic of Korea) daily for 14 days. Patients remained hospitalized for 7 days after diagnosis and each treatment dosage during that period was supervised and documented by the nursing staff during admission, with the remaining terminal primaquine regimens provided as an outpatient (undocumented) from Days 8–16 after initial treatment.

Microscopic examination of blood films. Thin blood films were prepared from venous blood in accordance with standard laboratory practice. Blood films were stained with Giemsa for 30 min at pH 7.2, air-dried, and slides reviewed by two pathologists who examined at least 200 fields at 1000×. Positive slides were defined as those with asexual stages (trophozoites/merozoites and schizonts) present in the thin blood films.

Hydroxychloroquine (HCQ) blood concentrations. Whole blood levels of HCQ and its active metabolite DHCQ were measured for malaria patients who were enrolled in the ROK Army chemoprophylaxis program. Whole blood (100 μL) collected from venous blood at D0 pre-treatment were blotted on filter paper (No. 1 Whatman, Fairfield, NJ). Filter paper blood spots were air-dried and stored in ziplock plastic bags at room temperature until analyzed. Blood level concentrations (ng/mL) were reported as THCQ (HCQ + DHCQ).

High-performance liquid chromatography (HPLC) was used to determine whole blood levels of HCQ and its active metabolite DHCQ. Hydroxychloroquine and DHCQ were extracted from filter paper using a liquid–liquid extraction technique. Analytical separation was achieved with a reverse-phase C18 column (4.6 × 150 mm, 5 μm; XTerra; Waters Corporation, Milford, MA) using a mobile phase consisting of 40% acetonitrile and 60% water containing 1% diethylamine at a flow rate of 1 mL/min. The analytes were detected using fluorescence with excitation and emission wavelength set to 330 and 380 nm, respectively. Standard curve samples were prepared by spiking whole blood at the following concentrations of HCQ and DHCQ: 0, 50, 100, 250, 500, and 1,000 ng/mL. The spiked blood (0.1 mL) was applied to filter paper and allowed to air-dry before extraction. Interassay precision (expressed as coefficient of variation [CV]) and accuracy (CV) were determined for both analytes. For nine standard curve runs (nine separate days), the precision (CV) was determined to be 42%, 23%, 11%, 13%, and 3% for HCQ and 40%, 17%, 11%, 14%, and 3% for DHCQ at concentrations of 50, 100, 250, 500, and 1,000 ng/mL, respectively. Interassay accuracy (CV) was 0%, 2.9%, 4.5%, and −1.4% for HCQ and 0.6%, 4.9%, 1.4%, and −0.6% for DHCQ respective to the concentrations stated previously. The limits of detection (3 × standard deviation of calculated baseline values) obtained for this assay were 67 and 38 ng/mL for HCQ and DHCQ, respectively.

Biological resistance was defined if the pre-drug level of THCQ whole blood level was > 100 ng/mL when patients self-administered chemoprophylaxis ≥ 4 days before vivax malaria diagnosis.[11,12] Suspected biological resistance was defined as pre-treatment whole blood concentrations of THCQ of > 100 ng/mL when patients self-administered chemoprophylaxis irregularly or within 3 days before diagnosis. Clinical resistance was defined if persistent parasitemia was demonstrated in blood films on D7 post-treatment or if soldiers presented with malaria within 28 days post-treatment.

RESULTS

A total of 142 ROK Army male soldiers presenting with vivax malaria were enrolled (informed consent) in the study on D0 with a median age of 21years (range 19–50 years), but by D7, only 108 of the malaria patients participated in the study (Figure 1). All patients had symptoms (e.g., recurrent fever, chills, and headache), with 128 (90.1%) showing fever at the time of admission. A total of 135 (95.1%) patients showed thrombocytopenia and none of the patients needed redosing during treatment because of drug side effects (e.g., vomiting

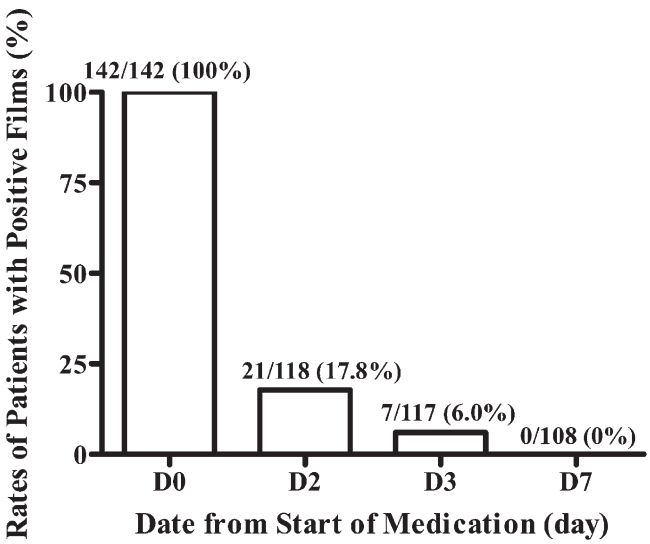

FIGURE 1. The number (percent) of patients with vivax malaria positive blood films, pre- and post-treatment with hydroxychloroquine. * The number of patients throughout the study: D0 = 142, D2 = 118, D3 = 117, D7 = 108.

within an hour after medication) (Table 1). For all 142 patients, temperatures returned to normal by D3 post-treatment. By D2 and D3, only 17.8% (21/118) and 6.0% (7/117), respectively, demonstrated parasites in their blood films (Figure 1). By D7, vivax malaria parasites were cleared from all patients examined (108/108). None of the patients reported for medical treatment of vivax malaria symptoms (e.g., fever, headache, and chills) within 28 days post-treatment.

A total of 10.6% (15/142) malaria patients enrolled in the study were not enrolled in the mandatory chloroquine/primaquine ROK Army chemoprophylaxis program for malaria high-risk areas, while 89.4% (127/142) were enrolled. A total of 14 (11.0%) patients had whole blood THCQ pre-treatment levels > 100 ng/mL and were classified as biological resistance (8) or suspected biological resistance (6), as defined previoiusly. Hydroxychloroquine and DHCQ were not detected in 58.3% (74/127) of the patients (Table 2). The ratio of HCQ/DHCQ for 8 patients classified as biological resistance ranged from 0.9 to 22.8 (Table 3).

DISCUSSION

This is the first clinical trial designed to identify HCQ-resistant vivax malaria using whole blood HCQ concentrations for soldiers diagnosed with *P. vivax* in the ROK. The study showed that some of the ROK Army soldiers admitted with malaria had high HCQ concentrations suggesting either an intrinsic, reduced susceptibly to HCQ, or biological resistance, all of which may be manifest as clinical resistance. This *in vivo* test on the use of HCQ for the treatment and prophylaxis of *P. vivax*, also showed chemoprophylaxis failures. This study shows evidence of biological resistance in at least eight ROK Army soldiers based on THCQ whole blood levels and patient chemoprophylaxis history, but may also reflect the vivax malaria situation in North Korea[13] based on the potential for the introduction of vivax malaria-infected mosquitoes along the DMZ or malaria infections through South Korean laborers working in Kaesong, North Korea.[11]

The whole blood concentration of CQ plus DHCQ to cure CQ-sensitive vivax malaria blood parasites is considered > 100 ng/mL over 4 days,[11,12,14–16] although determining the therapeutic range of CQ against *P. vivax* is problematic.[17] The ratio of molecular weight of HCQ/CQ is 1.05 and thus similar minimal effective concentrations can be applied to HCQ. In this study, 14 (11.0%) of the malaria patients had THCQ levels > 100 ng/mL and eight patients where parasites were estimated to be exposed to HCQ of higher than minimal effective concentration for ≥ 4 days at initial presentation. This suggests the presence of biological resistant vivax malaria parasites in the ROK. The CRPV is usually reported in high-burden and endemic areas where large amounts of CQ has been prescribed for considerable duration.[3,4,14,18] In these areas, biological resistance rates are much higher compared with our data, with previous studies in Papua New Guinea and Indonesia that reported biological resistance levels as high as and 53%[18] and 73%,[19] respectively, and where chloroquine is no longer effective for treatment or chemoprophylaxis for *P. vivax* blood infections. Considerations of costs and health benefits for continued extensive use of chemoprophylaxis in the ROK Army that may lead to the emergence of clinical resistant vivax malaria among the ROK Army and civilian components are necessary.[18,20,21] Perhaps a more limited application with observed compliance policies may be more effective for defined malaria high-risk areas that may reduce increased risks of drug resistance.

Hydroxychloroquine clinical-resistant vivax malaria was not observed, as febrile symptoms subsided within 3 days. Although 6.0% of the thin smears were positive on D3, post-treatment, all thin smears were negative on D7. This slow clearance may indicate the beginning of drug resistance to HCQ.[22,23] Although CQ can have a antipyretic effect,[24] none of the volunteers were readmitted to the hospital for fever within 28 days after therapeutic medication, except for one patient that was diagnosed with malaria (relapse) 31 days post-treatment. In this case, a recurrence of malaria cannot

TABLE 1

Patient characteristics at enrollment (N = 142)

Median age (range), years	21 (19–50)
Male (%)	142 (100)
No. patients symptomatic*	142 (100)
No. with fever† (%)	128 (90.1)
Complete blood count	
Mean white blood cell (SD‡)/mm^3	4,748 (1699)
Mean hemoglobin (SD‡) g/dL	13.6 (1.4)
Mean platelet (SD‡)	77.4 (66.3)
No. with thrombocytopenia (%)§	135 (95.1)
Side effects of treatment	
No. (%) with abdominal discomfort	11 (7.7)
No. (%) with diarrhea	7 (4.9)
No. (%) with nausea or vomiting	4 (2.8)
No. (%) with redosing because of side effects	0 (0)

*Defined as febrile, chills, headache, and/or myalgia.
†Defined as axillary temperature ≥ 37.3°C.
‡Standard deviation.
§Defined as < 150,000 cells/mm^3.

TABLE 2

The distribution of pre-treatment whole blood total hydroxychloroquine (hydroxychloroquine + desethylhydroxychloroquine) levels for malaria patients in the mandatory ROK Army chemoprophylaxis program

Total hydroxychloroquine (ng/mL)	Number of patients (%)
0	74 (58.3)
> 0–9.99	25 (19.7)
10–49.99	8 (6.3)
50–99.99	6 (4.7)
100–499.99	12 (9.4)
500–1000	2 (1.6)
Total	127

TABLE 3

The whole blood concentrations of total hydroxychloroquine for eight malaria patients with THCQ pre-drug levels > 100 ng/mL at D0 (pre-treatment) and that also reported chemoprophylaxis compliance*

	Drug level concentrations (ng/mL)			
Patient no.	HCQ	DHCQ	THCQ	HCQ/DHCQ
1	359.5	38.5	398.0	9.3
2	371.8	17.8	389.6	20.9
3	133.5	143.1	276.6	0.9
4	151.0	26.0	177.0	5.8
5	127.1	16.5	143.6	7.7
6	101.6	8.2	109.8	12.4
7	96.1	10.0	106.1	9.6
8	100.8	4.4	105.2	22.8

*HCQ = hydroxychloroquine; DHCQ = desethylhydroxychloroquine; THCQ = total hydroxychloroquine (HCQ + DHCQ).

be ruled out, especially with the anti-blood stage mode of action of primaquine.[25] However, as the patient was in non-compliance with terminal primaquine prophylaxis, this is highly suggestive that this is recrudescence caused by latent liver stage parasites. In a previous report, 2/86 patients that had recurrent malaria, demonstrated by *P. vivax* positive thin blood films and polymerase chain reaction (PCR)-based assays 14 days after medication, were successfully treated after additional therapy with the same regimen.[26] These cases were most likely a result of primaquine failure at doses of 15 mg for 14 days rather than HCQ failure.[27,28] Although there have been no reports showing clinical resistance to vivax malaria to CQ or HCQ in the ROK, 17.8% of patients on D2 and 6.0% on D3 post-treatment showed asexual parasites in thin blood films. This slow parasite clearance could be an important early clue to the development of clinical resistance, showing the need for a malaria surveillance program that includes clinical resistance to identify the minimal effective concentrations of THCQ.[29]

Hydroxychloroquine resistance is the main consideration for the chemoprophylaxis protocol. A prematurely terminated program can be one of the major causes of chemoprophylaxis failure, especially in military personnel.[30] Tolerability and efficacy vary between drugs.[31,32] In the ROK Army, HCQ is currently an effective prophylactic regimen because the number of malaria patients showing HCQ resistance are relatively low compared with the military personnel enrolled in the chemoprophylaxis program. However, the continued high usage or expansion of HCQ may lead to increased risks of chemoprophylaxis or clinical failures caused by parasite tolerance or resistance, especially where chemoprophylaxis is not carefully monitored to ensure proper dosage. Thus, studies need to be implemented that monitor blood level concentrations of HCQ among ROK Army soldiers on chemoprophylaxis and those developing malaria to identify early drug resistant trends.

This study has several limitations. First, only a thin smear was examined and this could have affected the decision of clinical resistance because thin blood films are not as sensitive as thick blood films for identification of low parasitemia levels. However, only one patient was readmitted for malaria (D31, post-treatment), which was most likely a result of incomplete terminal primaquine treatment. In addition, two experienced pathologists observed more than 200 high-power fields on at least two slides before considering slides negative for malaria parasites.[33] All of the enrolled patients had improved febrile symptoms after treatment, and none presented again with malaria within 28 days. Second, medication history depended on patients' memory. As patient identifiers were removed and questionnaires were anonymous with no retribution for non-compliance, the assumption was that the soldiers responded honestly to the questions regarding chemoprophylaxis compliance. Furthermore, we checked to determine if patient medication histories were in agreement with chemoprophylaxis program schedules for each military unit because soldiers usually took the medication on the same day of each week. However, this may be in error as 62.2% (46/74) soldiers indicated that they had taken hydroxychloroquine chemoprophylaxis weekly as directed, but did not show THCQ in their blood on admission at D0 before taking medication. Thus, a study that includes weekly "observed" compliance and weekly THCQ blood levels that shows the range of THCQ concentrations is justified. Despite this limitation, all eight patients showed biological resistance based on THCQ concentrations of 105 to 398 ng/mL, which indicated they had taken medication 2–7 days before hospital admission.[34] We also reconfirmed unit medication schedules for the eight patients that they were not given prophylactic medication on the same day of admission. Third, there is no data for Korean pharmacokinetics of CQ or HCQ and therefore data were correlated with previous reports elsewhere. Fourth, the cut-off of 7 days would only detect high grade parasite resistance and cases of recurrent parasitaemia after D7 could be missed. Finally, the use of primaquine, with its antiblood stage activity against vivax, also could limit the ability to define clinical resistance because CQ-resistant parasites might be subpatent because of primaquine.[35]

In conclusion, chemoprophylaxis data shows the potential for vivax malaria resistance or tolerance to HCQ when taken at 400 mg weekly in the ROK. To prevent the emergence of clinical resistance, careful consideration for the pros and cons for the use of extensive chemoprophylaxis is required.

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