Mutations in the Antifolate-Resistance-Associated Genes Dihydrofolate Reductase and Dihydropteroate Synthase in *Plasmodium vivax* Isolates from Malaria-Endemic Countries

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Abstract. Parasite dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) are known target enzymes of antifolate drugs used for the treatment and prophylaxis of persons with malaria. We sequenced the *Plasmodium vivax* dihydrofolate reductase (*pvdhfr*) and dihydropteroate synthase (*pvdhps*) genes to examine the prevalence and extent of point mutations in isolates from malaria-endemic countries. Double mutations (S58R and S117N) or quadruple mutations (F57L/I, S58R, T61M, and S117T) in the *pvdhfr* gene were found in isolates from Thailand (96.4%) and Myanmar (71.4%), but in only one isolate (1.0%) from Korea, where sulfadoxine-pyrimethamine has never been used. The *pvdhfr* point mutations correlated strongly with the *pvdhps* point mutations and ranged from single to triple mutations (S382A, A383G, and A553G), among isolates from Thailand, Myanmar, and Korea. These findings suggest that the prevalence of mutations in *pvdhfr* and *pvdhps* in *P. vivax* isolates from different malaria-endemic countries is associated with selection pressure imposed by sulfadoxine-pyrimethamine.

INTRODUCTION

Plasmodium vivax malaria is one of the most widely distributed infectious diseases in many parts of Central America, South America, and Asia.¹ Chemotherapy remains a key factor in the fight against malaria, but emerging and spreading resistance to an increasing number of antimalarial drugs is one of the greatest challenges for malaria control. Chloroquine (CQ) remains the first-line treatment for patients with *P. vivax* malaria in most malaria-endemic regions.^{2,3} Since the emergence of CQ-resistant *P. falciparum* in the late 1950s, sulfadoxine-pyrimethamine (SP) has been used extensively to counter the spread of CQ resistance in *P. falciparum*. However, treatment of patients with SP has inadvertently led to the simultaneous selection of SP-resistant *P. vivax*. In Thailand, SP was used alone or in combination with mefloquine as the first-line treatment of patients with *P. falciparum* malaria until the withdrawal of these drugs in 2001.⁴

Although SP has never been recommended for the treatment of patients with *P. vivax* malaria, the selection pressure exerted by the drug is expected to have continued progressively in both species.^{4,5} In Myanmar, high levels of *P. falciparum* resistance to CQ and SP were reported in the 1990s,⁶ and these drugs are considered to be completely ineffective for the treatment of patients with *P. falciparum* malaria in northern Myanmar.⁷ Several previous reports are also available regarding the current status of drug-resistant *P. vivax* malaria in Myanmar.^{8–11}

Since 1953, *P. vivax* malaria has reemerged in the Republic of Korea, and CQ/primaquine has been used for the treat-

ment of these patients with *P. vivax* malaria.¹² Sulfadoxime-pyrimethamine resistance in *P. falciparum* is well established because of selection for point mutations in the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes.¹³ However, studies from the 1950s have long been used to support the idea that *P. vivax* is intrinsically resistant to SP.¹⁴ When SP is used to treat patients with *P. falciparum* malaria, it also exerts a substantial selective pressure on the *P. vivax* population, providing a selective advantage to parasites carrying resistance-conferring mutations in the *P. vivax dhfr* and *dhps* genes (*pvdhfr* and *pvdhps*, respectively). It has been concluded that the clinical response to SP depends on the *pvdhfr* and *pvdhps* genotype.^{15,16} In Korea, SP has rarely been used to treat patients with *P. vivax* malaria, and no molecular investigations of SP resistance markers in *P. vivax* have been conducted.

To evaluate the presence and prevalence of mutations in the *pvdhfr* and *pvdhps* genes that are potentially associated with resistance to SP, the polymorphisms in these two genes were assessed in 140 isolates from four countries (Korea, Thailand, and Myanmar), which differ in their policies for the use of antimalarial drugs. The purpose of our study was to predict the status of SP for the treatment of patients with *P. vivax* malaria and to provide information for future decisions about drug selection on a regional basis in each country.

MATERIALS AND METHODS

***Plasmodium vivax*-infected blood samples.** Blood samples obtained from patients with *P. vivax* infections at local hospitals in malaria-endemic areas of Korea (n = 97) during 2007–2009. Samples from Thailand (n = 30) were obtained from malaria patients who were admitted to a local health center in Mae Sod, Thailand, during 2008, and samples from Myanmar (n = 15) were obtained from the Wet-Won Station Hospital, in Yangon, Myanmar, in 1999. All samples were collected

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according to protocols that were reviewed and approved by the Kangwon National University Hospital Human Ethics Committee, the Thai Ministry of Public Health Ethical Review Board, the Walter Reed Army Institute of Research Institutional Review Board, and the Department of Health, the Union of Myanmar.

DNA isolation. Genomic DNA was extracted from the whole blood samples by using a QIAamp DNA Blood Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions and from blood filter papers by using described methods.¹⁷

Sequencing of *pvdhfr* and *pvdhps* genes. The *pvdhfr* (GenBank accession no. X98123) and *pvdhps* (AY186730) gene sequences used as the reference wild type were amplified by polymerase chain reaction (PCR) using gene-specific primers. Amplifications were performed in a reaction mixture that contained 2 µL of 10 × buffer, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0.25 µM of each primer, 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 1 µL of genomic DNA or the amplicon from the first PCR. Primer Pvdhfr F1 (sense) 5'-ATGGAGGACCTTCAGATGTATT-3' and primer Pvdhfr R1 (antisense) 5'-CCACCTTGCTGTA AACC AAAAGTCCAGAG-3' (expected amplicon size = 715 basepairs, nucleotides 1–715) were used as primers for the first-round amplification of the *pvdhfr* gene.¹⁸ The PCR was performed with an initial denaturation at 94°C for 10 minutes and 35 cycles at 94°C for 50 seconds, 58°C for 50 seconds, and 72°C for 50 seconds.

Primer Pvdhfr F3 (sense) 5'-TTTGACATTTACGCCATC TGC-3' and primer Pvdhfr R3 (antisense) 5'-TACACCTC ACTGACGGACG-3' (expected amplicon size = 647 basepairs, nucleotides 22–668) were used for the second-round amplification of *pvdhfr*. The PCR cycling conditions for *pvdhfr* were an initial denaturation at 94°C for 10 minutes and 35 cycles at 94°C for 30 seconds, 60°C for 50 seconds, and 72°C for 1 minute.

Primer Pvdhps F1 (sense) 5'-AGGAAGCCATTTCGCTC AAC-3' and primer Pvdhps R1 (antisense) 5'-GGAACGCT GCAAACAACAC-3' (expected amplicon size = 1,700 basepairs, nucleotides 1188–2887) were used as the primers for the first-round PCR to amplify the *pvdhps* gene. The PCR was performed with a denaturation at 94°C for 10 minutes and 30 cycles at 94°C for 30 seconds, 60.7°C for 1 minute, and 72°C for 2 minutes.

The second-round *pvdhps* primers were PVDHPS-D (sense) 5'-GGTTTATTTGTCGATCCTGTG-3' and PVDHPS-B (antisense) 5'-GAGATTACCCTAAGGTTGATGTATC-3' (expected amplicon size = 1,301 basepairs, nucleotides 1367–2667).¹⁹ The PCR conditions were an initial denaturation at 94°C for 10 minutes and 30 cycles at 94°C for 30 seconds, 51°C for 1 minute, and 72°C for 1.5 minutes. The second-round PCR products were sequenced directly: primer Pvdhfr F3 was used to sequence the *pvdhfr* fragment (nt 22–668) and primers PVDHPS-D and PVDHPS-B were used to sequence the *pvdhps* fragment (nucleotides 1367–2667). The deduced amino acid sequences were aligned and analyzed with the Lasergene® software (DNASTAR, Madison, WI). Insertions and deletions were verified manually. The nucleotide sequences described in this paper have been deposited in GenBank under accession numbers GU224151–GU224162 and GU224164–GU224167 for *pvdhfr* and GU224168–GU224185 for *pvdhps*.

RESULTS

Detection of mutations in the *pvdhfr* gene. The mutation rates at amino acids 57, 58, 61, and 117 were higher in isolates from Thailand (89.3%, 96.4%, 85.7%, and 96.4%, respectively) and Myanmar (21.4%, 71.4%, 21.4%, and 71.4%, respectively) than in isolates from Korea (22.7%, 2.1%, 0.0%, and 15.5%, respectively; Table 1). Wild-type alleles were observed in isolates from Korea (61.9%) and Myanmar (21.4%). Double mutant alleles (S58R and S117N) were observed in isolates from Myanmar (50%), Thailand (10.7%), and ROK (1.0%). Quadruple mutant alleles (F57L/I, S58R, T61M, and S117T) were identified in isolates from Thailand (85.7%), and Myanmar (21.4%), although the alleles differed at amino acid 57 (F57I/L), and F57I was only observed in the isolates from Thailand (71.4%). No triple mutations (S58R, S117N, and I173F) were found in any isolates. The *pvdhfr* mutant allele at codon 173 was observed in two isolates from Korea and one isolate from Myanmar. In isolates from Korea, single mutant alleles at F57L (22.7%) and S117N (14.4%) and double mutant alleles at F57L and S58 (21.7%) of the *pvdhfr* gene were identified at high frequencies. Deletion genotypes within the *dhfr* tandem repeat region were observed in 66 isolates (68%) from Korea, 3 isolates (10.7%) from Thailand, and 7 isolates (50.0%) from Myanmar, which included the deletion at position 98–103 (THGGDN). A mutation at residue 99 (H99S), which is in the tandem repetitive domain, was also observed in some isolates from Korea (27.0%).

Detection of mutations in the *pvdhps* gene. The most prevalent mutant allele differed from the reference sequence at two positions (383G and 553G in 67.9% of isolates from Thailand and 26.7% from Myanmar), followed by an allele with a triple mutation (383G, 553G, with 382A, 512T, or 661V; 14.3% of isolates from Thailand) or a single mutation (383G in 10.7% of isolates from Thailand and 20% of isolates from Myanmar; 399I in 9.4% of isolates from Korea; and 525G and 661V),

TABLE 1
Prevalence of *pvdhfr* mutant alleles among *Plasmodium vivax* isolates from the Republic of Korea, Thailand, and Myanmar*

Allele†	Amino acid position, <i>pvdhfr</i> ‡					Repeat§	No. (%)	Region
	57	58	61	117	173			
Wild	F	S	T	S	I	W¶		
Wild	F	S	T	S	I	Del	35 (36.1)	Korea
Wild	F	S	T	S	I	M	25 (25.8)	
Single	L	S	T	S	I	Del	16 (16.5)	
Single	L	S	T	S	I	M	3 (3.1)	
Single	F	S	T	N	I	Del	12 (12.4)	Thailand
Single	F	S	T	T	I	Del	1 (1.0)	
Single	F	S	T	N	I	M	1 (1.0)	
Double	L	R	T	S	I	Del	1 (1.0)	
Double	L	S	T	S	F	M	2 (2.1)	
Double	F	R	T	N	I	Del	1 (1.0)	
Single	L	S	T	S	I	W	1 (3.6)	Myanmar
Double	F	R	T	N	I	Del	3 (10.7)	
Quadruple	I	R	M	T	I	W	20 (71.4)	
Quadruple	L	R	M	T	I	W	4 (14.3)	
Wild	F	S	T	S	I	W	3 (21.4)	
Single	F	S	T	S	F	W	1 (7.1)	
Double	F	R	T	N	I	Del	7 (50.0)	
Quadruple	L	R	M	T	I	W	3 (21.4)	

* *pvdhfr* = *Plasmodium vivax* dihydrofolate reductase.

† Tandem repeat sequence was not considered.

‡ Amino acids that differ from wild type are shown in bold.

§ Tandem repeat sequence including four types: W = wild type; Del = deletion of 292–309 basepairs; M = mutation at codon 99 (H99S).

¶ The wild type as reference (GenBank accession no. X98123).

TABLE 2
Prevalence of *pvdhps* mutant alleles among *Plasmodium vivax* isolates from the Republic of Korea, Thailand, and Myanmar*

Allele†	Amino acid position, <i>pvdhps</i> ‡									Repeat§	No. (%)	Region
	382	383	399	512	525	553	555	585	661			
Wild	S	A	M	K	R	A	K	V	A	W¶		
Wild	S	A	M	K	R	A	K	V	A	B	73 (76.0)	Korea
Wild	S	A	M	K	R	A	K	V	A	C	14 (14.6)	
Single	S	A	I	K	R	A	K	V	A	B	9 (9.4)	
Wild	S	A	M	K	R	A	K	V	A	A	1 (3.6)	Thailand
Single	S	G	M	K	R	A	K	V	A	A	3 (10.7)	
Double	A	G	M	K	R	A	K	V	A	B	1 (3.6)	
Double	S	G	M	K	R	G	K	V	A	A	19 (67.9)	
Triple	A	G	M	K	R	G	K	V	A	A	1 (3.6)	
Triple	S	G	M	T	R	G	K	V	A	A	1 (3.6)	
Triple	S	G	M	K	R	G	K	V	V	D	2 (7.1)	
Wild	S	A	M	K	R	A	K	V	A	B	1 (6.7)	Myanmar
Wild	S	A	M	K	R	A	K	V	A	W	2 (13.3)	
Wild	S	A	M	K	R	A	K	V	A	E	1 (6.7)	
Wild	S	A	M	K	R	A	K	V	A	F	1 (6.7)	
Single	S	A	M	K	G	A	K	V	A	B	1 (6.7)	
Single	S	A	M	K	R	A	K	V	V	D	1 (6.7)	
Single	S	G	M	K	R	A	K	V	A	W	1 (6.7)	
Single	S	G	M	K	R	A	K	V	A	A	2 (13.3)	
Double	S	G	M	K	R	G	K	V	A	A	4 (26.7)	
Double	S	G	M	K	R	A	R	V	A	A	1 (6.7)	

* *pvdhps* = *Plasmodium vivax* dihydropteroate synthase.

† Repeat sequence was not considered.

‡ Amino acids that differ from wild type are shown in **bold**.

§ For the repeat within the *dhps* coding region, see Table 3.

¶ The wild type as reference (GenBank accession no. AY186730).

each observed in 6.7% of isolates from Myanmar (Table 2). The *pvdhps* tandem repetitive domain, which is located between amino acid positions 603 and 665 of the reference sequence, was identified in seven genotypes (Table 3).

Distributions of *pvdhfr* and *pvdhps* alleles from different countries. To test for correlations between genotypes of the *dhfr* and *dhps* alleles among *P. vivax* isolates, we identified three *pvdhfr* alleles and five *pvdhps* alleles (Table 4). The most prevalent combination was a double mutant allele (383G and 553G) of *dhps*, combined with a quadruple mutant allele of *dhfr* (57I, 58R, 61M, and 117T; 57.1% of isolates from Thailand), followed by a quadruple mutant allele of *dhfr* (57L, 58R, 61M, and 117T; 7.1% of isolates from Thailand and

14.3% of isolates from Myanmar) or a double mutant allele of *dhfr* (58R and 117N; 10.7% of isolates from Thailand and 14.3% of isolates from Myanmar). Most (99.0%) isolates from Korea carried the wild-type alleles of both genes but only a few of the isolates from Thailand and Myanmar carried wild-type alleles of both genes and wild-type *pvdhps* coupled to double or quadruple mutant alleles of *pvdhfr*, respectively.

DISCUSSION

The *pvdhfr* gene is highly polymorphic compared with the *pfdhfr* gene.²⁰ In *P. vivax*, nonsynonymous single-nucleotide

TABLE 3
Polymorphisms of the amino acid sequences within the tandem repetitive domain of *pvdhps* gene among *P. vivax* isolates from ROK, Thailand, and Myanmar

Type	Repeat motif*	No. of isolates (%) from the following countries			
		ROK	Thailand	Myanmar	Frequency (%)
Wild				3 (20.0)	3 (2.1)
A			25 (89.3)	7 (46.7)	33 (23.6)
B		82 (85.4)	1 (3.6)	2 (13.3)	85 (60.8)
C		14 (14.6)			14 (10.0)
D			2 (7.1)	1 (6.7)	3 (2.1)
E				1 (6.7)	1 (0.7)
F				1 (6.7)	1 (0.7)
Total		96 (100)	28 (100)	15 (100)	140 (100)

* GEAKLTN/GEGKLTN; GDAKLTN/GDSKLTN/GEAKLTN; GEGKLTN/GEAKLTN; GEAKLTN/GEGKLTN/GEGKLTN; GEAKLTN/GEGKLMS.

TABLE 4

Distributions of *pvdhfr* and *pvdhps* allelic combinations among *Plasmodium vivax* isolates from the Republic of Korea, Thailand, and Myanmar*

<i>Pvdhfr</i> allele	Genotype			No. of isolates (%)			
	<i>Pvdhps</i> allele			Korea	Thailand	Myanmar	No. (%)
	S382A	A383G	A553G				
Wild type	S	A	A	95 (99.0)	1 (3.6)	4 (28.6)	100 (71.9)
58R-117N	S	A	A	1 (1.0)		2 (14.3)	3 (2.2)
	S	G	A			3 (21.4)	3 (2.2)
	S	G	G		3 (10.7)	2 (14.3)	5 (3.6)
57L-58R-61M-117T	S	A	A				1 (0.7)
	S	G	A		2 (7.1)	1 (7.1)	3 (2.2)
	S	G	G		2 (7.1)	2 (14.3)	4 (2.9)
57I-58R-61M-117T	S	A	A		1 (3.6)		1 (0.7)
	S	G	A		1 (3.6)		1 (0.7)
	S	G	G		16 (57.1)		16 (11.5)
	A	G	A		1 (3.6)		1 (0.7)
	A	G	G		1 (3.6)		1 (0.7)
Total				96 (100)	28 (100)	14 (100)	139 (100)

* *pvdhfr* = *Plasmodium vivax* dihydrofolate reductase; *pvdhps* = *P. vivax* dihydropteroate synthase. Amino acids that differ from wild type are shown in **bold**.

polymorphisms (SNPs) that alter amino acid positions 49, 57, 58, 61, 117, and 173, corresponding to similarly affected positions in *P. falciparum*, have been shown to confer resistance to pyrimethamine.²¹ The distributions of the double mutant allele (S58R and S117N) and the quadruple mutant allele (F57L/I, S58R, T61M, and S117T) of the *pvdhfr* gene in the isolates from different malaria-endemic countries are consistent with the findings of another study, in which high levels of resistance in *P. vivax* were associated with these double and quadruple mutant alleles in the isolates tested.^{18,22}

Surprisingly, single mutations in *pvdhfr* at codons 57 or 117 were identified at high frequencies in isolates from Korea in areas in which antifolate drugs have almost never been used for treatment of patients with *P. vivax* malaria. The first-line treatment for *P. vivax* malaria in Korea is still a combination of CQ and primaquine, although a recent study of chemoprophylaxis data confirmed CQ resistance in 2 of 484 enrolled *P. vivax* malaria patients.²³ It has been hypothesized that the S117N mutation represents the first step in the drug-resistance selection process that has occurred in the parasite.²⁴ In this study, because the single mutations in *pvdhfr* at F57L or S117N/T occurred independently in isolates from Korea, we can speculate that the F57L mutation performs a similar function to that of S117N/T.

The I173L mutation in the *pvdhfr* gene, which has been suggested to reflect the geographic subdivision of *P. vivax* between the Old World and the New World,²⁵ was observed in only two isolates from Korea and one from Myanmar. Isolates from Korea carrying the I173F mutation were collected in 2009. Because this mutation occurs at a high frequency (9.1%), the characteristics of this residue warrant further study. Regarding the combinations of mutations in *pvdhfr*, the observations that mutation 117N was not observed in the *pvdhfr* quadruple mutants in this study, although the quadruple mutants always included the mutation 117N/T in previous studies,^{5,11} and that mutation 61M was only seen in the quadruple mutants, are consistent with previous data.²⁶ However, the observation that 57L was not linked to 58R, but occurred alone in a small percentage of isolates from Korea (22.7%), is inconsistent with the results of a previous study.²⁶

A comparison was made of the prevalence of the various *pvdhfr* alleles in Myanmar at different times. In 1998, 11 of 12 isolates were 58R/117N, with 1 wild-type isolate, and no

triple or quadruple mutants.²² However, in 1999 (this study), 7 of 14 isolates were double mutants, 3 of 14 isolates were quadruple mutants, and 3 isolates carried only wild-type alleles; there were no triple mutants. In contrast, in 2005, 9 of 21 isolates were double mutants and 8 were quadruple mutants,¹¹ which indicated that these mutations may increase rapidly in isolates with highly mutated *pvdhfr* alleles. Therefore, the thorough and frequent surveillance of SNPs of drug-resistance genes is useful and important in monitoring drug resistance in malaria-endemic countries.

In *P. falciparum*, nonsynonymous SNPs of *dhps* have been shown to confer resistance to sulfadoxine.^{13,27} Based on a homology model, mutations of *pvdhps* may play a role in sulfadoxine resistance, insofar as polymorphisms at amino acid positions 382, 383, 512, 553, and 585 are homologous to *P. falciparum* amino acid positions 436, 437, 540, 581, and 613, respectively.^{27–30} All of the isolates examined in our study have a valine at amino acid position 585 of DHPS. Because there is an alanine at this position in the wild-type PfDHPS protein sequence, this alternative residue (valine) has been suggested to account for the low susceptibility of *P. vivax* to sulfadoxine.¹⁹ Sulfa drugs have been used in Korea as antibacterial drugs. Our results suggest that antifolate drug pressure may be carried on by this association in Korea. Mutations in codons 382, 383, 512, and 553 are more prevalent in regions with high SP use compared with areas of low SP use.³¹ Double mutant alleles (A383G and A553G) that are directly related to sulfadoxine resistance are considered to be associated with a reduction in the affinity between PvDHPS and sulfadoxine.¹⁹ In this study, 27 of 28 isolates from Thailand and 7 of 15 isolates from Myanmar carried mutations in 1 or more of codons 382, 383, and 553, but these were not found in isolates from Korea, which further supports the suggestion that these *pvdhps* mutations play important roles in sulfa drug resistance.

A tandem repetitive sequence has been described in *pvdhfr*,²² and a similar repeat motif has also been described in *pvdhps*.^{32,33} Whether polymorphisms in these repeat regions contribute to the *Plasmodium* resistance to antifolate drugs is still unclear. Both of these tandem repeat sequences show size polymorphisms, but these were not present in either the *pfldhfr* or the *pfldhps* gene.^{22,32} No wild-type of the tandem repeat sequences were determined in the *pvdhfr* and *pvdhps* genes

of isolates from Korea, but they are typically separated into two types: a deletion (nucleotides 292–309) and the mutation H99S in *pvdhfr*, and types B and C in *pvdhps* (Table 3). In this study, we found five types of tandem repeat motifs in *pvdhps* that have not been described in previous reports. These repeat sequences are complex and their functions warrant further study.

Relevant mutations in the *pvdhps* gene were observed only in parasites with mutations in the corresponding *pvdhfr* gene. In the *P. falciparum* parasite, the development of resistance mutations in *dhfr* and *dhps* is asymmetric, and mutations in *dhfr* appear to be selected before those in *dhps*.¹³ The data presented in this study indicate that a similar selection process occurs in *P. vivax*, in which mutant *pvdhps* alleles were observed only among isolates that also carried a highly mutant *pvdhfr*, which is consistent with the results of a previous study.¹⁵ The increased probability of coexisting combined mutations in the *pvdhfr* and *pvdhps* genes also contributes to the failure of SP treatment.³⁴ In our study, the distribution of combined mutations in *pvdhfr* and *pvdhps* is highly consistent with the use of SP in malaria-endemic countries.

The molecular data in your study provide an indication of the prevalence of *pvdhfr* and *pvdhps* mutations in countries in Southeast Asia and provide crucial information on the potential appearance of SP resistance in these areas. Notably, the surveillance of *pvdhfr* and *pvdhps* alleles in Korea should provide essential information about the association between SP resistance and the mutations in these two genes. These kinds of approaches, based on multilocus variant genotype analysis, could be used advantageously in molecular epidemiologic studies to evaluate the development and spread of drug resistance in *P. vivax*.

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