MOLECULAR MONITORING OF DIHYDROFOLATEREDUCTASE (DHFR) AND DIHYDROPTEROATE SYNTHETASE (DHPS) ASSOCIATED WITH SULFADOXINE-PYRIMETHAMINE RESISTANCE IN PLASMODIUM VIVAX ISOLATES OF PALAWAN, PHILIPPINES

BY

MR. ALISON PAOLO N. BARENG

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF MASTER
OF SCIENCE (BIOCLINICAL SCIENCES)
CHULABHORN INTERNATIONAL COLLEGE OF MEDICINE
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was approved as partial fulfillment of the requirements for
the degree of Master of Science in Bioclinical Sciences

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ABSTRACT

The emergence of drug-resistant *Plasmodium vivax* poses problems for malaria control and elimination in some parts of the world, especially in developing countries where individuals are routinely exposed to the infection. The aim of this study was to determine the single nucleotide polymorphisms (SNPs) in dihydropteroate synthase (*pvdhps*) and dihydrofolate reductase (*pvdhfr*) genes associated with sulfadoxine-pyrimethamine (SP) drug resistance among *P. vivax* isolates collected in Palawan, Philippines. Genetic polymorphisms of *pvdhps* and *pvdhfr* were analysed by nested PCR. Analysis at specific codons $I_{13}P_{33}F_{57}S_{58}T_{61}S_{117}I_{173}$ associated with pyrimethamine resistance in the *pvdhfr* gene revealed that most of the samples (66/87, 75.9%) carried double mutation at positions $I_{13}P_{33}F_{57}\mathbf{R_{58}}T_{61}\mathbf{N_{117}}I_{173}$, while only 18.4% (16/87) of the isolates carried the wild-type haplotype ($I_{13}P_{33}F_{57}S_{58}T_{61}S_{117}I_{173}$). For the *pvdhps* gene, the codons involved in sulfadoxine resistance $S_{382}A_{383}K_{512}A_{553}V_{585}$ were investigated. Single mutation $S_{382}\mathbf{G_{383}}K_{512}A_{553}V_{585}$ was most observed in 68.0% (68/100) of the samples, whereas wild-type haplotype was found in 26.0% (26/100) of samples. The *pvdhps* and *pvdhfr* combination $S_{382}A_{383}K_{512}A_{553}V_{585}/I_{13}P_{33}F_{57}S_{58}T_{61}S_{117}I_{173}$ (wild-type), $S_{382}\mathbf{G_{383}}K_{512}A_{553}V_{585}/\ I_{13}P_{33}F_{57}\mathbf{R_{58}}T_{61}\mathbf{N_{117}}I_{173}$, and $S_{382}A_{383}K_{512}A_{553}V_{585}$-$I_{13}P_{33}F_{57}\mathbf{R}_{58}T_{61}\mathbf{N}_{117}I_{173}$ were the most frequently observed combination haplotypes from the three study sites. The information on molecular markers associated with antifolate drug-resistance could help better understanding of the molecular epidemiology and situation of SP resistant *P. vivax* malaria in the country. Continuous surveillance of these genetic markers is necessary to monitor the evolution of SP resistance in the Philippines.

Keywords: *Plasmodium vivax*, *pvdhfr*, *pvdhps*, antifolate resistance, sulfadoxine-pyrimethamine, Philippines

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To God be the glory

Mr. Alison Paolo Namuco Bareng

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
ACT	Artemisinin-based combination therapy
ATP	Adenosine triphosphate
CQ	Chloroquine
crt	Chloroquine resistant transporter
DHFR-TS	Dihydroreductase-thymidylate synthase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dTMP	Deoxythymidylic acid
G6PD	Glucose-6-phosphate dehydrogenase deficiency
i.e.	That is
IPT	Intermittent preventive treatment
LGU	Local government unit
mdr1	Multi-drug resistance 1
MEGA	Molecular evolutionary genetics analysis
NBD	Nucleotide binding domain
nPCR	Nested polymerase chain reaction
pABA	p-aminobenzoic acid
QBC	Quantitative buffy coat
RBC	Red blood cell
RDT	Rapid diagnostic test
RITM	Research Institute for Tropical Medicine
SNP	Single nucleotide polymorphisms
SP	Sulfadoxine-pyrimethamine
SSU rRNA	Small subunit ribosomal RNA

TES	Therapeutic efficacy surveillance study
WHO	World Health Organization

CHAPTER 1
INTRODUCTION

1.1 Introduction

Sulfadoxine-pyrimethamine (SP) is an antifolate combination used for treatment of malaria infections in many countries for years. Sulfadoxine and pyrimethamine act synergistically to inhibit the folate biosynthesis pathway of the Plasmodium parasite. The combination has been shown to be more effective compared to either drug or chloroquine alone(1-4). It was introduced for clinical use as first-line treatment for P. falciparum infection after the worldwide spread of chloroquine resistance. However, the marked decline in efficacy of SP due to rapid spread of resistant parasite strains has become a major problem that limits its clinical utility in majority of malaria endemic areas(5). Sulfadoxine acts as an inhibitor of dihydropteroate synthase (DHPS) domain that catalyzes the condensation of p-aminobenzoic acid (pABA) to folic acid. Pyrimethamine, on the other hand, targets dihydroreductase-thymidylate synthase (DHFR-TS) which reduces deoxythymidine monophosphate (dTMP) levels and arrests DNA synthesis. Several mutations in the genes encoding for these enzymes have been identified in P. vivax. Most of these genetic markers have been analyzed and characterized based on comprehensive studies in P. falciparum. Specific mutations at the amino acid positions 382, 383, 512, and 553 of the pvdhps have been linked with sulfadoxine resistance(6), whereas mutations at the positions 13, 33, 57, 58, 61, 117, and 173 of the pvdhfr gene have been linked with pyrimethamine resistance(7). Moreover, the amino acid at position V585 of pvdhps has been predicted to inhibit the binding of sulfadoxine to DHPS making P. vivax intrinsically resistant to sufadoxine(6).

In the Philippines, SP was adopted as the second-line treatment for P. falciparum during 1990's until early 2000(8). It was later endorsed by the National Malaria Program of the Department of Health as first-line treatment in combination with chloroquine during 2002-2008. In 2009, based on the therapeutic efficacy studies demonstrating geographical

difference in the efficacy of SP-chloroquine combination, first-line treatment for P. falciparum was replaced with the artemisinin-based combination therapy (ACT), artemeter-lumefantrine(9). For P. vivax infection, chloroquine remains to be the first-line treatment with satisfactory efficacy(10). Despite the fact that SP has never been used for treatment of P. vivax in the Philippines, the co-infected P. vivax with P. falciparum has been exposing to SP for certain period. Limited facilities hinder correct species diagnosis in areas where there is a sympatric existence of both malaria species. These two species cannot be differentiated clinically and are accidentally treated with SP. The incidence of mixed infection in the Philippines was reported at about 0.9% in 2010(11). As a consequence of selective drug pressure during treatment of mixed infection of P. falciparum and P. vivax (with SP alone or the combination of SP and chloroquine), P. vivax isolates might have developed antifolate resistance. This is of particular concern because SP is still the recommended therapy for intermittent preventive treatment (IPT) for malaria infection among pregnant women and infants in areas with high malaria transmission(10). Determining the prevalence of point mutations on these markers (dhfr and dhps) would help provide information on P. vivax drug resistance mapping and future treatment policy with alternative antifolate drugs. Discovery of new inhibitors of enzymes involved in folate synthesis that are effective against both species would be useful especially in areas where P. falciparum and P. vivax coexist. Compared to P. falciparum, information regarding the prevalence and genetic markers of antimalarial drug resistance in the country has relatively been limited. The aim of the study was to monitor known single nucleotide polymorphisms (SNPs) in antifolate-associated genetic markers pvdhfr and pvdhps among P. vivax-infected blood samples collected during the period 2009-2011 in Palawan, a known malaria endemic area in the Philippines.

1.2 Objectives

The overall objective of the present study is to characterize P. vivax isolates in the Philippines through molecular genotyping of SP drug resistance-related markers. Specifically, it aims to determine the prevalence of mutation in genetic markers dhps and dhfr from the archived samples obtained from Research Institute for Tropical Medicine's (RITM) Therapeutic Efficacy Studies (TES) on Antimalarials in 2009-2011 in selected municipalities in Palawan, Philippines. Moreover, the presence and species identification of Plasmodium spp. in the collected blood samples can be verified through the use of nested PCR (nPCR).

1.3 Significance of the study

P. vivax has an equal share of malaria infections in many Asian countries. Although they are considered less virulent, vivax malaria can cause life threatening complications if ignored. Understanding the molecular epidemiology and the emergence of SP resistant vivax malaria would help provide information on mapping and future treatment policy with alternative antifolate drugs. Discovery of new inhibitors of enzymes involved in folate synthesis that are effective against both species would be useful especially in areas where P. falciparum and P. vivax coexist. This study will serve as a baseline research for the molecular assessment of SP resistant P. vivax in the country.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Introduction

2.1.1 Malaria

Malaria is still among the major health challenges, which hinders the development of some of the poorest countries today. They are endemic in 91 nations and globally, it affects an expected three hundred million individuals[12]. Moreover, the World Health Organization (WHO) considers malaria as one of the main health problems since its creation[13]. Malaria history goes back in early Chinese, Chaldean, Hindu, and Greek texts. People believe that malaria came from a bad atmosphere. The word malaria comes from the Italian words *"mala"* and "*aria"* meaning "bad air". Hence, during those times, if someone dies from an intermittent fever it was believed that they died because of the air (*d'aria*) or from bad air (*mal'aria*)[14]. Nowadays, due to the technology advancement on this disease, malaria is now recognized as a protozoan parasite that infects people, causing headache and pains in the back and limbs, nausea, chills, continous or remittent fever, and splenic enlargement[14-15]. Because of the symptoms and frequent relapses of malaria, this disease can greatly delay one country's economic development.

Malaria is a fatal disease caused by a eukaryote under *Plasmodium.* Parasites can spread through the bites of an infected female *Anopheles* spp. mosquito, which are usually present during dusk and dawn. Infection can also be transmitted by exposure to infected blood products and by congenital transmission, (*i.e.* mother to child transmission)[16]. The cellular structure of *Plasmodium* is the same for all mammalian malaria parasites which consist of nucleus, mitochondrion, and microtubules. The blood stage merozoite structure is the smallest cell in the life cycle of *Plasmodium* with approximately 1-2 μm in size, which is perfect for the invasion of red blood cells. Merozoites have a unique organelle found only in an apicomplexan cell. This apical complex of secretory organelles includes micronemes, rhoptries, and dense granules which

is believed responsible for the invasion of erythrocytes[17].

There are four species of *Plasmodium* that are parasitic only to humans: *P. falciparum, P. malariae, P. ovale,* and *P. vivax.* A fifth species that is infective to humans is the re-emerging parasite *P. knowlesi* which is a zoonotic disease. Each of these parasites have noticeable difference on its clinical symptoms, geographic locations, and basic biological characteristics[18]. Malaria protists are classified under the phylum Apicomplexa and can also parasitize reptiles, birds, and other mammals depending on the species of parasites[19].

2.1.2 Life cycle of *Plasmodium* spp.

The cycle starts when an infected *Anopheles* mosquito takes on a blood meal. Sporozoites from the salivary gland of the infected mosquitos goes into the host's bloodstream. An hour later, sporozoites start to penetrate and damage the liver. Thereafter, the parasites begin to divide into exoerythrocytic merozoites (tissue schizogony). Different species of *Plasmodium* have different modes of life cycle. For example, *P. vivax* and *P. ovale* remain quiescent in the liver until activated at a later time, these dormant forms are called hypnozoites. Other species such as *P. falciparum,* does not undergo hypnozoite stage instead, they leave the liver immediately and invade the host's erythrocytes or RBCs. Once inside the RBCs, merozoites will develop into early trophozoites. Trophozoites are ring shaped, vacuolated, and uninucleated cells. It will start to divide until daughter merozoites fill all the spaces of the cell (Blood schizogony). Eventually, the infected RBC will be lysed thaw will cause the release of the merozoites from the cell. Ultimately, it will infect other erythrocytes and will start another round of schizogony. The destruction of the erythrocytes are responsible for the clinical manifestation of this disease. After several cycles, some of the merozoites will develop into haploid gametocytes. These gametocytes cause no symptoms in humans but when they are ingested it may cause the spread of malaria in the area. Inside the mosquito gut, the gametocytes will develop into mature sex cells called male and female gametes. These gametes will fuse to form a diploid zygote and eventually forms into oocysts. After that, division of oocysts can yield thousands of sporozoites. These

sporozoites will be released in the body cavity of the mosquito. From there, sporozoites will travel to and stay in the mosquito's salivary gland. The cycle goes on when an infected mosquito takes another blood meal, injecting the sporozoites from the salivary glands into the mammalian bloodstream[16].

2.1.3 Different species of *Plasmodium*

Malaria is a parasitic disease that has a global clinical incidence estimated at 200 million cases per year with more than 2.6 billion at risk of infection[20]. Added to that challenge is that there are four species of *Plasmodium* that are parasitic to humans: *P. falciparum, P. malariae, P. ovale,* and *P. vivax.* Another re-emerging parasite is the *P. knowlesi* which is actually a zoonotic disease. Each of these parasites have noticeable difference on its clinical symptoms, geographic locations, and basic biological characteristics[18]. Malaria protists are classified under the phylum Apicomplexa and can also parasitize reptiles, birds, and other mammals[19].

2.1.4 Diagnosis of malaria: clinical and laboratory

Since traditional methods for diagnosing malaria are inaccurate and slow, advanced technologies have been developed for rapid and accurate identification of this infectious disease[21]. Rapid and precise diagnosis is important to reduce the mortality and complications brought about by these parasites. With the advent of drug resistant *Plasmodium* spp., many countries, especially where malaria is endemic lack the resources and expertise for rapid diagnosis. In order to effectively treat this disease, one must identify: malaria parasites or antigens in the blood, identify the different stages of erythrocytic schizogony, parasitemia, immunity, drug resistance, levels of transmission, epidemiology, signs and symptoms, problems with re-infection, relapses, and recrudescence, endemicity of different species, and possible treatment on the basis of clinical diagnosis[21].

Diagnosis of malaria can be divided into: clinical and laboratory. A clinical diagnosis is a traditional way of determining the disease based on the signs and

symptoms of the patient. Common symptoms of malaria infection are fever, headache, weakness, myalgia, chills, dizziness, abdominal pain, diarrhea, vomiting, anorexia and pruritus[21]. This kind of diagnosis is not accurate because it is based only on observation of the signs and symptoms, which is non-specific. On the contrary, laboratory diagnosis, provides rapid detection of parasites. There are a number of ways to diagnose malaria in a laboratory: conventional microscopic diagnosis (stained by Giemsa, Wright's, and Field's stains), rapid diagnostic test or RDT (commercially available kits such as OptiMAL, ICT, Para-HIT-f, ParaScreen, SD Bioline, Paracheck), Quantitative Buffy Coat or QBC (it involves staining of the parasite's DNA for easy detection)[21], and through PCR (amplification of a DNA sequence).

2.1.5 Recrudescence, relapse, and re-infection

Treatment failure is defined as the inability to eradicate parasitaemia or resolve clinical manifestations despite administration of antimalarial drugs[9]. Failure of malarial therapies are not always due to drug resistance. Other reasons for failure may be incorrect dosage, noncompliance of patients to administer the drug, and poor drug quality. This may result from the exacerbations of persistent, undetectable parasitaemias in the absence of an exo- erythrocytic cycle[22]. This is where parasite numbers begin to increase again because antimalarial drugs and/or the immune system did not succesfully eliminate the RBC infection. Relapses, which are common in *P. vivax* and *P. ovale* result from the reactivation of dormant stage parasites or hypnozoites found in the liver. Short term relapses are seen during the first 8 to 10 weeks while long term relapses are seen between 30^{th} to 40^{th} week after infection[22]. Re-infection happens when antimalarial drugs completely eliminated the parasites in the blood and/or in the liver, but invasion arises when another infected vector bites and transmits the parasite into the same person who were completely healed with malaria.

2.1.6 Malaria in the Philippines

The Philippines is one of the countries in the South East Asia that continues to experience the problem of a parasitic disease called malaria. In the year 1985, one third of the Filipinos in a population of 54 million are living in a malaria endemic region. *P. falciparum* is the predominant species followed by *P. vivax* with the ratio of 65% to 35%, respectively. And about 79% of falciparum infection are chloroquine resistant[23]. In the recent report by WHO in the Philippines, malaria impact has decreased to more than 75% in case incidence during the 2000 to 2010 study. Major *Plasmodium* spp. are still *P. falciparum* and *P. vivax* (75:25) with Anopheles mosquito species such as *An. flavirostris, An. maculatus, An. balabacensis,* and *An. litoralis* as vectors. First line treament of *P.vivax* is still the combination of chloroquine and primaquine which were adopted in 2002. Confirmed cases and deaths through malarial microscopy decreases from more than 50,000 cases (and more than 300 deaths) in the year 2000 down to less than 10,000 cases (and 100 deaths) in the year 2011. This huge drop in confirmed cases and deaths in the country was through the initiative of the Department of Health and the Malaria Control Services operating locally in endemic areas[24]. Control strategies consist of case finding and treatment such as barangay staffs providing consultations, diagnosis and providing medicines which are free of charge. Another strategy is through vector control that includes insecticide spraying and giving away insecticide-impregnated bednets[24].

However, cases of treatment failure were recorded among *P. vivax* collected samples in the therapeutic efficacy surveillance (TES) study during the year 2009 to 2012, wherein one sample had recurrence before day 28. In addition, three *P. vivax* samples had recurred in the 75 collected samples in the 2013-2014 study and one treatment failure was recorded in the 2017 collection. Although, the WHO encourages countries to shift treatment strategy from chloroquine if more than 10% of the collected samples had already treatment failure (WHO, 2017), the presence of recurred parasitaemia among *P.vivax* isolates may provide early signs of drug resistance against chloroquine drug.

2.2 *Plasmodium vivax:* the other malaria parasite

2.2.1 *Plasmodium vivax*

Recent studies on the vivax malaria research is the completion and analysis of its genomic sequence. *P. vivax* genome has a similar size and gene count as *P. falciparum* at about 27 megabases (Mb) with 5400 genes. The differences between the two are the repeat content and nucleotide bias of the vivax's 14 chromosomes. These chromosomes contain isochore like regions of high guanine and cytosine content interspersed with regions of high adenine and thymine bias mainly at the subtelomeric ends. Metabolic pathways remain the same between the two species. Hence, antimalarial drugs which initially target falciparum malaria can now be used to target vivax species. In addition, researchers already identified more than 150 microsatellites in the genomic sequence of *P. vivax* that are used to discern the genetic diversity and population structure of this parasite. This identification is an essential step towards the development of control measures against this disease[25].

2.2.2 Anti-vivax malaria efficacy and its drug resistance

There are two frontline drugs against vivax malaria which are widely available for more than 50 years, chloroquine and primaquine[26]. The drug primaquine targets parasites that remain dormant in the liver. On the other hand, numerous studies about chloroquine treatment have shown a sensitivity against malaria at nearly 100%[27]. Additional study by Ratcliff *et al.* (2007)[28] within that region reported that sensitivity exceeded at 50%. While a recent report in 2008 in India reported that chloroquine treatment has a 9% treatment failure[29] indicating the deterioration of efficacy of these drugs against vivax parasite[26].

Anti-vivax and other malarial drugs work by targeting a single segment in the life cycle of the parasite. However, some drugs such as primaquine targets not only the asexual liver stages but also to neutralize the mature sexual gametocytes to further prevent transmission of it to the vector mosquitoes. In classifying antimalarial drugs, one

must consider the therapeutic intent, dose, species, and target stage of the parasite because some drugs seem effective to the specific plasmodia stage but render ineffective in another species[26].

Chloroquine was the perfect drug for antimalaria before because it was cheap, effective against all plasmodia species, safe for all including pregnant women and small children. There is no antimalarial therapy would equal the effectiveness of chloroquine during its peak not until now where its efficacy is slowly vanishing[26]. The recommended adult treatment for chloroquine is 25 mg/kg body weight administered in 3 doses over 48 hours. Thus, a typical 60 kg adult may take in a total dose of 1.5 g chloroquine. This drug clears fever and vivax parasites within 72 hours of the first dose. It stays and is effective against blood vivax malaria until about 21 to 35 days after the start of the treatment[26].

People infected with vivax malaria is immediately given a dose of chloroquine drug. Chloroquine is the standard drug against *Plasmodium* parasitemia present in the blood. It remains the first-line treatment for patients with malaria, most especially with vivax malaria[30]. Not until the first case of *P. vivax* which are resistant to chloroquine which was reported in 1989[31]. The prevalence of chloroquine resistance in *P. vivax* grew and spread across Southeast Asia, including the Philippines, Africa and America. But the mechanism of chloroquine resistant vivax malaria is still unknown[32]. Unlike in vivax malaria, *P. falciparum* genes *pfcrt1* and *pfmdr1* polymorphisms have been shown to confer resistance to chloroquine[33]. The orthologous gene *pvmdr1* to the *pfmdr1* and *pfcrt* has been suspected as a possible genetic marker of chloroquine resistance[30].

Primaquine therapy is effective against hypnozoites, or those parasites that remain dormant in the liver, of the relapsing *P. vivax* and *P. ovale*[9]. Primaquine is an 8-amino-quinoline and is less toxic compared to pamaquine, the drug where it was derived. Primaquine may also have high risk to patients that have glucose-6-phosphate dehydrogenase deficiency (G6PD). G6PD is a hereditary disease that were previously observed among African men. A G6PD deficient person, when treated with primaquine may experience abdominal pains, nausea, vomiting, and headache[26,34]. This extereme

intravascular haemolysis, acute renal failure, and haemoglobinuria is commonly known as black water fever[34]. This may be avoided if people would be tested for G6PD deficiency before primaquine treatment.

Commercially available primaquine drugs come in 15 mg base. Fifteen mg are the recommended dosage of normal adult human. But there are some reports that a higher risk of treatment failure were observed among heavier patients that were given the standard 15 mg for 14 days. Thus, authorities now recommend doses of 15 mg for patients weighing 20-40 kg, 30 mg for people weighing 41-70 kg, and 45 mg for those weighing >70 kg[26].

Other drugs that are used to treat *P. vivax* are mefloquine, which are highly effective against chloroquine resistant vivax malaria. Sulfadoxine-pyrimethamine treatment which is effective against patients that are infected with wild type vivax malaria. While the artemisinin-based combination therapy (ACT) can clear vivax infections more quickly than chloroquine[9].

The WHO[35] defines drug resistance as the ability of the parasite strain to survive or reproduce despite the absorption of a drug given in doses equal to or higher than those usually recommended. Plasmodia drug resistance occurs when two or more drugs with different chemical classes and modes of action were taken in combination and does not affect the parasite. It also results in the delay or failure to eliminate asexual blood parasites thus, continues to reproduce gametocytes which are important for the transmission of the resistant genotype[9].

In the 2010 WHO global report[10], they discussed about the emergence and spread of resistance to antimalarial drugs. The formation of drug resistant parasite is a random event that starts with a genetic mutation caused by different external and internal factors. This mutation produces a resistant strain that favors the parasite giving them the edge to multiply or reproduce, giving rise to a population of parasites that have the resistant gene that are susceptible to the treatment.

The spread of antimalarial drug resistance occurs when the patient is treated with a subtherapeutic drug concentrations. Deficient concentrations of antimalarial

drugs can eliminate sensitive parasites only, leaving the resistant mutations that can subsequently spread to other people during malaria outbreak. In this case, treatment of the disease is considered a failure.

2.2.3 *P. vivax* dihydrofolate reductase *(pvdhfr)* and dihydropteroate synthase *(pvdhps)* gene

The spread of chloroquine resistance in *P. vivax* has been reported in some countries in Asia including Indonesia[27], Myanmar[36], Vietnam[37], and India[38]. And other parts of the globe such as in Turkey[39] and Ethiopia[40]. Antifolate drugs were used to replace chloroquine due to the rapid spread of chloroquine resistance. However, after more than 5 years of using these drugs, it gave rise to another antimalarial drug resistance in *P. falciparum* as well as in *P. vivax,* worldwide.

Antifolate dtugs were first used as a treatment for leukemia. It was then believed that inhibition of the folate pathway in humans can help stop the spread of this dreaded disease. There were different kinds of antifolate drugs used to treat leukemia. However, issues on toxicity and safety led a way to look for other antifolate drugs that can cause disruption of the folate pathway. The success of these drugs that target these specific pathways led to the adoption of it to treat other rapidly dividing cells such as bacteria and parasites, specifically *Plasmodium* spp. There are two classes of antifolate agents for the treatment of malaria. First is the dihydropteroate synthase pathway (DHPS) inhibitor, known as the class I antifolates, and the inhibitors of dihydrofolate reductase pathway (DHFR) commonly called as class II antifolates. Class I antifolates include the drugs Proguanil, chlorproguanil, clociguanil, BRL 6231, and pyrimethamine. This class targets the dihydrofolate reductase activity of malaria parasite's bifunctional DHFR-thymidylate synthetase (TS) protein. DHFR gene allows the constant supply of fully reduced forms of folate for essential one-carbon transfer reactions hence, essential to both hosts and parasites[41]. All the mentioned anti-DHFR drugs showed low bioavailability except for proguanil and pyrimethamine, which halted its development as an effective antimalarial agent. On the contrary, pyrimethamine has similar structure as proguanil, which led to the

screening of the former as a possible antimalarial drug. Today, pyrimethamine has been the most widely used antimalarial antifolate drug so far.

On the other hand, class II antifolates were found to alter the dihydropteroate synthetase of the bifunctional hydroxymethylpterin pyrophosphokinase (HPPK)-DHPS protein[41]. Unlike the class I antifolates, this gene is only found in parasite, which participates in the formation of *de novo* folate synthesis. The ability of this class to block the parasite's folate synthesis led to the use of these class against malarial parasites. Class II inhibitors or sulfadrugs are classified into two families: sulphonamide and sulphone. However, the use of these drugs as a lone treatment for malaria were withdrawn due to low efficacy and undesirable toxicity. Studies and trials on these drugs in combination with anti-DHFR were demonstrated and showed synergestic efficacy against malaria parasites. The most commonly used antifolate combination drug is the sulfadoxine-pyrimethamine (SP) agents or Fansidar. Fansidar has been used in majority of malaria endemic areas as a treatment for uncomplicated falciparum malaria and intermittent presumptive treatment in pregnancy or IPTp during the emergence of chloroquine resistance[42].

SP is used as a treatment against *P. falciparum.* It was not recommended against vivax malaria infection. The rapid use of SP in tropical areas especially in the Southeast Asian countries, where mixed infection of Pf-Pv is prevalent, gave way to the progressive selection pressure through the unintentional exposure of the drug to *P. vivax* during *P. falciparum* treatment[43-44]. Previous studies have been done to detect point mutations in the *pvdhfr* and *pvdhps.* Results showed that resistant parasites carry the mutation at positions I13, P33, F57, S58, T61, S117, and I173 of the *dhfr* gene. While resistance in *pvdhps* was determined at codons A383 and A553. Reports of SP resistant vivax parasites are present in Korea[44], Thailand-Myanmar; Thailand-Cambodia borders[43], China[45], Indonesia[42], India[30], Philippines, Bangladesh, and Nepal[46]. There were also studies on the absence of point mutation on these genes such as in Mauritania[47].

2.2.4 Other drug resistance gene markers for *P. vivax*

P. falciparum has developed resistance to chloroquine as early as the 1950's[48-49]. *P. vivax'*s resistance to chloroquine studies remain elusive not until a report by Rieckmann *et al.* in Pap ua New Guinea in 1989[31]. From there, chloroquine resistant vivax malaria spread in some countries in Asia, South America, and the Middle East. But despite these documented reports, prevalence of chloroquine-resistant *P. vivax* remains unclear[49]. *P. vivax* CQR *in vivo* is defined as the persistence of parasites in the blood despite adequate blood levels of chloroquine and chloroquine's metabolite desethylchloroquine[50].

Molecular mechanism underlying chloroquine resistance is not yet clear but the rise of genetic variation may contribute to this phenotype. *pvmdr1* gene is an orthologous gene to *pfmdr1* and *pfcrt* genes which are found in *P. falciparum*. These genes have been documented to confer resistance to the drug chloroquine when mutated[33]. Hence, *pvmdr1* has been suspected as a possible genetic marker for chloroquine resistance in *P. vivax* because of its characteristic similar to *P. falciparum*[30].

P. vivax Multi-Drug Resistance 1 protein or PvMDR1's structure is shown in Appendix Q. It encodes a P-glycoprotein of the family of ATP binding cassette transporters[50]. It has two hydrophobic domains which are homologous to each other. Each domains has six trans-membrane alpha helices that harbors a nucleotide-binding domain 1 (NBD1) and NBD 2. Each nucleotide binding domain carries an ATP binding site with walker motifs A and B and the S signature[51] .

A study by Orjuela-Sanchez *et. al* (2009)[51] shows the polymorphism positions and structure of *pvmdr1* gene (Appendix Q). Analysis of the sequence shows 24 small nucleotide polymorphisms (SNPs) present in the *pvmdr1* coding region. Seventeen are non-synonymous[51]. Eleven can be seen at the extracellular loops in the parasites digestive-vacuole cytosol[51] and 5 are present in the trans-membrane domains. White circles indicate small nucleotide polymorphisms that were described in some studies[49,52-54]. Dark colored circles are genes also described in previous studies. The most notable are the genes Y976F and F1076 which were believed to confer chloroquine resistance[49,52-54].

The Y976F mutation (TAC to TTC) is widespread in South East Asia but worldwide studies are still lacking and thus, *pvmdr1* as a molecular marker for chloroquine resistance remains unclear[50]. In addition, the mutated form of Y976 is interestingly been reported to be rare in alleles that do not carry the F1076L (TTT to CTT) mutation. This hypothesis leads to the two step mutation pathway, F1076 which is followed by Y976F, that could lead to chloroquine resistance[50].

A study conducted by Jovel *et al.* (2011)[55] in Honduras detected genetic polymorphisms associated with chloroquine resistance in a local *P. vivax* infection. *pvmdr1* 976F was found in 7 out of 37 samples. This might indicate chloroquine tolerance but not resistance in this region[55]. Double mutation was also found in 4/37 samples. But it must be noted that during 1998 and 2000, 100% of all the infected individuals with *P. vivax* were treated with the combination of chloroquine and primaquine (PQ). This indicates a low prevalence rate of chloroquine resistant *P. vivax* in this region.

Lu *et al.* (2012)[56] surveyed the prevalence of chloroquine resistant mutations in *P. vivax* in Central China and found out that there was no *pvmdr1* resistance conferring mutations in the region.

A separate study conducted by Imwong *et al.* (2008)[53] showed that amplification of the gene *pvmdr1* can be correlated with the use of the drug mefloquine, an antifolate drug which can be used as an alternative against highly chloroquine resistant *P. vivax.* This means that the use of mefloquine for mixed infections of falciparum and vivax malaria is enough to exert selective pressure on the gene *pvmdr*[57]. Additional studies on anti-folates were documented. It showed that mutations in several genes such as *pvdhfr* and *pvdhps* was observed when *P. vivax* isolates were exposed to anti-folate pressure[57].

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Study area

The study was conducted during February 2009 to 2011 in Palawan province in the western part of the Philippines (Figure 3.1). Infection occurs throughout the year with highest incidence in June or July. In 2015, the Local Government Unit (LGU) reported that 14 out of 24 municipalities had more than 6,000 cases of malaria in the province. *P. falciparum* was the dominant species and *P. vivax* cases accounted for about 30% in 2010[58]. The study sites were Rizal, Quezon, and Puerto Princesa. The municipality of Rizal recorded the highest number of infection at 40%, while Quezon and Puerto Princesa reported 5% and 0.1% of the total cases, respectively[59].

3.2 Ethical consideration

Approval of the study protocol was obtained from the Research Institute for Tropical Medicine-Institutional Review Board. Blood samples were collected from all patients presented at different barangays (smallest administrative division in the Philippines and is the native Filipino term for village/s) after obtaining written informed consents for study participation.

3.3 Sample collection

Approximately 100 µl of blood were collected from participants who fulfilled the inclusion (aged 12 to 65 and clinically and microscopy diagnosed with *P. vivax* infection) and had none of the exclusion (treatment with antimalarial drugs within the past two weeks, pregnant women, severe and complicated malaria, individuals who received treatment with potentially hemolytic drugs, patients with cardio-pulmonary conditions, and

those with limited availability during the investigation period) criteria. Blood samples were spotted on a Whatmann 3 MM filter paper and were kept in a ziplock plastic bag with desiccant packets and stored at -20^{o}C in the Research Institute for Tropical Medicine, Philippines for molecular analysis.

3.4 DNA extraction

The parasite DNA was extracted from dried blood spot samples collected during the first day of drug administration using commercially available QIAmp DNA Mini Kit (Qiagen) following manufacturer's instructions (see Appendix A) with some modifications. Scissors were used to cut the filter paper with blood spots. The same scissors were disinfected by washing with 10% bleach, then distilled water, and lastly 70% ethanol after cutting each samples. Also, instead of 150 µL, 100 µL of elution buffer was added to each samples to increase the yield of DNA product. The eluted DNA was then divided into stock and working solution.

Samples with confirmed *Plasmodium* spp. were used as positive controls in the nested PCR analysis.

3.5 Confirmation of *Plasmodium* spp. through nested PCR

Nested PCR was used to detect and differentiate all 5 human infecting *Plasmodium* spp. First round of nested PCR amplifies a large fragment (approximately 1200 bp) of small subunit ribosomal RNA (SSU rRNA) gene from all *Plasmodium* spp. Primers used for first round amplification were: rPLU1 (5'-TCA AAG ATT AAG CCA TGC AAG TGA-3') and rPLU5new (5'-CYT GTT GTT GCC TTA AAC TTC-3')[60-61]. The species-specific primers used for second round nested PCR are summarized in Table 3.1.

The PCR mixture consisted of: 10x PCR reaction buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl), 2 mM $MgCl_2$, 200 µM deoxynucleoside triphosphate (dNTPs), 250

nM primers, 0.4 U/µl of *Taq* DNA enzyme, of genomic DNA (5 and 1 µl for first and second round nested PCR, respectively). The first nested PCR amplification condition was as follow: initial denaturation at 95^{o}C for 5 min, 25 cycles of denaturation, annealing and extension at 94^{o}C (1 min), 58^{o}C (2 min), and 72^{o}C (2 min), respectively. The final extension was 10 min at 72^{o}C. The second nested PCR amplification condition was identical to the first nested PCR except denaturation, annealing and extension were performed for 30 cycles. Samples with confirmed *Plasmodium* spp. was used as PCR positive controls. Each sample was run in duplicates to ensure validity of the results.

3.6 Amplification of *pvdhps* and *pvdhfr* genes

The *pvdhfr* and *pvdhps* genes were amplified at the laboratory in RITM, Philippines using nested PCR following the previously described protocol and primers (Table 3.2). The components for Nest 1 and Nest 2 reactions were as follows: 1x PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer, 0.03U/µl of *Taq* DNA polymerase, and 2 µl of DNA template with a total volume of 20 µl for first round PCR. Two microliters of the primary PCR product was used for second round PCR with a final volume of 50 µl. The following PCR condition was used for both genes: initial denaturation at 94^{o}C for 5 min, denaturation at 94^{o}C for 30 sec, annealing at 53^{o}C for 1 min and extension at 72^{o}C for 1 min, and final extension period at 72^{o}C for 10 min. The reactions consisted of 25 and 45 cycles for the first- and second- round PCR, respectively.

3.7 Sequencing of *pvdhps* and *pvdhfr* genes

For the sequencing reactions, the amplified PCR products were purified using QIAquick PCR purification kit (Qiagen- see Appendix B). The second round primers were used for sequencing of *pvdhps* and *pvdhfr* genes. Concentration of DNA was confirmed by using Nanodrop before it was sent to First BASE laboratories (AsiaGel) in Singapore. Sequences were analysed by using Molecular Evolutionary Genetic Analysis version 6.0

(MEGA 6) sequence alignment software.

3.8 Data analysis

Point mutations of *pvdhfr* and *pvdhps* genes were analyzed through alignment of the sequenced regions using CLUSTAL W function in the Molecular Evolutionary Genetics Analysis (MEGA) 6 software[62]. The presence of polymorphisms at *pvdhfr* codons I13, P33, F57, S58, T61, S117, and I173 and *pvdhps* codons S382, A383, K512, A553, and V585 were recorded. The complete wild-type nucleotide sequences of *pvdhfr* and *pvdhps* genes were used as reference standards. The sequences were retrieved from GenBank with accession numbers X98123 for *pvdhfr*[63] and *pvdhps*[6], respectively (see Appendix D and E for full sequences). In addition, repeat variants between nucleotides 262 and 362 of *pvdhfr* gene were identified using Unipro UGENE[64]. The four allelic variants were designated as type A (GGDN TS GGDN TS GGDN AD), type B (GGDN TS GGDN TH GGDN AD), type C (GGDN TS GGDN AD), and type D (GGDN TS GGDN TR GGDN AD).

Figure 3.1 Sample collection sites in Palawan, Philippines. Puerto Princesa harbored the highest number of symptomatic participants at 63.9%, followed by Rizal and Quezon at 24.3 and 11.8%, respectively.

Table 3.1 Primer sequences and expected band sizes for nested PCR[60,65-66].

Plasmodium sp.	Primer Names	5' to 3' Sequence	Expected band size
P. falciparum	rFAL 1	5’-TTAAACTGGTTTGGGAAAACCAAATATATT-3’	
	rFAL2	5’-ACACAATGAACTCAATCATGACTACCCGTC-3’	~205 bp
P. vivax	rVIV1	5’-CGCTTCTAGCTTAATCCACATAACTGATAC-3’	
	rVIV2	5’-ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3’	~117 bp
P. ovale	PadPo	5’-CTGTTCTTTGCATTCCTTATGC-3’	
	rOVA2v	5’-GGAAAAGGACACTATAATGTATCCTAATA-3’	~787 bp
P. malariae	rMAL1	5’-ATAACATAGTTGTACGTTAAGAATAACCGC-3’	
	rMAL2	5’-AAAATTCCCATGCATAAAAAATTATACAAA-3’	~144 bp
P. knowlesi	Kn1f	5’-CTCAACACGGGAAAACTCACTAGTTTA-3’	
	Kn3r	5’-GTATTATTAGGTACAAGGTAGCAGTATGC-3’	~235 bp

Table 3.2 Primer sequences and expected band sizes for *pvdhfr* and *pvdhps*[47].

Gene	Reaction	Primers (forward and reverse)	Size (bp)	Annealing Temperatures
pvdhfr	1st PCR	5′-ACCCTTCCATAGGGAGTCCACTT-3′	961	53
		5′-CGCATTGCAGTTCTCCGAA-3′		
	2nd PCR, SR	5′-CCCCACCACATAACGAAGTAG-3′	632	65, 50
		5′-GCCGTTGATCCTCGTGAAG-3′		
pvdhps	1st PCR	5′-GGAAGCCATTCGCTCAACTTATAA-3′	970	53
		5′-CGTCAGTTTACCCTCCCCGTT-3′		
	2nd PCR, SR	5′-GATGGCGGTTTATTTGTCGAT-3′	767	65, 50
		5′-GCCTCCCCGCTCATCAGTCT-3′		

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Results

4.1.1 Study area and population

According to the Philippine Malaria Information System of the Department of Health, the number of malaria cases in Palawan decreased from more than 7,000 cases in 2009 to approximately 5,000 cases in 2011. *P. vivax* accounts for 18-20% of the total reported cases. In this study, 119 participants fulfilled all of the inclusion criteria and had none of the exclusion criteria were enrolled. All were treated with chloroquine at 25 mg/kg body weight over three days and primaquine at the dose of 15 mg body weight for 14 days. Among the three study sites, Puerto Princesa reported the highest number of cases (76 samples; 63.9%), followed by Rizal (29 samples; 24.3%) and Quezon (14 samples; 11.8%). Most of the participants were males (7:3) with a median age of 24 years.

The extracted DNA samples were amplified by nested PCR for confirmation of *Plasmodium* spp. Of the 119 samples, 107 and 3 samples were confirmed as *P. vivax* mono-infection and mixed-infection of *P. falciparum* and *P. vivax*, respectively. One sample each was confirmed as mono-infection of either *P. falciparum, P. malariae, P. ovale,* or *P. knowlesi*. The remaining five samples were PCR negative.

4.1.2 Species identification using nPCR

All 119 samples were amplified by nested polymerase chain reaction along with positive and negative controls (Figure 4.1). Nested PCR assay confirmed 90.0% (n=107) as *P. vivax,* while three subjects (2.5%) were identified to have mixed infections of *P. falciparum* and *P. vivax*. One percent (n=1) had monoinfections of either *P. falciparum, P. malariae, P. ovale,* or *P. knowlesi.* Five samples (4.2%) which were microscopically positive for *P. vivax* failed to amplify by PCR (Table 4.1). Re-run of samples were done for PCR and results were confirmed for all 119 samples.

4.1.3 *P. vivax* dihydropteroate synthase *(pvdhps)* gene analysis

The region of the *pvdhps* gene was successfully sequenced in 100 out of 107 samples (93%). Figure 4.2 shows gel electrophoresis results of some samples. Frequency distribution of the *pvdhps* mutation is summarized in Table 4.2. Isolates carrying wild-type at $S_{382}A_{383}K_{512}A_{553}V_{585}$ were found in 26 (26%) samples. The distribution of wild-type haplotype in blood samples from Rizal, Queson and Puerto Princesa were 20 (76.9%), 5 (19.2%) and 1 (3.9%) samples, respectively. On the other hand, single mutation $S_{382}\mathbf{G_{383}}K_{512}A_{553}V_{585}$ and $S_{382}A_{383}K_{512}A_{553}\mathbf{Y_{585}}$ were observed in 69 (69%) and 1 (1%) isolates, respectively. Nearly all A383G single mutation was observed in Puerto Princesa (57 samples, 83.8%), followed by Quezon (6 samples, 8.8%), and Rizal (5 samples, 7.4%). Double mutation $S_{382}\mathbf{G_{383}}K_{512}A_{553}\mathbf{Y_{585}}$ and $S_{382}A_{383}\mathbf{N_{512}}A_{553}\mathbf{S_{585}}$ were detected in Rizal, while $S_{382}\mathbf{G_{383}}K_{512}A_{553}\mathbf{I_{585}}$, and $S_{382}\mathbf{G_{383}}K_{512}A_{553}\mathbf{L_{585}}$ were observed in Puerto Princesa.

4.1.4 *P. vivax* dihydrofolate reductase *(pvdhfr)* gene analysis

The *pvdhfr* gene was successfully sequenced in 87% (87/100) of the isolates. The frequency of each *pvdhfr* SNP is summarized in Table 4.2. The wild-type gene corresponding to $I_{13}P_{33}F_{57}S_{58}T_{61}S_{117}I_{173}$ was found in 16 (18.4%) samples, with highest number in Rizal (13 samples, 81.3%), followed by Puerto Princesa (4, 4.6%) and Queson (2, 2.3%). Majority of the samples (66 samples, 75.9%) carried a double mutation at positions $I_{13}P_{33}F_{57}\mathbf{R_{58}}T_{61}\mathbf{N_{117}}I_{173}$. The highest number was found in Puerto Princesa (50 samples, 75.8%), followed by Rizal (9 samples, 10.34%), and Queson (7 samples, 8.05%). Single mutation ($I_{13}P_{33}F_{57}\mathbf{R_{58}}T_{61}S_{117}I_{173}$) was found only in Queson in 2 samples (2.23%). Triple mutation was recorded in 2 (2.3%) samples, *i.e.*, $I_{13}P_{33}F_{57}\mathbf{R_{58}M_{61}T_{117}}I_{173}$ (Quezon) and $I_{13}\mathbf{T_{33}}F_{57}S_{58}\mathbf{K_{61}N_{117}}I_{173}$ (Rizal). Quadruple mutation ($I_{13}P_{33}\mathbf{L_{57}R_{58}M_{61}T_{117}}I_{173}$) was detected in 1 sample (1.15%) from Quezon. Non-synonymous mutation at position H99S was also present in 63 (72.4%) isolates. Other haplotypes were also observed in some samples, especially in Quezon where two samples harbored the single mutation $I_{13}P_{33}F_{57}\mathbf{R_{58}}T_{61}S_{117}I_{173}$.

Table 4.3 summarizes the distribution of the allelic repeats found in samples collected from different study sites in each year in Palawan. High prevalence of type A repeat variant was found in majority of the samples in the three study sites

(64 samples, 73.6%), followed by type B variant (20 samples, 23.0%), type C (2 samples, 2.3%), and type D variant (1 sample, 1.1%).

4.1.5 *pvdhfr* and *pvdhps* haplotypes analysis

The distribution of the *pvdhps* and *pvdhfr* in *P. vivax* isolates in Palawan are summarized in Table 4.4. Of the 85 samples, 11 (12.9%) harbored the wild-type haplotype $S_{382}A_{383}K_{512}A_{553}V_{585}/I_{13}P_{33}F_{57}S_{58}T_{61}S_{117}I_{173}$. Single mutant *pvdhps* $S_{382}\mathbf{G_{383}}K_{512}A_{553}V_{585}$ coupled with double mutant *pvdhfr* $I_{13}P_{33}F_{57}\mathbf{R_{58}}T_{61}\mathbf{N_{117}}I_{173}$ was the most prevalent haplotype (54 samples, 63.5%). The highest number was found in Puerto Princesa (49 samples, 57.65%), followed by Queson (4 samples, 4.71%) and Rizal (1 sample, 1.18%). Another combination haplotype, wild-type *pvdhps* coupled with double mutation *pvdhfr* ($S_{382}A_{383}K_{512}A_{553}V_{585}/I_{13}P_{33}F_{57}\mathbf{R_{58}}T_{61}\mathbf{N_{117}}I_{173}$), was seen in Rizal (7 samples, 8.2%) and Quezon (3 samples, 3.5%).

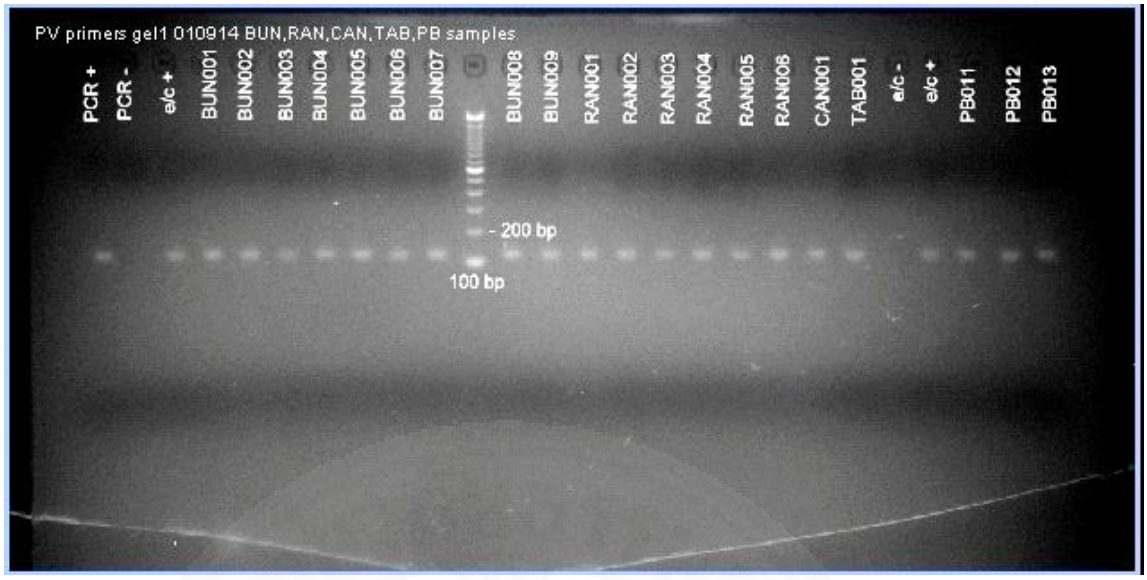

Figure 4.1 An example of gel electrophoresis result for species identification using nested PCR. Amplified products from Barangay Bunog (BUN001-BUN009), Ransang (RAN001-RAN005), Candawaga (CAN001), and Tabon (TAB001) yielded positive results for *P. vivax.* Based on the DNA marker (100 bp; Invitrogen), bands were between 100 to 200 bp. Target size for vivax malaria is approximately 117 bp.

Table 4.1 Species identification result of the 119 samples using nested PCR.

Species	**nPCR results**
P. falciparum	1 (0.8%)
P. vivax	107 (90.0%)
P. malariae	1 (0.8%)
P. ovale	1 (0.8%)
P. knowlesi	1 (0.8%)
Mixed infections	3 (Pf and Pv) (2.5%)
Negative	5 (4.2%)
Total	119

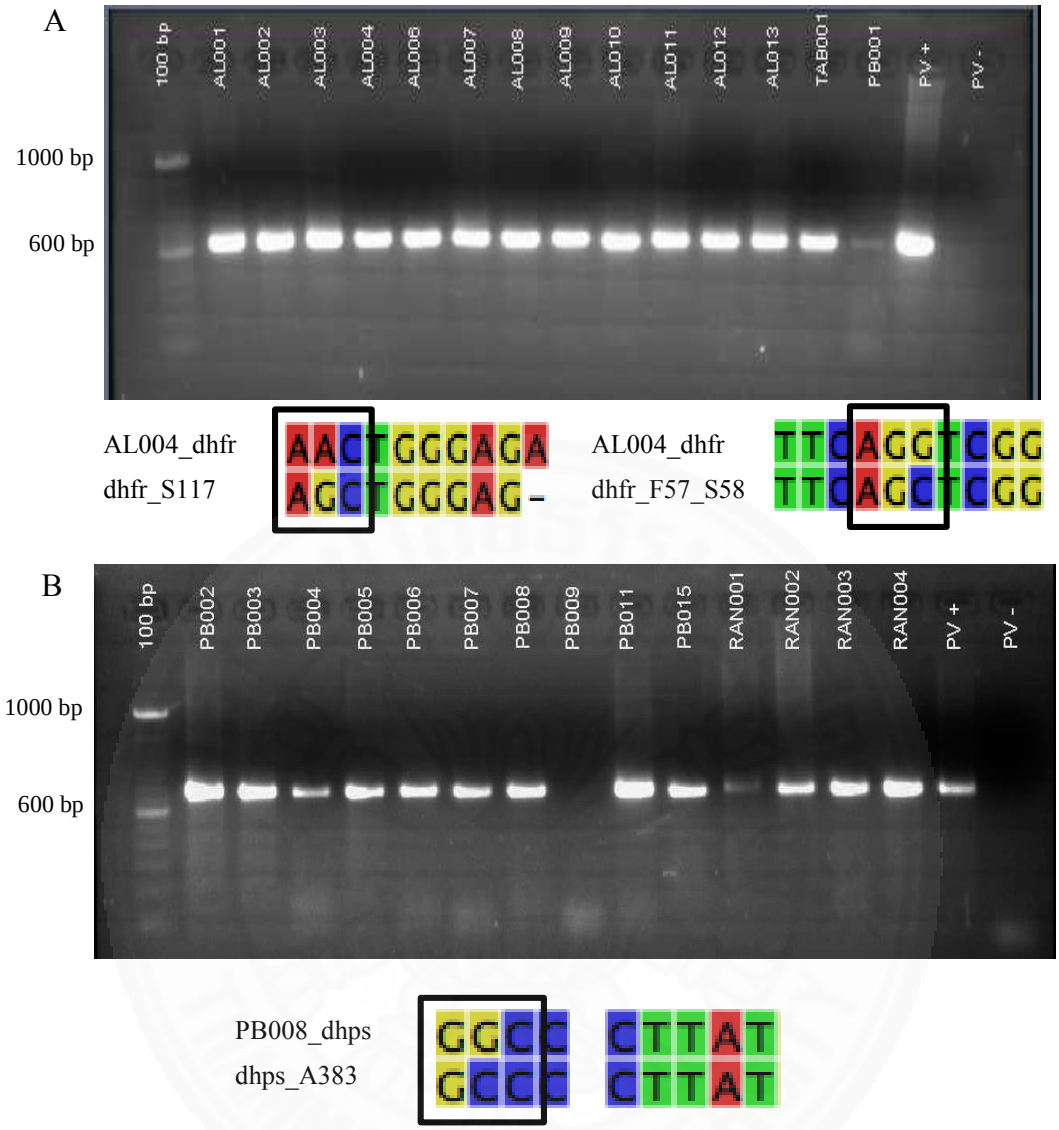

Figure 4.2 Gel electrophoresis results of *pvdhfr* (~632 bp) and *pvdhps* (~767 bp) amplification in selected samples. Gene analysis is done through alignment of the sequenced product to a reference sequence. (A) Selected samples positive for *pvdhfr* marker (above). An example of sequence alignment where sample AL004 has a mutation at positions 117 (Serine to Asparagine) and 58 (Serine to Arginine) of the *pvdhfr* marker (below). (B) Above are some samples which yielded positive for *pvdhps* marker. Shown below is the sequence alignment of sample PB008 and the reference sequence. At position 383, a change from Alanine (GCC) to Glycine (GGC) was observed.

Table 4.2 Frequency distribution of *pvdhps* and *pvdhfr* in *P. vivax* isolates collected in Palawan, Philippines.

Gene	osition	SNPs	Isolates
pvdhps (n=100)	382	S (Wild type)	100 (100%)
	383	A (Wild type)	28 (28.0%)
		G (Mutant)	72 (72.0%)
	512	K (Wild type)	99 (99.0%)
		N (Mutant)	1 (1.0%)
	553	A (Wild type)	100 (100%)
	585	V (Wild type)	95 (95.0%)
		Y (Mutant)	2 (2.0%)
		I (Mutant)	1 (1.0%)
		S (Mutant)	1 (1.0%)
		L (Mutant)	1 (1.0%)
pvdhfr (n=87)	13	I (Wild type)	87 (100.0%)
	33	P (Wild type)	86 (99.0%)
		T (Mutant)	1 (1.0%)
	57	F (Wild type)	86 (98.8%)
		L (Mutant)	1 (1.1%)
	58	S (Wild type)	17 (19.5%)
		R (Mutant)	70 (80.5%)
	61	T (Wild type)	84 (96.6%)
		M (Mutant)	2 (2.3%)
		K (Mutant)	1 (1.1%)
	117	S (Wild type)	18 (20.7%)
		N (Mutant)	67 (77.0%)
		T (Mutant)	2 (2.3%)
	173	I (Wild type)	87 (100.0%)

Table 4.3 Short repetitive sequences between nucleotides 262 and 309 within *dhfr* gene of *P. vivax* and summary of sequences in each study sites. (A) Tandem repeat sequence found in majority of the samples (63/87). (B) Corresponds to short repeats found in 21 of 87 isolates. This is also the consensus sequence deduced from an earlier study on *P. vivax*[63]. (C) Represents the deleted sequence found in 2 isolates from Rizal and Quezon. Dashes indicate deletions. (D) Corresponds to mutation in codon 305 (His to Arg). (E) Distribution of different repeat variation types in *dhfr* gene of *P. vivax* parasites from the three study sites in Palawan, Philippines.

(A)	GGG	GGT	GAC	AAC	ACA	AGC	GGT	GGT	GAC	AAC	ACA	AGC	GGT	GGT	GAC	GCC	GCC	GAC
	G	G	D	N	T	S	G	G	D	N	T	S	G	G	D	N	A	D
(B)	GGG	GGT	GAC	AAC	ACA	AGC	GGT	GGT	GAC	AAC	ACA	CAC	GGT	GGT	GAC	GCC	GCC	GAC
	G	G	D	N	T	S	G	G	D	N	T	H	G	G	D	N	A	D
(C)	---	---	---	---	---	---	GGT	GGT	GAC	AAC	ACA	AGC	GGT	GGT	GAC	GCC	GCC	GAC
							G	G	D	N	T	S	G	G	D	N	A	D
(D)	GGG	GGT	GAC	AAC	ACA	AGC	GGT	GGT	GAC	AAC	ACA	CGC	GGT	GGT	GAC	GCC	GCC	GAC
	G	G	D	N	T	S	G	G	D	N	T	R	G	G	D	N	A	D

Type	Puerto Princesa	Rizal	Quezon	Total
Type A	50	7	6	63 (72.4%)
Type B	1	14	6	21 (24.1%)
Type C	0	1	1	2 (2.3%)
Type D	0	1	0	1 (1.1%)

Table 4.4 Distribution of *pvdhps* and *pvdhfr* haplotypes in *P. vivax* isolates collected during 2009-2011 from the three study sites in Palawan, Philippines [Puerto Princesa (PP), Rizal, and Quezon].

Study sites	*pvdhps-pvdhfr* haplotypes																													
	S**G**KAV-IPF**R**T**N**I[a]			SAKAV-IPFSTSI (wild-type)[b]			SAKAV-IPF**R**T**N**I[c]			S**G**KAV-IPFSTSI[d]			SAKAV-IPF**R**TSI[e]			SAKA**Y**-I**T**FS**K**NI[f]			S**G**KA**Y**-IPF**R**TNI[g]			S**G**KAV-IP**LRMT**I[h]			SA**N**A**S**-IPFSTSI[i]			S**G**KAV-IPF**RMT**I[j]		
	2009	2010	2011	2009	2010	2011	2009	2010	2011	2009	2010	2011	2009	2010	2011	2009	2010	2011	2009	2010	2011	2009	2010	2011	2009	2010	2011	2009	2010	2011
PP	25	19	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rizal	1	0	0	6	4	0	6	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
Quezon	3	1	0	0	0	0	3	0	0	1	0	0	2	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0

[a] $S_{382}\mathbf{G}_{383}K_{512}A_{553}V_{585}$-$I_{13}P_{33}F_{57}\mathbf{R}_{58}T_{61}\mathbf{N}_{117}I_{173}$

[b] $S_{382}A_{383}K_{512}A_{553}V_{585}$-$I_{13}P_{33}F_{57}S_{58}T_{61}S_{117}I_{173}$

[c] $S_{382}A_{383}K_{512}A_{553}V_{585}$-$I_{13}P_{33}F_{57}\mathbf{R}_{58}T_{61}\mathbf{N}_{117}I_{173}$

[d] $S_{382}\mathbf{G}_{383}K_{512}A_{553}V_{585}$-$I_{13}P_{33}F_{57}S_{58}T_{61}S_{117}I_{173}$

[e] $S_{382}A_{383}K_{512}A_{553}V_{585}$-$I_{13}P_{33}F_{57}\mathbf{R}_{58}T_{61}S_{117}I_{173}$

[f] $S_{382}A_{383}K_{512}A_{553}\mathbf{Y}_{585}$-$I_{13}P_{33}F_{57}S_{58}\mathbf{K}_{61}\mathbf{N}_{117}I_{173}$

[g] $S_{382}\mathbf{G}_{383}K_{512}A_{553}\mathbf{Y}_{585}$-$I_{13}P_{33}F_{57}\mathbf{R}_{58}T_{61}\mathbf{N}_{117}I_{173}$

[h] $S_{382}\mathbf{G}_{383}K_{512}A_{553}V_{585}$-$I_{13}P_{33}\mathbf{L}_{57}\mathbf{R}_{58}\mathbf{M}_{61}\mathbf{T}_{117}I_{173}$

[i] $S_{382}A_{383}\mathbf{N}_{512}A_{553}\mathbf{S}_{585}$-$I_{13}P_{33}F_{57}S_{58}T_{61}S_{117}I_{173}$

[j] $S_{382}\mathbf{G}_{383}K_{512}A_{553}V_{585}$-$I_{13}P_{33}F_{57}\mathbf{R}_{58}\mathbf{M}_{61}\mathbf{T}_{117}I_{17}$

4.2 Discussions

4.2.1 Species identification using nPCR

Confirmation of microscopically identified *P. vivax* samples were done using nested polymerase reaction assay. There were samples which yielded negative for *P. vivax.* Four of them had monoinfection of either *P. falciparum, P. ovale, P. malariae,* or *P. knowlesi.* The discordance cold probably be the result of error in microscopy testing since some species are microscopically similar to other species of malaria hence difficult to identify. A study conducted by Ohrt *et al.* (2002)[67] discussed that in a blood smear, there are many artifacts which can resemble the shape of the malaria parasite. This could lead to false positive diagnosis of *Plasmodium* spp. In addition, false positive results are common in clinical settings and is probably due to the error of the microscopist. Another source of missed diagnosis is the quality of the prepared slide smears that may decrease over time[67]. On the other hand, PCR assay failed to detect DNA in 5 samples which were microscopically positive for *P. vivax.* In a study conducted by Parajuli *et al.* (2009) [68], DNA degradation during the process of extraction may give rise to false negative results. Reasons for the degradation of DNA include the repeated freezing and thawing of the samples and technical faults in storing the DNA samples. Technical faults such as the lack of silica gel crystals during strorage could contribute to the DNA damage[68]. Separate studies by Makler *et al.* (1998)[69], Puri *et al.* (2013)[70], and Barker *et al.* (1992)[71], discussed about the PCR inhibitors present in the blood that may also give rise to false negative or false positive results. Inhibitors such as EDTA, porphyrin ring of haeme, and intracellular substances could contribute to this discordance. In addition, failure of PCR to detect microscopically positive *P. vivax* may also be due to the low parasite density and the use of non-sensitive primers[70].

4.2.2 *pvdhfr* and *pvdhps* gene analysis

One of the factors hindering the control and elimination of malaria is the emergence and spread of resistance of *Plasmodium* parasite to antimalarial drugs. Determining drug resistance pattern has always been a challenge for most countries and surveillance plays an important role in the management of this problem[72]. The spread of chloroquine resistant *P. falciparum* in most malaria endemic regions has led to the extensive use of SP for treatment of *P. vivax* infection. However, this switch in strategy may have resulted in SP-resistant *P. vivax* whenever misdiagnosis and/or co-infection of *P. falciparum* and *P. vivax* occur as a consequence of selective drug pressure.

In the present study, prevalence of mutations at the genes responsible for SP resistance in *P. vivax* was investigated in isolates collected in Palawan during 2009-2011. Since SP is not used as treatment for *P. vivax* in the Philippines, it is assumed that the prevalence of mutations associated with SP resistance to this malaria species would be low. Results showed that majority of the collected samples had double mutation at positions $I_{13}P_{33}F_{57}\mathbf{R}_{58}T_{61}\mathbf{N}_{117}I_{173}$ (75.9%) in the *pvdhfr* gene. Some isolates also carried either single (2.3%), triple (2.3%), or quadruple (1.15%) mutation. Only 16 samples out of 87 (18.4%) harbored wild-type haplotype. The high prevalence of double mutation in the *pvdhfr* gene could be the result of the drug selection pressure toward the development of pyrimethamine resistance in *P. vivax* population. These results are consistent with the findings from other Asian countries including Myanmar[44], areas along the Thai-Cambodian and Thai-Malaysian borders[43], and East Timor[73], where double mutant *pvdhfr* haplotype was frequently observed among all genotypes analyzed. In spite of these observations, the presence of double mutation does not appear to result in clinical resistance of *P. vivax* to SP. Previous studies in Myanmar[74], India[75], Papua New Guinea[76] and Thailand[77] demonstrated that treatment failure was only associated with samples having quadruple or more mutations. The presence of quadruple *pvdhfr* mutation identified in an isolate from Quezon may also have expressed high level of resistance to SP. Furthermore, tandem repeats in the DHFR domain were also observed in the study. The three copies of GGDN (type A, B and D) repeat was the most common variant (85

samples, 97.7%). This observation was similar to that reported from China (77-97%)[72,45], Indonesia (66.7-100%)[43], India (87%)[78], Colombia (75%)[78], and Iran (88.7%)[79]. The prevalence of this variant in Lao was however lower (26.7-57.6%)[78]. The absence of four-copies of GGDN repeat type was contrast with other reports in Thailand[80], India[81], Myanmar[74] and Iran[79] where quadruple-mutant *dhfr* alleles were found to be exclusively associated with the four-copy repeat type. These variants however, did not appear to be clearly associated with antifolate resistance. Analysis of the crystal structure of PvDHFR enzyme suggests that the GGDN repeat region is located outside the binding pocket which not be essential for substrate binding[82].

Earlier studies suggest that *P. vivax* is innately resistant to sulfadoxine due to the presence of residue V585 in the *pvdhps* gene[83-84,6]. However, homology modeling showed that this residue had no effect on the sensitivity of *P. vivax* to other potential sulfa drugs[6]. In this study, the observed presence of V585 in majority of the samples further supports the claim of previous research that this is possibly a wild-type residue, thus solidifying evidence for the innate resistance of *P. vivax* to sulfadoxine[6,76]. Additional amino acid substitutions in PvDHPS are also associated with decreased sulfa sensitivity in *P. vivax* malaria[43]. In addition, consistent with the results from neighboring countries such as Myanmar and Thailand[44], the high distribution of single mutant $S_{382}\mathbf{G_{383}}K_{512}A_{553}V_{585}$ (64%) compared to wild-type haplotype (24%) observed in the present study may also be linked to acquired resistance due to the wide spread use of sulfadoxine and other sulfa drugs such as cotrimoxazole. This drug is used against bacterial infections and may have contributed to the drug selection of *P. vivax* populations[85,6].

More than half of the samples with mutations in *pvdhfr* gene also carried mutations in corresponding samples in the *pvdhps* gene. These coexisting combination mutations might be correlated with treatment failure to SP[86]. The most prevalent mutation observed in this study was the double mutation $I_{13}P_{33}F_{57}R_{58}T_{61}N_{117}I_{173}$ in *pvdhfr* coupled with single mutation $S_{382}G_{383}K_{512}A_{553}V_{585}$ in *pvdhps* gene (63.5%). The combination haplotype SNPs observed in this study were consistent with the selection

process occurring in *P. falciparum*, which supports that the development of resistance in *P. vivax* is asymmetric, *i.e.*, mutations in *pvdhfr* are initially selected before *pvdhps*[87,76].

This study was the first to monitor the molecular profile of antifolate resistance genes in Palawan, Philippines. Two previous investigation conducted in CARAGA Administrative Region (CARAGA) region (the northeastern part of the island of Mindanao designated as the Region XIII in the Philippines, Figure 3.1) showed conflicting results. The findings reported by Auliff *et al.*[76] in samples collected in Agusan del Sur, CARAGA region (n=15) in 2002 were similar to the present study where majority of the samples had double mutation in the *pvdhfr* gene and single mutation in the *pvdhps* gene[76]. On the other hand, results from the study reported by Thongdee *et al*. (2015) also in CARAGA collected in 2005 (n=33) showed the frequency of triple mutant *pvdhfr* of 81.8%, whereas that of single mutant *pvdhps* at position 553 was 100%[46]. However, it is of noted that CARAGA region is at a different geographical location in the Philippines and results obtained from both regions should not be directly compared. Movement of people for work or other activities between these two separate regions or provinces is minimal since they are situated far from each other. Therefore, the mutation patterns observed between these two regions are likely under similar drug pressure which was the consequence of the extensive use of SP rather than gene flow through population exchange. In Puerto Princesa, the $S_{382}\mathbf{G_{383}}K_{512}A_{553}V_{585}/I_{13}P_{33}F_{57}\mathbf{R_{58}}T_{61}\mathbf{N_{117}}I_{173}$ haplotype was most frequently found (49/50; 98%), with only one sample (2%) carrying wild-type haplotype. This may suggest that the genetic background of *pvdhps* and *pvdhfr* in *P. vivax* isolates in this area were rather homogeneous. In Quezon which is located next to Pueroto Princesa, seven *pvdhps/pvdhfr* combination haplotypes were reported with absence of wild type haplotype. In Rizal which is next to Quezon and far apart from Pueroto Princesa, the predominat haplotype was wild-type and five *pvdhps/pvdhfr* combination haplotypes were observed. This suggests that *P. vivax* isolates in Puerto Princesa may have extensively exposed to SP drug pressure, compared with Quezon and Rizal. Most isolates in Rizal has remained natural in genetic pattern, but the sign of resistance pattern was now distributed to this region.

The wide distribution of mutation in *pvdhfr* and *pvdhps* genetic markers may suggest emergence of SP resistant *P. vivax* in Palawan, Philippines. Although, SP has not been used against *P. vivax* infection due to its low efficacy and possible innate resistance, information of the patterns of drug resistant *P. vivax* in malaria endemic areas of the country is still essential as this could be utilized in updating malaria policy guidelines. This is particularly important since SP treatment is still used for IPT in most countries including the Philippines. In addition, development of new antifolate drugs capable of acting against the mutant enzymes requires in *P. vivax* isolates collected from different endemic areas. The urgent need to develop and discover alternative drugs against *P. vivax* is essential considering choloroquine resistance has started to emerge and become a problem in some endemic countries.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions and recommendations

High prevalence of point mutations was reported in the antifolate resistance associated genes *pvdhfr* and *pvdhps* in *P. vivax* isolates collected in Palawan, Philippines. This is despite of SP being not used as treatment against *P. vivax* infection. The results further support previous studies on the selective drug pressure caused by extensive use of SP which could result to drug resistance. Looking at the mutations in other drug resistance markers such as *pvmdr1* and *pvcrt-o* is recommended in order to provide valuable information on the molecular epidemiology of *P. vivax*. Most especially to gain insights on the chloroquine treatment failures recorded in the study area. This investigation and other related studies are needed to provide global data on *P. vivax* drug resistance.

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APPENDICES

APPENDIX A

DNA PURIFICATION FROM DRIED BLOOD SPOTS (QIAAMP DNA MINI KIT)

Procedure

- Place 3 punched-out circles from a dried blood spot into a 1.5 ml microcentrifuge tube and add 180 µl of Buffer ATL.
- Incubate at 85°C for 10 min. Briefly centrifuge to remove drops from inside the lid.
- Add 20 µl proteinase K stock solution. Mix by vortexing, and incubate at 56°C for 1 h. Briefly centrifuge to remove drops from inside the lid.
- Add 200 µl Buffer AL to the sample. Mix thoroughly by vortexing, and incubate at 70°C for 10 min. Briefly centrifuge to remove drops from inside the lid.
- Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing. Briefly centrifuge to remove drops from inside the lid.
- Carefully apply the mixture from step 5 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
- Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the collection tube containing the filtrate.
- Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
- Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.
- Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube and

discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 150 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min. Three punched-out circles (3 mm diameter) typically yield 150 ng and 75 ng of DNA from anticoagulated and untreated blood, respectively. If the yield from untreated blood is not sufficient, use 6 circles per prep instead of 3. The volume of the DNA eluate used in a PCR assay should not exceed 10%; for example, for a 50 µl PCR, add no more than 5 µl of eluate.

APPENDIX B

PCR PURIFICATION KIT (QIAGEN)

Procedure

- Add 5 volumes of Buffer PBI to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.
- Place a QIAquick spin column in a provided 2 ml collection tube.
- To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
- Discard flow-through. Place the QIAquick column back into the same tube.
- To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
- Discard flow-through and place the QIAquickcolumn back in the same tube. Centrifuge the column for an additional 1 min.
- Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
- To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
- If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

APPENDIX C

PROTOCOL: GEL EXTRACTION KIT (INVITROGEN)

Purifying DNA using a centrifuge:

- Pipet the dissolved gel piece conaining the DNA fragment of interest onto the center of a Quick Gel Extraction Column inside a Wash Tube. Note: Do not load >400 mg dissolved agarose per Quick Gel Extraction Column.
- Centrifuge at >12,000 × g for 1 min. Discard the flow-through and replace the Quick Gel Extraction Column into the Wash Tube.
- Add 500 µl Wash Buffer (W1), containing ethanol to the Quick Gel Extraction Column.
- Centrifuge at >12,000 × g for 1 min. Discard the flow-through and replace the column into the Wash Tube.
- Centrifuge the column again at maximum speed for 1–2 min to remove any residual Wash Buffer and ethanol. Discard the Wash Tube and place the Quick Gel Extraction Column into a Recovery Tube.
- Add 50 µl Elution Buffer (E5) to the center of the Quick Gel Extraction Column.
- Incubate the column for 1 min at room temperature.
- Centrifuge the Column at >12,000 × g for 1 min. The recovery tube contains the purified DNA. Discard the Quick Gel Extraction Column.
- Store the purified DNA or proceed to your downstream application.

APPENDIX D

P. VIVAX dhfr-ts GENE GENBANK ACCESSION NUMBER: X98123[63]

ATGGAGGACC TTTCAGATGT ATTTGACATT TACGCCATCT GCGCATGCTG CAAGGTCGCC
CCCACCAGTG AAGGGACAAA GAATGAACCG TTCAGCCCGC GGACCTTTAG GGGTCTGGGC
AATAAGGGGA CTCTCCCATG GAAATGCAAC TCCGTCGATA TGAAGTACTT CAGCTCGGTG
ACGACCTACG TGGATGAGTC AAAGTATGAG AAGCTAAAGT GGAAGAGGGA GAGGTACCTA
CGAATGGAAG CCTCACAGGG GGGGGGTGAC AACACAAGCG GTGGTGACAA CACACACGGT
GGTGACAACG CCGACAAGCT GCAAAACGTC GTGGTCATGG GGAGAAGCAG CTGGGAGAGC
ATCCCCAAGC AGTACAAGCC GCTCCCAAAC AGAATCAACG TCGTGCTTTC CAAGACGCTA
ACAAAGGAAG ACGTGAAGGA AAAGGTCTTC ATAATTGACA GCATAGATGA CCTACTGCTG
CTCTTAAAGA AGCTGAAGTA CTACAAATGC TTCATCATTG GGGGAGCACA AGTTTATAGG
GAATGCCTAA GTAGAAACTT AATCAAGCAG ATCTACTTCA CGAGGATCAA CGGCGCCTAC
CCGTGTGACG TCTTCTTCCC CGAGTTTGAC GAAAGCCAGT TTCGGGTGAC GTCCGTCAGT
GAGGTGTACA ACAGCAAGGG CACCACTCTG GACTTTTTGG TTTACAGCAA GGTGGGGGGG
GGAGTTGACG GGGGCGCCTC CAACGGGAGC ACTGCGACAG CGCTTCGGAG AACTGCAATG
CGTTCAACTG CAATGCGCCG AAATGTAGCG CCCCGAACTG CCGCTCCCCC AATGGGACCG
CACAGCAGGG CGAATGGGGA AAGGGCCCCG CCGCGTGCCC GTGCGAGAAG AACAACGCCG
AGGCAGAGGA AGACGACCTC GTGTACTTCA GCTTTAACAA CAAAGTGGGG GAGAAAAACC
CGGAGCACCT GCAAGATTTT AAAATTTACA ACAGCCTCAA GATTAATGCA GCACCCAGAG
TACCAATACC TAGGCATCAT ATACGACATC ATCATGAATG GAAACAAACA AGGAGACAGA
ACAGGTGTGG GAGTGATGAG CAATTTTGGC TACATGATGA AATTTAATTT AAGTGAATAC
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GCAAATGGAA CGAGGGAGTT CCTCGACAAC AGGAAATTAT TCCACAGAGA AGTGAATGAC
TTGGGGCCCA TTTATGGCTT CCAGTGGAGA CACTTCGGTG CTGAATACAC AAATATGCAT
GACAATTACG AAGATAAAGG TGTAGACCAA TTAAAAAATG TTATTCATTT AATAAAAAAT
GAACCAACAA GTAGGAGAAT TATTTTGTGT GCATGGAATG TAAAAGATTT GGATCAAATG
GCTTTACCTC CTTGTCATAT TTTATGCCAA TTTTACGTTT TCGATGGGAA ACTATCATGC
ATTATGTACC AGAGGTCTTG TGACTTGGGT CTTGGGGTCC CCTTCAACAT CGCTTCGTAT
TCCATATTCA CACACATGAT TGCGCAGGTG TGCAATTTGC AGCCTGCACA GTTCATACAC
ATTTTGGGCA ACGCGCACGT CTACAACAAC CATGTTGACA GCTTGAAAGT GCAGCTGAAC
AGGATCCCCT ACCCGTTCCC AACGCTTAAA CTGAACCCGG AGGTGAAGAA CATTGAGGAT
TTTACCATTT CGGATTTCAC AATAGAGAAT TACGTGCACC ACGATAAAAT AACCATGGAG
ATGGCCGCCT AA

APPENDIX E

P. VIVAX dhps GENE GENBANK ACCESSION NUMBER: AY186730[6]

AAAAAAAAGC GTAGCGACAG AAGAACGCAT ATAAACAGAA CAGCAGCAGT AACGATAGCG
GACCACCATG GAGGATTCAA ACACGGGGGG CACTACCCCA AGGAATATCG CCGTTTTAAA
CTTCGGAACG AATGACAAAA AGAACTGCGT AACGATTTTA GAAACGGCGC TCTATCTGAC
GGAGAAGTAC CTTGGTAAGG GCGAAAGGAG GGGACACTTT AGGGGGAATT TACCTTCCTC
TTTGAAAAGG GGCAACATTT AAGAAGCGTG AGAGTTCCGC TTTGCCCATT TCTTCCTACC
GGCTTATGTA CATTGAGGGG TGCATACCAT CCCCCAGTAG TAAACACACA CACGTGTAGA
CACATAGGTC CAAATGCCGC CACGATAGGT GTGACCCATT ACCCATTTTT TTCTCCCCCC
TTTTTCCTAA AACATGGTAG GCAAAATCAT CAACAGTTCG TACATTTACG AAACCGTCCC
GGAGTACATC GTCCTGGATG AAGAAAACAA AATTGGAGAG GTGACGGAGG GGGAGCCCCC
GAGGGACATC TCTTGGATAG GCGATTTGAT CCCAACTGTG GAAAACTCCA GATACGAAGA
AAGTGAAGAT TTAATTTATG AGTGCAAAGA GTTGGAGGTG TTTTTAAAAA ATGAAAAGAT
AAACGAAAGC ATTATTAGGG AAGTCAGCGT AGAGGATTAC GAAAATGAGG CAAGAAGGAT
AATCAAACGG AATGACGAAA TAATGAAGAA AAATTTAGAA CAGTCGAAAG ATAAATACTA
CACCAGTTAT TTTTTTAACT TAACGGTTGT AGTTAGGACC TTTGTTGAAG ACCCCCTCGC
CATGCTCGTA ATTTTAAAGT ACATTGAGCA AATCATGAAA AGAAGGGAAA GCAAAAAGGG
TCAGGGAGAA ATATTTCAAA ATCGCATGAT AGCCATTGAC ATTTTATTTT TTAACAACTA
CCCAATTTTT GAAAAGAGCA TATCTTTAAA AGGGGAAGAT ATTTATAAAA TCATCACAAA
GTATATTCAC ATAAACCACA CAAGTGATCA GAACCGTCTA GATATTATCC AAAATTTGGG
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CCTCTGCTTA AATGATATCA TTCCAGAGTA TAAGCACAGC ACATTTGAGG AAGCCATTCG
CTCAACTTAT AACAGTTATG TTGAAAGTTT TGAGGAGAAG TATCACATTA ATATTAGGAA
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CATCGGGGGG GAATCGTCCG CCCCTTATGT GGTCCCCAAT CCGAGCGTCA CTGAACGGGA
TTTGGTCATG CCTGTTTTGA AGCTCTTTAA GGAGGAGTGG CATAAGTTGG AGTGTGAGGT
TGGCGGTGGT GCCGTGTGCT GCGCTGCAGC AAGTGATGCC AGGAAAAACG CGCAGAGCTC
CCTACAGGGG AAACTACAAA AAGTGAGGGA CGCAAAACCG ATCATAAGCA TCGACACGGT
CAATTATGAT CTCTTCAAGG AGTGCGTGGA AGGCGAGTTG GTGGACATCC TAAACGATAT
CAGCGCGTGC ACGCACAACC CAGAGATTAT AAAATTGTTG AGGAGGAAAA ACAAGTTCTA
TAGCGTCGTT TTAATGCACA AGAGGGGAAA TCCACACACC ATGGATAAGT TAACAAATTA

CGATGACCTT ATAAGTGACA TTAAAAGGTA TTTAGAAGAT CGGCTACATT TTCTCGTTCT
AAATGGGGTA CCACGCTACC GAGTTCTCTT TGATGTCGGC CTGGGGTTTG CCAAAAAGCA
CGACCAGTCT ATTAAGCTGT TGCAGCATAT TCACGTTTAC GATGAGTACC CGCTGTTTCT
TGGCTACTCG AGGAAGCGCT TTATTGTCCA CTGCATGGGG AAGGGTGGCG CGGCCATCGG
GGAGTGCAGA CTGATGAGCG GGGAGGCCAA ACTGACCAAC GGGGAGGGTA AACTGACGAA
TGGGGAGGCC AAACTGACCA ACGGGGAGGG TAAACTGACG AATGGGGAGG CCAAACTGAC
CAACGGGGAG GGTAAACTGA CGAATGGTGA CGCCAAACTG ACTAACGGTG ACTCCAAACT
GACTAACGGG GAGGCCAAGC TGACCAACGG AGACCCATCA CAACTGTGGA GGTTCAAAAT
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TTTGTTTATC CTTTCATCAA CTCTAAGTTA TGTATGAGTC CCCATGTTTA ACACCTCCCC
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GTGGACCTTA TCAGAGTTCA CGACGTGTTA GAAACGAAGG CCGTTCTGGA TGTGCTCACA
AGGATACATC AACCTTAGGG TAATCTCCTG GCAGTGCACG AAAAGGCAAA ATAATCCCCC
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ATACCATAAT GCGTGTTTCA AATAAGCAAA TCTGCAGCTT TCATTTACTT TGCCCCTGTG
TACTTTTTAA GCTTTTCGCT TATATGCCGC TTTGTAGTTC CTCCTCCTGT GTTGTTTGCA
GCGTTCCAGA GCGGGCTCGC GGTCTGTATC TGCTTGCCTA TCTGAACGTT CAGATGATCC
CTTGTGCATC TATGTATGTG GGTGTATCAA TCCACTTTTT NTGCAGGCAA GCACAAATTG
CGCACGGNCA TTTTGAGCAT TTCGCTGAAA T

APPENDIX F

GEL ELECTROPHORESIS RESULTS FOR *pvdhfr* GENETIC MARKER

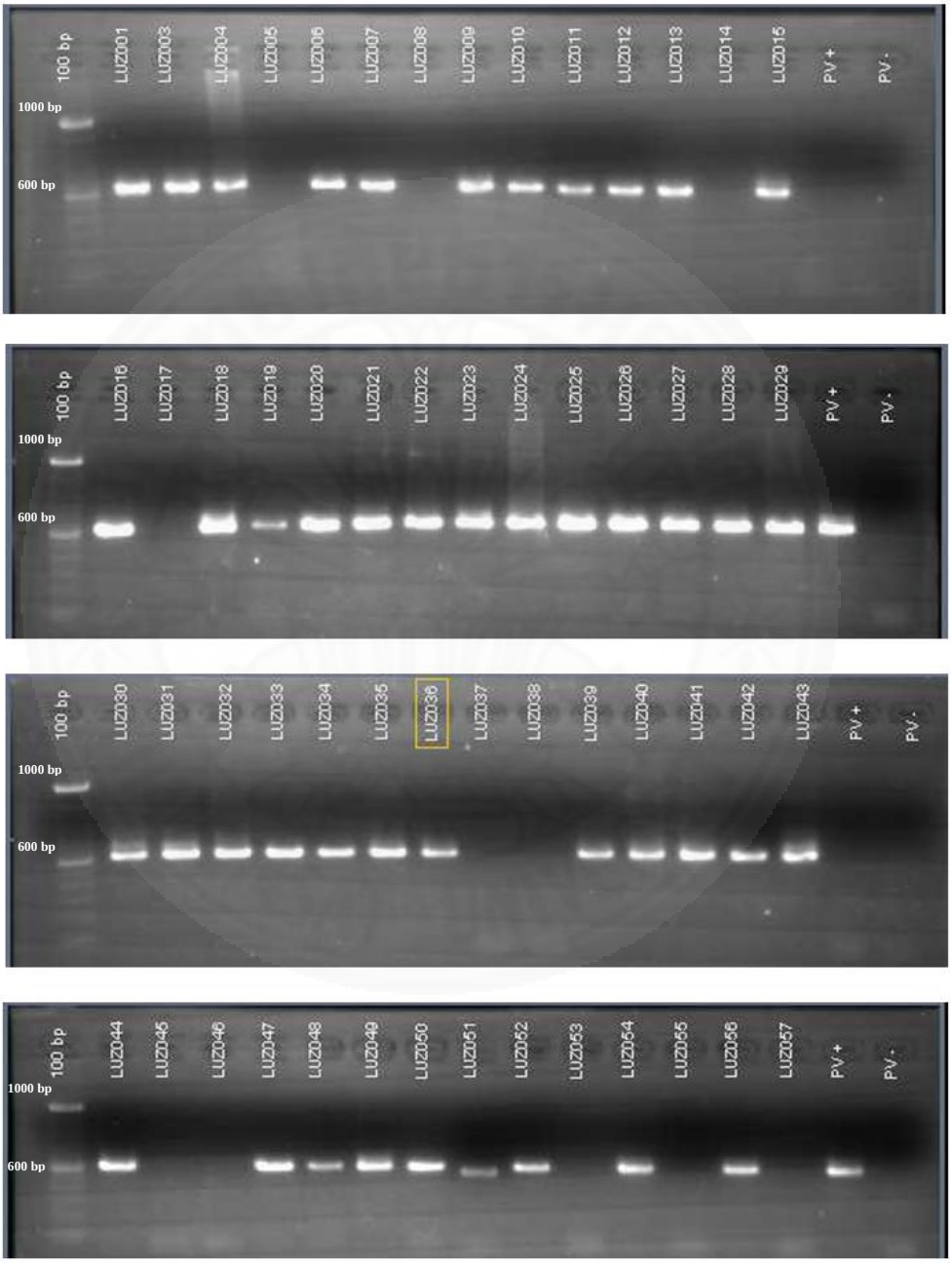


Figure F.1 First batch of gel electrophoresis results for *pvdhfr* genetic marker. Marker size at approximately 632 bp.

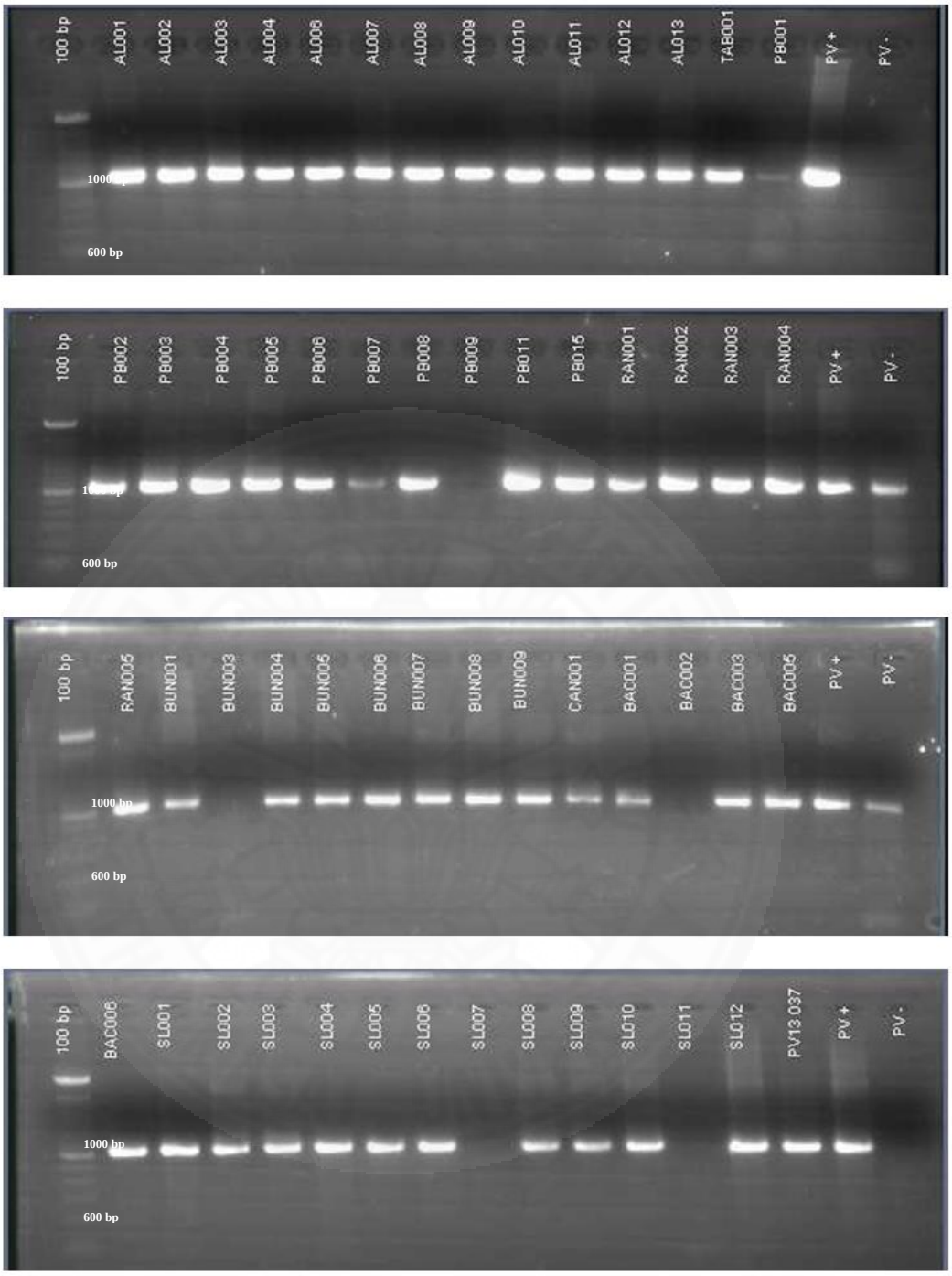


Figure F.2 Second batch of gel electrophoresis results for *pvdhfr* genetic marker. Marker size at approximately 632 bp.

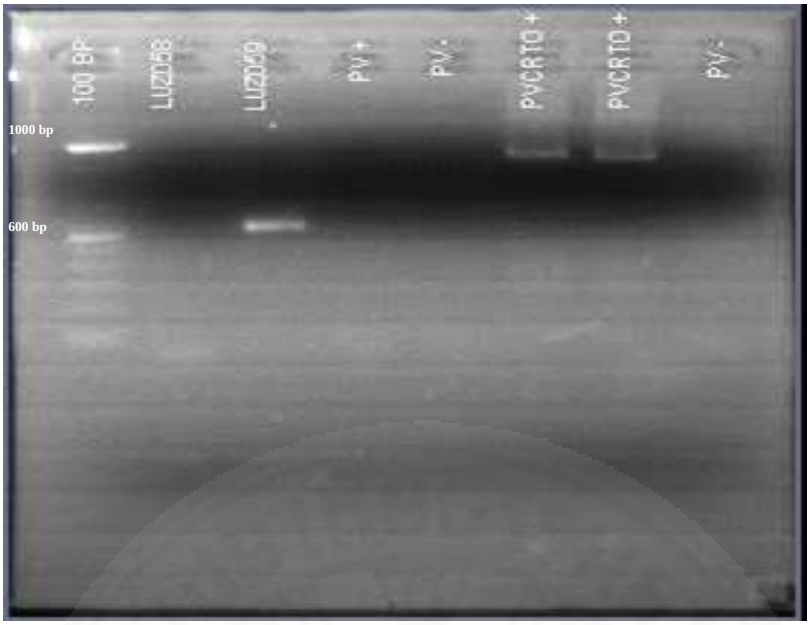


Figure F.3 Third batch of gel electrophoresis results for *pvdhfr* genetic marker. Marker size at approximately 632 bp.

APPENDIX G

GEL ELECTROPHORESIS RESULTS FOR *pvdhps* GENETIC MARKER

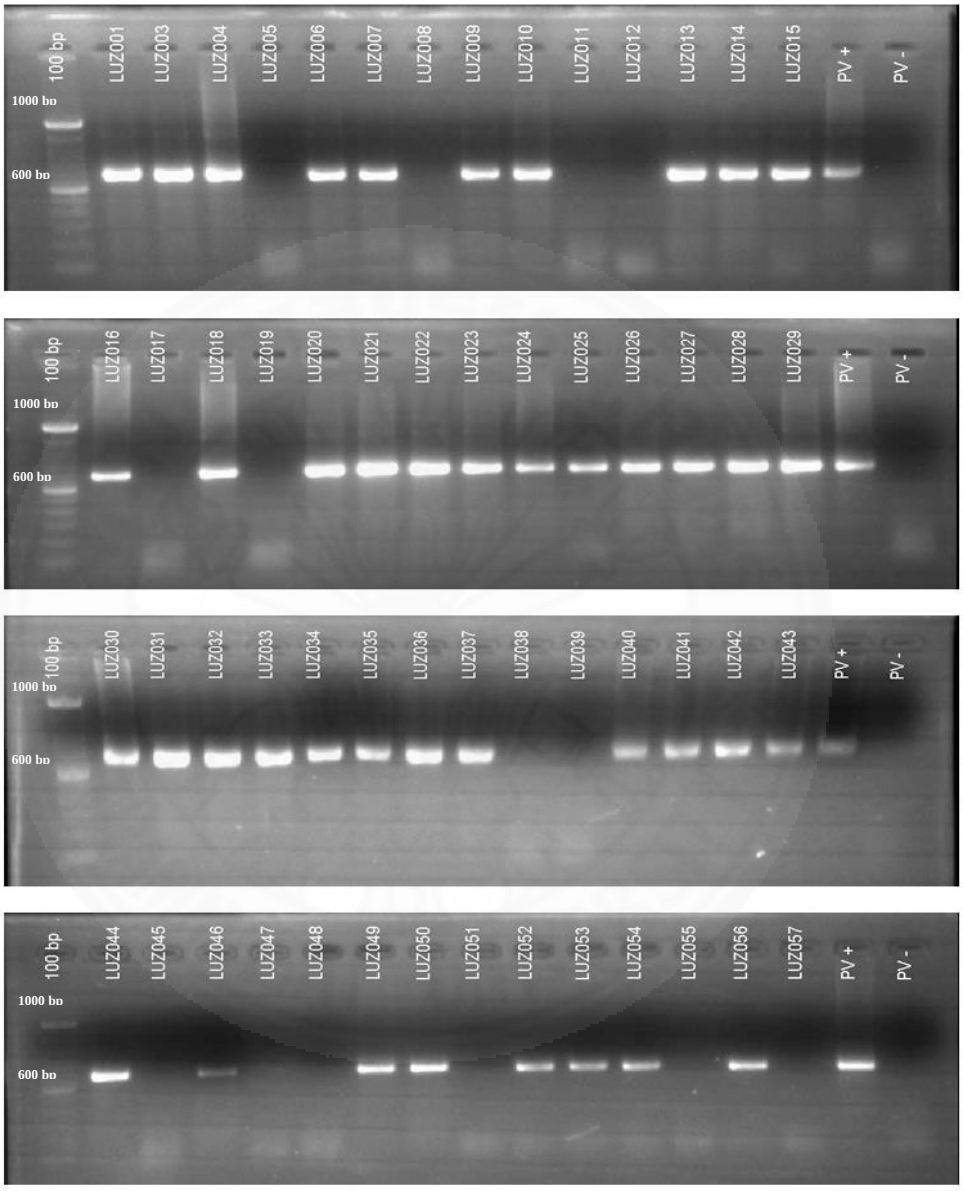


Figure G.1 First batch of gel electrophoresis results for *pvdhps* genetic marker. Marker size at approximately 767 bp.

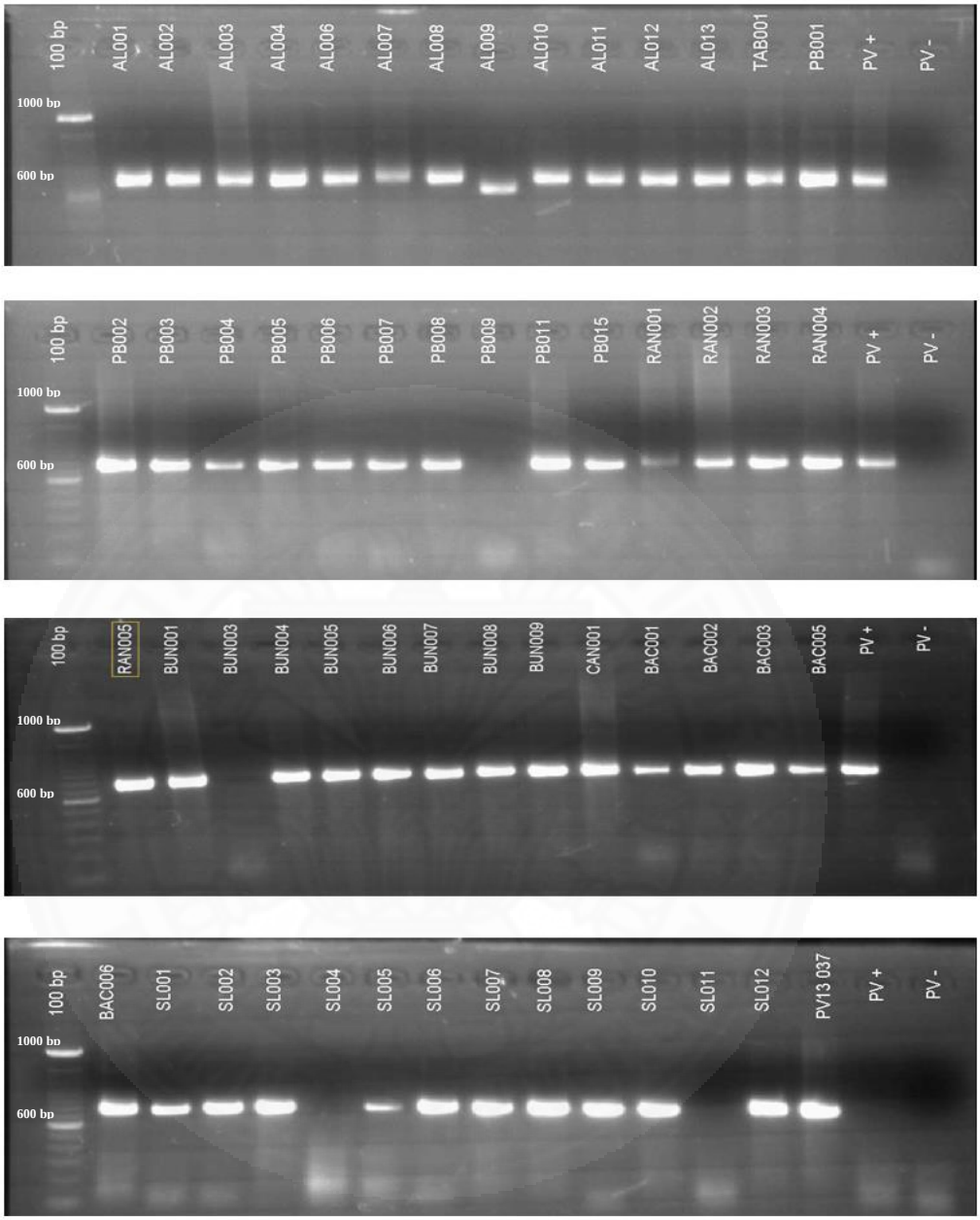


Figure G.2 Second batch of gel electrophoresis results for *pvdhps* genetic marker. Marker size at approximately 767 bp.

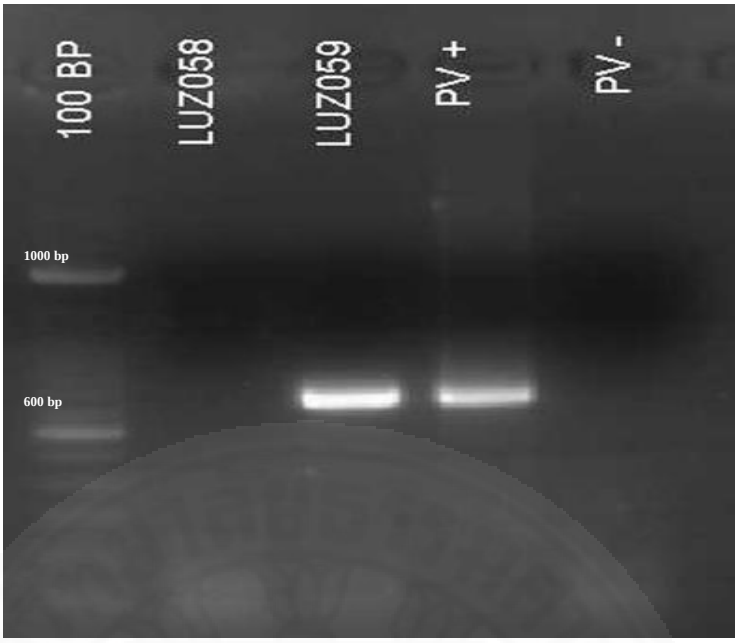


Figure G.3 Third batch of gel electrophoresis results for *pvdhps* genetic marker. Marker size at approximately 767 bp.

APPENDIX H

Table H.1 Summary of sequence analysis for the *pvdhfr* gene.

	Sample	**13**	**33**	**57**	**58**	**61**	**117**	**173**
1	LUZ001	WT	WT	WT	R	WT	N	WT
2	LUZ003	WT	WT	WT	R	WT	N	WT
3	LUZ004	WT	WT	WT	R	WT	N	WT
4	LUZ006	WT	WT	WT	R	WT	N	WT
5	LUZ007	NOT CLEAN						
6	LUZ009	WT	WT	WT	R	WT	N	WT
7	LUZ010	WT	WT	WT	R	WT	N	WT
8	LUZ011	WT	WT	WT	R	WT	N	WT
9	LUZ012	WT	WT	WT	R	WT	N	WT
10	LUZ013	WT	WT	WT	R	WT	N	WT
11	LUZ014	WT	WT	WT	R	WT	N	WT
12	LUZ015	WT	WT	WT	R	WT	N	WT
13	LUZ016	FAILED						
14	LUZ018	WT	WT	WT	R	WT	N	WT
15	LUZ019	FAILED						
16	LUZ020	WT	WT	WT	R	WT	N	WT
17	LUZ021	WT	WT	WT	R	WT	N	WT
18	LUZ022	WT	WT	WT	R	WT	N	WT
19	LUZ023	WT	WT	WT	R	WT	N	WT
20	LUZ024	WT	WT	WT	R	WT	N	WT
21	LUZ025	FAILED						
22	LUZ026	WT	WT	WT	R	WT	N	WT
23	LUZ027	WT	WT	WT	R	WT	N	WT
24	LUZ028	WT	WT	WT	R	WT	N	WT
25	LUZ029	WT	WT	WT	R	WT	N	WT
26	LUZ030	WT	WT	WT	R	WT	N	WT
27	LUZ031	WT	WT	WT	R	WT	N	WT
28	LUZ032	WT	WT	WT	R	WT	N	WT

	Sample	**13**	**33**	**57**	**58**	**61**	**117**	**173**
29	LUZ033	WT	WT	WT	R	WT	N	WT
30	LUZ034	WT	WT	WT	R	WT	N	WT
31	LUZ035	WT	WT	WT	R	WT	N	WT
32	LUZ036	WT	WT	WT	R	WT	N	WT
33	LUZ037	FAILED						
34	LUZ039	WT	WT	WT	R	WT	N	WT
35	LUZ040	WT	WT	WT	R	WT	N	WT
36	LUZ041	WT	WT	WT	R	WT	N	WT
37	LUZ042	WT	WT	WT	R	WT	N	WT
38	LUZ043	WT	WT	WT	R	WT	N	WT
39	LUZ044	WT	WT	WT	R	WT	N	WT
40	LUZ047	FAILED						
41	LUZ048	FAILED						
42	LUZ049	WT	WT	WT	R	WT	N	WT
43	LUZ050	WT	WT	WT	R	WT	N	WT
44	LUZ051	FAILED						
45	LUZ052	WT	WT	WT	R	WT	N	WT
46	LUZ054	WT	WT	WT	R	WT	N	WT
47	LUZ056	WT	WT	WT	R	WT	N	WT
48	LUZ059	WT	WT	WT	R	WT	N	WT
49	AL001	WT	WT	WT	R	WT	N	WT
50	AL002	WT	WT	WT	WT	WT	WT	WT
51	AL003	WT	WT	WT	R	WT	N	WT
52	AL004	WT	WT	WT	R	WT	N	WT
53	AL006	WT	WT	WT	S58R®	WT	WT	WT
54	AL007	WT	WT	WT	R	M	T	WT
55	AL008	WT	WT	WT	R	WT	N	WT
56	AL009	WT	WT	WT	R	WT	WT	WT
57	AL010	WT	WT	WT	R	WT	N	WT
58	AL011	WT	WT	WT	R	WT	N	WT

	Sample	**13**	**33**	**57**	**58**	**61**	**117**	**173**
59	AL012	WT	WT	WT	R	WT	N	WT
60	AL013	WT	WT	L	R	M	T	WT
61	SL001	WT	WT	WT	R	WT	N	WT
62	SL002	WT	WT	WT	R	WT	N	WT
63	SL003	WT	WT	WT	R	WT	N	WT
64	SL004	FAILED						
65	SL005	FAILED						
66	SL006	FAILED						
67	SL008	WT	WT	WT	WT	WT	WT	WT
68	SL009	WT	WT	WT	R	WT	N	WT
69	SL010	WT	WT	WT	R	WT	N	WT
70	SL012	WT	WT	WT	R	WT	N	WT
71	TAB001	WT	WT	WT	WT	WT	WT	WT
72	PB001	FAILED						
73	PB002	WT	WT	WT	R	WT	N	WT
74	PB003	WT	WT	WT	WT	WT	WT	WT
75	PB004	WT	WT	WT	R	WT	N	WT
76	PB005	WT	WT	WT	WT	WT	WT	WT
77	PB006	WT	WT	WT	WT	WT	WT	WT
78	PB007	FAILED						
79	PB008	WT	WT	WT	R	WT	N	WT
80	PB011	WT	WT	WT	R	WT	N	WT
81	PB012	WT	WT	WT	WT	WT	WT	WT
82	RAN01	WT	P33T	WT	WT	T61K	S117N	WT
83	RAN02	WT	WT	WT	WT	WT	WT	WT
84	RAN03	WT	WT	WT	WT	WT	WT	WT
85	RAN04	WT	WT	WT	WT	WT	WT	WT
86	RAN05	WT	WT	WT	WT	WT	WT	WT
87	BUN01	WT	WT	WT	WT	WT	WT	WT
88	BUN02	WT	WT	WT	R	WT	N	WT

	Sample	**13**	**33**	**57**	**58**	**61**	**117**	**173**
89	BUN003	WT	WT	WT	WT	WT	WT	WT
90	BUN004	WT	WT	WT	WT	WT	WT	WT
91	BUN005	WT	WT	WT	R	WT	N	WT
92	BUN006	WT	WT	WT	R	WT	N	WT
93	BUN007	WT	WT	WT	WT	WT	WT	WT
94	BUN008	WT	WT	WT	WT	WT	WT	WT
95	BUN009	WT	WT	WT	R	WT	N	WT
96	CAN001	WT	WT	WT	R	WT	N	WT
97	BAC001	WT	WT	WT	R	WT	N	WT
98	BAC002	WT	WT	WT	R	WT	N	WT
99	BAC003	WT	WT	WT	R	WT	N	WT
100	BAC005	WT	WT	WT	R	WT	N	WT
101	PVPOSITIVE	WT	WT	WT	WT	WT	WT	WT

APPENDIX I

Table I.1 Summary of sequence analysis for the *pvdhps* gene.

	Sample	382	383	512	553	585
1	LUZ001	WT	G	WT	WT	WT
2	LUZ003	WT	G	WT	WT	WT
3	LUZ004	WT	G	WT	WT	WT
4	LUZ006	WT	G	WT	WT	WT
5	LUZ007	WT	G	WT	WT	WT
6	LUZ009	WT	G	WT	WT	WT
7	LUZ010	WT	G	WT	WT	WT
8	LUZ011	WT	G	WT	WT	WT
9	LUZ012	FAILED				
10	LUZ013	WT	G	WT	WT	WT
11	LUZ014	WT	G	WT	WT	V585I®
12	LUZ015	WT	G	WT	WT	WT
13	LUZ016	WT	G	WT	WT	WT
14	LUZ018	WT	G	WT	WT	WT
15	LUZ019	FAILED				
16	LUZ020	WT	G	WT	WT	WT
17	LUZ021	WT	G	WT	WT	WT
18	LUZ022	WT	G	WT	WT	WT
19	LUZ023	WT	G	WT	WT	WT
20	LUZ024	WT	G	WT	WT	WT
21	LUZ025	WT	G	WT	WT	WT
22	LUZ026	WT	G	WT	WT	WT
23	LUZ027	WT	G	WT	WT	WT
24	LUZ028	WT	G	WT	WT	WT
25	LUZ029	WT	G	WT	WT	WT
26	LUZ030	WT	G	WT	WT	WT
27	LUZ031	WT	G	WT	WT	WT

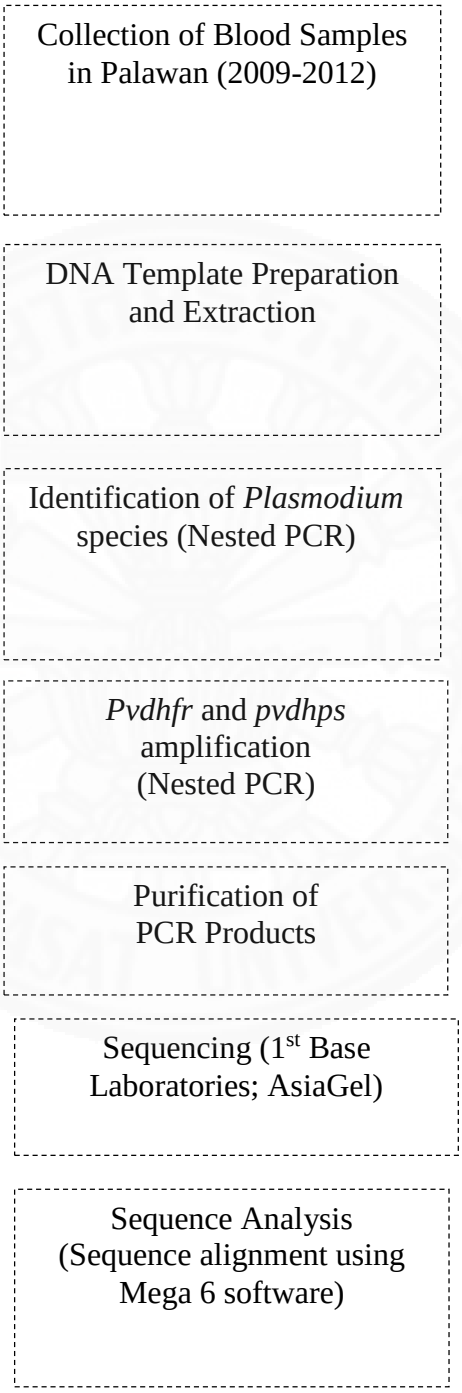
	Sample	382	383	512	553	585
28	LUZ032	WT	G	WT	WT	WT
29	LUZ033	WT	G	WT	WT	WT
30	LUZ034	WT	G	WT	WT	WT
31	LUZ035	WT	G	WT	WT	WT
32	LUZ036	WT	G	WT	WT	WT
33	LUZ037	WT	G	WT	WT	WT
34	LUZ039	WT	G	WT	WT	WT
35	LUZ040	WT	G	WT	WT	WT
36	LUZ041	WT	G	WT	WT	WT
37	LUZ042	WT	G	WT	WT	WT
38	LUZ043	WT	G	WT	WT	WT
39	LUZ044	WT	G	WT	WT	WT
40	LUZ046	WT	G	WT	WT	WT
41	LUZ047	FAILED				
42	LUZ048	FAILED				
43	LUZ049	WT	G	WT	WT	WT
44	LUZ050	WT	G	WT	WT	WT
45	LUZ051	FAILED				
46	LUZ052	WT	G	WT	WT	WT
47	LUZ053	WT	G	WT	WT	WT
48	LUZ054	WT	G	WT	WT	WT
49	LUZ056	WT	G	WT	WT	WT
50	LUZ059	WT	G	WT	WT	WT
51	AL001	WT	G	WT	WT	WT
52	AL002	WT	G	WT	WT	WT
53	AL003	WT	G	WT	WT	WT
54	AL004	WT	WT	WT	WT	WT
55	AL006	WT	WT	WT	WT	WT
56	AL007	WT	G	WT	WT	V585V
57	AL008	WT	WT	WT	WT	WT

	Sample	382	383	512	553	585
58	AL009	WT	WT	WT	WT	WT
59	AL010	WT	WT	WT	WT	WT
60	AL011	WT	G	WT	WT	WT
61	AL012	WT	G	WT	WT	WT
62	AL013	WT	G	WT	WT	WT
63	SL001	WT	G	WT	WT	WT
64	SL002	WT	G	WT	WT	WT
65	SL003	WT	G	WT	WT	WT
66	SL004	WT	G	WT	WT	WT
67	SL005	FAILED				
68	SL006	WT	G	WT	WT	WT
69	SL007	WT	G	WT	WT	WT
70	SL008	WT	WT	WT	WT	WT
71	SL009	WT	G	WT	WT	WT
72	SL010	WT	G	WT	WT	WT
73	SL012	WT	G	WT	WT	WT
74	TAB001	WT	WT	5512N(F)	WT	V585S®
75	PB001	WT	G	WT	WT	WT
76	PB002	WT	WT	WT	WT	WT
77	PB003	WT	WT	WT	WT	WT
78	PB004	WT	WT	WT	WT	WT
79	PB005	WT	WT	WT	WT	WT
80	PB006	WT	WT	WT	WT	WT
81	PB007	WT	WT	WT	WT	WT
82	PB008	WT	G	WT	WT	WT
83	PB011	WT	WT	WT	WT	WT
84	PB012	WT	G	WT	WT	WT
85	PB013	WT	WT	WT	WT	WT
86	PB014	WT	WT	WT	WT	WT
87	PB015	WT	G	WT	WT	WT
88	RAN001	WT	WT	WT	WT	V585Y®

	Sample	382	383	512	553	585
89	RAN002	WT	WT	WT	WT	WT
90	RAN003	WT	WT	WT	WT	WT
91	RAN004	WT	WT	WT	WT	WT
92	RAN005	WT	WT	WT	WT	WT
93	BUN001	WT	WT	WT	WT	WT
94	BUN002	WT	WT	WT	WT	WT
95	BUN003	FAILED				
96	BUN004	WT	WT	WT	WT	WT
97	BUN005	WT	WT	WT	WT	WT
98	BUN006	WT	WT	WT	WT	WT
99	BUN007	WT	G	WT	WT	WT
100	BUN008	WT	WT	WT	WT	WT
101	BUN009	WT	WT	WT	WT	WT
102	CAN001	WT	G	WT	WT	V585Y®
103	BAC001	WT	G	WT	WT	WT
104	BAC002	WT	G	WT	WT	WT
105	BAC003	WT	G	WT	WT	WT
106	BAC005	WT	G	WT	WT	WT
107	BAC006	WT	G	WT	WT	V585L®
108	PVPOSITIVE	WT	WT	WT	WT	WT

APPENDIX J

GENERAL FLOWCHART OF METHODS



APPENDIX K

PCR components and thermocycler profile for *pvdhfr* and *pvdhps* gene analysis using nested PCR[47,88].

N1 Component	Final [ ]	**N2** Component	Final [ ]
dH_2O	-	dH_2O	-
10X buffer:	1X	10X buffer:	1X
50Mm $MgCl_2$	2.5mM	50Mm $MgCl_2$	2.5mM
10mM dNTPs	0.2mM	10mM dNTPs	0.2mM
10uM Fwd P	0.5uM	10uM Fwd P	0.5uM
10uM Rev P	0.5uM	10uM Rev P	0.5uM
Invitrogen Taq (5U/uL)	0.03U	Invitrogen Taq (5U/uL)	0.03U
DNA	-	DNA	-

APPENDIX L

PCR components and thermocycler profile for the species identification (LSTHM protocol) using nested PCR[61].

Genus Specific Component	Final [ ]	**Species Specific** Component	Final [ ]
dH_2O	-	dH_2O	-
10X buffer:	1X	10X buffer:	1X
50Mm $MgCl_2$	2.0Mm	50Mm $MgCl_2$	2.0mM
10mM dNTPs	200µM	10mM dNTPs	200µM
10uM Fwd P	125Nm	10uM Fwd P	125nM
10uM Rev P	125Nm	10uM Rev P	125nM
Invitrogen Taq (5U/uL)	0.4U	Invitrogen Taq (5U/uL)	0.4U
DNA	-	DNA	-

Thermo-cycling conditions:
NEST1 species ID

94°C	5 mins	
58°C	2 min	
72°C	2 min	25x
94°C	1 min	
58°C	2 mins	
72°C	5 mins	
4°C	∞	

Thermo-cycling conditions:
NEST2 species ID

94°C	5 mins	
58°C	2 min	
72°C	2 min	30x
94°C	1 min	
58°C	2 mins	
72°C	5 mins	
4°C	∞	

PCR components and thermocycler profile for genus and species identification using nested PCR (RITM-MBL protocol).

RITM-MBL protocol Nest 1 Component	Final [ ]	**Pvmdr1N2** Component	Final [ ]
dH_2O	-	dH_2O	-
10X buffer:	1X	10X buffer:	1X
50Mm $MgCl_2$	1.75mM	50Mm $MgCl_2$	1.75mM
10mM dNTPs	200uM	10mM dNTPs	200uM
10uM Fwd P	1uM	10uM Fwd P	1uM
10uM Rev P	1uM	10uM Rev P	1uM
Invitrogen Taq (5U/uL)	0.4U	Invitrogen Taq (5U/uL)	0.4U
DNA	-	DNA	-

Thermo-cycling conditions:

NEST1 species ID (RITM)

94°C	7 mins	
58°C	2 min	35x
72°C	2 min	
94°C	40 sec	
72°C	7 mins	
20°C	2 mins	
4°C	∝	

Thermo-cycling conditions:

NEST2 species ID (RITM)

94°C	7 mins	
58°C	2 min	35x
72°C	2 min	
94°C	40 sec	
72°C	7 mins	
20°C	2 mins	
4°C	∝	

APPENDIX M
MUTATIONS (*pvdhfr)*

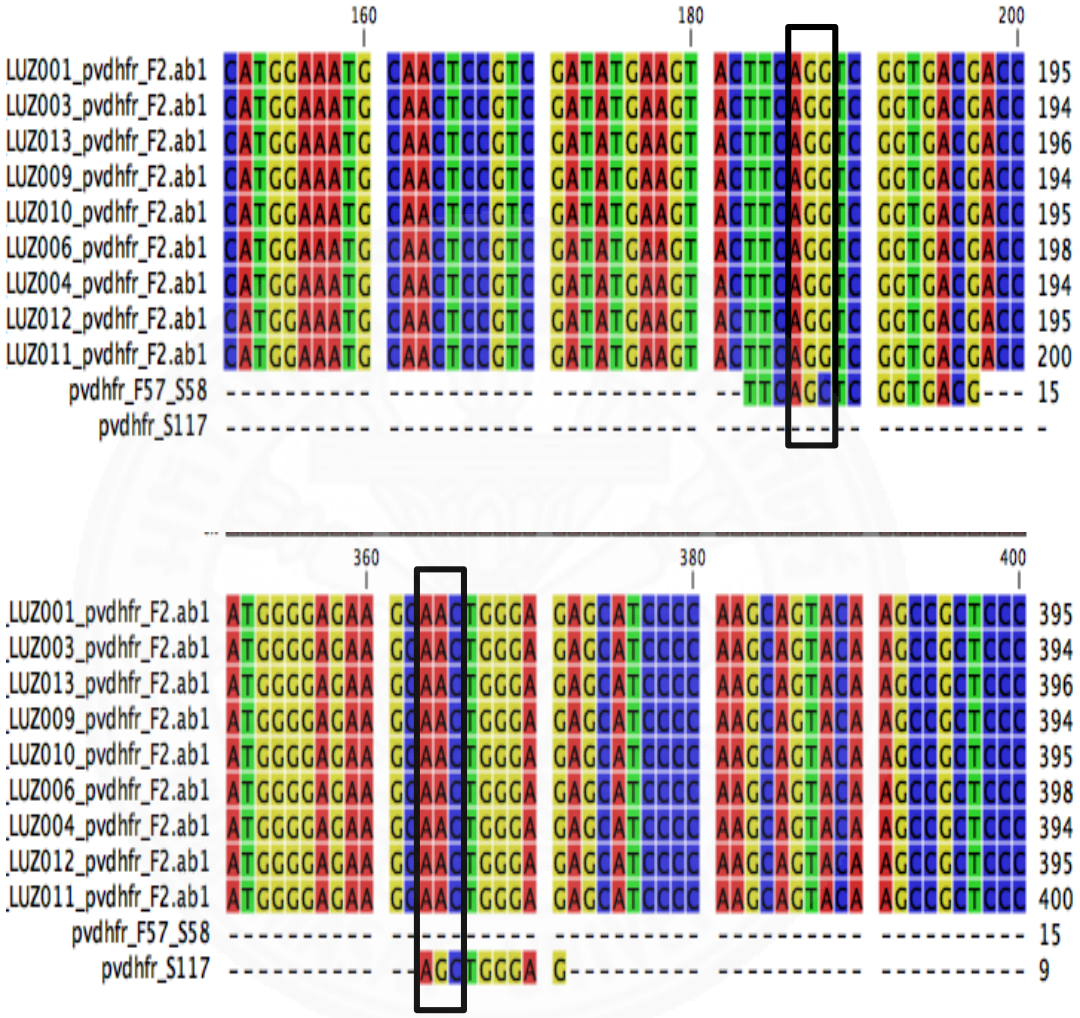


Figure M.1 Illustration showing the alignment of *pvdhfr* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

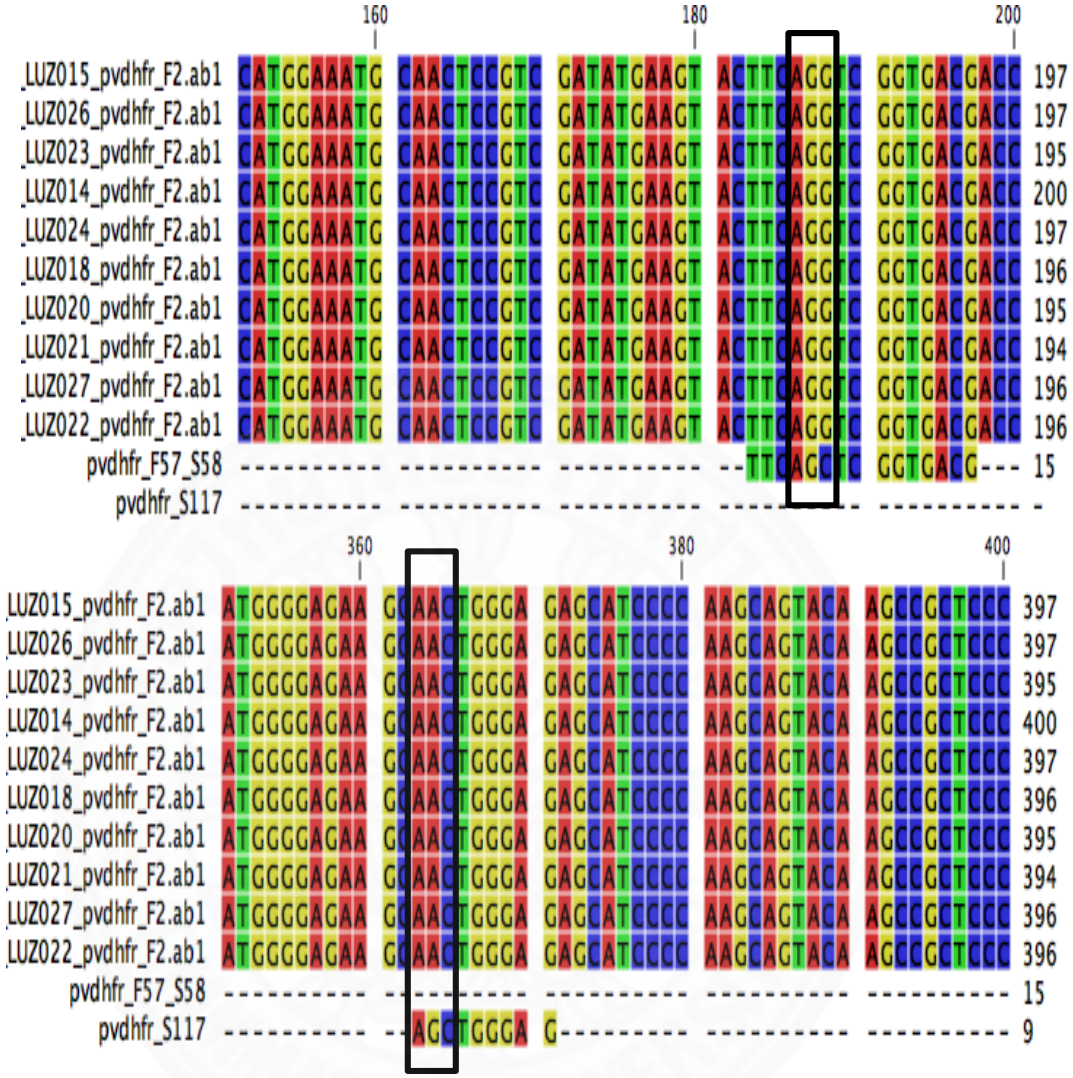


Figure M.2 Illustration showing the alignment of *pvdhfr* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

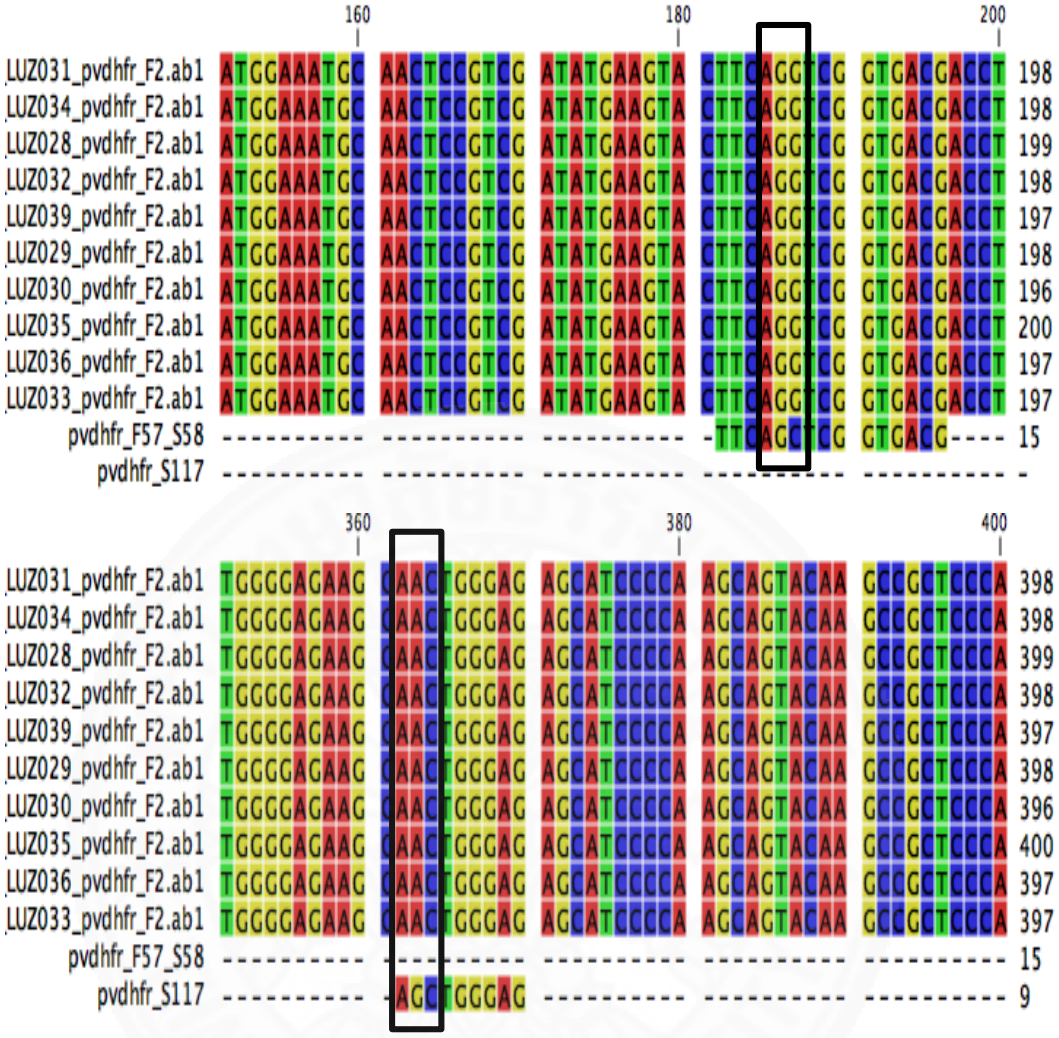


Figure M.3 Illustration showing the alignment of *pvdhfr* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

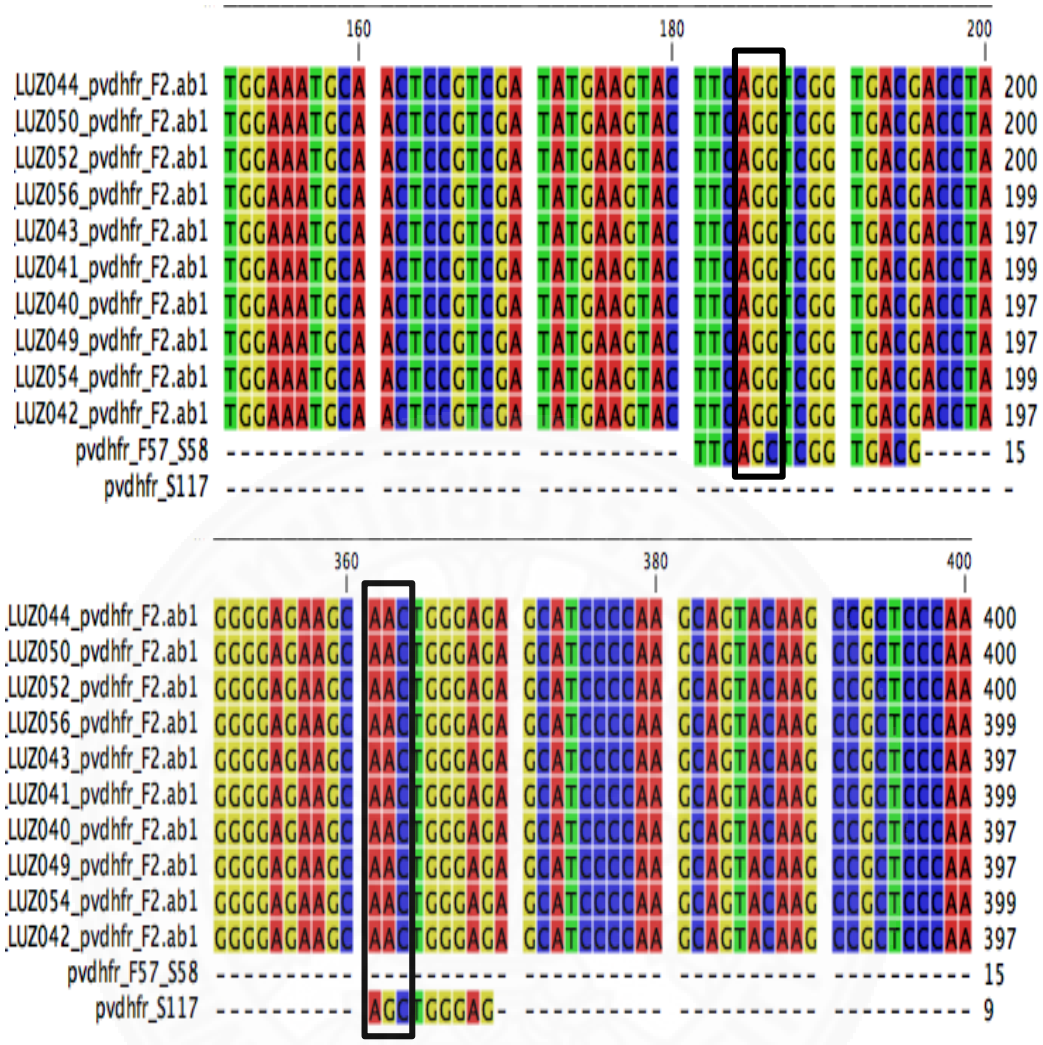


Figure M.4 Illustration showing the alignment of *pvdhfr* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

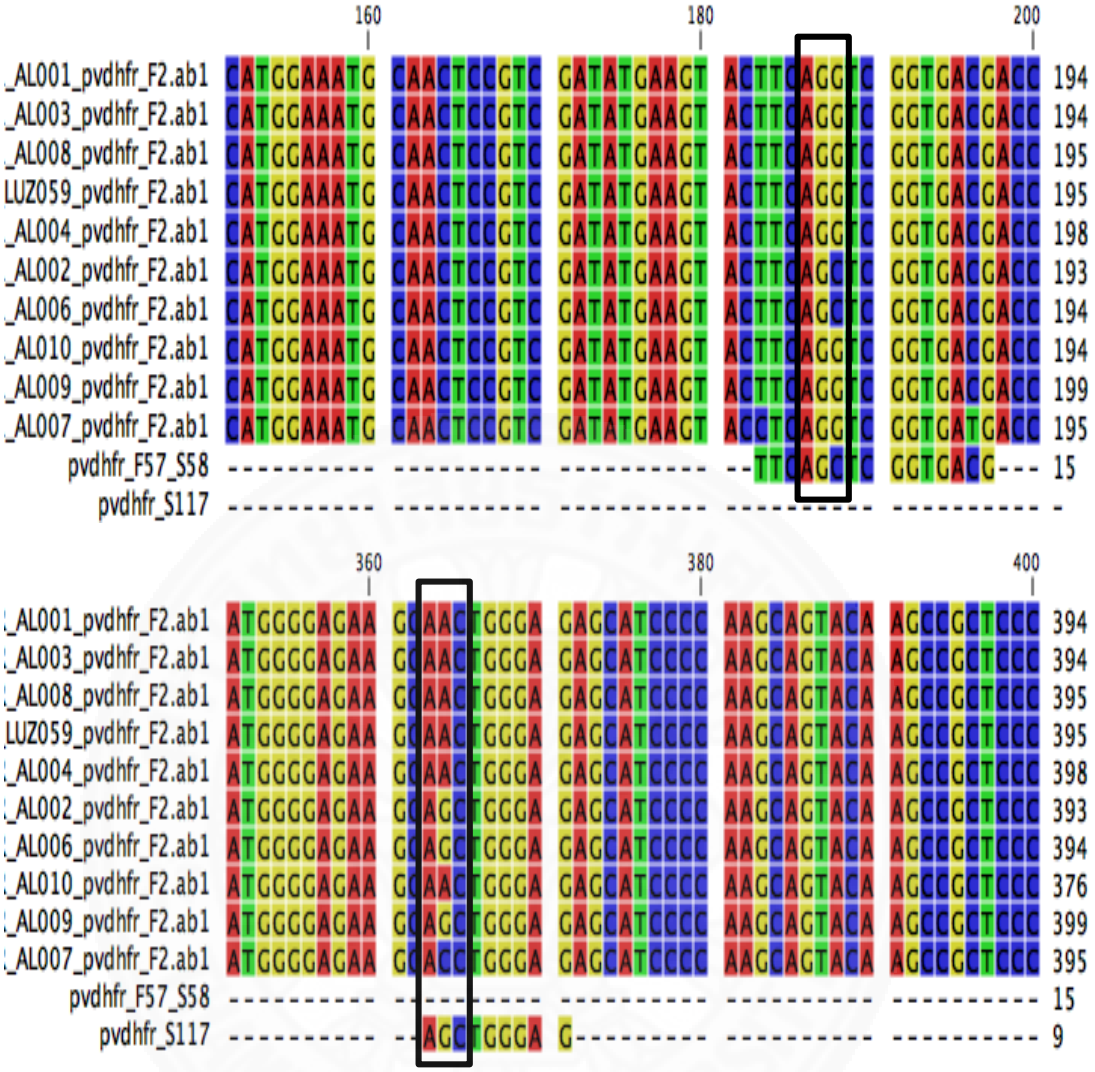


Figure M.5 Illustration showing the alignment of *pvdhfr* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

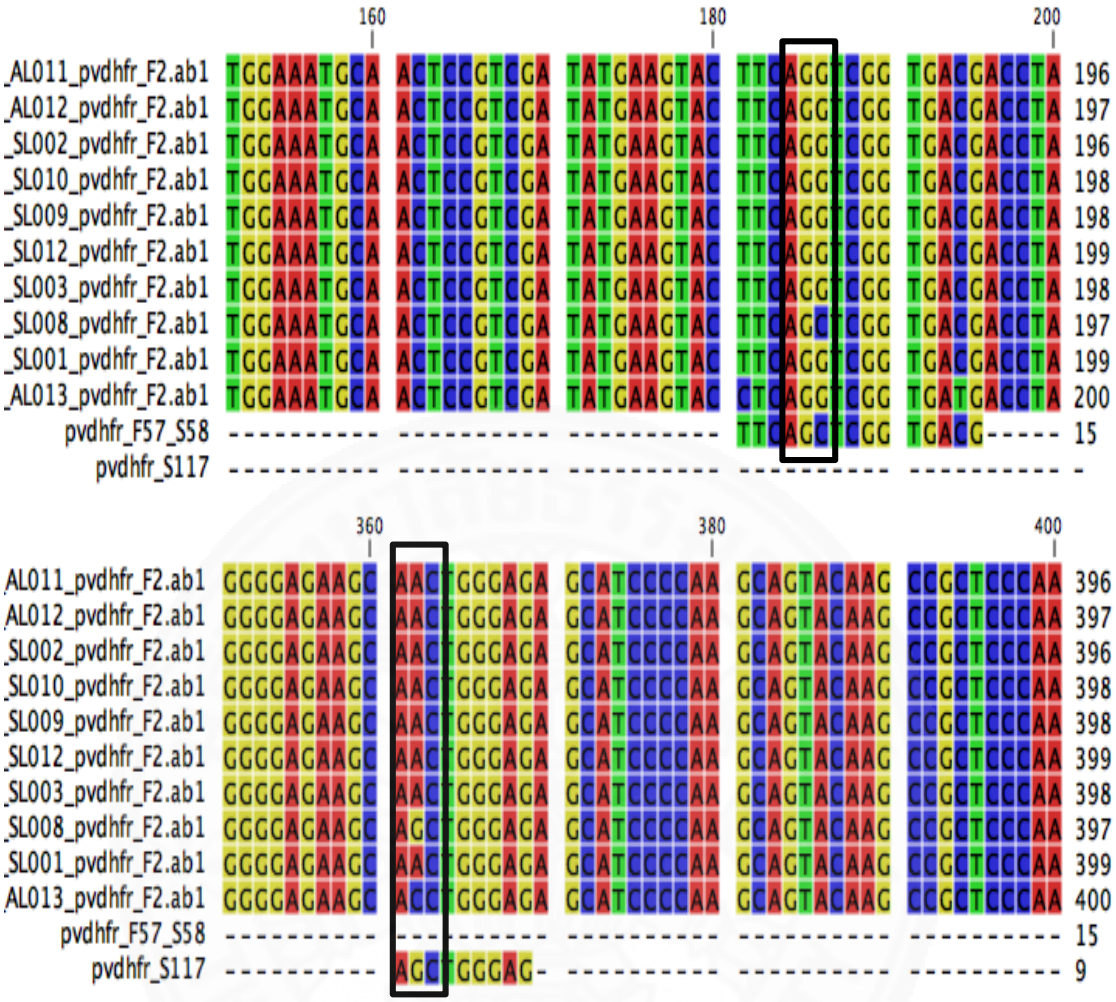


Figure M.6 Illustration showing the alignment of *pvdhfr* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

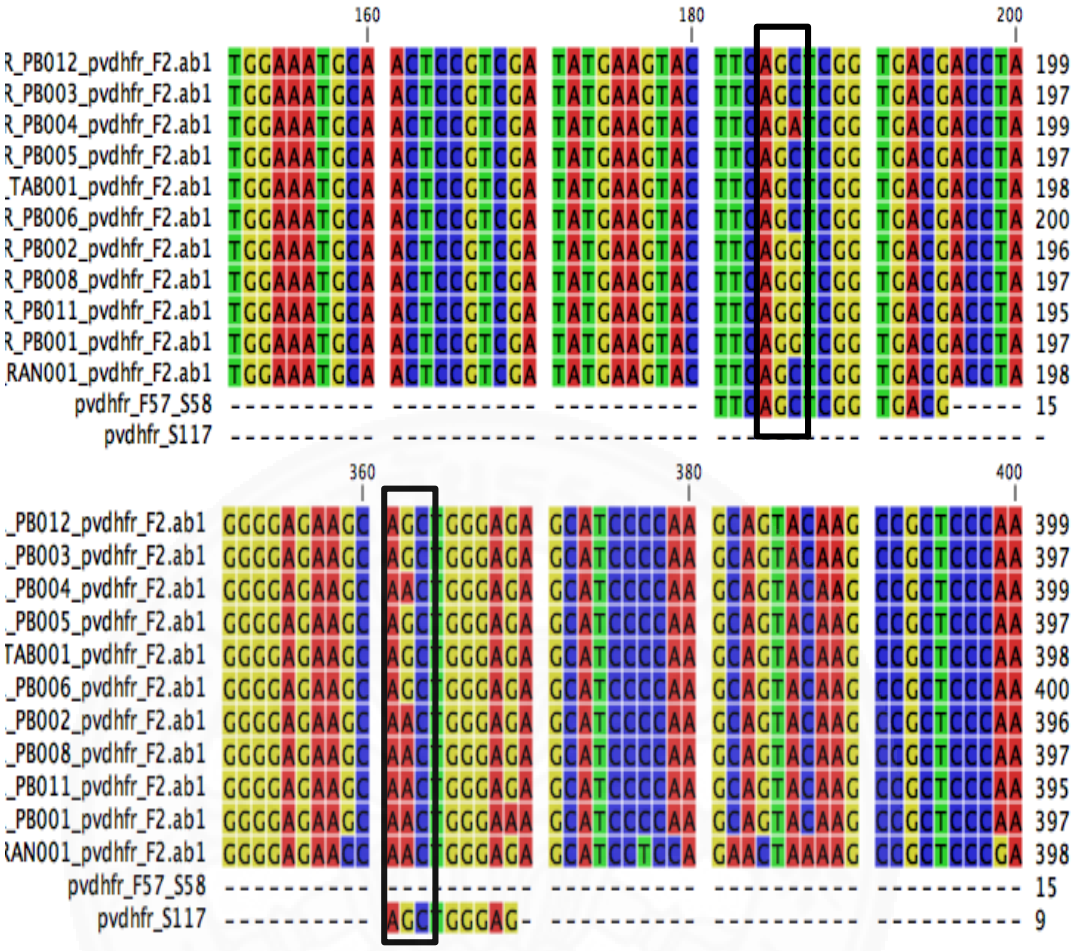


Figure M.7 Illustration showing the alignment of *pvdhfr* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

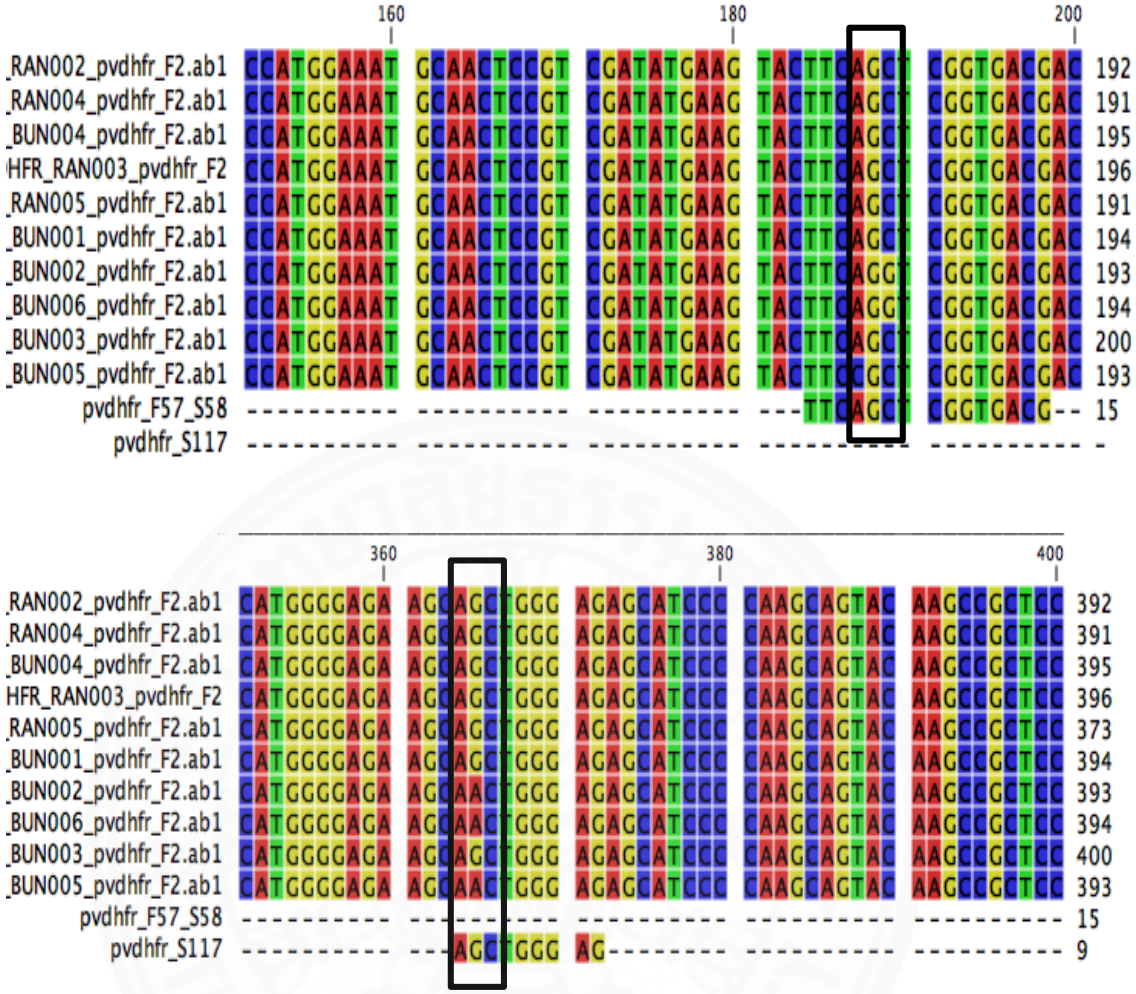


Figure M.8 Illustration showing the alignment of *pvdhfr* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

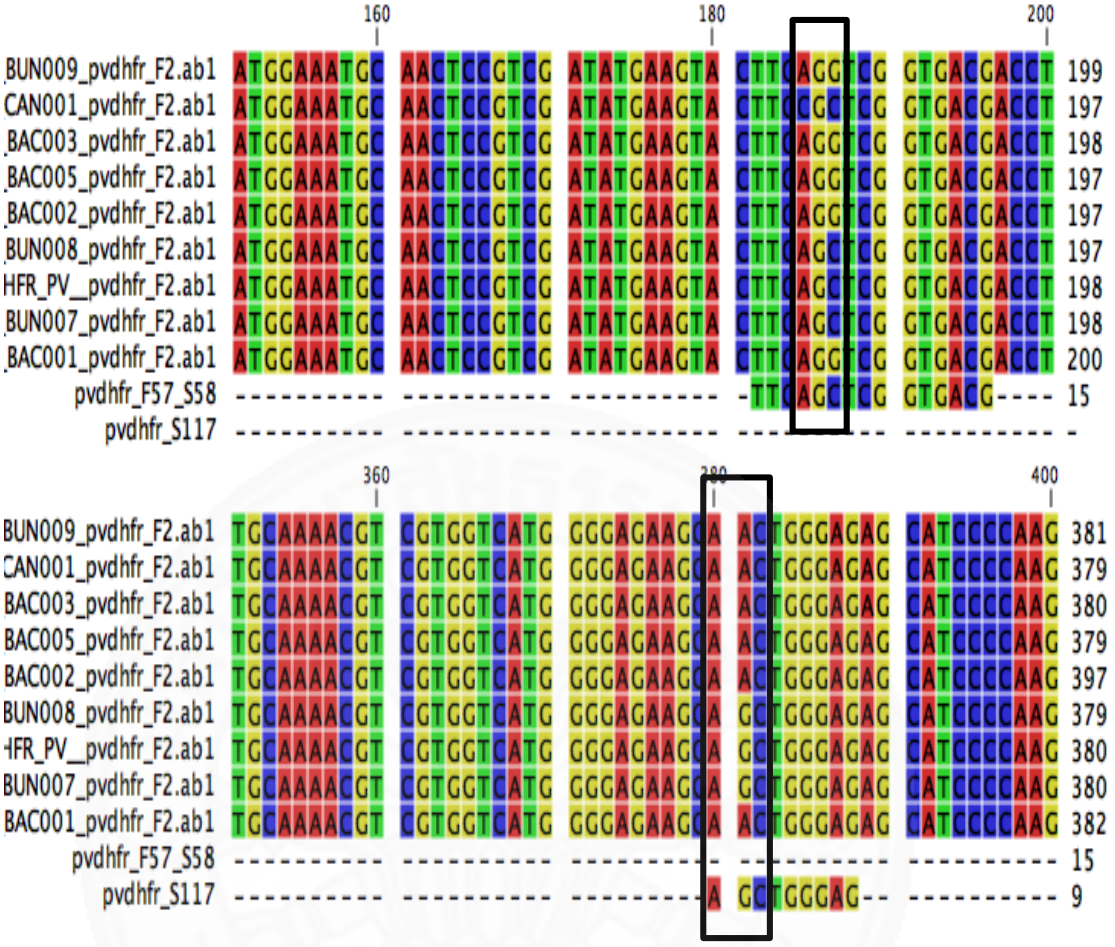


Figure M.9 Illustration showing the alignment of *pvdhfr* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

APPENDIX N

Blast sequence of one of the samples for pvdhfr gene confirmation**.**

Plasmodium vivax isolate PUR005 dihydrofolate reductase (dhfr) gene, partial cds

Sequence ID: AY772082.1 Length: 711 Number of Matches: 1

Range 1: 1 to 594 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1074 bits(581)	0.0	590/594(99%)	2/594(0%)	Plus/Plus

```
Query  12   ATGGAGGA-CTTT-AGATGTATTTGACATTTACGCCATCTGCGCATGCTGCAAGGTCGCC  69
            ||||||||  ||||  ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGGAGGACCTTTCAGATGTATTTGACATTTACGCCATCTGCGCATGCTGCAAGGTCGCC  60

Query  70   CCCACCAGTGAAGGGACAAAGAATGAACCGTTCAGCCCGCGGACCTTTAGGGGTCTGGGC  129
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CCCACCAGTGAAGGGACAAAGAATGAACCGTTCAGCCCGCGGACCTTTAGGGGTCTGGGC  120

Query  130  AATAAGGGGACTCTCCCATGGAAATGCAACTCCGTCGATATGAAGTACTTCAGGTCGGTG  189
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  AATAAGGGGACTCTCCCATGGAAATGCAACTCCGTCGATATGAAGTACTTCAGGTCGGTG  180

Query  190  ACGACCTACGTGGATGAGTCAAAGTATGAGAAGCTAAAGTGGAAGAGGGAGAGGTACCTA  249
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  ACGACCTACGTGGATGAGTCAAAGTATGAGAAGCTAAAGTGGAAGAGGGAGAGGTACCTA  240

Query  250  CGAATGGAAGCCTCACAgggggggggTGACAACACAAGCGGTGGTGACAACACAAGCGGT  309
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CGAATGGAAGCCTCACAGGGGGGGGGGTGACAACACAAGCGGTGGTGACAACACAAGCGGT  300

Query  310  GGTGACAACGCCGACAAGCTGCAAAACGTCGTGGTCATGGGGAGAAGCAACTGGGAGAGC  369
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GGTGACAACGCCGACAAGCTGCAAAACGTCGTGGTCATGGGGAGAAGCAACTGGGAGAGC  360

Query  370  ATCCCCAAGCAGTACAAGCCGCTCCCAAACAGAATCAACGTCGTGCTTTCCAAGACGCTA  429
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  ATCCCCAAGCAGTACAAGCCGCTCCCAAACAGAATCAACGTCGTGCTTTCCAAGACGCTA  420

Query  430  ACAAAGGAAGACGTGAAGGAAAAGGTCTTCATAATTGACAGCATAGATGACCTACTGCTG  489
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  ACAAAGGAAGACGTGAAGGAAAAGGTCTTCATAATTGACAGCATAGATGACCTACTGCTG  480

Query  490  CTCTTAAAGAAGCTGAAGTACTACAAATGCTTCATCATTGGGGGAGCACAAGTTTATAGG  549
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CTCTTAAAGAAGCTGAAGTACTACAAATGCTTCATCATTGGGGGAGCACAAGTTTATAGG  540

Query  550  GAATGCCTAAGTAGAAACTTAATCAAGCAGATCTACTTCACGAGATTCAACGGC  603
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  GAATGCCTAAGTAGAAACTTAATCAAGCAGATCTACTTCACGAGGATCAACGGC  594
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APPENDIX O

MUTATIONS (*pvdhps*)

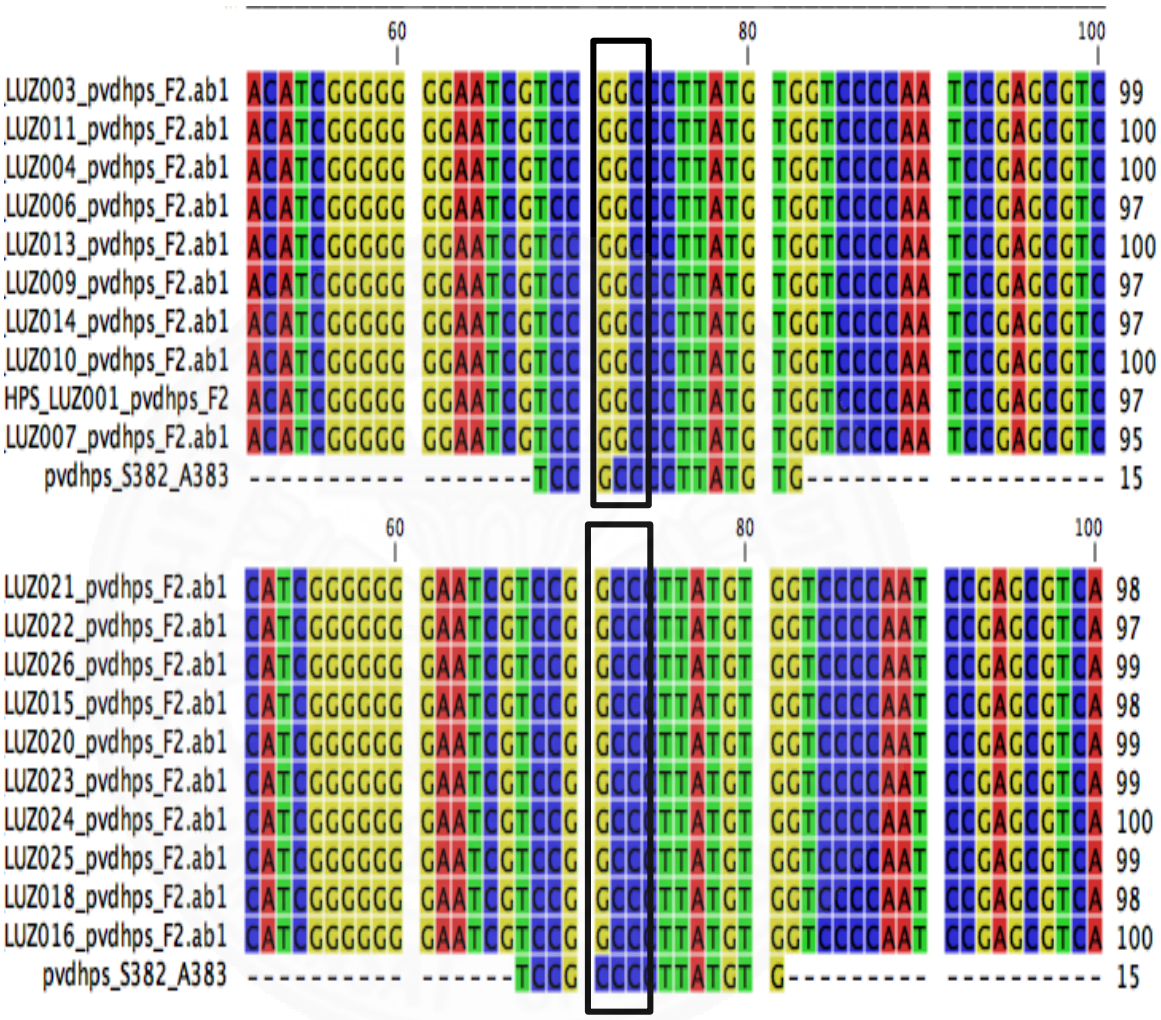

Figure O.1 Illustration showing the alignment of *pvdhps* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

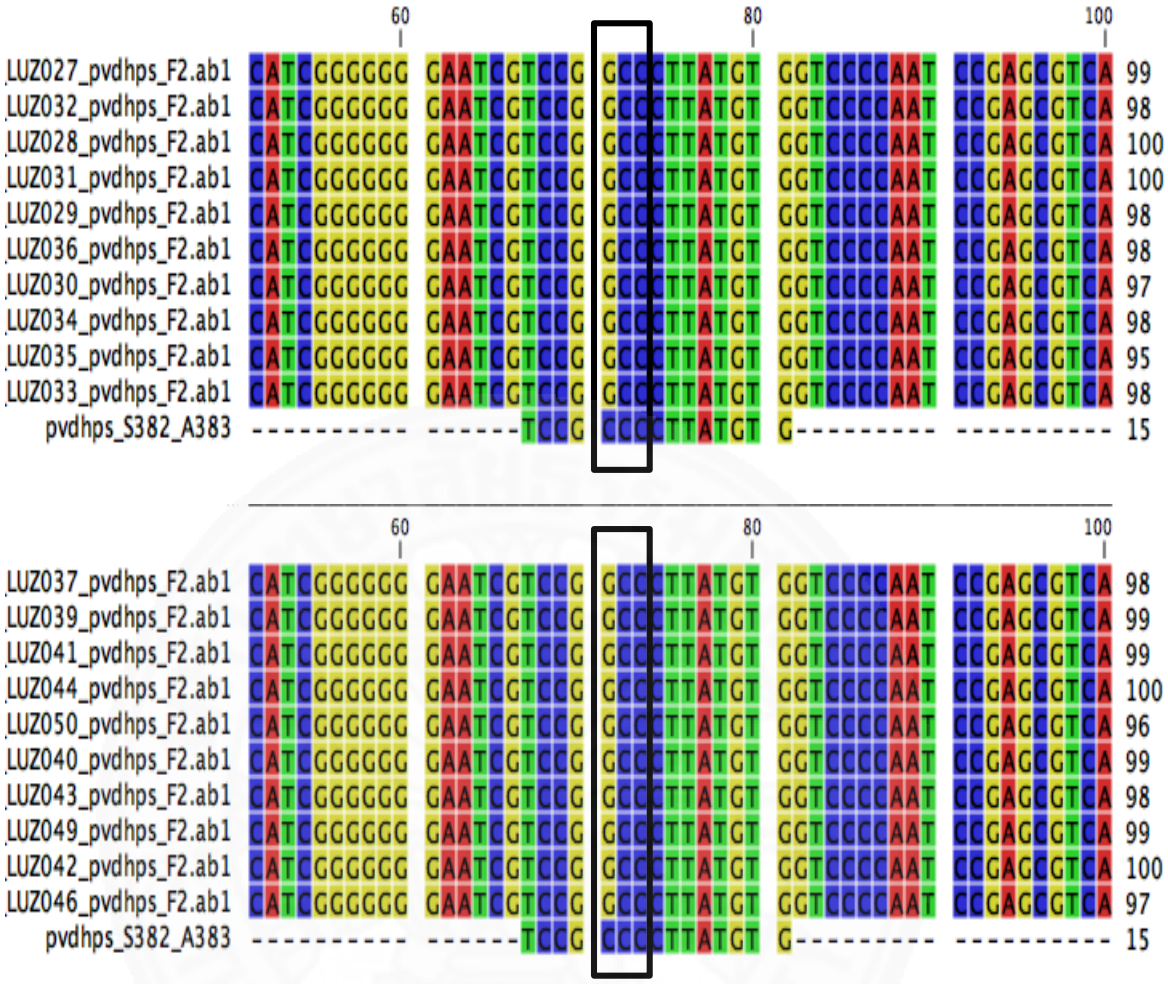

Figure O.2 Illustration showing the alignment of *pvdhps* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

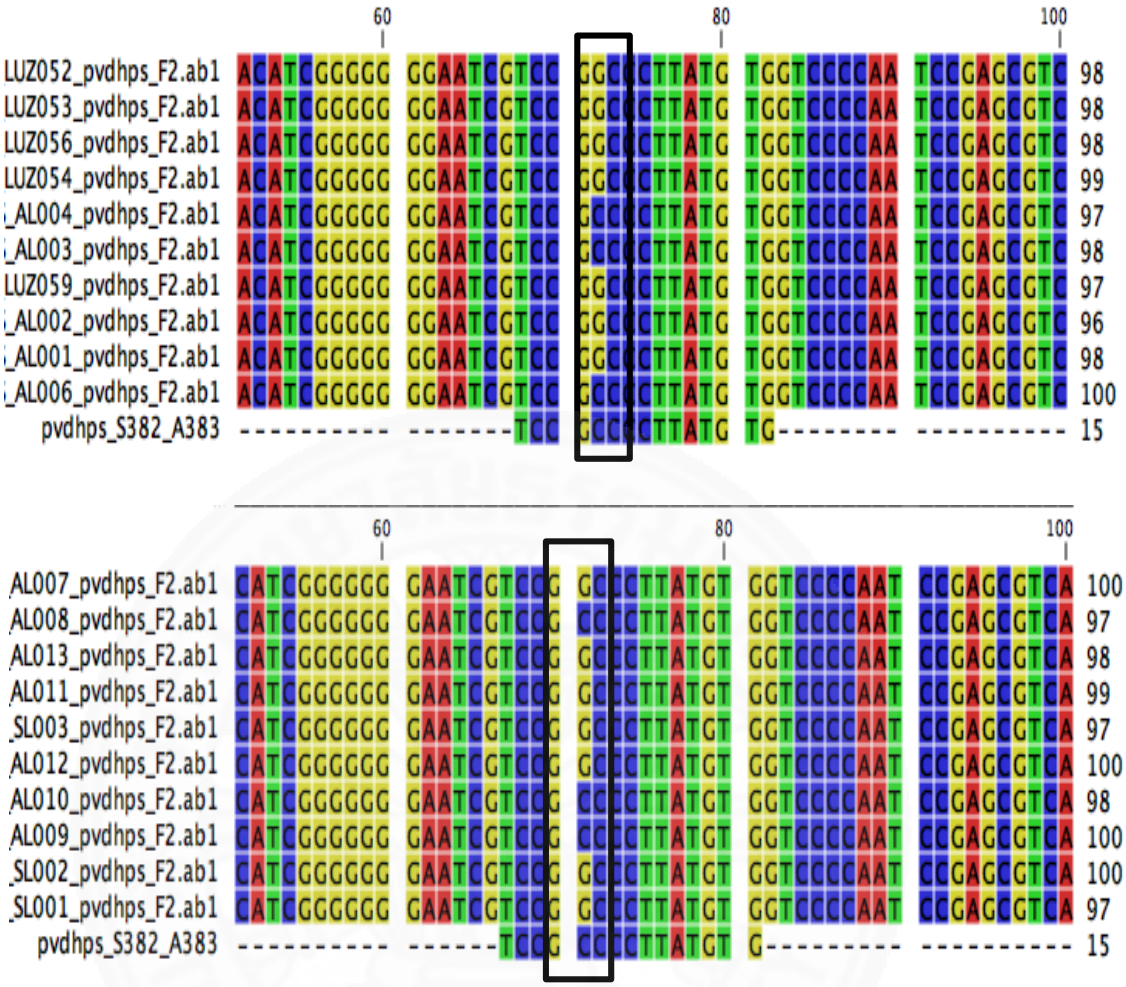

Figure O.3 Illustration showing the alignment of *pvdhps* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

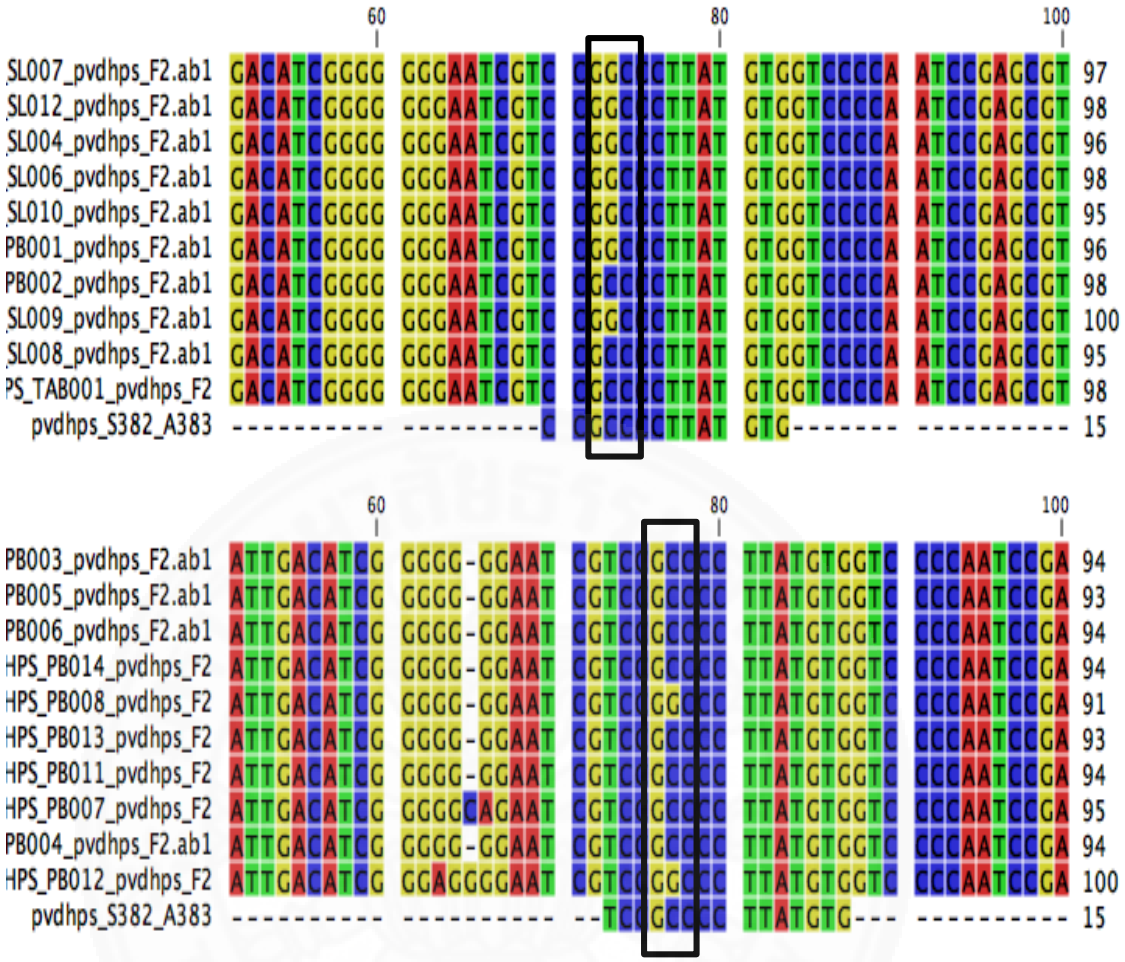

Figure O.4 Illustration showing the alignment of *pvdhps* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

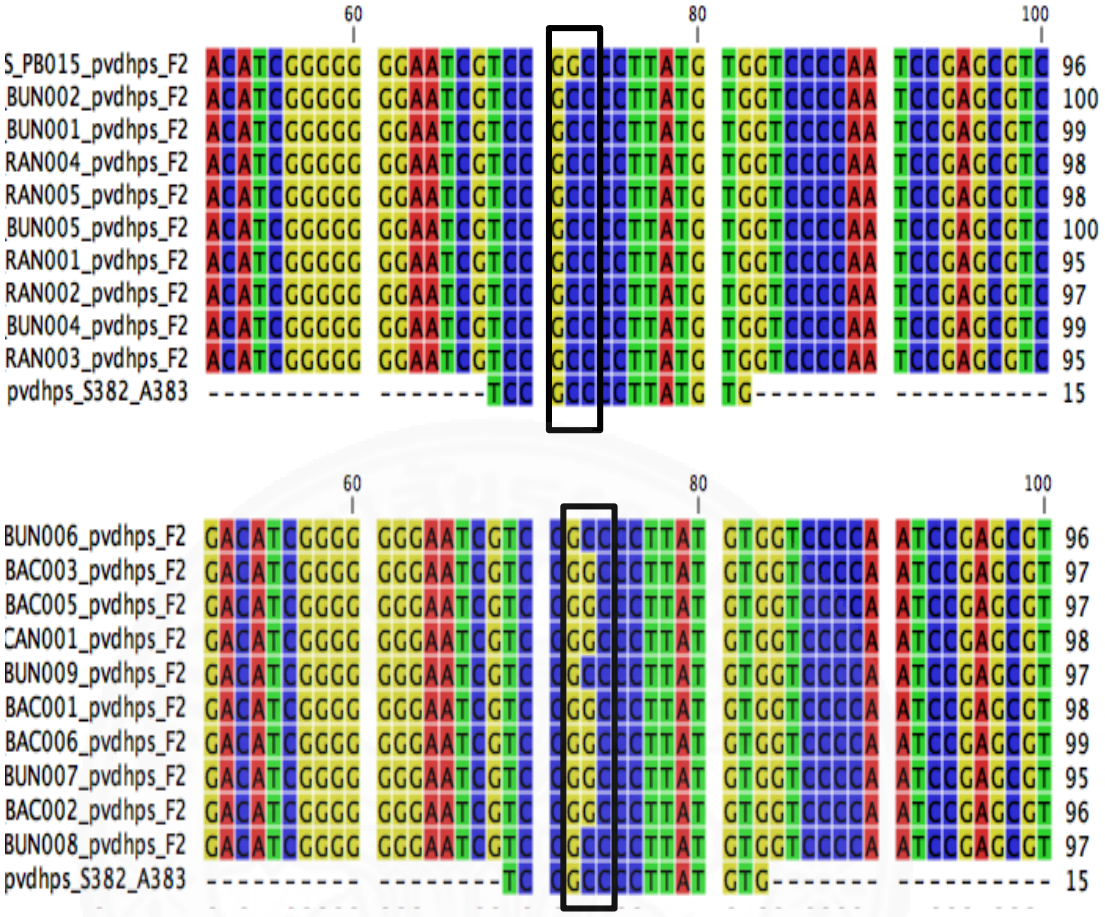

Figure O.5 Illustration showing the alignment of *pvdhps* reference sequences to the sequenced products using Molecular evolutionary genetic analysis software version 6.0.

APPENDIX P

Plasmodium vivax strain Shwegyin isolate D1130 putative hydroxymethylpterin pyrophosphokinase-dihydropteroate synthetase gene, partial cds

Sequence ID: KX000955.1 Length: 1250 Number of Matches: 1

Range 1: 28 to 761 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1312 bits(710)	0.0	727/734(99%)	5/734(0%)	Plus/Plus

```
Query  7    GTGGA-A-GA-GTTCG-AATGGC-AGTGATGGGGCGAGCGTGATTGACATCgggggggAA  61
            ||||| | || ||||| ||||||  ||||||||||||||||||||||||||||||||||
Sbjct  28   GTGGAGAGGATGTTCGAAATGGCAAGTGATGGGGCGAGCGTGATTGACATCGGGGGGGAA  87

Query  62   TCGTCCGGCCCTTATGTGGTCCCCAATCCGAGCGTCACTGAACGGGATTTGGTCATGCCT  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  88   TCGTCCGGCCCTTATGTGGTCCCCAATCCGAGCGTCACTGAACGGGATTTGGTCATGCCT  147

Query  122  GTTTTGAAGCTCTTTAAGGAGGAGTGGCATAAGTTGGAGTGTGAGGTTGGCGGTGGTGCC  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  148  GTTTTGAAGCTCTTTAAGGAGGAGTGGCATAAGTTGGAGTGTGAGGTTGGCGGTGGTGCC  207

Query  182  GTGTGCTGCGCTGCAGCAAGTGATGCCAGGAAAAACGCGCAGAGCTCCCTACAGGGGAAA  241
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Sbjct  208  GTGTGCTGCGCTGCAGCAAGTGATGCCAGGAAAAACGCGCAGAGCTCCCTACAGGGGAAA  267

Query  242  CTACAAAAAGTGAGGGACGCAAAACCGATCATAAGCATCGACACGGTCAATTATGATCTC  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  268  CTACAAAAAGTGAGGGACGCAAAACCGATCATAAGCATCGACACGGTCAATTATGATCTC  327

Query  302  TTCAAGGAGTGCGTGGAAGGCGAGTTGGTGGACATCCTAAACGATATCAGCGCGTGCACG  361
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Sbjct  328  TTCAAGGAGTGCGTGGAAGGCGAGTTGGTGGACATCCTAAACGATATCAGCGCGTGCACG  387

Query  362  CACAACCCAGAGATTATAAAATTGTTGAGGAGGAAAAACAAGTTCTATAGCGTCGTTTTA  421
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Sbjct  388  CACAACCCAGAGATTATAAAATTGTTGAGGAGGAAAAACAAGTTCTATAGCGTCGTTTTA  447

Query  422  ATGCACAAGAGGGGAAATCCACACACCATGGATAAGTTAACAAATTACGATGACCTTATA  481
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Sbjct  448  ATGCACAAGAGGGGAAATCCACACACCATGGATAAGTTAACAAATTACGATGACCTTATA  507

Query  482  AGTGACATTAAAAGGTATTTAGAAGATCGGCTACATTTTCTCGTTCTAAATGGGGTACCA  541
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  508  AGTGACATTAAAAGGTATTTAGAAGATCGGCTACATTTTCTCGTTCTAAATGGGGTACCA  567

Query  542  CGCTACCGAGTTCTCTTTGATGTCGGCCTGGGGTTTGCCAAAAAGCACGACCAGTCTATT  601
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Sbjct  568  CGCTACCGAGTTCTCTTTGATGTCGGCCTGGGGTTTGCCAAAAAGCACGACCAGTCTATT  627

Query  602  AAGCTGTTGCAACATATTCACGTTTACGATGAGTACCCGCTGTTTCTTGGCTACTCGAGG  661
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Sbjct  628  AAGCTGTTGCAGCATATTCACGTTTACGATGAGTACCCGCTGTTTCTTGGCTACTCGAGG  687

Query  662  AAGCGCTTTATTGTCCACTGCATGGGGAAGGGTGGCGCGGCCATCGGGGAGTGCACACTG  721
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Sbjct  688  AAGCGCTTTATTGTCCACTGCATGGGGAAGGGTGGCGCGGCCATCGGGGAGTGCAGACTG  747

Query  722  ATGAGCGGGGAGGC  735
            ||||||||||||||
Sbjct  748  ATGAGCGGGGAGGC  761
```

APPENDIX Q

CODON TABLE

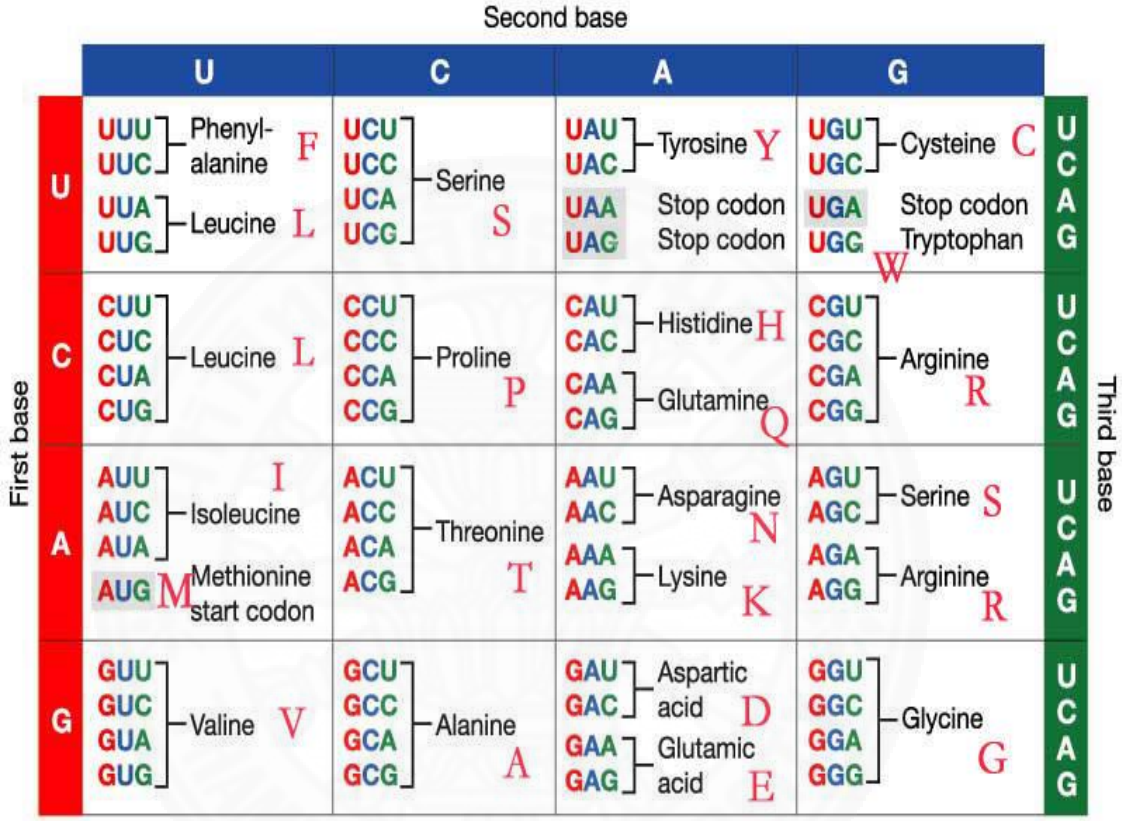

Figure Q.1 Codon table for reference.

APPENDIX R

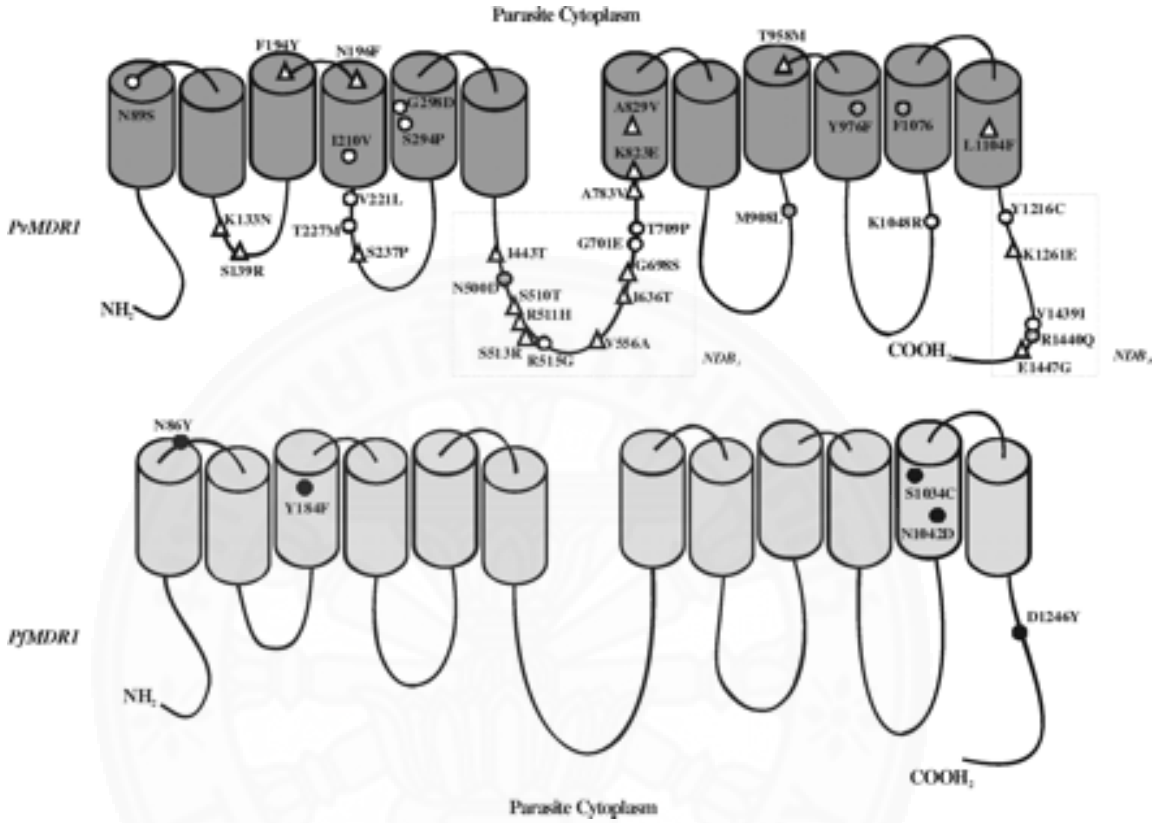

Figure R.1 Predicted structure of *pvmdr1* and *pfmdr1* gene as illustrated by Orjuela-Sanchez *et al.* (2009).

BIOGRAPHY

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