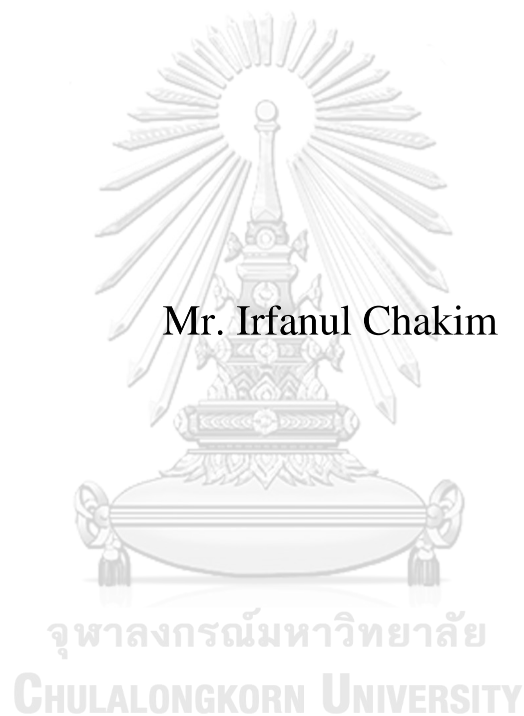
RISK FACTORS FOR MALARIA INFECTION AND DIHYD ROARTEMISININ+PIPERAQUINE RESISTANCE DISTRIB UTION BETWEEN HIGH AND LOW ENDEMIC AREAS IN INDONESIA

Mr. Irfanul Chakim



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ปัจจัยเสี่ยงในการติดเชื้อมาลาเรียและการกระจายของเชื้อมาลาเรียดื้อต่อยาผสมไดไฮโดรอาร์ติมิซินิน-ไพเพอราควินระหว่างพื้นที่การระบาดสูงและต่ำในประเทศอินโดนีเซีย

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อิฟรานู ชาคิม : ปัจจัยเสี่ยงในการติดเชื้อมาลาเรียและการกระจายของเชื้อมาลาเรียดื้อต่อยาผสมไดไฮโดรอาร์ติมิซินิน-ไพเพอราควินระหว่างพื้นที่การระบาดสูงและต่ำในประเทศอินโดนีเซีย. (
RISK FACTORS FOR MALARIA INFECTION AND DIHYDROARTEMISININ+PIPERAQUINE RESISTANCE DISTRIBUTION BETWEEN HIGH AND LOW ENDEMIC AREAS IN INDONESIA) อ.ที่ปรึกษาหลัก : อ. ดร.เทพนาฏ พุ่มไพบูลย์, อ.ที่ปรึกษาร่วม : ศ. ดร.Din Syafruddin

จำนวนผู้ป่วยด้วยโรคมาลาเรียทั่วโลกลดลงในช่วงกว่าสิบปีที่ผ่านมา จำนวนผู้ป่วยในประเทศอินโดนีเซียมีแนวโน้มลดลงเช่นเดียวกันแต่ยังไม่ถึงเป้าในการกำจัดโรคมาลาเรีย ในการควบคุมและกำจัดโรคมาลาเรียนั้นต้องการยุทธศาสตร์ที่ครอบคลุมและสอดคล้องทั้งการควบคุมปรสิตและยุงพาหะ อย่างไรก็ตามยุทธศาสตร์ในการกำจัดโรคมาลาเรียในหลายๆประเทศรวมถึงอินโดนีเซียยังคงมุ่งในการรักษาเพียงอย่างเดียวซึ่งนำไปสู่การเกิดเชื้อมาลาเรียดื้อต่อยาที่ใช้ในการรักษาหลายชนิด เมื่อไม่นานมานี้พบเชื้อมาลาเรียดื้อต่อยาอาร์ติมิซินินซึ่งเป็นยาที่ได้ผลในการรักษาเพียงชนิดเดียวที่เหลืออยู่ในปัจจุบันและเชื้อดื้อยานี้ได้กระจายไปในทวีปต่างๆทั่วโลก แต่เชื้อดื้อยานี้ยังพบไม่มากในประเทศอินโดนีเซีย นอกจากนั้นการขาดวินัยในการกินยาของประชากรนำไปสู่การรักษาที่ไม่ได้ผลก่อนที่จะเกิดการกระจายของเชื้อที่มีพันธุกรรมดื้อยา ด้วยเหตุนี้งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อหาปัจจัยที่ทำให้เกิดเชื้อมาลาเรียดื้อยาในประเทศอินโดนีเซีย การประเมินเพื่อหาปัจจัยเสี่ยงในการเกิดโรค ยุงพาหะ และความมีวินัยในการกินยาผสมอาร์ติมิซินินที่ใช้ในการรักษาโดยเปรียบเทียบระหว่างพื้นที่ที่มีการระบาดต่ำและสูงของประเทศอินโดนีเซีย การประเมินปัจจัยเสี่ยงในการเกิดโรคและความมีวินัยในการกินยาผสมไดไฮโดรอาร์ติมิซินินและไพเพอราควินทำโดยใช้แบบสอบถาม และเก็บข้อมูลพฤติกรรมการกินเลือดของยุงพาหะในแต่ละพื้นที่ในช่วงระหว่างสามสัปดาห์ด้วยวิธีจับยุงในขณะที่เกาะบนเหยื่อล่อ การประเมินประสิทธิภาพของยาผสมไดไฮโดรอาร์ติมิซินินและไพเพอราควินทำโดยติดตามคนไข้เป็นเวลา 42 วันตามแนวทางขององค์การอนามัยโลก รวมถึงการใช้เครื่องหมายโมเลกุลของยีน K13 สำหรับติดตามการดื้อยาอาร์ติมิซินินและยีนพลาสเมพซิน สำหรับยาไพเพอราควิน ผลจากการศึกษาสามารถจับยุงทั้งหมด 2,364 ตัว และพบว่าจำนวนของยุงพาหะที่แตกต่างกันระหว่างพื้นที่การระบาดต่ำและสูง และยังพบว่าช่วงเวลาในการออกหากินของยุงมีความแตกต่างกันอย่างมีนัยสำคัญ พบว่ามีปัจจัยเสี่ยงหลายปัจจัยที่มีผลต่อการเกิดโรค และระยะห่างระหว่างพื้นที่นั้นมีผลต่อปัจจัยเสี่ยงในการเกิดโรคระหว่างสองพื้นที่ นอกจากนั้นประชากรในสองพื้นที่มีระดับวินัยในการกินยาต่ำ แต่อย่างไรก็ตามยาผสมนี้ยังมีประสิทธิภาพในการรักษา ถึงแม้ว่าจะพบการกลายพันธุ์ในยีน K13 และการเพิ่มจำนวนชุดของยีนพลาสเมพซิน แต่ไม่พบความสัมพันธ์ของลักษณะพันธุกรรมดังกล่าวต่อความล้มเหลวในการรักษา ผลการประเมินทางกีฏวิทยาพบว่ามีความแตกต่างของเวลาในการออกหากินของยุงระหว่างพื้นที่ ไม่พบผลของโครงสร้างของบ้านที่อยู่อาศัยต่อการป้องกันโรคมาลาเรีย จากผลการศึกษาปัจจัยเสี่ยงในการเกิดโรคพบว่าลักษณะของพื้นที่ระบาดส่งผลถึงปัจจัยเสี่ยงที่พบในแต่ละพื้นที่ซึ่งอาจส่งผลถึงความแตกต่างของอัตราป่วยด้วยโรคมาลาเรีย เนื่องจากความมีวินัยในการกินยาต้านมาลาเรียอยู่ในระดับต่ำ ดังนั้นการติดตามที่ดีและยุทธศาสตร์ในการเพิ่มระดับความมีวินัยในการกินยาเป็นสิ่งที่จำเป็นเพื่อป้องกันการปรับเปลี่ยนยาในการรักษาเช่นเดียวกับประเทศอื่นในภูมิภาคเอเชียตะวันออกเฉียงใต้ ถึงแม้ว่ายาผสมไดไฮโดรอาร์ติมิซินินและไพเพอราควินจะยังคงมีประสิทธิภาพแต่การพบการกลายพันธุ์ในยีน K13 และการเพิ่มจำนวนชุดของยีนพลาสเมพซินซึ่งเป็นเครื่องหมายโมเลกุลของการดื้อยาผสมดังกล่าวทำให้เห็นว่าเชื้อมาลาเรียอาจจะมีการเปลี่ยนแปลงทางพันธุกรรมเพื่อให้มีความสามารถในการดื้อต่อยา ด้วยเหตุนี้การเฝ้าระวังติดตามประสิทธิภาพของยาผสมนี้ควรดำเนินการอย่างต่อเนื่อง โดยสรุปผู้กำหนดนโยบายเพื่อควบคุมโรคมาลาเรียจำเป็นต้องควบคุมปัจจัยเสี่ยงในการเกิดโรค วินัยในการกินยาของประชากรและติดตามประสิทธิภาพของยาผสมไดไฮโดรอาร์ติมิซินินและไพเพอราควินอย่างต่อเนื่องและกำหนดยุทธศาสตร์สำหรับแต่ละพื้นที่การระบาด

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Irfanul Chakim :
RISK FACTORS FOR MALARIA INFECTION AND DIHYDROARTEMISININ+PIPERAQUINE RESISTANCE DISTRIBUTION BETWEEN HIGH AND LOW ENDEMIC AREAS IN INDONESIA. Advisor: Tepanata Pumpaibool, Ph.D. Co-advisor: Prof. Din Syafruddin, M.D., Ph.D.

Globally, malaria case is decreasing over decades. This similar trend happens in Indonesia but it is still not even close to elimination. To control and eradicate malaria require a comprehensive and coincident strategy for the parasite and its vector. However, malaria elimination strategy of many countries, including Indonesia, is still considering only treatment which has led to resistance of *Plasmodium* species over various antimalarial drugs. Recently, resistance to the only and current available drug, artemisinin, has appeared and spread over continents in the world. However, it has not been extensively discovered in Indonesia. Additionally, it was widely known that a poor adherence in a population to antimalarial medication will lead to the development of treatment failure prior to the spread of parasite resistance genetically. Therefore, the objective of the current study is to discover extensively the factors contributing to the development of such resistance in *Plasmodium* parasite population in Indonesia. The assessment included identification of malaria risk factors and the vector and measurement of adherence level to artemisinin combination therapy in comparison between low and high endemicity areas in Indonesia. The assessment of malaria risk factors and dihydroartemisinin+piperaquine (DHP) population adherence were done by structured questionnaires. A three weeks longitudinal observation of vector biting dynamics was done using human landing catch method in each area. Efficacy study of DHP was done with 42-days follow up according to WHO guideline. Additionally, molecular marker for artemisinin (K13) and piperaquine (Plasmepsin II) were also included. In total, 2364 *Anopheles* mosquitoes were successfully collected and a difference in number of mosquitoes between low and high endemicity areas was also found. There was also an evidence that the biting time of each area differs significantly. We also found several risk factors of malaria between the two areas and discovered that there was a spatial effect of malaria risk factor between those two areas. Additionally, the population in those two areas had a low level of drug adherence. However, the efficacy of DHP in low and high endemiciy areas remained effective. Although several mutations occurred in K13 gene and multi-copy of Plasmepsin II gene were found, but no association was detected in accordance to treatment failure. The result of entomological assessment indicates that there is a difference in biting time of *Anopheles* mosquitoes between the studied locations and there is no effect of housing construction in preventing malaria. Malaria risk factor study suggested that the risk factor variable on malaria infection is influenced by area that may reflect the differences in annual parasite incidence (API). Since a low level of population adherence to antimalarial drug medication was found, it needs better monitoring and strategy to elevate adherence level to prevent the deployment of currently available antimalarial drug as seen in other South East Asian countries. Finally, although an efficacious DHP medication was found in study setting, but mutations and elevated number of multi copy of DHP marker was suggesting that the parasite may have developed the ability to resist the medication genetically. Therefore, a careful monitoring of DHP efficacy in Indonesia needs to be done continuously. In conclusion, it is imperative to public policy maker to control malaria case by controlling the risk factor and population adherence level in a population and continuous monitoring of DHP efficacy as well as the strategy needs to consider area-specific.

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Field of Study:	Public Health Sciences	Student's Signature ....
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		Co-advisor's Signature ....

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CHAPTER 1

INTRODUCTION

1.1. Background and rationale

The world malaria incidence has been reported to reach 212 million cases with a range of 148-304 million cases. It was also reported that between 2010 to 2015 there was a decrease of malaria cases as much as 14%. African countries have the largest number of malaria cases worldwidely followed by East Asia and Mediterranean. Of that 7% of all malaria cases worldwidely were from Indonesia. Based on WHO, the use of rapid diagnostic test (RDT) has supported the escalation of case detection from 40% suspected cases in 2010 to 76% suspected cases in 2015 (1).

In Indonesia, malaria can almost be found in all regions. According to the report of basic health research showed in the year of 2011 there are 374 districts which endemic for malaria and 256.592 estimated malaria cases with the total death caused by malaria in the number of 388 people. Nationally, the number of malaria morbidity during 2005-2015 tend to be gradually declined which is of 4.1 per 1000 people at risk in 2005 to be 0.85 per 1000 people in 2015 (2). Region with high endemicity declined from 17.4% in 2011 to 8.8% in 2015, likewise moderately endemic areas declined from 18.6% in 2011 into 17% in 2015, along with low endemic areas experienced sharp decline from 42.8% in 2011 to 28.8% in 2015. Otherwise malaria-free region have encountered significant escalation from 21.5% in 2011 to 45.4% in 2015 (3-5).

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To control and eradicate malaria require a comprehensive and coincident strategy for the parasite and its vector. For anopheline control strategy have been widely demonstrated using insecticide-impregnated bed-nets and indoor-residual spraying of insecticides (6), however, it would never be success story without effective antimalarial drug treatment against the parasite. There are two important aspects of drugs for malaria control. First, an effective treatment prevents progression to severe disease and limits gametocyte development that become transmission blocking agent to mosquitoes (7). Secondly, it can be used to prevent malaria in an endemic area with various strategies as chemoprophylaxis, intermittent preventive therapy and mass drug administration (8).

Antimalarial drug comprises of several drugs family containing some of currently used or removed due to resistance. Quinolines are the oldest class of antimalarial drugs. Quinine is the first drug in this group which has remained effective and still recommended for severe malaria and second line treatment. In 1934, first introduction of quinine derivatives known as chloroquine was used for chemoprophylaxis in 1950, unfortunately the spread of *Plasmodium* resistant to this drug prompted the change in policy and removal of this drug from antimalarial therapies. Amodiaquine and mefloquine were then appeared to counteract resistance to chloroquine and is currently recommended to be used in combination with artesunate to combat malaria. In response to the increasing prevalence of chloroquine-resistant parasites in Southern China (9), piperaquine was also then developed and adopted as the first-line treatment in 1978 (10), however, its used as monotherapy resulted eventual emergence of piperaquine-resistant parasites (10) and therefore reintroduced by WHO in combination with dihydroartemisinin that has undergone successful therapy in both Asia and Africa (9, 11-13). Another drug that especially target liver stages of

Plasmodium vivax and *Plasmodium ovale* in dormant form or hypnozoites causing relapse and recurrence of infection was known as primaquine (14, 15). Nowadays, WHO guidelines also recommend the addition of single-dose of primaquine to artemisinin combination therapy as gametocytocidal compound and should be considered in low-transmission regions that aim to control *Plasmodium falciparum* (16-18). The last quinoline member that has aryl alcohol form is lumefantrine, first synthesized in China in the 1970s and registered for antimalarial treatment in 1987. However its use never been recommended as monotherapy, rather in combination with artemether as the first-line treatment for uncomplicated malaria (19). The use of inhibitors of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHFS) as antifolates are the second family of antimalarial drugs (20). The widely used drug in this group is sulfadoxine/pyrimethamine (SP) that has been introduced in 1967 as a synergistic antimalarial drug as a first-line treatment of *P. falciparum* in Africa (21). The most prevalent and effectively antimalarial drug that is still in use broadly is artemisinin and its derivatives. Artemisinin is a potent and rapidly acting blood schizonticide originally isolated from the plant *Artemisia annua* from Chinese traditional medicine. The WHO recommended for the first-line treatment of malaria and combined of other group as combination therapy including: a combination of arthemeter plus lumefantrine, artesunate plus amodiaquine, mefloquine or sulfadoxine/pyrimethamine and dihydroartemisinin-piperaquine (17). The high efficacy, fast action and reduced likelihood of resistance development have made it widely adopted by most malaria-endemic countries for treating malaria. The last group antimalarial that include selected antibiotics was known since 1950s, comprises of tetracycline as the most active compound, doxycycline and clindamycin (22). Its use

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was recommended by WHO in combination with rapid acting drugs like artemisinin derivatives or quinine as a second-line antimalarial treatment (17).

The resistance problem of parasites to antimalarial drug have appeared to become major public health concern and routinely changed drug regime over time. Some drugs have been reported its failure to treat malaria, but the others still in investigation process. Quinine as the oldest drug from quinoline group has been reported to develop resistance in 1910 (23). Parasites that resist to amodiaquine have been reported from South America, Asia and East Africa (24-26), likewise the emergence of piperaquine-resistant parasites (10). Treatment failure to mefloquine has been occurred in Thailand in early 1990s (27, 28). In other hand, resistance to primaquine is a difficult entity to quantify separately, because primaquine is used with blood schizontocidal agent (15, 29) and also no report that have been convincingly demonstrated to lumefrantine. The complexity of antimalarial resistance became deteriorate since the main treatment of artemisinin directed against resistant parasites has begun to appear (30-33).

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Further the need of genetic marker for antimalarial drug resistance is notably considered as the main objective of various studies. Studies of field isolates and examination of candidate genes have led to the identification of several genes that responsible to antimalarial drugs resistance. The most recent discovered gene is K13 propeller domain has been identified as a key determinant for artemisinin resistance. Some nonsynonymous polymorphism of Kelch 13 propeller domain have lately been found to be responsible for artemisinin resistance (34). Since piperaquine drug has been widely used as an artemisinin combination in the form of coartem, researchers have

successfully found a genetic marker for piperaquine resistance which is *Plasmepsin II-3* copy number. The chance of parasite carrying multicopy-*Plasmepsin II* that could be survive against piperaquine is 20 times. After the introduction of dihydroartemisnin+piperaquine in 2002, the proportion of parasite carrying multicopy *Plasmepsin II* is steadily increasing. *Ex-vivo* study has demonstrated that piperaquine 50% inhibitory concentrations (IC^{50}) of field clinical isolates of *Plasmodium falciparum* is increased, hence it urgently needs surveillance application for piperaquine monitoring (35-37).

In part of malaria control strategies, it is imperative to monitor parasite resistance to antimalarial drugs. Instead of expensive and laborious *in vivo* treatment trials or *in vitro* drug susceptibility testing, surveillance of molecular marker can be alternative to detect drug-resistant strains as they enable analyzing large samples and assessing resistance to many antimalarial drugs simultaneously (38). For instance, several studies indicated F446I mutant allele of K13 domain of *Plasmodium falciparum* was highly prevalent in China-Myanmar border as well as in Myanmar especially northern region (39). C580Y mutant was also predominant in Cambodia, Myanmar, eastern and western Thailand (34, 39-41). Additionally, in an endemic region by which partner drug is widely used, discovery, validation and surveillance application of its marker has been done well especially in epicenter of drug resistance region. Excess amplification of *Plasmepsin II* as a marker for piperaquine has been documented in Cambodia (35-37).

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In Indonesia, there is limited information regarding to K13 and *Plasmepsin II* distribution and its correlation to ACT efficacy and even more parasite clearance half-

life. This is the issue since Indonesia has changed the policy of antimalarial utilization from non-ACT to be ACT in 2008, and followed by other South-East Asian country such as Thailand in 2015 (42). This information will be beneficial to inform public health policy makers to strengthen malaria control management by giving basic data of evolution of advantageous phenotypic trait. This is can be done by tracking the possibility of its spread and natural selection against them, population genetic approach can be extensively and effectively explored since a limited data have been provided.

Additionally, besides declining malaria cases but certain areas of Indonesia are still at high risk with high endemicity level and the spread of malaria is wider compared to the previous year, thus several attempts have been made by Indonesia government to prevent malaria with nation-wide scale malaria elimination program. These program are mass distribution of bed nets, vector control (although there are many attempts to do so, but indoor residual spraying and insecticide-treated net are more widely used), and diagnosis and treatment of infected patient (2). However, based on studies, at least there are 24 associated risk factors of malaria in Indonesia (43-45). These are divided into individual and environmental factors. Therefore, these such data will be imperative to explain any potential distinctive individual and/or environmental circumstances lead to different number of malaria incidence which is unique to every malarious region.

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Since ACT is known to be an effective prompt treatment and preventive as well as intermittent mass drug administration strategy, the deployment of ACT has been widely adopted. Previous systematic review has described how antimalarial drugs used among malaria infected patients (46). From these review, patient's adherence to ACT varied from 78% for a three-day regimen of artesunate plus sulfadoxine-pyrimethamine

(AS+SP) in Zambia (47) to maximum of 93% for artemether plus lumefanthrine (AL) in Uganda (48). In other hand, adherence level of ACT in Kenya is <30% (49) and in contrast 100% of AL adherence in Malawi (50). Age has been reported to be risk factor of poor adherence to non-ACT regimen (51), suggesting formulation of ACT and communication campaigns should take into account age related factor. Another associated risk factor of ACT medication is vomiting; however, vomiting is a difficult entity to measure. It is negatively correlated with Amodiaquine plus artesunate (AQ+AS) and AL (52, 53), though it is considered to be an exclusion criteria in some studies. The methods used to measure adherence to non-ACT drugs (46), yet they are still widely used to measure adherence to ACT today. Since self-report is subject to social desirability bias, which may overestimate adherence and current measurement tools of ACT adherence using questionnaire are not standardized, MEMS (medical event monitory services) and biological assays are more objective methods to measure adherence and may offer more precise adherence measurements (48, 54-58). On the other hand, primaquine is suggested to be the partner drug for ACT, since it can be gametocytocidal effect on *P. falciparum*. Thus, inhibit transmission and eventually eliminate malaria as is expected. Thus, it would be imperative to discover compliance to primaquine in the context of malaria elimination program. However, these such study have not been discovered or limited information in relation to adherence level of ACT and primaquine treatment in Indonesia.

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1.2.Research gap

1. Lack of information regarding the risk factors of malaria infection in the high and low endemic areas in Indonesia.

2. Lack of information regarding adherence of dihydroartemisinin+piperaquine and primaquine treatment in Indonesia.
3. Lack of information regarding epidemiological distribution of dihydroartemisinin+piperaquine resistance in Indonesia.

1.3.Research objective

General objective

This study aims to determine the risk factors for malaria infection in different endemic areas and also investigate the community adherence of current antimalarial medication and the ACT resistance distribution in Indonesia in the context of malaria elimination program

Specific objective

1. To investigate entomological circumstance of Anopheles vector in Indonesia.
2. To investigate individual and environmental risk factors of malaria incidence.
3. To compare malaria associated risk factors between low and high endemicity areas.
4. To investigate the level of adherence of dihydroartemisinin+piperaquine and primaquine treatment in Indonesia.
5. To investigate the efficacy of dihydroartemisinin+piperaquine medication against Plasmodium malaria in Indonesia.
6. To discover the sequence of K13-propeller domain and copy number of *Plasmepsin II* of *Plasmodium falciparum* in Indonesia.

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1.4.Benefit of study

The finding of this current research will be beneficial for malaria disease prevention effort in Indonesia. There is several beneficial information for public health importance:

1. For public health preventive program, it is essential to understand about associated risk factors related to malaria incidence. This study will confirm whether government can apply nation-wide scale prevention program or area-specific. As well as the information of the adherence level of Indonesian to ACT and primaquine treatment which may partly a factor of drug resistance. It is also can be used to allow public health management to plan prevention program in order to avert the emergence and spread of antimalarial drugs. Additionally, public health policy maker needs to consider, according to our result, that the intervention to area-specific is better than nation-wide due to different area may have different situation of such factors.
2. To provide database of molecular marker of currently used antimalarial drugs. Previously confirmed marker will support epidemiological and surveillance data regarding current situation of the impact of antimalarial drug usage in Indonesia.

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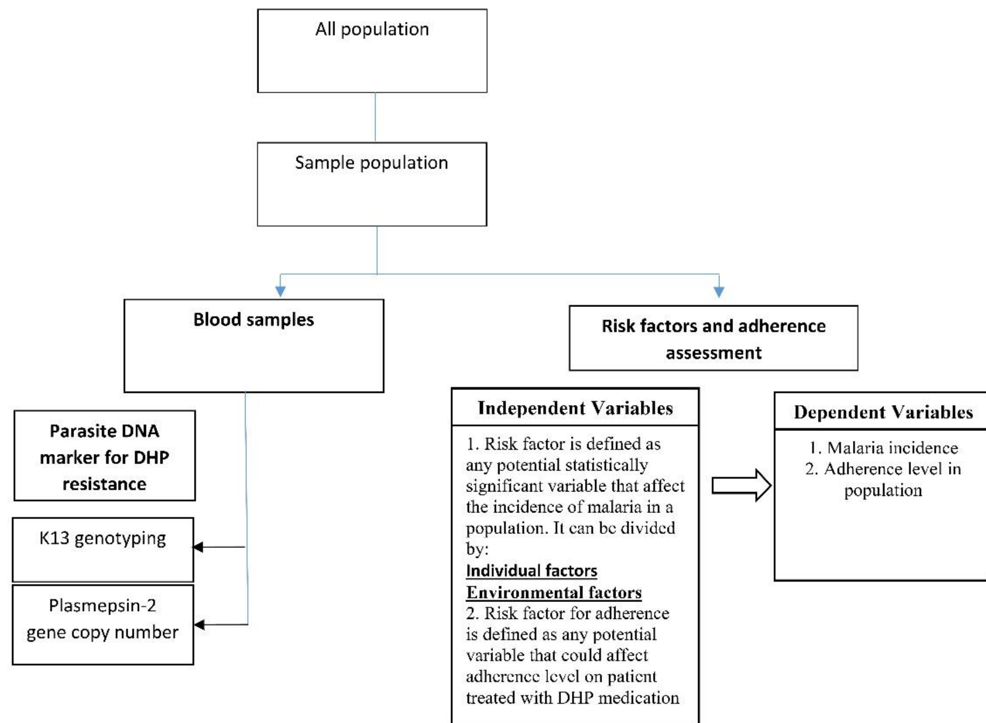

Figure 1. Conceptual Framework

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CHAPTER 2

LITERATURE REVIEW

2.1.Malaria

2.1.1. Biology of malaria

The genome of four *Plasmodium* species – *Plasmodium falciparum*, *Plasmodium knowlesi*, *Plasmodium vivax* and *Plasmodium yoelii* – have been sequenced. All these species have genomes of about 25 megabase organized into 14 chromosomes consistent with earlier estimates. The chromosomes vary in length from 500 kilobases to 3.5 megabases and it is presumed that this is the pattern through the genus (59).

Taxonomically, *Plasmodium* as part of the family Plasmodium, order Haemosporidia and phylum Apicomplexa. There are presently 450 recognized species in this order. Out of those are still under investigation for further cross examination of their taxonomy with DNA analysis. It seems more likely that many of these species will be re-assigned after these studies have been completed (60, 61).

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The evolution of *Plasmodium* probably started from its *Leucocytozoon* ancestor of about 130 million years ago, a moment by which it occurred accidentally with the prompt deployment of the angiosperms (flowering plants). A plausible explanation of these phenomenon is that the escalation in the number of flowers have led to an amplified amount in the number of mosquitoes and accordingly their contact with vertebrates (62). While evolution of mosquitoes has begun in what is now South America approximately 230 million years ago. There are over 3500 recognized species, but until recently their evolution has not been convincingly demonstrated, thus several

knowledge gaps in our knowledge of the evolution of *Plasmodium* remain. For instance, there is evidence of a recent expansion of *Anopheles gambiae* and *Anopheles arabiensis* populations in the late Pleistocene in Nigeria (63). Another circumstance of vector dynamic that is the emergence of cryptic species of *Anopheles* mosquitoes, it just complicated the vector control strategy very recently. This is important, since specifying species of *Anopheles* is considered to be very detrimental to discriminate between vector and non-vector (64). However, until presently, there is limited number of researches that discovered the reason why a relatively finite number of mosquitoes should be such successful vectors of multiple diseases, and it is not yet known clearly. On other hand, the symbiont bacterium *Wolbachia* was known to be present in a mosquito-free pathogen (virus or parasite) which known as *Wolbachia*-strain specific. It is more likely to be this bacteria can reduce the ability of mosquito to be a vector (65, 66).

The life cycle of *Plasmodium* has similarity for those of other species in the Haemosporidia. Transmission of human malaria *Plasmodium* species is carried out by mosquito species of the genus *Anopheles*. The rest of non-human malaria parasite can be carried out by other mosquito genera; *Aedes*, *Culex*, *Mansonia* and *Theobaldia*, as exhibited by bird malaria which commonly transmitted by species belonging to the genus *Culex*. This habit is naturally explained by the demand of the mosquito to produce egg which need protein content of human blood instead of nectar’s protein. Biting female mosquito transmits sporozoites from saliva to either the blood or the lymphatic system of the recipient. Occasionally parasites can block salivary ducts of the mosquito and as a consequence the mosquito normally necessitates multiple efforts to obtain blood. However, the reason behind this phenomenon is still unclear. Thus, whether this

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multiple attempts by the mosquito may assist to immunological tolerance of the parasite remain unclear (67). The main mode of entry of the parasite is injected to subcutaneous tissue from which they migrate to capillaries. Partly they are captured by macrophages and some others are caught by the lymphatic system where they are probably destroyed. Only 10% of injected parasites can remain in the skin where they may develop into infective merozoites (59, 68, 69).

Successfully injected sporozoites will travel to the liver and invade hepatocytes, although it is still unclear of specific behavior of them when penetrating several hepatocytes before selecting one to reside within (70). Afterwards, when sporozoites matures in the hepatocyte to a schizont, it will produce dozen of merozoites and release it into bloodstream. However, in other *Plasmodium* species, such as *Plasmodium vivax* and *Plasmodium ovale*, they are more likely to delay the maturation during residing in the hepatocyte or remain as a latent or dormant from called a hypnozoite. Although *Plasmodium falciparum* is not considered to have this kind of latency form, but in fact they can reside inside hepatocyte as short as 48 hours in the rodent parasite and as long as 15 days in humans (71, 72).

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There is considerable variation in the maturity if the hepatic forms visibly on liver biopsy, even within a single experimental individual (73). Re-emergence of the hypnozoites have been documented up to 30 years after initial infection in human. The factor(s) that accelerate this reactivation is still poorly demonstrated (74, 75). The exact mechanism and time period by which parasite develop from the hepatic stages to the erythrocyte stages have, until recently, been obscure. It has been denoted that the parasite buds off the hepatocytes in merosomes containing hundreds of thousands of

merozoites. These merosome lodge in the pulmonary capillaries and slowly crumble there over 48-72 hours releasing merozoites (76, 77).

After introduction of merozoites in the erythrocyte, its nucleus begins to be lobulated. Initial form of developing merozoite is ring-shaped form and then to a larger trophozoite form. The terminal growth of it is schizont stage, which subsequently several divisions occur to produce new merozoites before freely traveling to bloodstream to infect new red blood cells. The source of their nutrient is hemoglobin and other materials from red blood cells and serum (77). Infected red blood cells tend to form clumps, called rosettes, and these have been linked to pathology by vascular occlusion. This rosette formation may be hindered by heparin, although in practical pharmaceutical use of this natural product is known to be risky due to heamorrhage (78).

Almost all the merozoites will continue an explicative cycle but a proportion of it experience sexual differentiation, being male or female (gametocytes), which will be carried out by the female mosquito. Five disparate morphological stages have been clearly documented (stage I-V). Female gametocytes are more likely to be dominant in production than male. Gametocytes emerge in the blood after a number of days post infection. Particularly, *Plasmodium falciparum* gametocyte arise after 7 to 15 days while it takes only 1-3 days on other species. Gametocyte half-life has been estimated to be between 2-3 days, although longer period of half-life has been reported for up to four weeks (79, 80).

Inside mosquito's midgut, the gametocytes undergo further maturation called gametes and self-fertilization, which producing motile zygotes called ookinetes. Of

those ookinetes, almost 50% of them encounter apoptosis within the midgut. In order to communicate each other, they form thin cytoplasmic extensions in the mosquito midgut. The ookinetes then escape from the midgut, invested themselves onto the exterior of the gut membrane. As in the liver, selection of appropriate cell will be more likely to be occurred. Division of ookinetes happen repeatedly until they successfully produce large number of tiny elongated sporozoites. After sufficient number of sporozoites have been produced, they travel to salivary gland of the mosquito where they are inoculated into subcutaneous tissue of the next human host (81).

Natural protection generated by inherent immune system restricts the asexual parasite density which eventually result in limited gametocyte production. However, direct effect of plasmodium induced host-immune response on gametocytogenesis have been evidenced. It was showed that addition of lymphocytes and sera from malaria-infected person have been successfully increased gametocyte production (82), as well as after supplemental anti-*Plasmodium falciparum* antibodies produced by hybridoma cell lines (83). Epidemiological studies have proposed evidence of triggering gametocytogenesis by a decrease in total number of red blood cells (RBCs) or hemoglobin in the blood (anemia) as showed by high proportion of gametocyte carriers observed among anemic individuals (84-87). However, these association may be underpowered by a bias of longer duration of infection which led to late gametocyte development. More conclusive data are from in vitro studies in which promotion of *Plasmodium falciparum* is supported by the presence of young RBCs or reticulocytes (88, 89).

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Level of severity of disease, probably lead to death, is highest among naïve young children and declines rapidly. Susceptibility of dangerous (life-threatening) clinical episodes of malaria continues but falls steadily so that when the person become adulthood febrile episodes are slight. Although the risk of being infected from malaria is continuously exposed throughout life. Occasionally, anti-disease immunity (acquisition of immunity to disease) precedes anti-parasite immunity (acquisition of immunity to parasite replication), however this phenomenon obscures the established insight of malaria biology: a naïve subject who experiences and survives the initial wave of parasitemia, subsequently followed by a balancing circumstance inside the host body but with persistent low-level asymptomatic parasitemia for long time periods. Thus, though with high susceptibility status of being infected with clinically severe malaria disease in children at the time, their normal state is to be asymptomatically parasitized. It means that at this stage acquisition of immunity is based on the survival of the parasite isolate after immunity clearance stage (strain specific immunity), leading to the idea that throughout life, exposure of parasite which lead to the increase of immunity is relatable to the repertoire of responses to many different isolates or to the development of a cross-protective response to shared antigenic targets. However, the development of acquisition of protective immunity is slowed down by antigenic polymorphism which is naturally occurred, but there is still considerable evidence that malaria infection modulates the host immune response (90-92).

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2.1.2. Epidemiology of malaria

In 2015, it was documented that worldwide malaria cases reached 212 million cases (range 148-304 million). Most of the cases are from African region (accounted for 90% of total cases), followed by the South-East Asia region (7%) and the Eastern

Mediterranean Region (2%). While death associated with malaria were an estimated 429.000 (range 235.000-639.000) worldwide. Hierarchical pattern of death related malaria is similar to the morbidity, whereby the African region possessed the most abundant deaths (92%), followed by the South-East Asia Region (6%) and the Eastern Mediterranean Region (2%). Incidence rate of malaria, between 2010-2015, dropped down by 21% globally. In line with global incidence rate of malaria, significant reduction by 31% was documented in the African Region. Similar phenomenon of remarkable reduction of malaria burden has been reported in other regions. Since 2010, the mortality rate of malaria experienced a gradual and continuous loss of number which is 58% in the Western Pacific Region, 46% in the South-East Asia Region, 37% in the American Region and 6% in the region of Eastern Mediterranean. The European Region have reported in 2015 that there was malaria-free in all 53 countries. Additionally, 5-aged year children are notably vulnerable to malaria infection and death. Malaria has killed, in 2015, an estimated 303.000 under-fives worldwidely, of those 292.000 deaths came from the African Region. Between 2010-2015, mortality rate of malaria among 5-aged year children dropped by approximately 35%. However, malaria remains a major killer of under-fives, the rate at which 1 child death every 2 minutes (1).

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In Indonesia, there is a marked reduction of population at risk from 4.1 per 1000 population in 2005 to 0.85 per 1000 population in 2015. The five highest number of annual parasite incidence (API) are Papua (31.93), Papua Barat (31.29), Nusa Tenggara Timur (7.04), Maluku (5.81) and Maluku Utara (2.77). There also decreased number of endemic areas from 17.4% in 2011 to 8.8% in 2015. A downward trend of the number of mesoendemic area also surprisingly happened from 18.6% in 2011 to 17% in 2015.

Followed by extreme reduction of hyper endemic area from 42.8% in 2011 to 28.8% in 2015. However, on the contrary, the number of malaria free areas were increased from 21.5% in 2011 to 45.4% in 2015 (3).

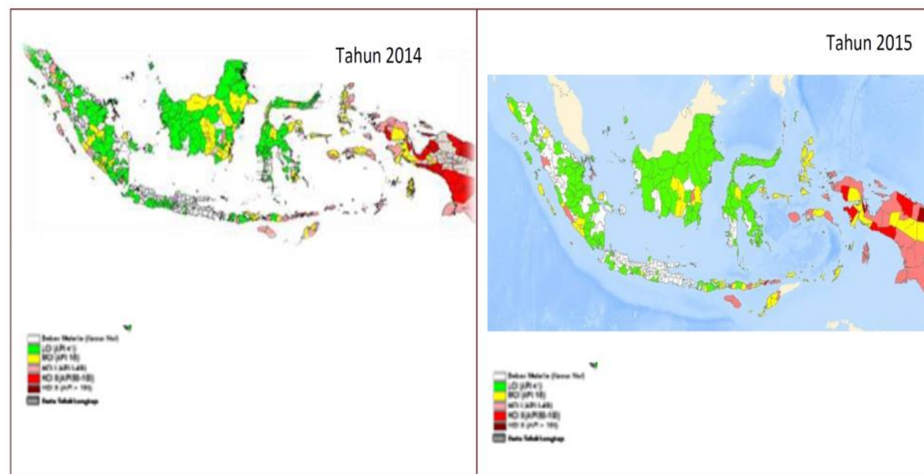

Figure 2. The map of endemic area of malaria in Indonesia from 2014-2015.

2.1.3. Indonesian policy to malaria treatment (93)

2.1.3.1.Treatment of *Plasmodium falciparum* and *Plasmodium vivax*

Currently, the treatment for *P. falciparum* and *Plasmodium vivax* is using dihydroartemisinin+piperaquine (DHP) plus primaquine. The dose of DHP to *Plasmodium falciparum* is similar to *Plasmodium vivax*. Primaquine is only given for the first day of treatment only for *Plasmodium falciparum* with 0.25 mg/kg body weight dose, while for *Plasmodium vivax* is 0.25 mg/kg body weight for up to 14 days. Primaquine is prohibited to be given to a baby aged < 6 months.

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2.1.3.2.Treatment of *Plasmodium vivax* relapse case

The treatment given for relapse case of *Plasmodium vivax* is with the same dose of DHP but with increased dose of primaquine to 0.5 mg/kg body weight.

2.1.3.3.Treatment of *Plasmodium ovale*

Currently, for the treatment of *Plasmodium ovale* is by giving DHP along with 14 days of primaquine medication. The dose of treatment is the same to those given for *Plasmodium vivax*.

2.1.3.4.Treatment of *Plasmodium malariae*

The treatment for *Plasmodium malariae* is by giving a single dose of DHP for up to 3 days of medication with the same dose as the other malaria parasite.

2.1.3.5.Treatment of mixed infection between *Plasmodium falciparum* and *Plasmodium vivax/ovale*

In the case of mixed infection, the patient needs to be given a regular dose of 3 days DHP treatment with addition of primaquine with dose of 0.25 mg/kg body weight for up to 14 days.

2.1.3.6. Second line treatment

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Intravenous artesunate is the main choice to treat an adverse infection of malaria in Indonesia. If it is not available, then the patient can be treated with quinine drip.

2.2.Antimalarial drug

2.2.1. History of antimalarial drugs

2.2.1.1. Quinine

The bark of cinchona trees was known as natural source of quinine compound, which primarily found in high altitudes of South America. Initial introduction of cinchona bark into Europe as an ague treatment was in the early 17th century. Indeed, existence of malaria is unknown in the New World prior to Columbus (94, 95). There

have been an endless debate regarding to the medical use of cinchona bark in those moment due to lack of knowledge which limited to symptom manifestations (96).

A Spanish countess, her name is Chinchon, are credited with introducing the bark to Spain, as hypothesized, this story might be fallacious like many other myths. Therefore the name of cinchona was given to the bark by Linnaeus in 1742 (97). Various European physicians and quack doctors had exceptional success with the bark (98), though initially faced a various skepticism by which its original initiator and discrepancy of other species of the trees (99, 100). Those discrepancy of cinchona species have led to broad variation in quinine content and thus caused inconsistent consideration of an administration of the bark.

Richard Morton, who published his *pyretologia* in 1692, claimed that the bark was a “Herculean antidote” which if it is given in proper dosage, usually reverted the patient to health shortly. Further, his idea was developed by Francesco Torti which indicates only certain fevers could be treated by cinchona and became remarkable invention (101). By the late 18th century, standardized formulations are reported and cinchona was more worldwidely admitted as a treatment for particular fevers or its manifestation (96, 102).

In the 18th century, cinchona had become prevalent and in that time few of cinchona trees begin to be extinct (99, 103). The alkaloids quinine and cinchonine from cinchona bark have managed to be isolated by two young French in 1820 and followed by successful treatment for variety of intermittent fever by using pure quinine. Afterwards, high quinine content of *Cinchona ledgeriana* have been specified and within a short time Dutch government were producing 97% of the world’s supply of

quinine. Eventually, between mid-19th to the 1940s, quinine widely used as a standard therapy for intermittent fever worldwide (102, 104).

2.2.1.2. Synthetic antimalarials

A broad revolution of synthetic organic chemistry was partly in reaction to the necessity of new antimalarials. William Henry Perkins, a British chemist, have failed to synthesize quinine (which indeed its discovery was not accomplished until 1944) but he managed to produce “mauve”, the first synthetic textile dye (99). Since the new dye industry has merely raised, methylene blue was mainly effective in staining malaria parasites, and those parasites known to avidly take up the dye and being poisoned *in vivo*. Thus, Paul Ehrlich, a German scientist, cured two patients of malaria using methylene blue, and this was the first time a synthetic drug ever used in humans.

Bayer, one of the leading German dye companies, had tried to develop new synthetic antimalarial using methylene blue as prototype. In 1925, the developed plasmoquine (aslo called pamaquine). The first compound that able to prevent relapses in vivax malaria have been discovered, it is called plasmoquine, the first 8-aminoquinoline. Another successful discovery is mepacrine (atebrine) which was effective against falciparum malaria in 1932. In subsequent year, in 1934, Bayer again developed a compound known as resochin, though it is too toxic. A synthesized derivate of resochin known as sontochin which was less toxic (105, 106).

During World War II, world supply of quinine was cut off, and thus have led to a large-scale attempt to discovery new synthetic antimalarials. Those efforts yielded to sixteen thousand compounds which were synthesized and tested. For the second time allied scientists have tried to test resochin (it had acquisition number SN-183) which

still was considered too toxic. However, scientist's interest to resochin have led to production of chloroquine (and renumbered SN-7618). Us clinical trials in 1946 exhibited that this particular compound was far superior to atebrine (105, 106). The ultimate discovery of chloroquine as a remarkable antimalarial is one of the most fascinating stories in the history and development of synthetic drugs.

Chloroquine verified to be the most effective and main antimalarial ever and was used worldwidely. These success followed by wide scale prophylactic use of chloroquine known as "Pinotti's method" (a strategy of putting chloroquine into common cooking salt), though its main choice in the WHO Global Eradication Programme is overshadowed by the widespread use of residual insecticide DDT, chemoprophylaxis or chloroquine-mediated salt and eventually hampered by a widespread of chloroquine resistance (107).

After introduction of chloroquine which is known to be remarkably advance made during World War II, another attempt has proposed to produce more effective versions of the 8-aminoquinoline plasmaquine. Thereafter, those efforts resulting in introduction of primaquine which proven to be the standard drug for vivax relapses prevention (106). 50 years after, another promising 8-aminoquinoline have been discovered that is WR 238605 or tafenoquine (108). After that, several other promising compound have raised to the discovery of proguanil (paludrine), which is served as prototype of pyrimethamine (Daraprim) development in 1950 by Burroughs-Wellcome (106). Thus, a combination of sulfadoxine and pyrimethamine called fansidar have been surprisingly produced (109).

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During the American war effort later led to discovery of several other compounds, one such compound, SN 10275, was a prototype for mefloquine which is introduced in the mild-1970s (110). Subsequently, the 2-hydroxynapthoquinones also have been found during World War II. These provided as prototypes for a recently discovered drug, Atovaquone which is used in combination with proguanil and sold as Malarone (111).

2.2.1.3. Artemisinin

Sweet wormwood or qinghao which its species name is *Artemisia annua* was initially utilized by Chinese herbal medicine practitioners for up to 2000 years to medicate hemorrhoids (112). The government of the People's Republic of China, in 1967, have conducted a large-screening of traditional remedies in an attempt to leverage traditional medicines (113). Thus, qinghao have been found to be potential as antimalarial drug which have a high activity and therefore in 1972, the active ingredient was purified and named qinghaosu (essence of qinghao). Various studies that have been published between 1970s and early 1980s have been managed to test these quinghaosu derivatives on thousands of patients (113, 114). Artemisinin derivatives are now widely used in South-East Asia and indeed, widely spread through the world (115).

2.3.The spread of antimalarial drug resistance

The emergence of resistance in Plasmodium depends on multiple factors. The mutation rate of the parasite has a direct influence on the frequency at which resistance can occur, thereby the higher mutation is then the faster resistance can emerge and could also lead to an accumulation of advantageous mutation (116, 117). Since mutations correlated with resistance to a particular drug regime often impart a fitness cost, the

selective advantage acquired by becoming drug resistant is balanced by the biological cost arising from the altered function of the mutated protein, and such a fitness cost can be alleviated by the acquisition of compensatory mutations during prolonged drug pressure (118). The existence of newly emerged drug-resistant can also be accelerated by strong drug selection pressure, improper dosing (yielded from inadequate drug exposure), poor pharmacokinetic properties and fake drugs, which decrease the prevalence of competing drug-sensitive wild type parasites (119). Transmission is another critical circumstance which its intensity has a remarkable role in determining if parasites are effectively transmitted during a mosquito blood meal (120).

Quinine is currently used for treatment of severe malaria cases, as a second line treatment, and in combination with antibiotics to treat resistant malaria. Relative short half-life of 8-10 hours probably contributed to the dearth of widespread quinine resistance; though, indication of quinine resistance have already been reported in vivo (121, 122). These reports of resistance are rarely found, but isolated cases have been notified from Thailand and East Africa (123, 124). Recent report of quinine resistance was from North India, where a 5 days failure after treatment was experienced by severe malaria patients (116). However, the sporadic observation of quinine resistance might be related to improper dosing or quality of drug rather than parasite resistance (125).

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Introduction of Chloroquine in the late 1940s, which is known as 4-aminoquinoline derivatives, became a gold standard of malaria for many years, it is because its relative longest half-life among other antimalarial (60 days) (126). These long half-life can be advantageous by becoming chemoprophylactic effect during the drug elimination phase, but simultaneously could be deleterious by exposing parasite

with prolonged time period and thus may enhance the number of selective drug-resistant parasites (127). Teen years after chloroquine introduction, parasites resistant to this drug begin to emerge and spread rapidly, which is its first report came along the Thai-Cambodian border and also in Colombia in the late 1950s (128). Wide transmission of these particular resistant strain from South-East Asia to Africa have been confirmed by genetic epidemiological data in the late 1970s. Moreover, independent emergence of resistance to this drug have come from other foci, including Papua New Guinea and the Philippines (128). And thus, chloroquine resistant parasites now being widely distributed in nearly all malaria endemic regions.

Another a 4-aminoquinoline which is structurally similar to chloroquine that is Amodiaquine, with short half-life (3 hours, and thus it hypothesized that monodesethylamodiaquine have exerted primary metabolite up to 9-18 days) have been used for more than 70 years (127-129). It might be due to its similarity to chloroquine then cross-resistance between these two drugs have been reported and associated to mutations in *PfCRT* and *PfMDR1*, however, the cross-resistance seem to be partial because some chloroquine resistant parasites remain susceptible to amodiaquine (129). Currently, Amodiaquine is recommended to be applied in combination with artesunate for malaria treatment (17).

Mefloquine is included as a 4-methanolquinoline with a long half-life of 14-18 days (127), which have been first introduced in the 1970s (130). Copy number of *pfmdr-1* is known to be associated with mefloquine resistance, which in turn leading to overexpression of this resident DV membrane transporter (131, 132). Exact mechanism of action is yet fully understand, which *in vivo* study suggested that mefloquine can

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bind to heme and exert some antimalarial activity by inhibiting heme detoxification (133, 134).

The bis-4-aminoquinoline piperaquine has a prolonged half-life of roughly 5 weeks. Its resemblance to chloroquine have led to hypothesis that their mode of action is similar, though the mechanism is yet precisely defined, studies have suggested that piperaquine accumulates in the DV and that is a potent inhibitor of heme polymerization (135, 136). In the late 1980s, due to its use as monotherapy, eventual emergence of Piperaquine-resistant parasites have been reported and thus diminished its utilization (10). Subsequently, it is combined with ACT program consisted of dihydroartemisinin (DHA), piperaquine (PPQ) and primaquine (PQ) which resulted in high cure rates (9). Revised version of WHO guideline have recommended PPQ administered with DHA, and therefore this combination has undergone successful clinical evaluation in both Africa and Asia (9, 11-13).

Lumefantrine (previously known as bunflumetol) is structurally allied to the hydrophobic arylamino alcohol antimalarials which has 3-5 days half-life and absorption of this lipophilic drug could vary between individuals (137), necessitating the high-fat meal co-ingestion to elevate its oral bioavailability (138). The field resistant isolates to this drug is yet convincingly demonstrated, though inverse correlation between lumefantrine and chloroquine susceptible is quite interesting (139).

First introduction of sulfadoxine-pyrimethamine (Fansidar) was in 1967 to many parts of Arica which has approximately 4-5 days, known as a highly effective, inexpensive, well tolerated drug with good compliance rates as a single dose (21). Unfortunately, resistance developed within few years due to point mutations in both

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target enzymes, facilitated by the slow elimination of Fansidar from the body (140, 141). Therefore, sulfadoxine-pyrimethamine is now mainly utilized as intermittent preventative malaria treatment during pregnancy and its use as combination therapy with other drugs is no longer recommended (142).

Since the emergence of resistance to almost all quinolone and antifolate drugs in Plasmodium malaria, a unique trioxane structure with an endoperoxide bond derived from *A. annua* plant have been produced (134). Due to the low solubility of artemisinin, several semi-synthetic derivatives are used clinically (artemether, artesunate and dihydroartemisinin) with combination strategy as follows: artemether plus lumefantrine, artesunate plus amodiaquine, mefloquine or Fansidar and DHA-PPQ (17, 143). Initial widespread utilization of artemisinin derivatives as monotherapy in South-East Asia has likely become a major cause for the reduced efficacy in recent years in the Thai-Cambodian border characterized as delayed parasite clearance time (30, 32, 144). Special strategy has been established by WHO in the Cambodian affected area to prevent the potential widespread across borders and for containment of the resistance (145). Strategic planning for frequent screening at sentinel sites and resistance management is hampered by limited understanding in terms of molecular mechanism of resistance and molecular markers (146).

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2.4.K13-propeller domain

PfK13 is located in chromosome 13 of *Plasmodium falciparum* and encodes a 726-amino acid protein (PfK13) consisting of a poorly conserved Apicomplexa-specific N-terminal region and three annotated, highly conserved domains: a coiled-coil-containing (CCC; amino acids 212-341), a BTB (Broad-complex, tramtrack and bric-

a-brac; also known as BTB/POZ; amino acids 350-437) and a C-terminal Kelch-repeat propeller (KREP; amino acids 443-726) which harbors nearly all PfK13 alleles associated with artemisinin resistance (147). The Apicomplexa-specific N-terminal domain is predicted to exhibit a random-coil formation. The crystal structure of BTB and KREP domains was solved; the CCC domain is expected to form two helices coiling together (147).

Putative gene (*PfKelch13*) for artemisinin resistance have been described by Ariey *et al* in 2014. These researchers have been managed to produce artemisinin-resistant parasite from a 5 years study through 125 repeated cycle of artemisinin selection generated from the artemisinin-susceptible F32 Tanzania clone. Subsequently, the produced resistant parasite and its germinal line recognized eight SNPs in seven genes by whole genome sequencing. Causative agent of artemisinin resistance, among these SNPs, was concluded to the M476I mutation in *PfKelch13*. Significant correlation further confirmed between four mutation of *PfKelch13* (Y493H, R539T, I543T and C580Y) were closely associated with elevated RSA survival rate (34).

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Afterwards, straight evidence of relationship between *PfKelch13* alteration with artemisinin resistance was noticed (148, 149). These report explained a single mutational alteration (M476I, Y493H, R539T, I543T or C580Y) of *PfKelch13* gene in an artemisinin-susceptible *Plasmodium falciparum* clone (Dd2) enhanced RSA survival rates, likewise revertant of relevant mutational sites (R539T, I543T and C580Y) affected a significant decrease in the RSA survival rates (149).

Generating *PfKelch13* genotypes map represents a geographic-motif of distribution, which so far there are around 100 nonsynonymous mutations of *PfKelch13* have been

reported. Five abovementioned mutational alteration of *PfKelch13* are exclusively distributed in Cambodian lines. In this context, due to earlier introduction of artemisinin to these regions may be the plausible explanation. C580Y has been prominently prevalent in the field population which showed phenotypically a 13.6-fold increase in the level of artemisinin resistance (149). Eventually, WHO have reported 15 mutational sites of K-13 which is validated or candidate, the validated genotypes are N458Y, Y493H, R539T, I543T, R561H and C580Y. They concluded that though the delayed response to artemisinin in some areas but generally the drug is still acceptable by the fact most of the patients with delayed parasite clearance are cured, and as long as the partner drug remains effective.

There are four major hypothesis of artemisinin resistance currently described. (1) elevated PIP3 production; initially, the PfPI3K wild-type parasite, during exposure of artemisinin, will undergo 48-linked ubiquitination and subsequent proteasome degradation of PfPI3K caused by a binding of PfKelch13 to PfPI3K, on the contrary, resistant-lines will be bindless and no such degradation occur. These mechanism of PIP3 hypothesized to have a contribution to cell survival signaling pathway (150, 151). (2) Up-regulation of ubiquitin/proteasome system; generally, inhibition of protein translation followed by protein degradations is particularly regulated by the ubiquitin/proteasome system (152, 153). Up-regulated ubiquitin/proteasome system was occurred in the artemisinin-resistant parasites rather than susceptible one (154, 155). (3) Activation of unfolded protein response pathway; most up-regulated genes in the resistant strain can export proteins, these up-regulation was associated with unfolded protein response to react the endoplasmic reticulum stress, defining a mitigation of potential protein damage by artemisinin (156-158). (4) Lower production

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of damaged protein; the accumulation of damaged protein can be beyond of parasites capacity to maintain proteostasis and lead to death. Therefore, growth retardation of resistant strain parasites in the ring stage could lead to less activated artemisinin by less hemoglobin digestion thus damaged protein are under proteostasis and survival rate of parasite can achieve higher than susceptible one (155).

2.5.Plasmepsin II

In *P. falciparum*, up to ten Plasmepsins have thus far been identified, namely *Pf*PMs 1, 2, 4–10 and *Pf*HAP (*H*isto-*A*spartic *P*roteinase). These PMs, encoded by genes located in five different chromosomes, are composed of the pro-segment and the mature enzyme domain. Of note, *pfpm4, pfpm1, pfpm2* and *pfhap* cluster in a 20-kb-long region of chromosome 14, and share a high amino acid sequence identity (159). Studies have suggested expression of Plasmepsin I, II, IV, V, IX and X in the erythrocytic stage whereas Plasmepsin VI, VII, and VIII being expressed in exo-erythrocytic stage. Plasmepsin I, II, and IV belong to A1 family aspartic proteases having dual aspartate active site configuration. Like all aspartic proteases, Plasmepsin involve a catalytic water molecule and generate a "tetrahedral intermediate" while acting upon their substrates. Plasmepsin along with cysteine proteases and metalloproteases function in acidic food vacuole to participate in orderly Hb degradation pathway. Plasmepsin I and II are 75% identical in sequence and cleave the Phe33-Leu34 peptide bond of Hb which is considered to be the critical first step in the degradation pathway. Plasmepsin I readily cleave native Hb and Plm II has a preference for acid-denatured globin (160).

Derived from Cambodian isolates, in the Greater Mekong subregion where clinically treatment failure of concurrent artemisinin along with partner drugs have been

noticed (161-166), *Plasmepsin II* and *plasmepsin 3* gene amplifications on chromosome 14 successfully described in accordance to piperaquine resistance. Additionally, another marker aslo has been reported which resides on chromosome 13 (*exo-E415G*) to be associated to piperaquine that undergo significant linkage disequilibrium with *Plasmepsin II-3* (36). Genome wide study was then used to achieve greater degree of association, which resulting in significant correlation between resistant parsites in vitro with *Plasmepsin II*. Strengthening the fact, dihydroartemisinin-piperaquine failure clinically was associated with *Plasmepsin II* multi copy (35). However, thought the invention of *plasmespin 2-3* as clinically and in vitro associated marker, *exo-E415G* also came with identical evidence of piperaquine resistance (36).

These reports evidenced that amplification of *Plasmepsin II-3* becoming such a robust marker for piperaquine. Transfection study of *Plasmepsin II-3* through laboratory strains will enhance current knowledge of exploring underlying mechanism of piperaquine resistance. A plausible explanation of emergence of piperaquine resistance laid on the fact that previous emerging resistant strain to relatively short half-life (3 days) artemisinin have led to a greater residual parasite biomass exposed to the partner drugs (167) which eventually allowing drug resistant strain selection (161). This also due to the pharmacokinetics properties of piperaquine that has an initial relatively rapid plasma clearance followed by a long terminal half-life (2-4 weeks) (168), these sequence of exposure by which minimal increase of parasiticidal concentrations come after initial concentrations will yield in a much shorter period of time parasites undergoing adequate parasiticidal concentrations, and then eventually to selection of resistant.

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2.6.Dihydroartemisinin+piperaquine failure and its spread

Initial investigation of efficacy of dihydroarteminin-piperaquine (artekin) in Cambodia provided successful rate of clearing parasite in natural population (169). However, this satisfactory result is not last until reduction of efficacy of artekin have been observed in the western of Cambodia several years after (162). Although from recent report in China-Myanmar border, satisfying efficacy result have been documented, though gametocyte clearance rate need to be increased with additional dosage of primaquine (170). It is likely the China-Myanmar border not affected by recent circulation of *Plasmodium falciparum* resistant lineage from the epicenter of drug resistance. This is due to the fact of recent circulating *Plasmodium falciparum* resistant lineage to artemisinin in the epicenter of drug resistance which its report has been increasing during the year of 2016-2017.

In 2009, initial signature of artemisinin resistance was described by clinical investigation of field isolate in Western Cambodia and northwestern Thailand. This study convincingly demonstrated slow parasite clearance in their study sites which is an indication of reduced susceptibility to artemisinin derivative (32). Afterwards, in order to obtain more detail information, a study was conducted to measure parasite clearance rate in field population of the same region and resulted in a finding of delayed parasite clearance with additional information regarding certain suspected chromosome that responsible for the phenotypic trait of the parasites (171). Confirmative result of a study in Central Vietnam a year after suggested that artekin was satisfactory in the area and no indication of resistant parasite expansion from its neighboring (172). It was hypothesized that these delayed parasite clearance phenomenon is multifactorial including the host, parasite and drug factors which have equal contribution to delay the

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clearance and subsequent failure of treatment (173). Additionally, this delaying event was associated with increased gametocyte carriage and, therefore, presumably in line with increased transmissibility of drug resistant phenotype, particularly in the epicenter of drug resistance (84, 174, 175).

First attempt to emphasize the detrimental effect of rapid evolution of resistant parasite was satisfactorily exhibited by the fact that therapeutic failure of artemisinin in vivo was considerably high in Western Cambodia which shows initial finding for subsequent investigation afterwards (165). This was strengthened by a multisite prospective cohort study in Cambodia indicating elevated parasite capacity to resist to both artemisinin and its partner drug and resistant parasite in this area was well established (166). Subsequent report indicated that piperaquine IC_{90} in the area of Cambodia was greater than the upper limit in 2013, and this rate doubled to 80% by 2015, contrary to mefloquine which exhibits IC_{90} declining to median level. This rapid progression of piperaquine resistance was also closely associated with dihydroartemisinin-piperaquine treatment failures in northern Cambodia limits alternative medicine in this region (176). Though pharmacokinetics studies suggested that small children more likely to have lower piperaquine exposure than larger children or adults which may play a role in delaying parasite clearance, however this is true only to African children not Asian children where slower parasite clearance have been evenly scattered (177, 178). Surprisingly, though Cambodia was the epicenter of drug resistance low parasite prevalence was confirmed in this region which mutation in the K13- propeller domain gene (C580Y) were highly prevalent in both symptomatic and asymptomatic cases. Thus, asymptomatic individuals could be an important reservoir for artemisinin resistance (179).

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2.7.Flanking region of artemisinin resistance gene

Over the past decades, several antimalarial drug resistance gene as a potential marker have been developed successfully to respond essential needs against development of resistant parasite. It is clearly demonstrated that *Plasmodium falciparum* have been resistant to all partner drugs of ACT (artemisinin combination therapy) including abandoned drugs due to highly ineffective (quinine or even synthetic antimalarials). In the early of twentieth century, massive regimen changes and deployment of ACT was highly recommended by WHO due to avoiding development of resistance by implementing different mode of action of artemisinin and its partner drug thus complicates the progress of parasite to resist. As indicated by previous section, it was successfully and satisfactorily proven to be highly effective against plasmodium in natural population until resistance arisen in the recent years. Previously, molecular studies have been monitoring the progress of partner drug resistance marker of ACT and its possibility of spread into larger territorial areas. This is due the fact that resistant parasite can be reverted back to susceptible when the pressure is relaxed. However, this direction of research in the past decade have been underestimated since the main and the only effective drug against multidrug resistant plasmodium, that is artemisinin, known to be highly ineffective. Therefore, during the year of 2016-2017, the researchers' works have focused on the evolutionary forces against artemisinin resistance and finding the fact of positive selection.

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Drug resistance is a common inhibitor of the success of malaria elimination all this time. Once a newly arisen mutant has been introduced in the population, and when favorable circumstance of the environment (stable transmission, stable drug pressure, etc.) is exist, it will be rapidly spread throughout the population. Mutant allele that

under selection will cause recombination of flanking region of the mutant associated gene. Hence, genetic hitchhiking will play a role in reducing genetic diversity in the flanking region of the coding region of the resistant gene, both upstream and downstream. At this point, these advantageous mutations will increase linkage disequilibrium of the flanking region. A common pattern starting from soft selective sweep and then eventually become hard selective sweep, when the beneficial mutation gets fixed in the population and leaves no genetic diversity. Therefore selective sweep and hitchhiking are used to track the emergence and spread of drug resistant alleles in the natural population by using various molecular marker including microsatellite (the most popular marker for population genetic) (180-183).

Some of molecular markers were already widely known and the spread of them also was already well documented. *Plasmodium falciparum* chloroquine resistance (*PfCRT*), *Plasmodium falciparum* dihydropteroate synthase (*Pfdhps*) and *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) genes, responsible to chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) resistance respectively, were worldwidely well-reported regarding their mutational epidemiological spread and evolution. Microsatellite analysis around these alleles revealed that CQ and SP resistance independently originated starting from limited range of areas and later spread throughout the world, suggesting the role of gene flow in the evolution of drug resistance. Microsatellite also can be beneficial for validation of hypothetically selected allele responsible for resistance caused by evolution, as exhibited by 76T mutation inside *PfCRT* which act as a full-proof ‘genetic marker’ for classifying CQR in *Plasmodium falciparum* displaying lesser genetic diversity than those present in far-away flanking regions on both sides of this gene (184, 185). Different levels of selection

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pressure can affect to the extent of hitchhiking of microsatellites surrounding *PfCRT* gene in natural population (185). Independent emergence of CQR have been inferred from population genetic data which suggest independent lineage of South America (186, 187), PNG (188) and Philippines (189). These data have exhibited the similarities of the parasites from Asian and African origins, but they differ from parasites from South America and PNG, supporting the previous hypothesis that parasite migration must have played a critical role in the distribution and spread of CQR (190). Other evidence come from sulfadoxine-pyrimethamine resistance associated gene, which revealed that there are three independent origins of triple mutant *pfdhfr*, one from South-East Asia and two from South America (185, 187, 191-194). A vast majority of the triple mutant alleles found in Africa originated from South-East Asia suggesting that, like CQ resistance, pyrimethamine resistance associated alleles was also migrated and introduced into Africa from South-East Asia (191, 192, 194-200). Unlike South-East Asian parasite which have single origin, local evolution of the *dhfr* alleles has been reported in Africa, thus African lineage originated twice (185, 191, 194, 197-199, 201, 202). Recent evidence suggested another independent origin of the double mutant *dhfr* alleles (59R/108N) in Papua New Guinea (203). Therefore, taken together, similar to CQ resistance at least four major distinct origins of pyrimethamine resistance have been well-documented worldwidely.

Recent report of scientific works has revealed the emergence of artemisinin resistance in the South-East Asian countries, especially the Great Mekong subregion (epicenter of antimalarial drug resistance), thus severely threatens worldwide malaria control. This serious threat is particularly underlined by previously widely spread of chloroquine and sulfadoxine-pyrimethamine resistant *Plasmodium falciparum* lineages

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from their origins in South-East Asia to India and Sub-Saharan Africa (204). Additionally, previous works suggested, resembling chloroquine and sulfadoxine-pyrimethamine resistant lineages, that multiple lineage of artemisinin resistant *Plasmodium falciparum* were circulating (204, 205), which led to alteration of strategy focusing on containment approach to elimination only (206). However, as exhibited by evolution and expansion of CQ and SP resistant strain in the past, most probably a single parasite strain eventually dominated in the GMS, before spreading to India and Africa (128). In order to test the hypothesis of a single origins of artemisinin resistant parasite, a group of researchers examined the microsatellite loci flanking *PfKelch13* (40, 207) as well as the partner drug piperaquine marker through evaluation of copy number of *Pfplasmepsin2* gene which recently identified (35). The dominant haplotype of C580Y was present at rate of exceeding 73% in Southern Laos, Northeastern Thailand and Western Cambodia. Furthermore, a unique mutational pattern of *Pfplasmepsin2* amplification only present in conjunction with the C580Y mutation in Western Cambodia and Northeastern Thailand, where *Pfplasmepsin2*, where multi-copy of *Pfplasmepsin2* was present in 71% and 100% of isolates, respectively. This is supporting the previous hypothesis about the domination of one lineage of artemisinin-resistant *Plasmodium falciparum* C580Y which probably arose in western Cambodia and then spread to Thailand and Laos, outcompeting other parasites and acquiring piperaquine resistance (42). Therefore, the finding of a dominant artemisinin resistant haplotype associated with partner drug resistance has implications for malaria control and ACT resistance containment strategies.

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2.8.Gametocyte in parasitized patient and antimalarial drug resistance transmissibility

Advantageous features are possessed by certain malaria parasite carrying antimalarial drug resistance gene. Drug transport and binding capacity is affected by mutational location in certain genes of plasmodium species, establishing phenotypic ability of the parasite to resist against antimalarial drugs (207-210). Advantageous selection of such mutant genotypes is responsible for the multiplication of resistant parasites (128). Besides conferring resistance to antimalarial drugs, the emergence and presence of specific/key mutation in the parasite may magnify the possibility of gametocyte transmissibility (211-213). Various findings have suggested that there is an increase in gametocytemia following treatment with antimalarial drugs which supported the previous hypothesis of increased transmissibility (214-230). Artemisinin itself, the active principle of artemisinin derivatives, has a gametocytocidal effect on *Plasmodium falciparum*; however, its activity is restricted to only early stages of gametocytes (231). Although artemisinin-based combination therapy (ACT) proposes higher clinical efficacy against *Plasmodium falciparum*, reduces the rate of resistance development and lowers gametocyte carriage and density (232-235), its effect on malaria transmission is moderate and limited to the duration of gametocyte carriage and the proportion of mosquitoes infected by carriers (236). Sutherland et al. (2002) (221) reported increased *in vivo* gametocyte production with increased infectivity by drug-resistant parasites. Another supportive fact has shown a 5-fold increase of *in vitro* gametocytogenesis of *Plasmodium falciparum* in response to chloroquine treatment (215). Increased gametocyte density yields in elevated mosquito infectivity and hence enhancing parasite transmission (214, 237, 238). There is possibility of higher

gametocyte prevalence and density in resistant than sensitive *Plasmodium falciparum* strains, assisting to the survival advantage and spread of resistant strains (219, 220, 239, 240). A host infected with resistant genotypes tend to be of a mixed type, containing both wild-type and mutant genotypes. Antimalarial treatment allows the resistant strains to fill in the ecological space offered by the elimination of sensitive strains, contributing to the competitive spread of drug resistance (240). Taylor et al. (1997) (238) reported greater infectiousness of mixed-genotype than of a single-genotype infection.

Taken together, suggested evidences of the effect of mixed genotypes on the infectivity of gametocytes, incomplete clearance of resistant *Plasmodium* parasites and increased gametocytemia after antimalarial chemotherapy, hence it is required to formulate a new calculation of the potential resistant-genotype transmissibility. Previous correlation has been demonstrated regarding the presence of resistant genotype of *Plasmodium* species with a parasite density that takes into account the asexual stages of the parasite but not gametocyte. However, in order to reduce disease transmission and for prevention strategy of advantageous selection of resistant parasites in its spread, gametocyte clearance should be a specific target (228, 241). Evaluation of the impact of antimalarials on gametocytemia, studies commonly use the gametocyte carriage rate on a weekly basis for a month after treatment (86, 211, 220, 222-224, 242-244). Mendez et al. (2006) (241) utilized a further developed approach to evaluate *Plasmodium falciparum* transmission potential, based on the area under the curve for posttreatment gametocyte levels, to incorporate their length of time and magnitude with transmission.

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Insights of the genetic basis of antimalarial drug resistance have been provided by genome scanning of malaria parasite (245). Since molecular markers for certain antimalarial drug resistance have been established and validated, it is now possible for designing a simpler formula that readily used in field setting to be epidemiological tools for antimalarial resistance. Gametocyte is often difficult to be detected since its lower densities than asexual stages, however by adopting such molecular approach the detection of sexual stage of malaria parasite is enhanced significantly (246). Therefore, highly sensitive gametocyte-specific molecular detection technique makes it feasible to use such technique in conjunction with epidemiological investigation of the spread of antimalarial drug resistance (246-252). Given that infectious reservoir is larger than revealed by microscopy at various endemic setting (226, 248, 249, 253), molecular technique would enhance the detection of submicroscopic gametocyte carriage, which could be of great interest in interventions targeting malaria transmission (251, 254-256). Although there is a direct correlation between gametocyte density and infectiousness of mosquitoes (257-259), gametocyte infectivity to mosquitoes at low densities remains higher than that predicted by simple linear relationships (246). Detection of silent gametocyte carriers in conjunction with validated resistance alleles to antimalarial drugs could be a great opportunity to provide significant information regarding adoption of appropriate strategies toward the control and elimination of malaria. Therefore, designing feasible and accurate formula to determine the potential transmission of resistant *Plasmodium* genotypes based on parameters generated through the use of molecular technique, genotypes and gametocytes, would be valuable and beneficial for epidemiological studies.

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Abdul-ghani et al. (2014) (260) have proposed a simple and sophisticated formula without using complicated mathematical model to measure the potential transmission of drug-resistant-genotype of *Plasmodium* for surveillance purposes. Since various molecular marker for antimalarial drug resistance, including artemisinin the newest one, have been well-documented, it is now possible to combine parameters that could theoretically predict the potential transmission of resistant genotypes. In these present perspective, two variables that particularly should be considered: presence of resistant genotypes with gametocytes and presence of resistant genotypes without gametocytes. Therefore, the suggested formula will be as follows:

Potential transmission of resistant genotype at a particular point in time = $\frac{Rg}{Rt}$

Where Rg = frequency of resistant genotypes in samples with gametocytes and Rt = total number of samples with resistant genotypes; Rt = Σ Rg + Rn, where Rn is the frequency of resistant genotypes in samples without gametocytes. The output of the above formula could be expressed as a fraction of 1.0, where 1.0 denotes 100% potential transmission and 0.0 denotes no potential transmission.

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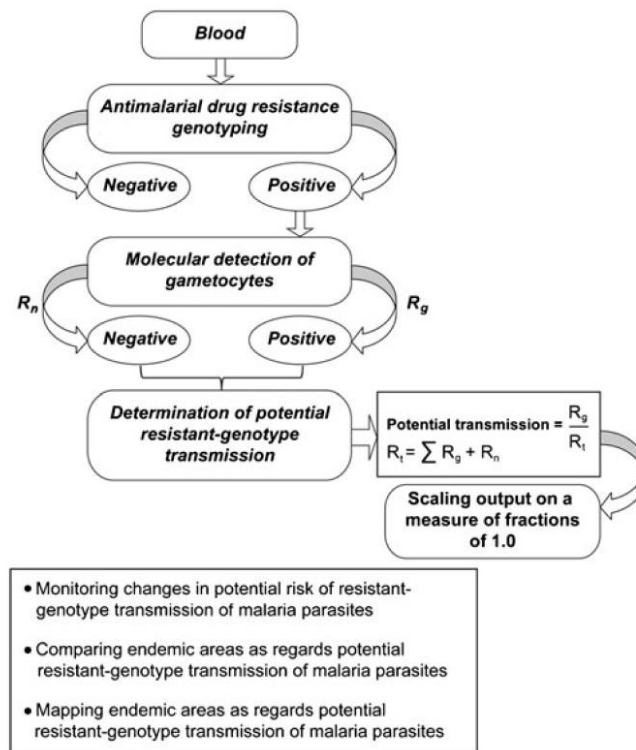

Figure 3. Flowchart for determining potentially transmissible resistant genotypes of malaria parasites using Rashad's devised formula

2.9.Individual and environmental determinants of malaria incidence

2.9.1. Global finding of risk factors associated with malaria incidence

Malaria is such a disease that is not solely transmitting themselves, rather require a specific vector to successfully inject themselves to the host body. Theoretically, based on causal concept of epidemiology that the occurrence of diseases depends on three necessary factors: (i) the host, (ii) the agent, (iii) the environmental factors. All factors related to the agent have been widely described in the abovementioned chapter. Therefore, this chapter will particularly discuss about the factors related to the host and the environmental constrain.

Studies in Amhara region, northwestern part of Ethiopia, suggested an insight of key factors deriving malaria risk. In general, there are three factors have been

recognized to be the key factors deriving malaria risk: climate variable, entomological parameter, and human population dynamic. The impact of climate is inevitably accounted as a potential circumstance in malaria transmission, which has known to be enhancement factor of malaria transmissibility due to increasing vector capacity by providing potential source of breeding place, increasing mosquito longevity and feeding rates. More detail information, climate change tend to alter above-average temperature and precipitation, which in case of precipitation can result in prolonged occurrence of water bodies that support laid eggs of female mosquitoes for successful larval development (261, 262). Although in a reverse way, excessive precipitation could also reduce mosquito population density by providing harmful environmental circumstance for the larvae (263). Additionally, increased temperatures from an impact of climate change is more likely to enhance the development rate of both the mosquito larvae and Plasmodium parasites (264). As a result of elevated development rates, greater abundance and fitness of the mosquito in a given population would be achieved. Similarly, to what can be observed of malaria transmission rates will be higher as in consequence of climate change (265). Subsequently, the more likelihood of being increased of malaria transmission and risk is redoubled by elevated temperatures and humidity (266). Although the presence of increased temperatures without humidity, reversely, reduce the mosquito capacity to transmit the parasite by degrading mosquito longevity (267). Previous studies have documented the occurrence of time lags ranging from weeks to months between climate anomalies and elevated malaria transmission rates (261, 268-270). These time lags following a climatic anomaly are due to the time needed for mosquitoes to reach the adult stage, to then acquire and transmit the malaria parasite, and eventually, for malaria symptoms to manifest in the human host (261).

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Another point, in the case of warmer weather than normal years, there is an increase of altitudinal distribution of malaria, and hypothetically suggesting that climate change may elevate the altitudinal distribution of malaria (271). It is plausible that interconnection of cofactor such as environmental constrain affecting the entomological and parasitological constituents enhance the transmissibility of malaria. However, the variable over which plays an important role as driver of transmission is not limited only to temperature. In fact, malaria is a disease whose transmission pattern is varyingly driven by a range of factors such as access to health care, lifestyle, vector behavior, deployed vector control measures, and human behavior and migration (271, 272). Thus, the effect of climate change on malaria may likely be minor in comparison to the potential impact of public health interventions and better socioeconomic conditions (273).

Besides climate change which the alteration on various aspect have been well documented, another important variable driving malaria transmission are entomological parameter and human population dynamic. The variations related to behavioral and biological importance of mosquito species, such as habitat associations and associated climatic and environmental drivers of population abundance, host-seeking behavior, and resistance to insecticides, influence the nature of malaria outbreaks (267, 274). Endophilic mosquito tend to feed and rest indoors, thus a poorly constructed dwellings and in a close proximity with vector breeding sites along with human's behavior attracting mosquito such as sleeping and tethering their livestock inside their homes increase the chance of mosquito contact (275). However, there is an observed behavioral change of feeding habit of mosquito from endophilic to be exophilic and its feeding time from late evenings to early evenings (276-278). It is also

possible that vector control intervention could change natural behavior of mosquito shifting from endophilic to exophilic caused by avoiding such control strategy such as insecticide exposure usually utilize inside human dwelling (276, 277, 279). In other hands, human population dynamic also become a potential source of malaria transmission by providing likelihood to spread the disease mainly through import case. This import case can be either from recent migration or short-term travel (280-282). A study in Ethiopia found evidence suggesting that high transmission rates of malaria in the 1980s were due to large population movement from non-malarious highlands to lowland malarious region (283). These large movement of human population might be in a close relationship with agricultural work (284), and these migrant workers tend to live in temporary housing unfit for protection against mosquitoes and occasionally sleeping outside as well as inherent awareness of malaria risk (280, 281). A difference in practical agricultural activities which is suitable habitats of vector mosquitos may be the predisposing factor of malaria transmission (276). Additionally, small and mobile sub-population groups that are difficult to tract, might be at higher risk of malaria infection and may delay its elimination from certain areas, or may even re-introduce malaria in areas where it had been eliminated (281).

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Moreover, besides of evidence from Ethiopia, plenty of studies have associated several other risk factors influencing malaria transmission, mostly published in a recent year. In the late 90's, it was known that the older the patient the less the incidence of malaria as well as the knowledge of malaria prevention. Several other associated factors such as exposure to the forest and receiving previous antimalarial treatment. Bednets could not be very effective protective measure in setting of such this study was done, rather environmental intervention may be better applied (285). In pregnant woman, the

associated factors of malaria infection are lack of education and non-possession of insecticide treated nets (ITNs) followed by a decrease of parasite density as age increased (286). Children under the age of five years also particularly at risk of being infected by malaria parasite, especially in sub-Sahara Africa (287). The associated risks of this particular at-risk population are mostly sociodemographic related factors such as main floor material and main wall material of the house and availability of electricity. However, indoor residual spraying (IRS) significantly reduces a child's risk of malaria, with additional information of the more older of the children apparently have more risk of malaria, notwithstanding their risk decreases with an increase in cluster altitude and an increase in their caregiver's education level (288). Another study showed an interesting method to discover the associated factors of malaria infection. Pinchoff *et al* used a case-control approach based on positively detected incidence by rapid diagnostic test (RDT) with a sophisticated statistical method. They found that, in multivariate model generalized by generalized estimating equations (GEE), the odds being RDT positive are highest in 5-17 years old (8.83 odds compared to 18 years old (or more)) and do not vary between season. Additionally, there was an interaction between age and report of symptoms, with an almost 50% increased odds of report of symptoms with decreasing age category (289). Besides using case-control approach, Elijah chirebvu *et al* rather uses a more convenient method over which history of malaria infection as independent variable and found that the correlated factors of malaria are household income, late outdoor activities, time spent outdoor, travel outside study area, non-possession of ITNs, hut/house structure, and homestead location from water bodies. In addition, close proximity of a health facility and low vegetation cover are being advantageous protective factors (290). Another interesting study by Lawrene

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et al which used a spatial regression analysis to estimate risk factors. These finding based on regression estimation found that the children who visited rural area have 6 odds of magnitude being infected by malaria parasite, as previous finding the more the age of the children the more the likelihood of being infected notwithstanding the risk reduces as higher sociodemographic status. Nearness to a garden, river and standing water are not associated but act as cofactor of increased risk. Furthermore, it showed a spatial clusters of household of the infected patients affect the risk of transmission which may explain by variability of the environmental factors (291). The last, interestingly, a group of researchers using secondary database of national wide scale with regression model found that wealth status is the first socio-economic factor which contributed more to the difference of malaria risk among African children. They did not find any demographic factor among the associated variables. In detailed information, some factors are associated with the risk of malaria such as age of child, education level of mother, use of bednets, anemia status, fever status, house with electricity, household size, wealth status, children in rural areas are at higher risk, quantity of rainfall reduces the risk of malaria, and additionally living near areas with conflict events increases the risk of malaria. In other hand, sex of child and river or water body are not associated with the risk of being infected with malaria. Country of resident and temperature could be cofactor in the analysis with supplementary information of negative association between population density and malaria incidence. One should be noted, these study had done a comparison study of differing malaria risk and found there were several differences in associated variables between low and high risk countries (292).

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2.9.2. Risk factors of malaria in Indonesia

In Indonesia, the associated risk factors of malaria have been extensively studied across individual island of Indonesian archipelago. Different Island may have distinct risk factors associated with malaria. Based on systematic review with random study sites (43), out of 14 variables (mean variables of pooled study), 5 variables are associated (mean of pooled associated variables). However based on similarity of variables, there are 21 associated variables (the presence of breeding place, no barrier of ventilation, non-possession of repellent, nearness with garden, the presence of puddle, nearness with rice field, the presence of household waste, the presence of livestock stall, poor hygiene and sanitation of household, wall structure, without household ceiling, impermanent household floor, night outdoor activity, non-possession of mosquito net, hanging clothes indoor, non-possession of ITNs, non-possession of repellent, low education level, low income, noncompliance of drugs, malnutrition status) within all studies with mean OR = 5.3, and 2 protective associated variables (the use of mosquito nets with OR = 0.26 and the presence of household ceiling with OR = 0.69). Subsequently, other studies are based on a specific location across Indonesian archipelago or using a unique methodological approach. In the western of Kalimantan (44), the identified risk factors are no mosquito net in the ventilation (OR = 10.5), the presence of water pool (OR = 2.5), the presence of bush near household (OR = 5.4), the presence of livestock (OR = 4.0), the presence of puddle (OR = 2.7), the habit of using mosquito net (OR = 2.6), the habit of night activity outdoor (OR = 5.2), while in multivariate analysis, out of 7 aforementioned variables, combination of 5 contribute to 71% likelihood of being infected by malaria parasite. In the city of Jayapura (45), Papua, Indonesia, by case-control epidemiological survey

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design, there are 6 associated factors of malaria, namely do not give burden at every ventilation (OR = 2.14), wall made by wood (OR = 3.14), livestock cage presence near the house (OR = 2.44), the outdoor activity at night (OR = 5.54), low income (OR = 3.24) and education less than primary high school (OR = 3.56). While another study in Sijunjung district, West Sumatra (293), behavior and practice of preventing malaria are the significant risk factors of malaria infection but not for environmental condition, which is against the previous finding. In case of pregnant woman in Maluku province (294), the associated risk factors of malaria are education (OR = 2.78), knowledge (OR = 2.66), the presence of breeding sites (OR = 3.56), and livestock cage presence nearby household (OR = 2.69), while attitude towards malaria prevention plays a role as protective factor (OR = 0.12). In North Sumatra (295), annual malaria incidence (AMI) are associated with poor action (OR = 6.9), mosquito breeding place (OR = 5.1), lower knowledge (OR = 5.5), lower education (OR = 3.3), being farmer (OR = 2.8), the distance from breeding places to residential area < 2 km (OR = 3.7), house/environmental spraying (OR = 4.3). In Belitung province as part of Sumatra island (296), the factors are divided into individual and environmental factors namely the habit of night activity outdoor (OR = 4.4), recent visit to endemic area (OR = 4.9), the presence of bush nearby household (OR = 2.16), the presence of puddle (OR = 3.2). In Lampung, Sumatra island (297), there are two types of associated factors of malaria, individual factor (knowledge, perception, use of mosquito nets, use of anti-mosquito drugs, the use of wire gauze, cover the body, night activity outdoor, and Job), and environmental factor (housing condition, environmental breeding site of mosquitoes, cattle raising, and the distance of household to breeding places), although these factors are only associated with cross sectional method. Another cross sectional study from

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Nunukan, East Kalimantan (298), the associated factors of malaria are the habit of mosquito bed net use, the habit of insecticide use, the distance of household to breeding place, and the distance to cocoa/coffee plantation. In East Java (299), in terms of areas divided by administrative boundaries, two significant factors are identified namely, contact with infected patient (OR = 2.4) and mobility of the person (OR = 3.045).

Additionally, several other studies were using a unique methodological approach to discover the associated factors of malaria. Based on active and passive surveillance assessing three common species of malaria in Aceh (300) found that the associated factors are male (AOR = 12.5), adult (OR = 14.05), visiting forest from previous month regardless of the reason (OR = 5.6), working place in forest with overnight stays (OR = 7.9). In Papua (301), adopting Bayesian hierarchical logistic model found that rural Papuans as well as those who live in poor, densely forested, lowland districts are at higher risk of being infected of malaria with additional information of nine districts of the island have higher than-expected malaria risks. And the last, using geographically weighted negative binomial regression (302), the study found that the variables affecting malaria cases are the percentage of healthy household, the percentage of house with healthy household waste, the percentage of health center, the percentage of health socialization, the percentage of household with sufficient hygiene and sanitation condition, the percentage of household with clean water facility, and the percentage of healthy food processing.

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Table 1. Summary of risk factors. The same color indicates the same variable though from different study.

Research study	Statistically significant variables	OR	95% CI	Status of risk factor	Type
Researcher 1	The distance of breeding place	5.02	1.74 – 14.45	Risk factor	Outdoor environmental factor
	Without gauze or barrier on ventilation	2.47	1.34 – 104.14	Risk factor	indoor environmental factor
	Night activity outdoor	4.25	1.71 – 10.55	Risk factor	Individual factor
	Not bed net user	0.26	0.77 – 0.90	Protective factor	Individual factor
	Less nutritional status	8.28	1.09 – 62.72	Risk factor	Individual factor
Researcher 2	Non-user of mosquito repellent	12.4	1.33-13.18	Risk factor	Individual factor
	the existence of shrubs	7.3	1.50-35.38	Risk factor	Outdoor environmental factor
	No predator fish in the stagnant water	4.2	2.28-66.91	Risk factor	indoor environmental factor
	Not bed net user	3.5	1.42-10.11	Risk factor	Individual factor
Researcher 3	Without gauze or barrier on ventilation	10.67	0.11-0.81	Risk factor	indoor environmental factor
	Not bed net user	8.09	1.99-32.79	Risk factor	Individual factor
	The presence of livestock cage	13.89	3.7-51.8	Risk factor	indoor environmental factor
	Not insecticide/pesticide user	9.53	1.89-47.93	Risk factor	Individual factor
	Not mosquito repellant user	9.53	4.33-62.23	Risk factor	Individual factor
Researcher 4	Hygine and sanitation of household	3.63	1.41-9.28	Risk factor	indoor environmental factor
	Household wall	2.89	1.14-7.28	Risk factor	indoor environmental factor
	Without gauze or barrier on ventilation	2.87	1.09-7.49	Risk factor	indoor environmental factor
	The presence of puddle/stagnant water	4.12	1.63-10.39	Risk factor	Outdoor environmental factor
	The presence of rice field	6.56	2.57-16.72	Risk factor	Outdoor environmental factor
	The presence of puddle/stagnant water	7.35	2.53-21.26	Risk factor	indoor environmental factor
	Not bed net user	3.15	1.33-7.44	Risk factor	Individual factor
	Not mosquito repellant user	3.37	1.41-8.04	Risk factor	Individual factor
Researcher 5	Low education level	4.28	1.98-18.72	Risk factor	Individual factor
	House floor construction is not permanent	5.182	1.18-22.23	Risk factor	indoor environmental factor
	The presence of the ceiling of the house	0.69	0.53-0.91	Protective factor	indoor environmental factor
	The presence of puddle/stagnant water	3.68	1.06-12.71	Risk factor	Outdoor environmental factor
	Not bed net user	5.18	1.33 – 20.05	Risk factor	Individual factor
	Hanging clothes inside the house	16.92	1.93-147.76	Risk factor	Individual factor
	noncompliance of taking medication	5.18	1.33-20.05	Risk factor	Individual factor
	Night activity outdoor	4.68	1.29-16.98	Risk factor	Individual factor
Researcher 6	Without gauze or barrier on ventilation	3.71	1.80-7.59	Risk factor	indoor environmental factor
	Not bed net user	5.82	2.72-12.43	Risk factor	Individual factor
	Not mosquito repellant user	3.43	1.66-6.97	Risk factor	Individual factor

Research study	Statistically significant variables	OR	95% CI	Status of risk factor	Type
Researcher 7	Without gauze or barrier on ventilation	10.5	3.4-32.3	Risk factor	indoor environmental factor
	The presence of water pool	2.5	1.3-4.9	Risk factor	Outdoor environmental factor
	the existence of shrubs	5.4	2.5-11.4	Risk factor	Outdoor environmental factor
	The presence of livestock cage	4	2-8.3	Risk factor	indoor environmental factor
	The presence of puddle/stagnant water	2.7	1.3-5.4	Risk factor	Outdoor environmental factor
	Not bed net user	2.6	1.2-5.5	Risk factor	Individual factor
	Night activity outdoor	5.2	2.4-11.1	Risk factor	Individual factor
Researcher 8	Without gauze or barrier on ventilation	2.14	1.02-4.47	Risk factor	indoor environmental factor
	the wall material of the house made by wood	3.14	1.43-6.88	Risk factor	indoor environmental factor
	The presence of livestock cage	2.44	1.21-4.9	Risk factor	indoor environmental factor
	Night activity outdoor	5.54	2.37-12.98	Risk factor	Individual factor
	Salary <1.000.000 IDR	3.24	1.62-6.50	Risk factor	Individual factor
	Education level less than primary high school	3.56	1.37-9.27	Risk factor	Individual factor
Researcher 9	Environmental factor	1.1	0.32-4.08	Risk factor	Outdoor environmental factor
	The level of knowledge	22.5	4.9-103.6	Risk factor	Individual factor
	the attitude of the family	7	2.61-18.33	Risk factor	indoor environmental factor
	The action of the family	19.6	4.27-9.93	Risk factor	indoor environmental factor
Researcher 10	Education level	2.78	1.06-7.32	Risk factor	Individual factor
	Knowledge	2.66	1.02-6.91	Risk factor	Individual factor
	Attitude	0.12	0.03-0.520	Risk factor	Individual factor
	The presence of breeding place	3.56	1.21-10.49	Risk factor	Outdoor environmental factor
	The presence of livestock cage	2.69	0.33-22.24	Risk factor	indoor environmental factor
Researcher 11	Low education level	3.3	1.5-6.9	Risk factor	Individual factor
	High risk occupation	2.8	1.3-6.1	Risk factor	Individual factor
	Low level of knowledge	5.5	2.6-11.8	Risk factor	Individual factor
	low level of action	6.9	3.2-14.8	Risk factor	Individual factor
	The presence of breeding place	5.1	2.4-10.8	Risk factor	Outdoor environmental factor
	The distance of the house to the breeding place (high risk)	3.7	1.7-7.7	Risk factor	Outdoor environmental factor
	Insecticide spraying	4.3	2.0-9.1	Risk factor	Individual factor
Researcher 12	Contact with malaria patient	2.496	1.053-5.921	Risk factor	Individual factor
	High mobility	3.045	1.248-7.216	Risk factor	Individual factor
Researcher 13	Men	12.5	3.0-52.1	Risk factor	Individual factor
	Adult (16-45 year)	14	2.2-89.6	Risk factor	Individual factor
	Visited a forest from previous month for any reason	5.6	1.3-24.2	Risk factor	Individual factor
	Working place is in the forest and requiring overnight stay	7.9	1.6-39.7	Risk factor	Individual factor

2.10. Compliance and adherence of taking ACT medication in population

Since ACT is known to be an effective prompt treatment and preventive as well as intermittent mass drug administration strategy, the deployment of ACT has been widely

adopted. Increasing trend of ACT adoption occurred annually. By 2010, 84 countries had adopted ACT, with 60 countries providing ACT free-of-charge to all ages in the public sector and eight have piloted provision of subsidized ACT in the private sector through the Affordable medicine facility – malaria (AMFm) (487-489).

Previous systematic review has described how antimalarial drugs used among malaria infected patients (490). From these review, patient's adherence to ACT varied from 78% for a three-day regimen of AS+SP in Zambia (491) to maximum of 93% for AL in Uganda (492). In other hand, adherence level of ACT in Kenya is <30% (493) and in contrast 100% of AL adherence in Malawi (494). Adherence was found to be generally better when "interventions focusing on provider knowledge and behavior, packaging and provision of correct dosage" were implemented (490). A poor homogeneity between studies and large range of adherence level can be directed to the variability of the study settings, study design and ACT formulation, as well as studies measurement tools (questionnaire or interview), blinding protocol and the features of study design (RCT vs observational). Additionally, it would be imperative to consider about standardized definition of adherence, importantly a definition which incorporates duration, timing and frequency of dose. Without any standardized definition of adherence, each study will not be comparable.

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Findings of each published factors was inconsistently described. Demographic background seems to be not associated with adherence of ACT, such as sex, socio-economic status and age (495-507). However, the age factors seem to be underpowered by some studies, due to other findings found that age was a risk factor of adherence. Younger people more likely to be not adherent. Children less than five were more likely

not to be adherent to take ACT treatment in Malawi (496), while in Kenya children aged more than 15 years were less adherent than older patients (495). Although age has been reported to be risk factor of poor adherence to non-ACT regimen (508), suggesting formulation of ACT and communication campaigns should take into account age related factor. Another associated risk factor of ACT medication is vomiting; however, vomiting is a difficult entity to measure. It is negatively correlated with AQ+AS and AL (505, 507), though it is considered to be an exclusion criterion in some studies. As vomiting could be influenced by treatment regimen and severity of the disease, thus care should be taken when attributing vomiting to non-adherence.

Generally, study design of discovering adherence level in takin ACT treatment is mostly prospective observational or randomized controlled trial (RCT). Some issues have appeared to those study designs. The limitation of retrospective observational study is lack of accuracy due to sampled persons mostly was derived several weeks after treatment. Similarly, for cross-sectional design, recall bias more likely to appear thus under- or over-estimating adherence level. The adherence level is also commonly obtained from Malaria Indicator Survey (MIS), though indication of adherence could be obtained notwithstanding such of study do not include the suitable measurement. Additionally, patient knowledge or recognition of the drugs may not be sufficient through these types of survey. The nomenclature of local term of the drugs also need to be considered for ensuring a correct understanding for local inhabitant. Prospective observational studies that interviewed patients or caregivers the day following the last treatment, should have better recall, however, the accuracy of the measurement is dependent on how patients/caregivers were recruited and whether they knew they would be followed up at a later date (504, 505, 508). RCT and pre-post designs also

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have similar challenges, as patients are enrolled and consent to participation prior to taking part in the study. In majority of study, patients/caregivers already knew that they were enrolled in a study and therefore may have altered their behaviour to be more adherent (i.e. Hawthorn effect).

Plenty of studies have been conducted to discover adherence to anti-malarials for over a decade, but in fact, their methodologies are still lack standardization. A crude and imprecise definition of adherence (probably adherent, probably non-adherent and non-adherent), which have been used for almost studies, can result in misclassification of individual's adherence lead to over-or underestimation of adherence. These methods used to measure adherence to non-ACT drugs (490), yet they are still widely used to measure adherence to ACT today.

Most of studies also used self-report, due to less expensive and ease to implement. However, though with all these conveniences, it is subject to social desirability bias, which ay overestimate adherence. Currently measurement tools of ACT adherence using questionnaire are not standardized and follow more complex household survey structure. For instance, both HIV and TB treatment regimens standardized questionnaire have been utilized to measure treatment adherence, with some of these tools are long and detail while the others are short or abbreviated versions, which it is still considered to be relatively accurate adherence measurement (e.g. the brief medical questionnaire (BMQ) and Morisky scale) (509-511). In other hand, MEMS (medical event monitory services) and biological assays are more objective methods to measure adherence and may offer more precise adherence measurements. However, further consensus is needed with regard to translating bioassay data into a

measurement of adherence/non-adherence. Although the interpretation of this method which objective regarding measuring drug metabolites in the blood may be problematic (512-514, 492, 498-499).

2.11. Population pharmacokinetics of piperaquine and primaquine

2.11.1. Piperaquine

As mentioned in the previous section, a number of antimalarials have been introduced and known to be resistance. All family of antimalarials has its pharmacokinetic property which is unique and may partly explain their failure in treating malaria infected patient. Artemisinin combination therapy is suggested due to avoiding resistance by combining two different property of pharmacokinetic of the drugs to expand effectivity and efficacy. Mainly, the most highlighted property is half live of the drug. Since artemisinin has short half live and concentration dependent, these drug needs additional long half live partner drug which is time dependent in order to achieve greater clinically significant and avoid unexposed parasite to remain in the patient's body after artemisinin treatment. Another important pharmacokinetic property of a drug is maximum concentration (Cmax), time at which maximum concentration is achieved (Tmax), and area under curve which is a function of time and concentration (AUC) which these are denoted as secondary result of pharmacokinetic study. The primary and secondary definition of pharmacokinetic parameter will be described as follows:

Table 2. Description of pharmacokinetics parameter

No	Abbreviated parameter (unit measurement)	Definition
PRIMARY OUTCOMES		
1	CL/F (l/h)	It is the apparent elimination clearance
2	Vc/F (l)	It is the apparent volume of distribution of the central compartment
3	Q1/F (l/h)	These are the inter-compartment clearances between the central and the peripheral compartment
4	Q2/F (l/h)	
5	VP1/F (l)	These are the apparent volumes of distribution of the peripheral compartments
6	VP2/F (l)	
7	MTT (h)	It is the mean transit time of the absorption
8	Number of transit compartments	It is the number of transit compartments used in the absorption model
9	F	It is the relative bioavailability
10	RUV	It is the variance of the unexplained residual variability
COVARIATE RELATIONSHIP		
11	Scale	It is the difference between venous and capillary predictions
12	MF50 (years)	It is the maturation age (years) to reach 50% of the full elimination clearance
13	$Hill_{MF}$	It is the hill function in the maturation equation, with an upper limit of 10
14	$Dose_{F}$	It represents the increase in relative bioavailability between dosing occasion
SECONDARY OUTCOMES		
15	Cmax (ng/ml)	It is the maximum concentration
16	Tmax (h)	It is the time after dose to reach the maximum concentration
17	$T_{1/2}$ (d)	It is the terminal elimination half life
18	AUC∞ (h x ng/ml)	It is the area under the concentration-time curve from time 0 to infinity
19	Day 7 concentration	It is the venous plasma concentration at day 7 after dosing
ADDITIONAL INFORMATION		
20	Coefficient of variation for inter-individual variability (IIV)	These are calculated as 100 x $(e^{variance} - 1)^{1/2}$
21	Inter-occasion variability (IOV)	

A meta-analysis of a group of researchers aimed to identify and enhance piperaquine dosing from a pooled analysis has found several important points (Table 3) (516). A three-compartment disposition model with a transit compartment absorption model was found to adequately describe a pooled data of meta-analysis as a final model of piperaquine pharmacokinetics. The effect of a disease on pharmacokinetic would be present only during the early assessments (day 1-3) due to patient's body recovery. Furthermore, a disease could effect on relative bioavailability, resulting in an increase in the exposure to piperaquine in healthy volunteers compared to infected patient. A 24% increase in relative bioavailability is observed between dose occasions, whereas the total daily milligram/kilogram dosage does not influence absorption. Secondary results are Cmax (ng/ml) is estimated to be 248 (24.3-1070 (minimum-maximum), Tmax (h) is 3.49 (1.13-10), $T_{1/2}$ (d) is 22.5 (9.15-52.3), AUC∞ (h*ng/ml) is 28.800 (2650-116.000) and day 7 concentration (ng/ml) is 28.1 (2.35-115).

Table 3. Concentration of piperaquine at day 60

No	Reference	Sites	Study population	Dose regimen	Malaria infection	Drug concentration Day 60 (approximate minimum-maximum)
1	516	Pooled analysis	Pooled analysis	Pooled analysis	Pf & healthy volunteers	>1 ng/ml (±1.6-20 ng/ml)
2	517	-	adults	Single dose	Healthy	<1 ng/ml (±0.3-0.9 ng/ml)
3	517	-	adults	3 days dose	Healthy	>1 ng/ml (±4-7 ng/ml)
4	518	Thailand	adults	3 days dose	Pf	>1 ng/ml (±1.15-17 ng/ml)

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No	Reference	Sites	Study population	Dose regimen	Malaria infection	Drug concentration Day 60 (approximate minimum-maximum)
5	515	Thailand	Children and adults	3 days dose	Pf & Pv	Pf: 1.2 ng/ml Pv: 1.3 ng/ml
6	519	Vietnam	adults	3 days dose	Healthy	6 ng/ml

Pf: *Plasmodium falciparum*, Pv: *Plasmodium vivax*

2.11.2. Primaquine

Primaquine is known as either radical cure for hypnozoite form of *Plasmodium vivax* and gametocytocidal effect to *Plasmodium falciparum*. Thus, it prevents relapse of *Plasmodium vivax* and disease transmission of *Plasmodium falciparum*. Therefore, it is imperative to understand the basic of pharmacokinetic properties of primaquine.

Basically, after primaquine dosing, there are two important metabolites of primaquine in the patient's body, primaquine and carboxy-primaquine. Primaquine is rapidly absorbed attaining peak plasma concentration (median & range) of 167 (113-532) µg l^{-1} in 2 (1-4) hours. Afterwards. It decreases rapidly with an apparent terminal half-life of 6.1 (1.7-16.1) hours. And there is no effect of partner drug on the values of any pharmacokinetics parameters of primaquine. On the other hand, the carboxylic acid metabolite of primaquine attain maximum concentrations (median & range) of 890 (553-3634) µg l^{-1} at 6 (3-16) hours. Afterwards, it declines to 346 (99-918) µg l^{-1} at 24 hours. Similar to primaquine, partner drug has also no effect on carboxy-primaquine. However, acute malaria does have the effect significantly on the reduction of oral clearance of primaquine from 21.3 (15.9-73,0) to 19.4 (9.3-24.7) hours. The area under curve for the carboxylic acid metabolite of primaquine is significantly greater following the administration of primaquine alone relative to the combination of quinine and

primaquine (520). Additionally, the primaquine pharmacokinetics data suggest that women have increased exposure to primaquine, which may put them at increased risk for toxicity when administered the same maintenance as men (521). However, a short-higher dose primaquine regimen is safe and well tolerated, which could improve primaquine compliance and effectiveness (522).

Table 4. Concentration of primaquine at 15, 25 and 100 hours

No	Authors	Site	Infection	Metabolite	Drug Cconcentration at 15 h	Drug concentration at 25 h	Drug concentration at 100 h
1	520	Thailand	Healthy	1. PQ 2. Carboxy-PQ	1. ±35 $\mu g\ l^{-1}$ 2. ±790 $\mu g\ l^{-1}$	1. – 2. ±510 $\mu g\ l^{-1}$	1. – 2. -
2	520	Thailand	Pf	1. PQ 2. Carboxy-PQ	1. ±89 $\mu g\ l^{-1}$ 2. ±240 $\mu g\ l^{-1}$	1. – 2. -	1. - 2. -
3	521	Vietnam	Healthy	1. PQ 2. Carboxy-PQ	1. - 2. -	1. ±4 ng/ml 2. ±1000 ng/ml	1. – 2. ±80 ng/ml

PQ: Primaquine, Pf: *Plasmodium falciparum*

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CHAPTER 3

RESEARCH METHODOLOGY

3.1. Risk factor of malaria

3.1.1. Research design

This study was conducted in two localities representing low and high endemicities, namely Jambi and Sumba, respectively. The study was done for 6 months in Jambi between January to June 2018, and 6 months in Sumba between July to December 2018. Risk factors of malaria incidence generally was following case-control design over which case if defined by positively detected RDT or control as negatively detected RDT regardless of their originated island. Therefore, sampling calculation and method followed case-control design related to the number of persons that necessarily to be tested by RDT. The number of controls was determined by as much as twice as the number of cases (1:2).

3.1.2. Variable and operational definition

Variable	Definition	Measurement	Measuring scale, category
Risk factors of malaria infection	Risk factor is defined as any potential statistically significant variable that affect the incidence of malaria in a population. It can be divided by individual and environmental factors	Questionnaire	**Environmental factors:** 1. The presence of breeding place nearby the house * 2. The distance of breeding place to household # 3. Without gauze or barrier on ventilation * 4. The existence of shrubs * 5. No predator fish in the stagnant water * 6. The presence of livestock cage nearby household * 7. The presence of any livestock nearby house * 8. Hygine and sanitation of the house * 9. Household wall * 10. The presence of puddle/stagnant water * 11. The presence of rice field * 12. House floor construction is not permanent * 13. The presence of ceiling of the house * 14. The presence of water pool * 15. The wall material of household made by wood * 16. The attitude of the family * 17. The action of the family *

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			18. The distance of household to stagnat water (divided by stream based on stahler classificarion) # 19. The distance of household to the nearest health facility (delineation of the distance) # **Individual factor:** 1. Night activity outdoor * 2. Using bed nets * 3. Nutritional status # 4. Using mosquito repellant * 5. Using any kind of insecticide/pesticide * 6. Education level * 7. Hanging clothes inside the house * 8. Noncompliance of taking medication * 9. Salary less than 1.000.000 * 10. The level of knowledge * 11. The level of attitude * 12. Type of occupation * 13. The level of action * 14. Insecticide spraying * 15. Contact with malaria patient * 16. High mobility * 17. Type of sex * 18. Age # 19. Visited a forest from previous month for any reason * 20. Working place is in the forest and requiring overnight stay *

* denotes nominal or ordinal data, # denotes ratio or interval data

3.1.3. Sample size calculation

$$n1=n2=\frac{(Z\alpha\left(\sqrt{2PQ}\right)+Z\beta\left(\sqrt{P1Q1+P2Q2}\right))^2}{(P1-P2)^2}$$

Description:

n1=n2= sample size

Zα= level of confidence

Zβ= Power

P= 0.5(P1+P2)

Q = 1-P

P1= Proportion of case (Literature = table 1)(43)

P2= P1/(OR x (1-P1) + P1) (Proportion of control)

Q1= 1-P1

Q2= 1-P2

Based on proportion of case in previous report equal to 0.5, while the pooled odds ratio in literature review section indicate the mean effect of 2.5. Thus P=0.39, Q=0.61, P1=0.50,

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P2=0.28, Q1=0.50 and Q2=0.72. As following abovementioned formula, the minimum total sample size is 75. With addition 10%, the total sample size is 82.

3.1.4. Data collection technique

3.1.4.1. Entomological survey

The survey was taken to observe the pattern of blood feeding of the potential vector between low and high localities by taking a series of entomological observations. It was a three-week observation in each area. We used a human landing catch (HLC) method to gain female *Anopheles* vector (303). In detail, the method requires human bait to collect the mosquitoes over a 12-hour period (6 pm to 6 am). The bait is required to stay still in their preferred location. The bait is only allowed to collect mosquito that laid down on their body. Following the previous study, the present study used an indoor and outdoor catcher in each house (303). There were six houses per day for HLC collection and there were six days in a week. In total, we had three weeks of collection in each study area. For attaining a same pattern of an indoor and outdoor collection for each house, we used random selection for repetition. For avoiding biases of potential differences of mosquito species abundance, the distance of each houses was set up to not more than two kilometers. The collected mosquitoes were sent and identified in Eijkman institute for molecular biology, Jakarta, Indonesia.

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3.1.4.2. Comparison between Non-malaria house (NH), Malaria house (MH) and Non-malaria permanent house (PH)

The entomological survey of the current study was in the purpose of observing the possible difference of mosquito bites between MH, NH and PH. MH and NH were the same house type, non-permanent or not well-constructed house. Beside of the observational measurement of malaria cases, this entomological survey will support the finding of the malaria risk factors in which is often correlated with human dwellings.

This survey was initially started by a week of pre-observational HLC and then followed up by up to 3 weeks of a comparative observational HLC survey between the three types of houses. To avoid disparity of mosquito species and abundance and indeed biases, the distance of the three kinds of houses has been set up not to exceed 2 km. The result of the initial screening was used to differentiate between MH, NH and PH. NH and PH were defined as the absence of malaria infection at least one year. Additionally, to make sure the presence or absence of malaria infection, weekly screening was conducted. If malaria infection was detected in NH and PH, then the house was excluded and changed to another house. After initial screening, the selected houses were numbered and picked randomly for weekly HLC. There were two houses per house types per day (2 for MH, 2 for NH and 2 for PH). And there were 12 houses in total per house types (6 days of collection per week). However, with three repetitions (3 weeks), the total sample was 36 houses per house types per sites.

3.1.4.3. Risk factor assessment

The design of the current study was case control. The case was those who positively detected for malaria by RDT or microscopic observation or combination of both. The selection of control was by criteria of an absence of malaria infection of at least 1-year period and the closest location by the distance to the selected case due to avoiding the possible difference in vectorial capacity. Risk factors of malaria were examined by structured questionnaire comprised of both individual and environmental variables that previously had been found to correlate with malaria incidence. There were 11, and 15 environmental and individual variables examined in the current study, respectively. The environmental factors are as follows: without gauze or barrier on ventilation, the existence of shrubs, the presence of puddle/stagnant water, the presence of livestock

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inside household, the presence of any livestock nearby house, household wall material, the presence of rice field, house floor construction, the presence of ceiling of the house, hanging clothes inside the house and the presence of water pool. On the other hand, the individual factors are comprised of: night activity outdoor, possession of ITNs, using mosquito repellant, using any kind of insecticide/pesticide, education level, previous antimalarial drug consumption, salary less than 1 million Rupiah, type of occupation, contact with malaria patient, mobility, sex, age, visited a forest from previous month for any reason, working place is in the forest and requiring overnight stay.

Study population of control group

Since case-control design also possesses bias if not appropriately applied, then it necessitates a tight selection of the control group. A number of detected case and control was also regarded as the case. The number of controls was twice as many as the case. Therefore, for the selection of control, a matching strategy had to be applied. In this case, a matched control has been taken in the basis of approximate distant to the case.

3.1.5. Processing and data analysis

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The variables included in this section were: (a) nominal, (b) ordinal, and (c) ratio. To perform association analysis of determinant of malaria incidence, we used Chi-square statistical test. Since this was nonparametric, there will no need to assess the normality of data. The threshold of association in Chi-square analysis was set up at 0.05%.

Validity was assessed by two independent experts (1. Medical doctor specialized in internal medicine which is expert in clinical malaria; 2. Ph.D. in tropical medicine which is expert in malariology). Back translation was performed by independent professional editor.

3.2. Patient adherence to dihydroartemisinin+piperaquine and primaquine medication

3.2.1. Research design

This study was conducted in two localities representing low and high endemicities, namely Jambi and Sumba, respectively. The study was done for 6 months in Jambi between January to June 2018, and 6 months in Sumba between July to December 2018. In order to understand the adherence level of ACT and primaquine treatment, we interviewed our patient using previously described questionnaire with cross-sectional design. The confirmed-RDT patient was our population.

3.2.2. Variable and operational definition

Variable	Definition	Measurement	Measuring scale, category
Adherence of ACT and primaquine treatment	**Certain non-adherence** (pills remaining; Blister packaging (present); intake (incomplete)) **Probable non-adherence** (patient describes incomplete number of pills taken; Blister packaging (not present); intake (incomplete)), **Probable non-adherence** (patient describes incorrect time schedule or dosage; Blister packaging (present or not), intake (incorrect)), **Probable adherence** (patient describes correct number of pills taken, time schedule and dosage; Blister packaging (Present or not), intake (correct)).	Questionnaire	1. Classification of adherence (ordinal scale) 2. Reason of ACT/primaquine intake (nominal scale) 3. ACT/primaquine treatment education and understanding (ordinal and nominal scale) 4. Concentration of drug in plasma (ratio scale)

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3.2.3. Sample size calculation

The estimation of sample size was based on formula of $n = \frac{z^2 P(1-P)}{d^2}$, where Z^2 is the level of confidence at 99%, d^2 is the 4% precision and P is the following assumed adherence level. We assumed that the level of adherence in the population is 70%. With addition of 10% for contingencies, the minimum sample is 138.

3.2.4. Data collection technique

Initially, active and passive case detection was carried out to detect any malaria case in the area. Active case detection (ACD) was performed to those who had fever >37.5° C. Passive case detection (PCD) was implemented by local health worker for those who visited the local health care center with suspected clinical sign and symptom related to malaria. Laboratory performance was carried out by collecting finger prick on slide glass and was detected under light microscopy. The person who positively detected for any *Plasmodium* malaria was immediately prescribed with standard dose of DHP or primaquine drugs. There is two type of questionnaires in our study based on previous published paper with minor modification (304):

1. Center questionnaire

All person who positively detected by *Plasmodium* malaria was then treated with standard DHP and primaquine treatments by either direct visit or in the local health center. At the time after prescription (day 0), all patients were interviewed using center questionnaire containing patients/ caretakers detail including name, age, sex, the number and type of prescriptions and information regarding the understanding of patient/caretaker towards ACT and primaquine and pharmacy dispensing practices.

2. Home questionnaire

After the completion day of DHP and primaquine medications (day 3 and day 14), patients were visited to have "home-questionnaire" interview. It specifically assessed the adherence of the patient to DHP and primaquine medications. Any socio-demographic of the patients/caretakers was sought at this time followed by a systematic question of how pills were taken. Besides of the answer of each patient/caretaker, blister

package was observed to find whether the pills were taken correctly, or any remaining pills found. Any reason for not complying with the treatment regimen was recorded. There are some additional questions assessing patients/caretaker's understanding about knowledge of malaria cause and prevention. Any patient who was not getting better after treatment has been referred back to the local health facility.

The definition of adherence was following previous paper (304). Adherence was defined by either the answer of the patient/caretaker or the presence of any DHP or primaquine pills inside the blister package. Accordingly, there is 3 classification of adherence: certain non-adherence, when the remaining DHP or primaquine pills have been seen; probable non-adherence, if the blister empty and patient/caretaker have given incorrect answer of necessary intake (pill count or time schedule); probable adherence, if the blister empty and patient/caretaker have given correct answer of necessary intake (pill count and time schedule).

3.2.5. Processing and data analysis

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Level of adherence of ACT medication

A structured questionnaire was subjected to both retrospective and prospective patient. The population was defined by all patients that can be found based on medical record of local health facilities and active surveillance using RDT. The operational definition of each variables in the questionnaire was as aforementioned definition.

Validity was assessed by two independent experts (1. Medical doctor specialized in internal medicine which is expert in clinical malaria; 2. Ph.D. in tropical medicine which is expert in malariology). Back translation was performed by independent professional editor.

3.3. Efficacy study of dihydroartemisinin+piperaquine medication against *Plasmodium falciparum* and *Plasmodium vivax* in Indonesia

3.3.1. Research design

We selected 2 regions of either high and low endemicity; namely Papua and Jambi, respectively. This study was done in Papua starting from April 2017 to January 2018, while in Jambi starting from October 2017 to April 2018. From the two areas, we selected several villages that had the highest annual parasite index or prevalence of cases. Active surveillance was carried out by rapid diagnostic test (RDT) to actively screening an incubation period of the parasite inside host body or pre-symptomatic host and those who are asymptomatically infected by the parasite in the selected population. Thereafter, field blood survey has been done, laboratory confirmation was carried out for ascertaining the previously confirmed *Plasmodium falciparum* cases by RDT. K13 propeller domain, a molecular marker for artemisinin, was performed by standard polymerase chain reaction (PCR) and sequencing, while *Plasmepsin II* was performed using real time PCR which datasets are the amount of copy number.

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3.3.2. Variable and operational definition

Variable	Definition	Measurement	Measuring scale, category
K13	Moleucular marker for artemisinin resistance, which is exhibited by several candidate or validated loci (7 candidate and 6 validated)	Laboratory testing of PCR	Nominal: 1. Mutated 2. Susceptible
Plasmepsin II	Molecular marker for piperaquine resistance of *Plasmodium falciparum* which is located on chromosome 14.	Laboratory testing of Real time PCR	Nominal: 1. Single copy 2. Multiple copy

Efficacy of DHP	It is defined as a successful cure rate of DHP against *Plasmodium falciparum* and *Plasmodium vivax* according to WHO classification for resistance stage following below classification: **1. Early treatment failure (ETF)** If the patient develops one of the following during the first three days of follow up: - Development of danger signs or severe malaria on day-1, day-2 or day-3, in the presence of parasitemia; - Parasitemia on day 2 higher than day 0 count irrespective of axillary temperature; - Parasitemia on day-3 with axillary temperature > 37.5 - Parasitemia on day 3 >25% of count on day 0 **2. Late clinical failure (LCF)** (if the patient develops one of the following during the follow-up period from day 4 to day 28) - Development of danger or signs or severe malaria after day 3 in the presence of parasitemia, without previously meeting any of the criteria of early treatment failure (ETF) - Presence of parasitemia and axillary temperature >37.5 (or history of fever) on any day from day 4 to day 28, without previously meeting any of the criteria of ETF **3. Late parasitological failure (LPF)** (if the patient develops one of the following during follow-up period from day 7 to day 28) - Presence of parasitemia on any day from day 7 to day 28 and axillary temperature <37.5, without previously meeting any of the criteria of ETF or LCF	Microscopic examination and PCR assay	Nominal

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	4. Adequate clinical and parasitological response (ACPR) (if the patient shows one of the following during the follow up period (up to day 28) - Absence of parasitemia on day 28 irrespective of axillary temperature without previously meeting any criteria of ETF or LCF or LPF	

3.3.3. Procedure of field sampling

- **Study population**

The population was including of a number of patients with uncomplicated *P. falciparum* malaria attending the study health clinic for passive case surveillance. Population of active surveillance was all society in the certain villages that have been selected. All adult patients were requested to fulfill an informed consent form for their participation. On other hands, parents or guardians that have full responsible of the child was given informed consent on behalf of them. Children over 12 years of age signed an informant assent form.

- **Inclusion criteria**

a. All suspected case of *Plasmodium falciparum* or *Plasmodium vivax* regardless man and woman
b. Infected with a single-infection of *P. falciparum* or *P. vivax* by RDT
c. experienced parasitemia in range of 1000-10000/ µl asexual forms
d. Axillary or tympanic temperature ranged ≥ 37.5 °C or temperature of oral rectal ranged ≥ 38 °C or history of symptoms or signs of fever during the past 24 h

e. Ability and willingness to complete the protocol of the study for the duration and visiting schedule including taking oral medication scheme that have been specified by the researcher/principal investigator

f. Willingness to fulfill informed consent from the patient or from a parent or guardian in the case of children

- **Exclusion criteria**

a. General danger symptoms and signs of severe and complicated falciparum malaria detected early according to the definitions of WHO

b. Another infection of *Plasmodium* species or mixed infection detected by microscopy

c. Acquired severe malnutrition (defined as a child whose growth standard is below -3 z-score, has symmetrical oedema involving at least the feer or has a mid-upper arm circumference <110 mm)

d. In case of other infection or febrile illness rather than malaria (e.g. measles, acute lower respiratory tract infection, severe diarrhea with dehydration) or other known underlying chronic or severe diseases (e.g. cardiac, renal and hepatic diseases, HIV-AIDS)

e. Secondary or regular or routine medication, which may interfere with antimalarial pharmacokinetics

f. Hypersensitivity reactions or contraindications to any ingredients of antimalarial treatment tested and used during the study

g. Breastfeeding mother and a positive pregnancy test those women-bearing age

h. Unwilling to take contraceptives (for women of child-bearing age)

- **Loss to follow-up**

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Loss to follow-up was defined as all of enrolled patients who have been included in the study in case, they could not attend the scheduled visits, despite all reasonable events. The principal investigator has made decisions regarding to those who be definitely classified as lost to follow-up or was to be maintained for the analysis.

- **Patient discontinuation or protocol violation**

Any patients who meet a part or more of the following criteria below have been characterized as withdrawal subjects.

a. Consent withdrawal. A patient is free to withdraw any time, without obligation to attend the follow-up or treatment
b. Failure to complete treatment of the study, due to:
    a. Unable to present at the scheduled visits; or
    b. Worst condition of the patient due to adverse effects that necessarily need to be terminated before the study is completed
    c. Enrolment violation:
        i. An accident of severe plasmodium malaria on day 0; or
        ii. Patient is recognized as exclusion criteria
    d. Involuntary protocol violation:
        i. During follow up, there is a new infection of another species of malaria; or
        ii. An error of laboratory results that necessitates to administration of rescue treatment

- **Treatment**

a. Antimalarial treatment

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Dihydroartemisinin+piperaquine will be administered as follows:

Body weight (kg)	Total administration of DHP (Tablets)
81-100	5
61-80	4
41-60	3
31-40	2
18-30	1.5
11-17	1
6-10	0.5
=5	0.25

Tablets of dihydroartemisinin and piperaquine was obtained from Ministry of health, the republic of Indonesia.

A selected group of qualified members was administered the drug supervised by the principal investigator. There was a 30 minutes observation after administration to monitor adverse reactions or emesis. The patient who had vomited during the observation process was re-treated with the same dose and being observed again for additional 30 minutes. If the patient may have experienced vomiting again, he/she should have been withdrawn and administered for rescue treatment.

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a. Microscopic blood examination

Thick and thin blood films for parasite counts have been obtained and examined at screening on day 0, 2, 3, 7, 14, 21, and 28 (35 and 42) or on any other day if the patient returns spontaneously and parasitological reassessment is required. Giemsa-stained thick and thin blood films was examined at a magnification of 1000x to identify the parasite species and to determine the parasite density. The study number of the patient, the date and the day of follow up was recorded either on the frosted edge of the slide or on the glass with a permanent glass pen.

b. Genotyping of malaria parasites

In order to differentiate a recrudescence (same parasite strain) from a newly acquired infection (different parasite strain), a genotype analysis was conducted. This was based on the extensive genetic diversity among antigenic genes of the malaria parasite, i.e. MSP1, MSP2 and GLURP. The genotype profiles of pre- and post- parasite strains were compared. The PCR technique used was nested PCR in Eijkman laboratory, Jakarta. Paired filter papers were used for parasite DNA extraction and genotyping only in cases of treatment failure.

c. Molecular markers for antimalarial drug resistance

Two to three drops of blood were collected on filter paper on day 0 (and day of failure) to study the polymorphism of K13 propeller domain and copy number of *Plasmepsin II*, which were considered as markers of resistance to artemisinin and piperaquine. We used all collected samples that successfully being extracted for the DNA. The technique used was nested PCR and real-time PCR, respectively. The laboratory testing was done in Eijkman institute for molecular biology, Jakarta. Specimen was labelled anonymously (study number, day of follow up, date), kept in individual plastic bags with desiccant pouches and protected from light, humidity and extreme temperature until analyzed.

3.3.4. Laboratory protocol

1. DNA extraction

Parasite and human host DNA (from the day of enrollment to the study-end point) was extracted from blood samples using Chelex-100 ion exchanger (Bio-Rad laboratories, Hercules, CA) according to a previously published procedure (305). Briefly, the method

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needs an overnight stay of saponin addition, than 3 times of washing solution of PBS and lastly addition of *Chelex*-100 to obtain the solely extracted DNA from the blood. Extracted DNA have been either used immediately for PCR assays or stored at -20° C for later analysis.

2. Method for discriminating species of *Plasmodium*

PCR analysis to confirm further the result of positively or negatively detected *Plasmodium* using RDT was similar to previously published protocol (306, 307). There were a general ribosomal sequence of genus *Plasmodium* and specific nested-PCR for *P. falciparum* and *P. vivax* (rPLU, rFAL and rVIV) (306, 307).

Table 5. Primer pairs for rPLU, rFAL and rVIV

Type of marker	Primers (5’-3’)
Genus specific: rPLU	TTAAAATTGTTGCAGTTAAAACG
	CCTGTTGTTGCCTTAAACTTC
Species specific: rFAL	TTAAACTGGTTTGGGAAAACCAAATATATT
	ACACAATGAACTCAATCATGACTACCCGTC
rVIV	CGCTTCTAGCTTAATCCACATAACTGATAC
	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA

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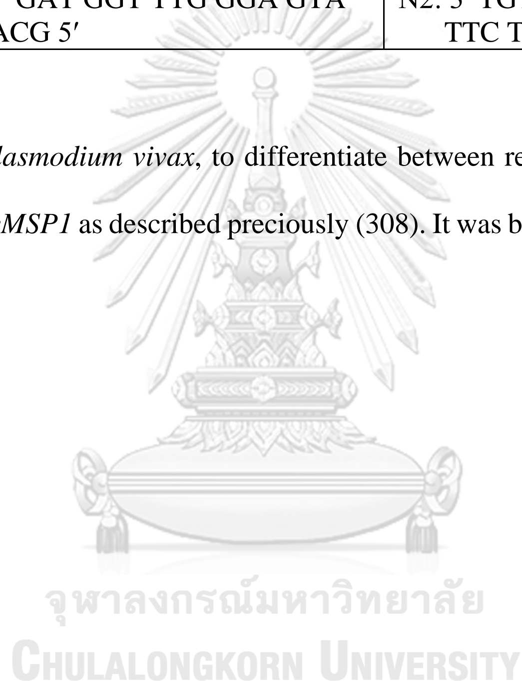
3. Method for discriminating a recrudescence or reinfection

In order to differentiate between reinfection and recrudescence, we used genotyping procedure. There were 3 loci genotype of *P. falciparum* tested (MSP1, MSP2 and GLURP) [480]. The primary (P) and nested (N) primers are as following:

Table 3. Primer pairs for MSP1, MSP2 and GLURP

Markers	Primer for primary PCR	Primer for nested PCR
MSP1	P1: 5′ CAC ATG AAA GTT ATC AAG AAC TTG TC 3′	N1: 5′ GCA GTA TTG ACA GGT TAT GG 3′
	P2: 3′ GTA CGT CTA ATT CAT TTG CAC G 5’	N2: 3′ GAT TGA AAG GTA TTT GAC 5′
MSP2	P1: 5′ GAA GTT AAT TAA AAC ATT GTC 3′	N1: 5′ CTA GAA CCA TGC ATA TGT CC 3′
	P2: 3′ GAG GGA TGT TGC TGC TCC ACA G 5′	N2: 3′ GAG TAT AAG GAG AAG TAT G 5′
GLURP	P1: 5′ ACA TGC AAG TGT TGA TCC 3′	N1: 5′ TGA ATT CGA AGA TGT TCA CAC TGA AC 3’
	P2: 3′ GAT GGT TTG GGA GTA ACG 5′	N2: 3′ TGT AGG TAC CAC GGG TTC TTG TGG 5′

Additionally, For *Plasmodium vivax*, to differentiate between reinfection and recrudescence, we used *Pvcs* and *PvMSP1* as described preciously (308). It was based on nested or semi-nested PCR approach.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4. Method for genotyping K13 (41)

High-fidelity PCR was applied for the full-length K-13 gene using Advantage HD DNA polymerase mix (Clontech Mountain View, CA, USA) with following primers:

<table>
<tr><th>Primary PCR</th><th>Sequencing primer</th></tr>
<tr><td rowspan="3">KP13-F:
5’- TATAACAAGGCGTAAATATTCGTG-3’)</td><td>KP13-65 F: 5’- GGGAATCTGGTGGTAACAGC-3’</td></tr>
<tr><td>KP13-640R: 5’- CACTAGCATCACTTAATTCCGTT-3’</td></tr>
<tr><td>KP13-517 F: 5’- GATGCAGCAAATCTTATAAATGATG-3’</td></tr>
<tr><td rowspan="3">KP13-R:
5’- TGTGCATGAAAATAAATATTAAAGAAG-3’</td><td>KP13-759R: 5’- GGAAAGAGTACGATTGTACAAAG-3’</td></tr>
<tr><td>KP13-1363R: 5’- CTACACCATCAAATCCACCTATA-3’</td></tr>
<tr><td>KP13-1595F: 5’- GTGGTGTTACGTCAAATGGTAG-3’</td></tr>
</table>

5. Method for real time PCR for detecting copy number of *Plasmepsin2*

Standard RT-PCR method was carried out using newly developed protocol by *Witkowski, et al 2017* (35).

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Table 7. Primer pairs for Plasmepsin-2 gene

qPCR	Primers	Sequences	Tm (ºC)	Product size (bp)	Range of melting temperature (ºC)
PfPM2	PfPM2_CN_F	5'-TGGTGATGCAGAAGTTGGAG-3'	59.8	79	76.8-77.2
	PfPM2_CN_R	5'-TGGGACCCATAAATTAGCAGA-3'	59.4		
	Pf β-tubulin_CN_F	5'-TGATGTGCGCAAGTGATCC-3'	61.9	79	79.0-79.2
	Pf β-tubulin_CN_R	5'-TCCTTTGTGGACATTCTTCCTC-3'	60.5		

6. PCR protocol

For this molecular examination, we used Mytaq^{TM} HS Red Mix. The PCR mix contains purified DNA samples (1 µl per sample), double distilled water (10.7 µl), primers (0.4 µl each) and Mytaq^{TM} HS Red Mix (12.5 µl). The PCR condition is as follows: 95°C, 1 minute of initial denaturation, 95°C, 15 seconds of denaturation, 54°C, 15 seconds of annealing, 72°C, 10 seconds of extension, 72°C, 15 minutes of final extension and 4°C for holding. The amplified product of PCR was then ran in gel electrophoresis and visualized in Gel documentation system.

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7. Electrophoresis protocol

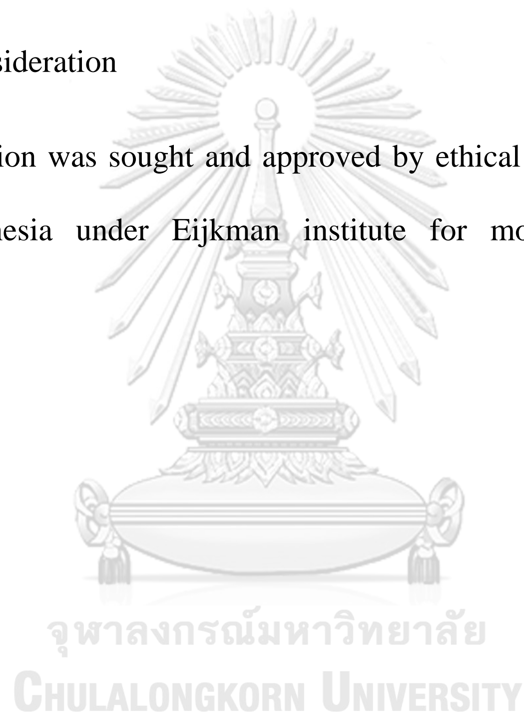
After PCR protocol was done, the PCR product then was run on gel electrophoresis apparatus. Initially, we made gel agarose by combining 2 gram of agarose powder into 1X TAE buffer and was heated in microwave for 30 seconds. After that, the gel was placed into electrophoresis apparatus and was left on for approximately 45 minutes. The PCR product was then loaded into the well of the gel and was run for approximately 60 minutes with 100-volt electricity. The gel was then entered in gel documentation (*Quantityone*) for visualization.

8. Pre-sequencing protocol

Prior sent to sequencing facility, the single-band PCR product was then purified using ExoSAP-IT™ cleanup reagent to remove primers and dNTPs. The PCR reaction was run with Big-Dye terminator RR Mix and purified to remove dye-ddNTPs. Eventually, the samples were sent to Biochem sequencing facility.

9. Ethical consideration

Ethical consideration was sought and approved by ethical committee of Hasanuddin University, Indonesia under Eijkman institute for molecular biology, Jakarta, Indonesia.



CHAPTER 4

RESULT

4.1.Risk factor of malaria

4.2.1. Entomological assessment

In order to better understand the risk factor of being infected with *Plasmodium* infection, thus our study discovered the differences of behavioural pattern of *Anopheles* species that may reflect the difference in annual parasite index between the two locations. In total 216 houses and catchers (108 at each location) have been done for collection of 2435 *Anopheles* mosquitoes. There was a statistically different number of *Anopheles* caught in Jambi and Sumba (*p value*= <0.0001). A total of 71 *Anopheles* has been obtained from Jambi, while a total of 2364 *Anopheles* has been obtained from Sumba. *Anopheles aconitus* (relative abundance: 40.02; Human Landing Rate (HLR): 8.76) and *Anopheles sundaicus* (relative abundance: 58.50; HLR: 12.81) were the predominant *Anopheles* mosquitoes found in Sumba. We also found other *Anopheles* in Sumba with minor number of collection i.e. *Anopheles farauti* (relative abundance: 0.04 and HLR: 0.01), *An. barbirostris* (relative abundance: 0.09 and HLR: 0.02), *An. maculatus* (relative abundance: 1.06 and HLR: 0.23), *Anopheles leucosphyrus* (relative abundance: 0.04 and HLR: 0.01), *Anopheles subpictus* (relative abundance: 0.17 and HLR: 0.04) and *Anopheles vagus* (relative abundance: 0.09 and HLR: 0.02) (**Table 8**). In Jambi, four species *Anopheles* have been found i.e. *Anopheles barbirostris*, *Anopheles balabacensis*, *Anopheles maculatus* and *Anopheles sinensis*. A leucosphyrus group (*Anopheles balabacensis*) was the most abundant species in the location followed by *Anopheles maculatus*, *Anopheles barbirostris* and *Anopheles sinensis* (**Table 8**).

Table 8. Species, total collection, relative abundance and human landing rate of *Anopheles* collected from Jambi and Sumba

Jambi (low endemicity)			
Species	**Total collection**	**Relative abundance (%)**	**Human landing rate**
An. balabacensis	56	78.87	0.52
An. barbirostris	1	1.41	0.01
An. maculatus	13	18.31	0.12
An. sinensis	1	1.41	0.01
Total	71		0.66
Sumba (high endemicity)			
Species	**Total collection**	**Relative abundance (%)**	**Human landing rate**
An. aconitus	946	40.02	8.76
An. barbirostris	2	0.09	0.02
An. farauti	1	0.04	0.01
An. leucosphyrus	1	0.04	0.01
An. maculatus	25	1.06	0.23
An. subpictus	4	0.17	0.04
An. sundaicus	1,383	58.50	12.81
An. vagus	2	0.09	0.02
Total	2,364		21.90

Anopheles biting time between Jambi and Sumba is different (**Figures 4 and 5**). Early evening (6 pm) was the time when *Anopheles balabacensis* reached its peak time (decreasing trend to midnight), while an irregular pattern of biting time has been found in *Anopheles maculatus*. *Anopheles aconitus* and *Anopheles sundaicus* shared a similar trend of biting time which is gradually increasing with peak biting time 21.00-22.00 and 01.00-02.00. After 01.00-02.00, it is progressively decreasing until 05.00-06.00. An irregular pattern of biting time has been found in *Anopheles maculatus* from Sumba.

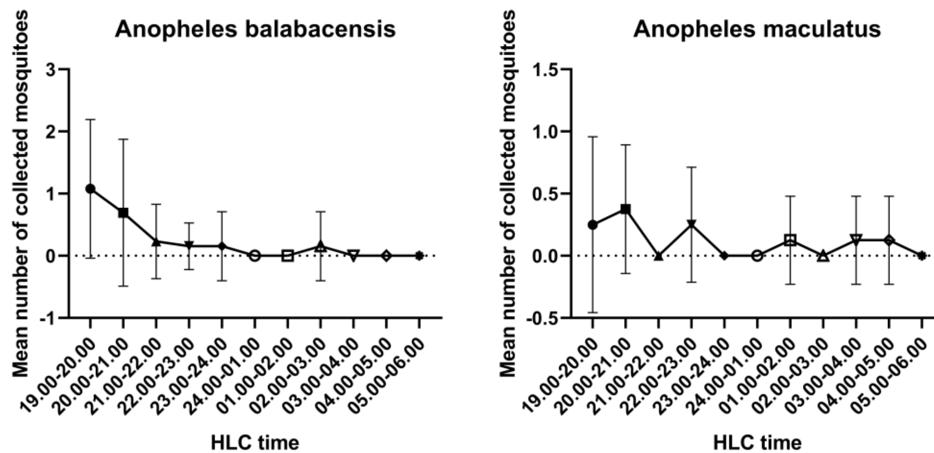


Figure 4. Biting time pattern of *Anopheles balabacensis* and *An. maculatus* collected from Jambi

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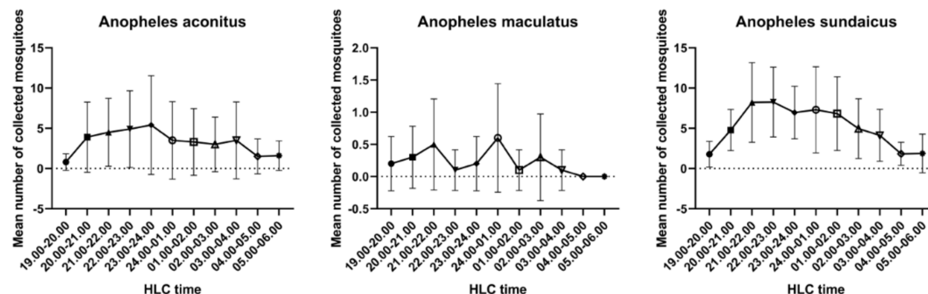

Figure 5. *Anopheles aconitus*, *An. maculatus* and *An. sundaicus* biting times in Sumba

A comparison of indoor and outdoor collection was done to discover biting preference (**Figures 6 and 7**). In case of *Anopheles balabacensis* from Jambi, the indoor collection was outnumbered that of outdoor collection significantly. However, no such significant difference has found in *Anopheles maculatus* from Jambi. Similarly, *Anopheles aconitus*, *Anopheles sundaicus* and *Anopheles maculatus* have been identified to be having no significant different between indoor and outdoor collection, suggesting indoor and outdoor activity in Sumba have the same risk for getting bitten by local *Anopheles vector*.

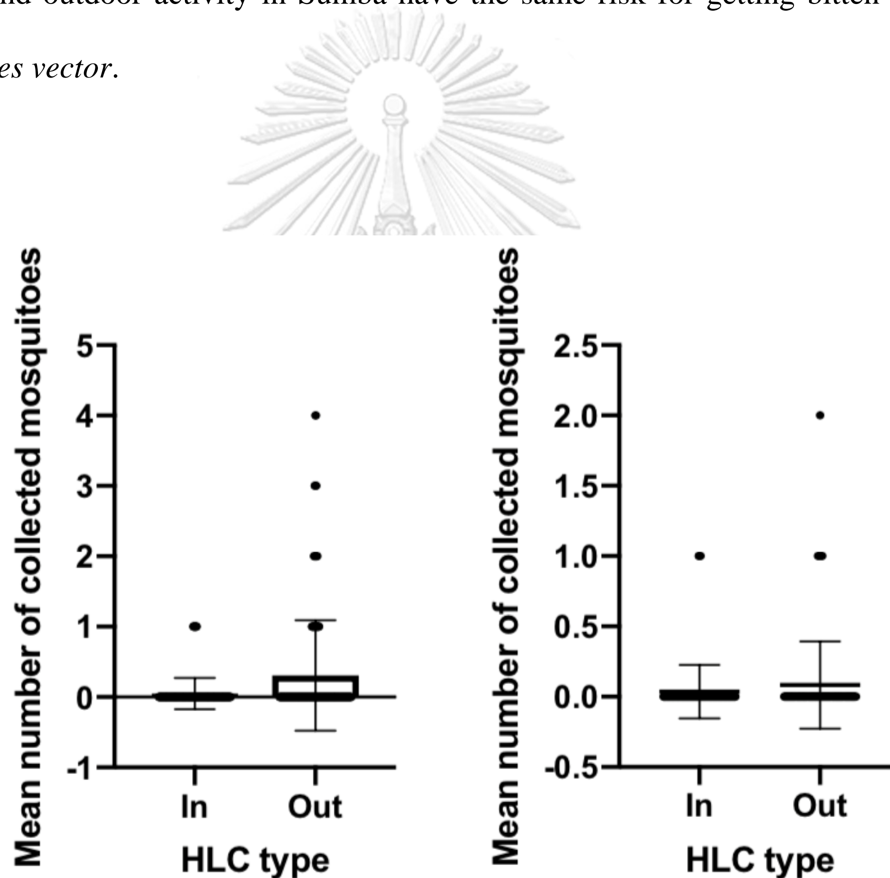

Figure 6. Indoor and outdoor biting preference of *Anopheles balabacensis* (left) and *An. maculatus* (right) in Jambi

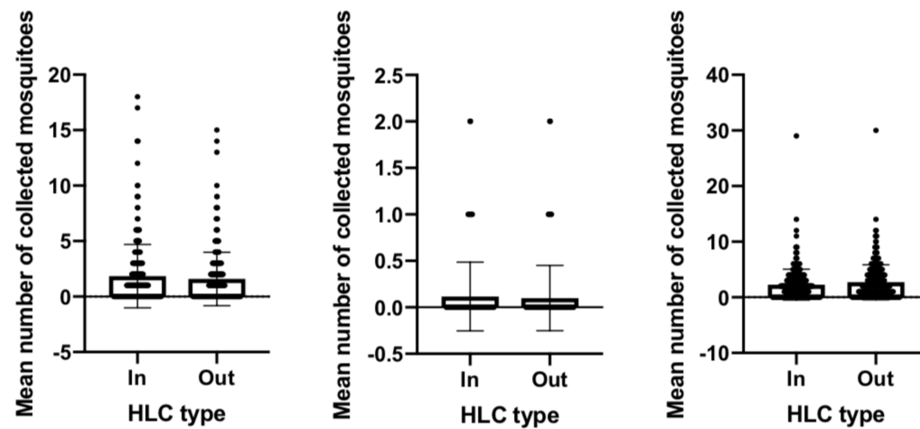

Figure 7. Mean number of *Anopheles aconitus* (left), *An. maculatus* (center) and *An. sundaicus* (right) indoors and outdoors in Sumba

A multiple comparison analysis for pooled data was done to discover the difference in biting time between Jambi and Sumba (**Table 9 and Figure 8**). In Jambi, biting time of early evening (18.00-19.00) was statistically different with that of 21.00-22.00 to 05.00-06.00 (*p value*= <0.00001). Moreover, the number of mosquitoes at 19.00-20.00 statistically outnumbered that of 24.00-01.00 and 05.00-06.00 (*p value*= 0.0435). In Sumba, the number of mosquito bites at 18.00-19.00 (early evening) significantly differed with all of other biting times, in exception to 19.00-20.00, 04.00-05.00 and 05.00-06.00 (*p value*= <0.0001-0.0069). Moreover, several statistically different biting times based on post hoc statistic test are: 19.00-20.00 vs 21.00-22.00; 22.00-23.00 vs 23.00-24.00; 21.00-22.00 vs 04.00-05.00; 22.00-23.00 vs 04.00-05.00 and 05.00-06.00, 23.00-24.00 vs 04.00-05.00 and 05.00-06.00; and 24.00-01.00 vs 05.00-06.00. The finding indicates that, in Jambi, early evening (18.00-20.00) is the vulnerable time for the people to get bitten by *Anopheles*. While in Sumba, a gradual increase trend of *Anopheles* biting pattern has been found from early evening (18.00-21.00) to early morning (02.00) with eventual declining trend to 6 am.

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Table 9. Summary of significant multiple comparisons between different mosquito biting times in Jambi and Sumba

Jambi			
Dunn's multiple comparisons test	Mean rank difference	Summary	Adjusted P value
18.00-19.00 vs. 21.00-22.00	77.81	****	<0.0001
18.00-19.00 vs. 22.00-23.00	67.15	***	0.0004
18.00-19.00 vs. 23.00-24.00	83.48	****	<0.0001
18.00-19.00 vs. 24.00-01.00	89.85	****	<0.0001
18.00-19.00 vs. 01.00-02.00	84.17	****	<0.0001
18.00-19.00 vs. 02.00-03.00	83.48	****	<0.0001
18.00-19.00 vs. 03.00-04.00	84.17	****	<0.0001
18.00-19.00 vs. 04.00-05.00	84.17	****	<0.0001
18.00-19.00 vs. 05.00-06.00	89.85	****	<0.0001
19.00-20.00 vs. 24.00-01.00	50.52	*	0.0435
19.00-20.00 vs. 05.00-06.00	50.52	*	0.0435

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Table 9. Summary of significant multiple comparisons between different mosquito biting times in Jambi and Sumba (continue)

Sumba			
Dunn's multiple comparisons test	Mean rank difference	Summary	Adjusted P value
18.00-19.00 vs. 20.00-21.00	-195.5	****	<0.0001
18.00-19.00 vs. 21.00-22.00	-238.1	****	<0.0001
18.00-19.00 vs. 22.00-23.00	-238.3	****	<0.0001
18.00-19.00 vs. 23.00-24.00	-232.0	****	<0.0001
18.00-19.00 vs. 24.00-01.00	-214.9	****	<0.0001
18.00-19.00 vs. 01.00-02.00	-204.8	****	<0.0001
18.00-19.00 vs. 02.00-03.00	-180.1	***	0.0001
18.00-19.00 vs. 03.00-04.00	-147.6	**	0.0069
19.00-20.00 vs. 21.00-22.00	-145.1	**	0.0090
19.00-20.00 vs. 22.00-23.00	-145.4	**	0.0088
19.00-20.00 vs. 23.00-24.00	-139.1	*	0.0168
21.00-22.00 vs. 04.00-05.00	150.2	**	0.0052
21.00-22.00 vs. 05.00-06.00	155.0	**	0.0030
22.00-23.00 vs. 04.00-05.00	150.5	**	0.0050
22.00-23.00 vs. 05.00-06.00	155.2	**	0.0030
23.00-24.00 vs. 04.00-05.00	144.2	**	0.0099
23.00-24.00 vs. 05.00-06.00	149.0	**	0.0059
24.00-01.00 vs. 05.00-06.00	131.9	*	0.0347

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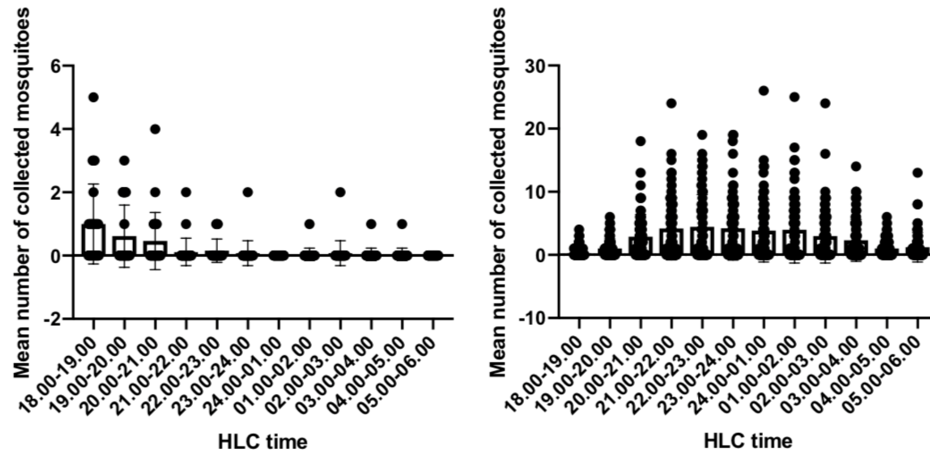

Figure 8. Mean number of *Anopheles* mosquito at different biting times in Jambi (left) and Sumba (right)

4.2.2. Malaria risk factors

This research had a duration of 4 months period in Jambi, the western part of Indonesia, and another four months in Sumba Island, the eastern part of Indonesia. It was started from February to October 2018. The total of 157 cases of both locations has been successfully collected during the field sampling time. The proportion of case and control was following a 1:2 ratio. Therefore, of 157 cases (109 cases from Sumba and 48 cases from Jambi), there are 328 controls (223 and 105 controls from Sumba and Jambi, respectively) with a percentage of 32.3% and 67.7%, respectively. Basic demography of each location is presented in **Table 10**. The portion of sex between Jambi and Sumba have a slightly similar pattern of male and female. Age strata from the two sites are identical at 6-24 years unless Sumba has more cases at early childhood (0-5 years).

Table 10. Basic characteristics of cases and controls from Sumba and Jambi.

Study site	Variable	Case		Control	
		Frequency	Percentage	Frequency	Percentage
Sumba	Sex				
	Male	63	57.7%	180	55.6%
	Female	46	42.3%	144	44.4%
	Age (years)				
	0-5	37	34.9%	37	11.3%
	6-24	59	55.7%	79	24.2%
	25-80	10	9.4%	211	64.5%
Jambi	Sex				
	Male	29	60.4%	58	37.9%
	Female	19	39.6%	95	62.1%
	Age (years)				
	0-5	9	18.8%	9	5.9%
	6-24	23	47.9%	34	22.4%
	25-80	16	33.3%	109	71.7%

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Several individual and environmental factors from both Sumba and Jambi have been associated with malaria incidence **Table 11** and **Table 12**. There are seven associated individual variables with malaria from Sumba. The individual risk factors of malaria in Sumba are not having ITNs (OR = 2.55; 95%CI: 1.52-4.29), low education level (OR = 6.09; 95%CI : 2.12-17.47), ever consumed antimalarial drug (OR = 4.16; 95%CI : 2.09-8.28), ever contacted with malaria person (OR = 17.33; 95%CI: 8.04-37.32), frequent traveling outside residential area (OR = 5.38; 95%CI: 2.39-12.09), ever visited forest in a previous month (OR = 1.96; 95%CI: 1.03-3.73) and requiring

overnight stay in the forest (OR = 2.88; 95%CI: 1.22-6.81). Additionally environmental risk factors associated with malaria are existence of shrubs surrounding house (OR = 20.99; 95%CI: 8.24-53.46), existence of puddle or stagnant water surrounding house (OR = 39.98; 95%CI: 13.86-115.32), existence of livestock inside house (OR = 3.24; 95%CI: 1.70-6.18), existence of livestock nearby house (OR = 9.44; 95%CI: 2.87-31.07), non-permanent house wall (OR = 5.22; 95%CI: 1.81-15.06) and non-permanent floor construction (OR = 20.79; 95%CI: 2.81-153.79) and not having a ceiling of the rooftop (OR = 19.72; 95%CI: 1.18-330.29). In Jambi, night activity outdoor (OR = 0.32; 95%CI: 013-0.79) and history of visiting forestry area in the previous month (OR = 0.35; 95%CI: 0.15-0.84) working place is inside forest (OR = 0.17; 95%CI: 0.07-0.43) are being protective factors against malaria infection. The individual risk factor for malaria infection in Jambi are not having ITNs (OR = 2.09; 95%CI: 1.04-4.18), education (OR= 12.2; 95%CI: 2.52-59.02) occupation (*p value* < 0.001) and contact with malaria-infected patient (OR =. 3.37; 95%CI: 1.62-7.01). The observed environmental factors that are associated with malaria in Jambi are the existence of shrubs around house area (OR = 28.00; 95%CI: 6.45-121.59), the existence of puddle around house area (OR = 2.49; 95%CI: 1.05-5.98), the presence of livestock nearby house area (OR = 6.36; 95%CI: 2.94-13.79) and the proximity of house to forestry area (OR = 10.84; 95%CI: 3.97-29.58). Since Sumba and Jambi have different endemicity level, the GLM univariate was applied to discover the spatial effect of risk factor variable on malaria infection. Due to any difference in the number of cases of both locations, only prospective cases from Sumba was included in the analysis. Prospective cases are those who enumerated within 5-7 days after confirmed by RDT or the result from two independent microscopists or combination of both. The result of GLM

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indicates that there is an effect of area in risk factor variable on malaria infection (*p value*= 0.002) (**Table 13**).

Table 11. Variables associated with malaria infection in Sumba.

Variable	**Case** (n=109)	**Control** (n=223)	***p-value***	**OR [95%CI]**
Night activity outdoor Yes No	 94 15	 216 29	0.605	1.189 [0.609, 0.364]
ITNs possession No Yes	 39 70	 40 183	<0.001*	2.55 [1.52, 4.29]
Education Low educated High educated	 100 4	 178 64	<0.001*	8.989 [3.179, 25.416]
Occupation Farmer Teacher Civil servant Others	 10 2 0 8	 199 5 3 38	0.004*	
Antimalarial drug consumption Yes/ever No	 25 83	 15 207	<0.001*	4.16 [2.09, 8.28]
Contact with malaria person Yes No	 101 8	 94 129	<0.001*	17.33 [8.04, 37.32]
Ever visiting outside village area Yes No	 71 38	 135 109	0.102	0.663 [0.415, 1.058]
Frequency of traveling outside residential area Once a day Once a week Once a month[ref]	 39 24 8	 18 64 56	<0.001*	5.38 [2.39, 12.09]
Visited forest in a previous month Yes No	 95 14	 173 50	0.038*	1.96 [1.03, 3.73]

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Variable	**Case** (n=109)	**Control** (n=223)	***p-value***	**OR [95%CI]**
Requiring overnight stay in the forest Yes No	13 96	10 213	0.012*	2.88 [1.22, 6.81]
Working place is inside forest Yes No	24 85	190 54	<0.0001*	12.461 [7.228, 21.483]
The existence of barrier on ventilation No Yes	103 2	225 18	0.046*	4.120 [0.938, 0.031]
The existence of pool nearby house Yes No	0 106	1 234	1.000	
Existence of shrubs Yes No	104 5	111 112	<0.001*	20.99 [8.24, 53.46]
Existence of puddle or stagnant water Yes No	46 63	4 219	<0.001*	39.98 [13.86, 115.32]
Existence of livestock inside house Yes No	96 13	155 68	<0.001*	3.24 [1.70, 6.18]
Existence of livestock nearby house Yes No	106 3	176 47	<0.001*	9.44 [2.87, 31.07]
Type of house wall Made by wood Made by cement[ref] (permanent construction) Made by bamboo	4 1 104	68 37 118	<0.001*	5.22 [1.81, 15.06]

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Variable	**Case** (n=109)	**Control** (n=223)	***p-value***	**OR [95%CI]**
House is in a close proximity to rice field Yes No	 3 106	 0 223	0.0764	14.69 [0.75, 286.96]
House floor construction Non-permanent Permanent	 108 1	 187 36	<0.001*	20.79 [2.81, 153.79]
Ceiling in the rooftop No Yes	 109 0	 205 18	0.002*	19.72 [1.18, 330.29]

***** Statistically significant at *p value*<0.05; ref: reference

Table 12. Variables associated with malaria infection in Jambi.

Variable	**Case (48)**	**Control (105)**	***p-value***	**OR [CI]**
Night activity outdoor Yes No	36 12	95 10	0.011*	0.32 [0.13, 0.79]
ITNs possession No Yes	27 21	40 65	0.036*	2.09 [1.04, 4.18]
Education Low educated High educated	9 38	2 103	0.0019*	12.2 [2.52, 59.02]
Occupation Farmer Miners Teacher Civil servant Others	 14 1 1 0 28	 100 0 1 1 3	<0.001*	-
Antimalarial drug consumption Yes/ever No	4 44	5 100	0.463	0.550 [0.141, 2.147]
Contact with malaria infected patient Yes No	34 14	44 61	0.001*	3.37 [1.62, 7.01]

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Variable	Case (48)	Control (105)	*p-value*	OR [CI]
Ever visiting outside village area			0.862	1.108 [0.559, 2.194]
Yes	23	53		
No	25	52		
Frequency of traveling outside residential area			0.680	
Once a day	13	18		
Once a week	4	10		
Once a month[ref]	6	11		
Visited forest in a previous month			0.016*	0.35 [0.15, 0.84]
Yes	35	92		
No	13	12		
Requiring overnight stay in the forest			0.225	0.636 [0.319, 1.27]
Yes	28	49		
No	20	55		
Working place is inside forest			<0.001*	0.17 [0.07. 0.43]
Yes	32	96		
No	16	8		
The existence of barrier on ventilation			0.307	
No	48	99		
Yes	0	4		
The existence of pool nearby house			0.579	1.494 [0.449, 4.969]
Yes	4	11		
No	44	81		
The existence of shrubs around house area			<0.001*	28.00 [6.45, 121.59]
Yes	46	46		
No	2	56		
The existence of puddle around house area			0.035*	2.49 [1.05, 5.89]
Yes	13	13		
No	35	87		

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Variable	Case (48)	Control (105)	*p-value*	OR [CI]
Existence of livestock inside house Yes No	2 46	10 93	0.340	2.473 [0.520, 11.754]
The presence of livestock nearby house area Yes No	36 12	33 70	<0.001*	6.36 [2.94, 13.79]
Type of house wall Made by wood Made by cement (permanent construction)	39 9	80 20	1.000	1.083 [0.452, 2.598]
House is in a close proximity to rice field Yes No	0 48	1 101	1.000	
House floor construction Non-permanent Permanent	39 9	83 21	1.000	0.912 [0.383, 2.174]
Ceiling in the rooftop No Yes	29 19	54 38	0.859	1.074 [0.527, 2.189]

* Statistically significant at *p value*<0.05; ref: reference

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Table 13. Result of type III analysis of GLM univariate assessing the effect of area on risk factor variable of malaria infection.

Response variable	Variable's name	Num DF	F value	P value
Area	Risk factor	1	9.865	.002

In order to prove the finding of better construction element could prevent malaria, then we extensively performed a comparison study among different housing construction from entomological point of view. As described in the method section, in

this particular study, we enrolled a three types of housing construction; Malaria house (MH), Non-malaria house (NH) and Permanent house (PH). MH and NH shared the same housing condition which is non-permanent. However, to better understand the effect of housing to malaria infection, we differentiated MH and NH into two different types. While, PH was a housing type that is not having malaria infection.

There is a significant difference between the total number of mosquitoes collected from Sumba and Jambi (*p value* <0.0001) (**Figure 9**). There is no significant difference in each house types from Jambi (*p value*= 0.1856). Although MH from Jambi have the highest mean collected mosquitoes (0.64) than the other house types, yet, PH (0.34) is higher of mean collected mosquitoes than the NH (0.29) (**Figure 10**). On the contrary, there is a significant difference between MH vs NH (*p* value= 0.0143) and PH (*p value*= 0.0351) in Sumba as presented in **Figure 11**. However, no difference was observed between NH and PH (*p value* >0.9999). Additionally, if both sites are combined (**Figure 12**), only MH and NH that have a significant difference in the number of collected mosquitoes (*p value*= 0.0301). PH is slightly higher in the mean number of collected mosquitoes compared to NH (5.6 and 5.032, respectively).

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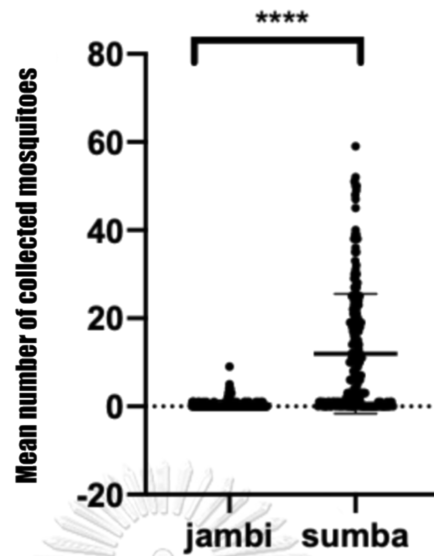

Figure 9. The mean number of mosquitoes in Jambi and Sumba. Asterisks (****) indicated statistically significant at *p value* <0.0001.

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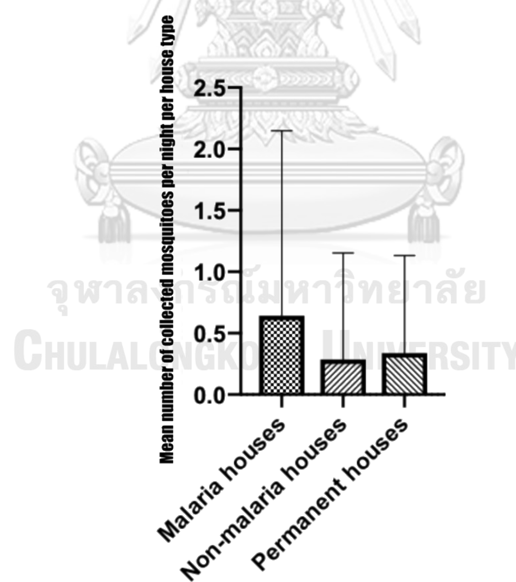

Figure 10. The mean number of mosquitoes collected in different house types in Jambi.

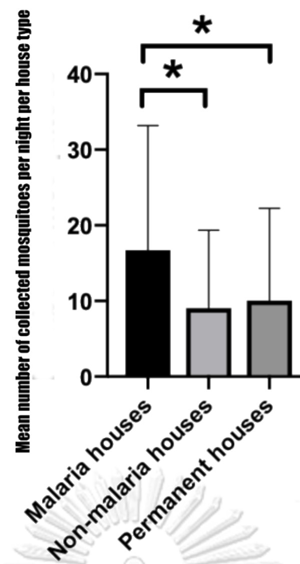

Figure 11. The mean number of mosquitoes collected in different house types in Sumba. An asterisk (*) indicated statistically significant at *p value* <0.05.

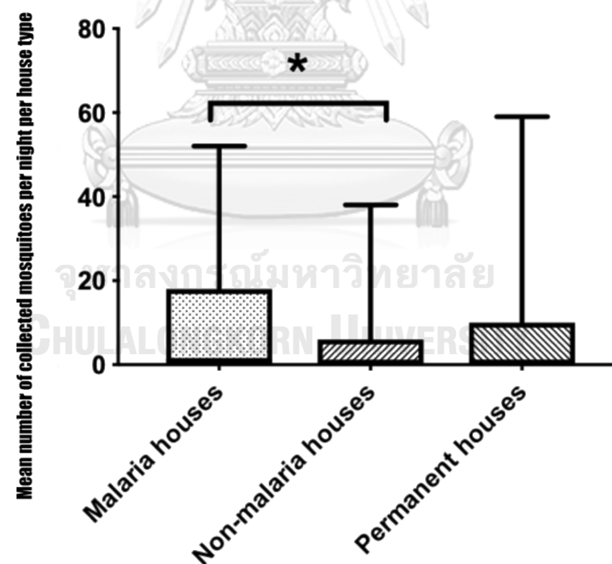

Figure 12. The mean number of mosquitoes collected in different house types in Jambi and Sumba. An asterisk (*) indicated statistically significant at *p value* <0.05.

Malaria has detected only one in the first week from Jambi. Otherwise, 10 cases were detected during three weeks of observation from Sumba (**Table 14**). If the number

of cases is transformed into an incidence rate per collection method per year, then MH from Jambi have 1.4 incidence rate per year and null for other types of houses. However, considering the difference in the endemicity level in Sumba, MH has 8.7 incidence rate per year while NH and PH have the same rate of 2.9. Additionally, based on the calculated odds ratio, the odd ratio of MH compared to another house type is 3.77 (95%CI: 0.76-18.81) while NH versus PH have the odd ratio of 1 (95%CI: 0.14-7.30).

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Table 14. The result of weekly screening of malaria infection per house types in Jambi and Sumba.

Weekly screening report									
Week	Area	Type of house	Number of infections	Species	Incidence rate Cases per collection method-week	Incidence rate Cases per collection method-year	OR (CI)		
							MH vs NH	MH vs PH	NH vs PH
1	Jambi	MH	1	Pv	0.08	1.4			
2	Jambi	-	-	-	0	0			
3	Jambi	-	-	-	0	0			
1	Sumba	MH	3	Pf R102, Pf R80, Pf R2	0.25				
		PH	1	Pf R2 G1	0.08		3.77 [0.76, 18.81]	3.77 [0.76, 18.81]	1.00 [0.14, 7.30]
2	Sumba	-	-	-	0				
3	Sumba	MH	3	Pf R568, Pm R8 Tr10, Pm R1 Tr1	0.25	8.7			
		NH	2	Pf R5, Pf	0.17	2.9			
		PH	1	Pf R232	0.08	2.9			

R : *ring stage*

Tr : *Trophozoite stage*

G : *Gametocyte stage*

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4.3. Population adherence to dihydroartemisinin+piperaquine and primaquine medication in Indonesia

4.3.2. Dihydroartemisinin+piperaquine

Survey profile

In total, 200 patients were positively detected for malaria parasite and given DHP medication. Out of 200, 62 was detected in Jambi province and the other 138 was from Sumba Island. In Jambi, out of 62 malaria patients, 47 (75.8%) patients were able to be visited and interviewed at the day of the completion day of the medication. The remaining 15 (24.2%) patients were unable to be visited due to traveling outside study area. In Sumba Island, 138 patients were given DHP treatment. However, only 65.9% (91 patients) that was successfully collected for home-visit interview. The rest of 47 patients (34.1%) was unable to reach because of either working inside forestry area or traveling to unknown area. No patient has ever refused to be our study participant.

Table 15. The description of socio demographic variable of the patients and the caretakers in Jambi and Sumba

Socio-demographic factor	Jambi (%)	Sumba (%)
Age group		
< 5 years (young children)	9 (19.1)	22 (24.2)
5-14 (school age children)	19 (40.4)	52 (57.1)
15 years and above (independent adults)	19 (40.4)	17 (18.7)
Sex		
Male	30 (63.8)	52 (57.1)
Female	17 (36.2)	39 (42.9)
Caretaker relation to patient		
Patient	18 (38.3)	30 (33)
Father/mother	25 (53.2)	54 (59.3)
Grandfather/grandmother	1 (2.1)	2 (2.2)
brother/sister	2 (4.3)	3 (3.3)
Uncle/aunty	1 (2.1)	2 (2.2)

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Highest education of patient		
Can't read/write	8 (17.4)	51 (56)
Primary incomplete	16 (34.8)	18 (19.8)
Primary complete	13 (27.7)	12 (13.2)
Secondary incomplete	1 (2.2)	0
Secondary complete	4 (8.7)	6 (6.6)
Higher incomplete	0	0
Higher complete	4 (8.7)	4 (4.4)
Highest education of caretaker		
Can't read/write	1 (3.4)	21 (34.4)
Primary incomplete	1 (3.4)	13 (21.3)
Primary complete	14 (48.3)	13 (21.3)
Secondary incomplete	0	4 (6.6)
Secondary complete	4 (13.8)	6 (9.8)
Higher incomplete	0	0
Higher complete	9 (31)	4 (6.6)

*Table 1Figure 1**Socio-demographic description***

In Jambi, the majority of study participants were 5-14 years (40.4%) and >15 years (40.4%), while the rest was under 5 years (19.1%). In the other hand, the participants in Sumba Island were dominated with 5-14 years (57.1%) and under 5 years (24.2%), while the rest was above 15 years (18.7%). Sex ratio in Jambi was 1.8 (male/female; 30/17), while in Sumba island was 1.3 (male/female; 52/39). In Jambi, the majority of the patients was non-educated (52.2%; can't read/write and primary incomplete) followed by poor-educated (38.6%) and high-educated (8.7%). Similar condition has been observed in Sumba where the majority of the patients was non-educated (75.8%; can't read/write and primary incomplete) followed by poor-educated (19.8%) and high-educated (4.4%). It was inversely proportional between Jambi and Sumba Island related to caretaker education where Jambi was dominated with poor- and high-educated persons (93.1%) while Sumba Island was dominated with non- and poor-educated persons (93.4%) (**Table 15**).

In Jambi, mostly the household member was proportional (1-4 household members; 76.6%) while Sumba island was dominated with non-proportional household member (5-8 household members; 72.5%) (**Table 16**). The majority of patients in Jambi was not having children under five (40.4%) or having only 1 child under five (38.3%). Contrarily, all the household of the patients was having at least 1 child under five (range: 1-6). Jambi and Sumba island shared a similar pattern of profession of heads of households which is farmer for trading (Jambi: 95.7% and Sumba: 75.8%).

Table 16. The description of socio demographic information of household in Jambi and Sumba

Socio demographic of household	Jambi (%)	Sumba (%)
Number of household member		
1-4	36 (76.6)	21 (23.1)
5-8	10 (21.3)	66 (72.5)
9-12	1 (2.1)	4 (4.4)
Number of children < 5 years old in the house		
0 children	19 (40.4)	0
1 children	18 (38.3)	47 (51.6)
2 children	10 (21.3)	15 (16.5)
3 children	0	3 (3.3)
4 children	0	1 (1.1)
5 children	0	0
6 children	0	1 (1.1)

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Socio demographic of household	Jambi (%)	Sumba (%)
Profession of head of household		
Farmer for trading	45 (95.7)	69 (75.8)
Trader	1 (2.1)	0
Employee	0	2 (2.2)
Odd jobs	0	1 (1.1)
Unemployed	0	10 (11)
Other	1 (2.1)	5 (5.5)

Patient adherence

In Jambi, there is one (2.1%) patient that the pills were visible, or the tablets remained in the blister package at the day of the completion day of DHP medication (**Table 17**). Fifteen (31.9%) of the patients were considered probable non adherence since no blister seen and they answered incorrectly the dose and time they should have taken. The remaining 31 (66%) patients were considered probable adherence since no blister seen and the patients answered correctly the dose and time they should have taken. Additionally, out of those probable adherence patients, two (4.25%) of them had no pills inside the blister package with correct answer. In Sumba island, two (2.2%) of the total interviewed patients still had DHP pills in their blister and was considered certain non-adherence. Seventeen patients were considered probable non adherence since no blister seen and they have incorrectly answered the dose and time they should have taken. The rest 72 (79.1%) patients were categorized as probable adherence (no blister seen and correct answer of dose and time). Two patients from Jambi and four patients from Sumba Island vomited the pills at the first day of the treatment regimen (day 0 and day 1).

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Table 17. Community adherence to dihydroartemisinin+piperaquine regimen in Jambi province and Sumba island, Indonesia

	Jambi		Sumba	
Calculation of adherence	Incomplete/incorrect intake described	Complete/correct intake described	Incomplete/incorrect intake described	Complete/correct intake described
No blister	15	25	17	68
Blister empty	0	6	0	4
Blister with pills	1	0	2	0
Classification of adherence	Number of patients	Proportions (%)	Number of patients	Proportions (%)
Certain non-adherence	1	2.10	2	2.20
Probable non-adherence	15	31.90	17	18.70
Probable adherence	31	66	72	79.10
Adherence status	Number of patients	Proportions (%)	Number of patients	Proportions (%)
Non-adherent	16	34	19	20.90
Adherent	31	66	72	79.10

Reason for incomplete, incorrect and correct intake

Reason for incomplete, incorrect and correct intake of the patients were recorded (**Table 18**). In Jambi, the reason of incomplete intake was patient was cured didn't need to continue the medication, while in Sumba was patient forgot to take the pill/caretaker forgot to give the pill and other reason. The major reason of incorrect intake of ACT medication was similar between Jambi and Sumba which is patient/caretaker claims that incorrect instruction was given. Similarly, for the reason of correct intake between the two localities was correct instruction was given in the clinic/primary health facility/sampling location (Jambi: 88% and Sumba: 98.6%). However, six (12.8%) patients from Jambi was missing for the answer of correct intake.

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Table 18. Reason for incomplete, incorrect and correct intake given by the patients

	Jambi		Sumba	
Reasons given for incomplete intake (pills remaining)	**N**	**Percent (%)**	**N**	**Percent (%)**
Patient was cured and didn't need to continue the medication	1	100	0	0
Patient forgot to take the pills/ caretaker forgot to give the pills	0	0	1	50
others	0	0	1	50
Reasons given for incorrect intake	**N**	**Percent (%)**	**N**	**Percent (%)**
Patient/caretaker thought that the patient will cure faster	1	6.7	0	0
Patient/caretaker claims that incorrect instruction was given	13	86.7	15	88.2
Patient can't swallow the pills	1	6.7	0	0
Others	0	0	2	11.8
Reasons given for correct intake	**N**	**Percent (%)**	**N**	**Percent (%)**
Patient/caretaker/household member have taken the same pills before, so understood how to take it	2	8	1	1.4
Correct instruction was given in the clinic/primary health facility/sampling location	22	88	71	98.6
others	1	4	0	0

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Assessment of possible risk factors

Univariate and multivariate analysis for assessing possible risk factors for non-adherence behavior have been done (**Table 19**). The possible risk factors included sex, patient age group, education of caretaker, understanding of malaria cause and preventive including bed nets can prevent malaria, the presence of bed nets inside household and the understanding of ACT use. Age group was associated with non-adherence behavior (*p value*=0.020). Children blow 5 years old were more likely to be not adhering with ACT medication (OR= 2.79 [95%CI; 1.188-6.552]). The other risk factor of non-adherence behavior was the understanding of the patient to malaria cause and prevention; bed net prevents malaria (*p value*= 0.025). The understanding of malaria cause and prevention (bed net prevents malaria) was more likely a protective factor for adherence (OR= 0.29 [95%CI; 0.09-0.9]).

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Table 19. Associated risk factors of adherence to dihydroartemisinin+piperaquine medication in Indonesia

Risk factors	Adherence (n)	Non-Adherence (n)	OR	95% CI	P value
Patient Sex					
Male	62	20			
Female	41	15	0.882	0.405-1.918	0.751
Patient age group					
Under 5 child	18	13			
Above 5 years	85	22	2.79	1.188-6.552	**0.020**
Education of caretaker					
Illiterate	25	11			
Any education	40	14	1.26	0.49-3.20	0.631
Bed net prevents malaria					
No	96	28			
Yes	7	7	0.29	0.09-0.90	**0.025**
Bed nets observed					
No	4	2			
Yes	98	32	1.53	0.27-8.76	0.63
Understanding of ACT use					
No	82	28			
Yes	21	7	1.02	0.39-2.67	0.961

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4.3.3. Primaquine

Malaria situation in Lembah masurai sub-village, Jambi province

Malaria caused by *Plasmodium vivax* started to be prevalent from December 2014 with an incidence rate of 0.5. The case was highest in January 2015 with an incidence rate of 0.75 and maintained until the end of the year 2015. Limited cases have been observed during the year 2016 with the highest incidence rate of 0.2. At the beginning of the year 2017, *Plasmodium vivax* started to increase throughout the year (**Figure 13**). No *Plasmodium falciparum* has been detected in the area based on the report of local primary health care personnel. The data indicated that malaria foci have been maintained in the area in the past five years.

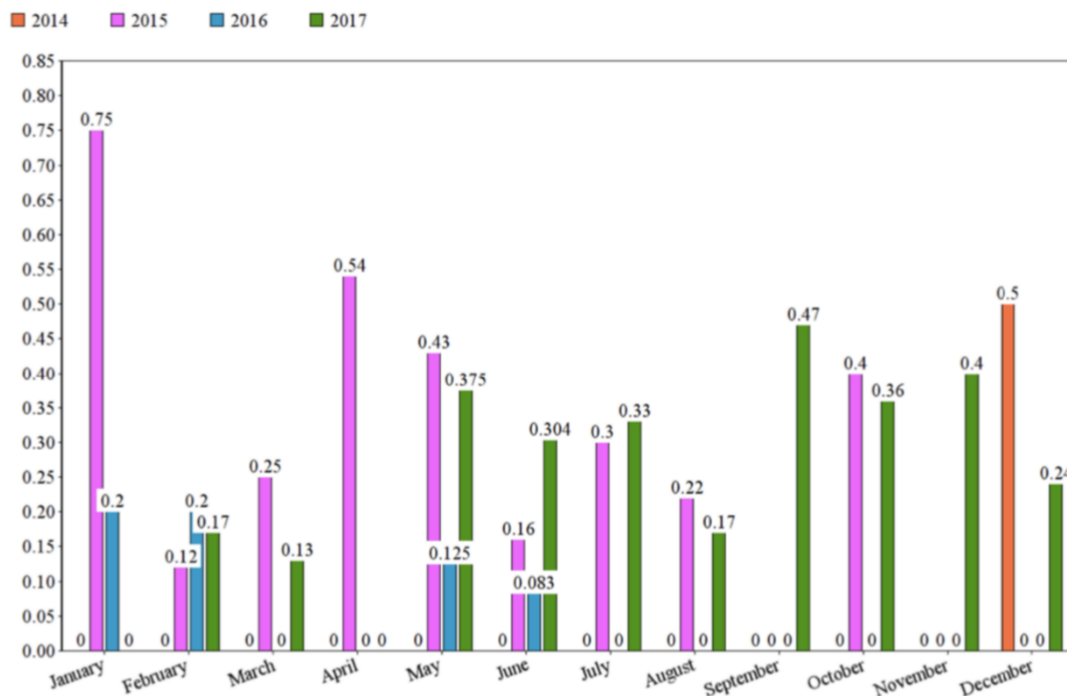

Figure 13. Incidence of malaria caused by *Plasmodium vivax* in Lembah masurai sub-district between 2014-2017

General characteristics of the samples

A total of 44 individuals were recruited in the current study. There were 26 (59.1%) male and 18 (40.9%) female individuals. The vast majority of samples were independent adults (45.5%) and the rest was young child (18.2%) and school age children (36.4%). Approximately half of the patients (43.2%) were able to take medication by themselves and the other half were given by their caretakers (50% by father or mother and 2.3% by grandfather/grandmother or brother/sister or uncle/aunty). The majority of education of the samples were either complete or incomplete primary school (63.6%), 15.9% of them were unable to write or read, or the rest were higher than primary school (20.5%). The highest education of caretakers was primary school (27.3%), higher education (15.9%), secondary high school (9.1%) and primary incomplete and unable to write or read (2.3%) (**Table 20**).

Table 20. Socio-demographic information of the population

Socio-demographic characteristics	Total (%)
Age group	
Under 5 years (young children)	8 (18.2)
5-14 years (school age children)	16 (36.4%)
15 years (independent adults)	20 (45.5%)
Sex	
Male	26 (59.1%)
Female	18 (40.9%)
Caretaker relation to patient	
Patient	19 (43.2%)
Father/mother	22 (50%)
Grandfather/grandmother	1 (2.3%)
brother/sister	1 (2.3%)
Uncle/aunty	1 (2.3%)
Highest education of patient	
Can't read/write	7 (15.9%)
Primary incomplete	15 (34.1%)
Primary complete	13 (29.5%)
Secondary incomplete	1 (2.3%)
Secondary complete	4 (9.1%)

Socio-demographic characteristics	Total (%)
Higher incomplete	0
Higher complete	4 (9.1%)
Highest education of caretaker	
Can't read/write	1 (2.3%)
Primary incomplete	1 (2.3%)
Primary complete	12 (27.3%)
Secondary incomplete	0
Secondary complete	4 (9.1%)
Higher incomplete	0
Higher complete	7 (15.9%)

Level of adherence

Of the 44 samples recruited, 5 (11.3%) had remaining primaquine pills on their blister defined as certain non-adherence. There were 13.6% defined as probable adherence as no remaining pills found in their blister package. Out those whose blister unable to see, 47.7% were considered probable adherence as they were able to describe correctly time and number of pills taken and 27.3% were considered probable non-adherence as they were unable to describe correctly time and number of pills taken. In total, 11.4 % of the patients were certain non-adherence, 27.3% were probable non-adherence and 61.4% were probable adherence. Therefore, if we restrict the classification into adherent and non-adherent only, there were 38.6% non-adherent and 61.4% adherent individuals (**Table 21**).

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Table 21. Adherence level to primaquine medication in Lembah masurai sub-district

Calculation of adherence	Incomplete/incorrect intake described	Complete/correct intake described
No blister	12	21
Blister empty	0	6
Blister with pills	5	0
Classification of adherence		
Certain non-adherence	5	11.40%
Probable non-adherence	12	27.30%
Probable adherence	27	61.40%
Adherence status		
Non-adherent	17	38.60%
Adherent	27	61.40%

Risk factors of adherence

Out of six variables, there is only one variable associated with non-adherence behavior. The variables are sex, age group, primaquine prescription, understanding of malaria cause, the existence of bed nets and understanding of primaquine use. Primaquine prescription is associated with non-adherence behavior (*p value* = 0.044) (**Table 22**). The odd ratio of primaquine prescription with other drugs is 8 indicating that a person who has been given primaquine medication with other drugs may have 8 times higher of possibility to be non-adherent.

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Table 22. Associated factors of non-adherence behavior to primaquine medication

Risk factors	Adherent (%)	Non-Adherent (%)	OR	95% CI	P value
Patient Sex					
Male	18	8			
Female	9	9	0.44	0.13-1.54	0.198
Patient age group					
Under 5 years	4	4			
Above 5 years	23	13	1.77	0.38-8.28	0.690
Primaquine prescription					
Primaquine only	26	13			
Primaquine with other drugs	1	4	8.00	0.81-79.02	**0.044**
Malaria caused by mosquito bites					
No	15	13			
Yes	12	4	2.6	0.67-10.06	0.16
Bed nets observed					
No	0	2			
Yes	27	15	10.19	0.46-227.32	0.068
Understanding of primaquine use					
No	15	14			
Yes	11	3	3.42	0.79-14.88	0.092

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Reason of incomplete, incorrect and correct intake

The major reason of incomplete intake of the pills is patient felt unwell/the medication was not working properly (60%) (**Table 23**). The other reasons are patient was cured and didn’t need to continue the medication (20%) and patient was cured and saved the pills for other occasions (20%). Reasons given for incorrect intake are patient/caretaker claims that incorrect instruction was given (70.6%), patient/caretaker thought that the patient will cure faster (11.8%), patient can’t swallow the pills (5.6%) and others (11.8%). Additionally, correct instruction was given in the clinic is the major reason for correct intake (84%).

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Table 23. Reasons given by patients for incomplete, incorrect and correct primaquine intake.

Reason for primaquine intake	No	Percent (%)
Reasons given for incomplete intake (pills remaining)		
Patient was cured and didn't need to continue the medication	1	20
Patient was cured and saved the pills for other occasion	1	20
Patient felt unwell/ the medication wasn't working properly	3	60
Reasons given for incorrect intake		
Patient/caretaker thought that the patient will cure faster	2	11.8
Patient/caretaker claims that incorrect instruction was given	12	70.6
Patient can't swallow the pills	1	5.6
Others	2	11.8
Reasons given for correct intake		
Patient/caretaker/household member have taken the same pills before, so understood how to take it	3	12
Correct instruction was given in the clinic/primary health facility/sampling location	21	84
Patient was helped by local community health volunteers	1	4

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4.4. Efficacy of dihidroartemisinin+piperaquine for *Plasmodium* malaria treatment in Indonesia

The study was conducted in collaboration with Eijkman institute for molecular biology, Jakarta and World Health Organization. The study was located in Jambi (western part of Indonesia, Sumatra Island) and Keerom province (eastern part of Indonesia, Papua Island). The study was initially taken from Keerom in April 2017-January 2018 and followed by in Jambi between October 2017-April 2018. In general, no *Plasmodium falciparum* detected in Jambi, contrarily both *Plasmodium falciparum* and *Plasmodium vivax* have been detected and enrolled for efficacy study in Keerom.

Efficacy of dihydroartemisinin+piperaquine against *Plasmodium falciparum* and *Plasmodium vivax* in Keerom province, Papua, Indonesia

The efficacy study was conducted in three localities in keerom, namely primary health care (PHC) of Arso barat, PHC of Arso kota and PHC of Waris.

4.4.1. Plasmodium falciparum

Baseline characteristics

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In total, there 114 patients that were successfully enrolled in the study. The ratio of male/female was 56/58. The study was dominated with adults (56) followed by adolescents (49) and young child under-5 (8). The mean age of the patients in the study was 22 years old (SD: 16.8) ranged between 1 to 65 years old. The mean weight of the patients in the study was 41.1 kg (SD: 18.4) ranged between 8.5 to 98.8 kg. The mean temperature of the patients in the study was 37.6 ºC (SD: 1.2) ranged between 35 to 39.9 ºC. Finally, mean geometric parasitemia of all enrolled patients was 10777 parasites/µl ranged between 600 to 213.000 parasites/µl.

Table 24. Characteristics of study patients infected with *Plasmodium falciparum* in Keerom

Number of patients	114
Ratio male/female	56/58
Age group (years)	
Adults	56
5 to 15	49
under 5	8
Age (years)	
mean (SD)	22 (16.8)
range (min-max)	1-65
Weight (kg), day 0	
mean weight (SD)	41.1 (18.4)
range (min-max)	8.5-98.8
Temperature (°C), day 0	
mean temperature (SD)	37.6 (1.2)
range (min-max)	35-39.9
Parasitemia (parasites/µl), day 0	
mean (geometric) parasitemia	10777
range (min-max)	600-213000

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Summary of follow up

At enrolment day, there was 114 patients that met our inclusion criteria with 11 patients considered as loss of follow up. No early treatment failure (ETF) was detected across all enrolled patients. There was one patient who experienced late clinical failure (LCF). Microscopically, six patients were classified as late parasitological failure (LPF). However, after considering for PCR-corrected, the number of LPF was decreased to 1. The remaining 95 patients were classified as adequate clinical and parasitological response (ACPR).

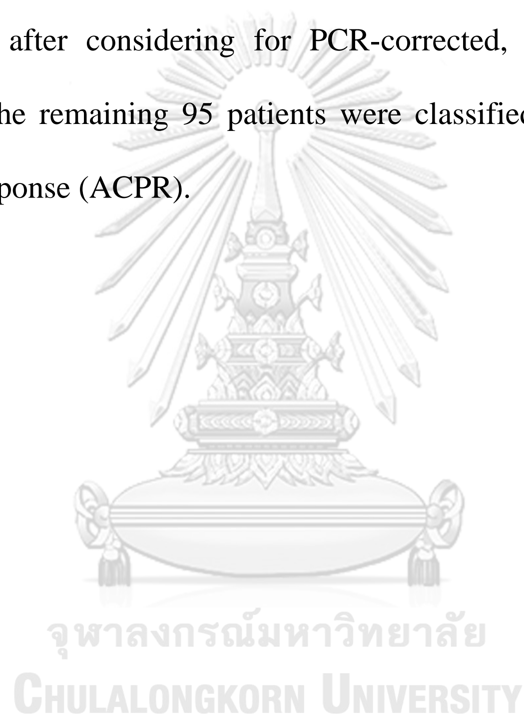
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Table 25. Summary of follow up of efficacy study of dihydroartemisinin+piperaquine against *Plasmodium falciparum* in Keerom, Papua, Indonesia

Without PCR correction	number	%	lower 95%CI	upper 95% CI	With PCR correction	number	%	lower 95%CI	upper 95% CI
ETF	0	0.0	0.0	3.6	ETF	0	0.0	0.0	3.7
LCF	1	1.0	0.0	5.3	LCF	1	1.0	0.0	5.6
LPF	6	5.9	2.2	12.4	LPF	1	1.0	0.0	5.6
ACPR	95	93.1	86.4	97.2	ACPR	95	97.9	92.7	99.7
Total patients per protocol	102				Total patients per protocol	97			
WTH	1				WTH	6			
LFU	11				LFU	11			
Total patients LFU/WTH	12	10.5			Total patients LFU/WTH	17	14.9		
Total patients at baseline	114				Total patients at baseline	114			
Day 3 parasite clearance	number	%	lower 95%CI	upper 95% CI					
Day 3 positive	0	0.0	0.0	3.3					
Total analysis at day 3	110								

ETF: early treatment failure; LCF: Late clinical failure; LPF: Late parasitological failure; ACPR: adequate clinical and parasitological response; WTH: Withdrawal; LCU: loss of follow up.

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Kaplan-Meier survival cure rate of *Plasmodium falciparum* against Dihydroartemisinin+piperaquine medication

As previously mentioned, without PCR correction, the number of patients classified as ACPR at day 42 was 95 with 12 loss of follow up and withdrawals and 7 treatment failures. The cumulative incidences of success at day 42 was 0.9333 (95%CI: 0.865-0.968). Contrarily, the number cumulative incidences of failure at day 42 was 0.067 (95%CI: 0.032-0.135). With PCR-corrected, the number of loss of follow up, withdrawals and reinfections were 17 with 2 treatment failures. Therefore, the number of cumulative incidences of success at day 42 has increased to 0.982 (95%CI: 0.928-0.995). Additionally, the number of cumulative incidences of failure at day 42 has decreased to 0.018 (95%CI: 0.005-0.072).

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Table 26. Kaplan-Meier analysis of survival rate of *Plasmodium falciparum* against Dihydroartemisinin+piperaquine medication in Keerom, Papua, Indonesia.

Number of patients		
ACPR day 42	95	
Censored (lost to follow-up, withdrawals)	12	
Treatment failures	7	
		95% CI
Cumulative incidence of success, day 42	0.933	(0.865-0.968)
Cumulative incidence of failure, day 42	0.067	(0.032-0.135)

Number of patients		
ACPR day 42	95	
Censored (lost to follow-up, withdrawals, reinfections)	17	
Treatment failures	2	
		95% CI
Cumulative incidence of success, day 42	0.982	(0.928-0.995)
Cumulative incidence of failure, day 42	0.018	(0.005-0.072)

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4.4.2. Plasmodium vivax

Efficacy study of dihydroartemisinin+piperaquine against *Plasmodium vivax* was conducted in two different places, Lembah masurai sub-district (Jambi province) and Keerom district (Papua island). Therefore, in this section, it is discussed simultaneously.

Baseline characteristics

The total number of enrolled patients across all study sites was 124 with 33.1% (41 patients) from Jambi and 66.9% (83 patients) from Papua. In Jambi, the ratio of male/female was 19/22, while in Papua was 42/41. In Jambi, the proportion of age was dominated by adult (17) followed by adolescent and young child (15) and under 5 children (9). Contrarily, in Papua, it was dominated by adolescent and young child (44) followed by adult (26) and under 5 children (13). The mean age was 16.6 (SD: 14) and 15.1 (SD: 11.9) in Jambi and Papua, respectively. The mean weight was 33 (SD: 17.4) and 33.8 (SD: 17.8) in Jambi and Papua, respectively. The mean temperature was 36.8 (SD: 1) and 37.4 (SD: 1.4) in Jambi and Papua, respectively. The mean geometric parasitemia from Papua was higher (3918) compared to Jambi (1512).

Table 27. Baseline characteristic of the enrolled patients infected with *Plasmodium vivax* in Jambi and Papua, Indonesia

Jambi		Papua	
Year	2017/18	**Year**	2017/18
Months	Oct-Apr	**Months**	Apr-Jan
Number of patients	41	**Number of patients**	83
Ratio male/female	19/22	**Ratio male/female**	42/41
Age group (years)		**Age group (years)**	
adults	17	adults	26
5 to 15	15	5 to 15	44
under 5	9	under 5	13

Jambi		Papua	
Age (years)		**Age (years)**	
mean (SD)	16.6 (14)	mean (SD)	15.1 (11.9)
range (min-max)	2-58	range (min-max)	2-45
Weight (kg), day 0		**Weight (kg), day 0**	
mean weight (SD)	33 (17.4)	mean weight (SD)	33.8 (17.8)
range (min-max)	9.2-62	range (min-max)	3.4-72
Temperature (°C), day 0		**Temperature (°C), day 0**	
mean temperature (SD)	36.8 (1)	mean temperature (SD)	37.4 (1.4)
range (min-max)	36-41	range (min-max)	35-40.3
Parasitemia (parasites/µl), day 0		**Parasitemia (parasites/µl), day 0**	
mean (geometric) parasitemia	1512	mean (geometric) parasitemia	3918
range (min-max)	40-9320	range (min-max)	40-31800

Summary of follow up

Jambi

At enrolment day, there was 41 patients that met our inclusion criteria with 2 patients considered as loss of follow up. No early treatment failure (ETF) was detected across all enrolled patients. Microscopically, one patient was classified as late parasitological failure (LPF). However, after considering for PCR-corrected, the number of LPF was decreased to 0. The remaining 37 patients were classified as adequate clinical and parasitological response (ACPR).

Papua

At enrolment day, there was 83 patients that met our inclusion criteria with one patient considered as loss of follow up. No early treatment failure (ETF) was detected across all enrolled patients. There was one patient who experienced late clinical failure (LCF). Microscopically, eight patients were classified as late parasitological failure (LPF).

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However, after considering for PCR-corrected, the number of LCF and LPF was decreased to 0. The remaining 73 patients were classified as adequate clinical and parasitological response (ACPR).

Table 28. Summary of follow up of efficacy study of dihydroartemisinin+piperaquine against *Plasmodium vivax* in Jambi and Papua, Indonesia

Jambi									
Non-PCR-corrected	number	%	lower 95%CI	upper 95% CI	PCR-Corrected	number	%	lower 95%CI	upper 95% CI
ETF	0	0.0	0.0	9.3	ETF	0	0.0	0.0	9.5
LCF	0	0.0	0.0	9.3	LCF	0	0.0	0.0	9.5
LPF	1	2.6	0.1	13.8	LPF	0	0.0	0.0	9.5
ACPR	37	97.4	86.2	99.9	ACPR	37	100.0	90.5	100.0
Total patients per protocol	38				Total patients per protocol	37			
WTH	1				WTH	1			
LFU	2				LFU	2			
Total patients LFU/WTH	3	7.3			Total patients LFU/WTH	3	7.5		
Total patients at baseline	41				Total patients at baseline	40			
Day 3 parasite clearance	number	%	lower 95%CI	upper 95% CI					
Day 3 positive	0	0.0	0.0	8.8					
Total analysis at day 3	40								
Papua									
Non-PCR-corrected	number	%	lower 95%CI	upper 95% CI	PCR-Corrected	number	%	lower 95%CI	upper 95% CI
ETF	0	0.0	0.0	4.4	ETF	0	0.0	0.0	4.9
LCF	1	1.2	0.0	6.6	LCF	0	0.0	0.0	4.9
LPF	8	9.8	4.3	18.3	LPF	0	0.0	0.0	4.9
ACPR	73	89.0	80.2	94.9	ACPR	73	100.0	95.1	100.0
Total patients per protocol	82				Total patients per protocol	73			
WTH	0				WTH	5			
LFU	1				LFU	1			
Total patients LFU/WTH	1	1.2			Total patients LFU/WTH	6	7.6		
Total patients at baseline	83				Total patients at baseline	79			
Day 3 parasite clearance	number	%	lower 95%CI	upper 95% CI					
Day 3 positive	0	0.0	0.0	4.3					
Total analysis at day 3	83								

ETF: early treatment failure; LCF: Late clinical failure; LPF: Late parasitological failure; ACPR: adequate clinical and parasitological response; WTH: Withdrawal; LCU: loss of follow up

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Kaplan-Meier survival cure rate of *Plasmodium vivax* against Dihydroartemisinin+piperaquine medication

Jambi

As previously mentioned, without PCR correction, the number of patients classified as ACPR at day 42 was 37 with 3 loss of follow up and 1 treatment failure. The cumulative incidences of success at day 42 was 0.975 (95%CI: 0.835-0.996). Contrarily, the number cumulative incidences of failure at day 42 was 0.025 (95%CI: 0.004-0.165). With PCR-corrected, the number of treatment failure was 0. Therefore, the number of cumulative incidences of success at day 42 has increased to 1. Additionally, the number of cumulative incidences of failure at day 42 has decreased to 0.

Papua

As previously mentioned, without PCR correction, the number of patients classified as ACPR at day 42 was 73 with 1 loss of follow up and 9 treatment failures. The cumulative incidences of success at day 42 was 0.891 (95%CI: 0.8-0.942). Contrarily, the number cumulative incidences of failure at day 42 was 0.109 (95%CI: 0.058-0.2). With PCR-corrected, the number of treatment failure was 0 and loss of follow up was 6. Therefore, the number of cumulative incidences of success at day 42 has increased to 1. Additionally, the number of cumulative incidences of failure at day 42 has decreased to 0.

Table 29. Kaplan-Meier survival cure rate of *Plasmodium vivax* against dihydroartemisinin+piperaquine medication in Jambi, Indonesia

Number of patients			**Number of patients**		
ACPR day 42	37		ACPR day 42	37	
Censored (lost to follow-up, withdrawals)	3		Censored (lost to follow-up, withdrawals, reinfections)	3	
Treatment failures	1		Treatment failures	0	
		95% CI			95% CI
Cumulative incidence of success, day 42	0.975	(0.835-0.996)	Cumulative incidence of success, day 42	1.000	#DIV/0!
Cumulative incidence of failure, day 42	0.025	(0.004-0.165)	Cumulative incidence of failure, day 42	0.000	#DIV/0!

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Table 30. Kaplan-Meier survival cure rate of *Plasmodium vivax* against dihydroartemisinin+piperaquine medication in Papua, Indonesia

Number of patients		
ACPR day 42	73	
Censored (lost to follow-up, withdrawals)	1	
Treatment failures	9	
		95% CI
Cumulative incidence of success, day 42	0.891	(0.8-0.942)
Cumulative incidence of failure, day 42	0.109	(0.058-0.2)

Number of patients		
ACPR day 42	73	
Censored (lost to follow-up, withdrawals, reinfections)	6	
Treatment failures	0	
		95% CI
Cumulative incidence of success, day 42	1.000	#DIV/0!
Cumulative incidence of failure, day 42	0.000	#DIV/0!

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4.5. Distribution of K13 mutation and Plasmepsin II copy number of *Plasmodium falciparum* in Indonesia

4.5.1. K13 mutation

It was previously known that 124 mutations of K13 have been discovered across all continents of the world. It is divided into 6 blades. There was 46 K13 mutations originated from South-East Asian countries, 62 from Sub-Saharan Africa and 16 found in both regions (309). We have examined the mutation of K13 in Papua. However, we could not process the genotyping of K13 in Jambi due to no *Plasmodium falciparum* found in the area. We have successfully obtained 72 sequence of *Plasmodium falciparum* samples. Out of total 124 K13 mutations across the world, we have found 20 mutations. At codon 485, 469, 515, 525, 528, 609 and 667, there was found only a single mutation (1 mutation) out of 72 samples analyzed. Contrarily, at codon 474, there was 67 mutant-type and 5 wildtypes of this particular K13 codon. Similarly, at codon 490, 68 mutants found by alteration of Asparagine (N) to Threonine (T). At codon 449, 464, 501, 575, 581, 613 and 626, there was only a single mutation found. In contrast, at codon 464, there was 69 mutants out of 72 samples analyzed. Similarly, at codon 520, 67 mutants found by alteration of Valine (V) to Isoleucine (I). At codon 459, there was 67 samples that change the base of Serine (S) to Leucine (L). In contrast, there was only found a single mutation at codon 495.

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Table 31. Summary of K13 mutation found in Papua

Blade	Codon	Number of mutants	ACT failure	OR [95%CI]	*p value*
1	G449S	1	0	2.82 [0.11, 75.67]	0.5242
1	N458I	1	0	2.82 [0.11, 75.67]	0.5242
1	S459L	67	6	0.39 [0.04, 4.11]	0.8767
1	D464H	69	6	0.19 [0.01, 2.42]	0.8333
1	D464Y	1	1	30.23 [1.11, 820.07]	0.0516
1	C469Y	1	0	2.87 [0.11, 76.85]	0.5242
1	T474I	67	6	0.39 [0.04, 4.11]	0.4358
2	N490T	68	7	1.10 [0.05, 22.46]	0.9518
2	F495L	1	0	2.87 [0.11, 76.85]	0.5242
2	D501G	1	0	2.87 [0.11, 76.85]	0.5242
2	R515T	1	0	2.87 [0.11, 76.85]	0.5242
2	V520I	67	6	0.39 [0.04, 4.11]	0.8767
2	N525D	1	0	2.87 [0.11, 76.85]	0.5242
3	R528T	1	0	2.87 [0.11, 76.85]	0.5242
4	R575G	1	0	2.87 [0.11, 76.85]	0.5242
4	V581F	1	0	2.87 [0.11, 76.85]	0.5242
4	N609S	1	0	2.87 [0.11, 76.85]	0.5242
4	Q613L	1	0	2.87 [0.11, 76.85]	0.5242
5	A626T	1	0	2.87 [0.11, 76.85]	0.5242
6	P667L	1	0	2.87 [0.11, 76.85]	0.5242

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4.5.2. Copy number of *Plasmepsin II*

Failure in efficacy study of ACT that causes resistance based on WHO protocol is when after 72 hours of drug prescription (Day 3) the number of parasites does not decrease, or in the period of 5 hours after half-live of the drug, the number of parasites does not decrease. For DHP, the standard protocol recommended by WHO is monitoring the efficacy of the drug for 42 days period. If before 42 days, infection appeared, it could indicate a recurrent infection caused by a new parasite and should be proved by PCR.

In this study, we used several patient's data from data that showed a recurrent infection at day-21, 35 or 42. Based on previous study, the hypothesis of this study is the sample of the patients possess a multi-copy of *Plasmepsin II*.

Of 15 samples there was only 7 samples that had data of D0 and D21. Therefore, the total samples considered to be valid was 7/15 (46.7). Based on the result, two samples (13.3%) was considered to have multi-copy of *Plasmepsin II*.

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Table 32. The result of *Plasmepsin II* genotyping of Papua samples by real-time PCR

No	Sample code	Concentration (ng/µl)	Parasitemia	CT				Tm (ºC)				Delta CT	PfPM2 CN (3D7)
				PfMAP		PfTub		PfMAP		PfTub			
				Average	St. dev	Average	St. dev	Average	St. dev	Average	St. dev		
1	F01 (D0)	18.5	Pf R2020	25.42		26.28		75.80		77.30		-.086	2
2	F01 (D21)	19.7	Pf R321	28.33	0.13	28.43	0.12	75.40	0.00	77.03	0.23	-0.11	1
3	F08 (D0)	21.8	Pf R2458	25.24		25.76		75.40		76.90		-0.52	1
4	F112 (D0)	20.3	Pf R1768	24.42		25.31		75.80		77.30		-0.89	2
5	F115 (D0)	19.9	Pf R2512	26.99		27.28		75.40		77.30		-0.29	1
6	F133 (D0)	20.5	Pf R1212	27.07		27.29		75.40		76.90		-0.22	1
7	F19 (D0)	20.4	Pf R41	31.01		31.46		75.80		77.30		-0.44	1

CHAPTER 5

DISCUSSION, CONCLUSION, AND RECOMMENDATION

5.1.Entomological survey

According to the Malaria Atlas Project (310), for API <0.1, *Plasmodium falciparum* and *Plasmodium vivax* distributions are similar across the Indonesian archipelago. *Plasmodium falciparum* is more stable in distribution, where each part of Indonesian archipelago has the same pattern of low to moderate API. Meanwhile, *Plasmodium vivax* is more intense in the eastern part of Indonesia and unstably distributed in the western part of Indonesia. However, only *Plasmodium vivax* was found in Jambi, and more diverse *Plasmodium* species have been observed in Sumba, suggesting a different diversity of *Plasmodium* species distribution in the two localities. A discrepancy was also found in the calculated API between this study and the basic health report by the Ministry of Health of Indonesia, which might be explained by the different ways of presenting the data. The national health report (311) used the provincial population and the larger the area, the larger the population involved in the calculation, as API is calculated by dividing the total cases and the total population. API at a sub-district level is often observed to vary from one district to another and variation between districts is observed at a provincial level (312, 313).

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There are 20 *Anopheles* species known to be vectors for malaria in Indonesia. In this study, four and eight species have been found in Jambi and Sumba, respectively. The student *t-test* suggested a different abundance in the number of *Anopheles* mosquitos between the two sites. This difference is often explained by environmental

conditions. A distinct sampling time may cause this difference in mosquito abundance; however, since rainfall anomalies have been observed in Indonesia, this may not be the case (314). Since the existence of *Anopheles* breeding sites depends on rainfall providing a sufficient water bodies for the mosquitos to lay eggs, rainfall anomalies in Indonesia may lead to an irregular pattern of mosquito abundance across time and place in Indonesia. The limited number of water bodies or humidity conditions may affect the habitat and abundance of *Anopheles* mosquitos in Jambi (315, 316). The difference in mosquito abundance may also reflect the annual incidence rate of malaria infection in different endemic areas. However, no correlation may be found if the correlation of mosquito abundance and annual incidence rate takes into account the species of *Plasmodium* (317).

The main *Anopheles* vector and biting preference differs between Jambi and Sumba. *An. balabencis,* which belongs to leucosphyrus group, is the primary vector in Jambi, as determined from its highest relative abundance and HLR. Moreover, *An. aconitus* and *An. sundaicus* are the primary vectors in Sumba, along with other minor *Anopheles* species found. Only *An. balabacensis* in Jambi was found to be exophagic, as previously known from the biting preference of this peculiar species (318). *An. maculatus* has been found to be both endophagic or exophagic similar to the finding of Elyazar *et al* (318). However, previous studies have found that *An. aconitus* has an irregular pattern of biting preference while *An. sundaicus* is mainly exophagic (318). This study found that there was no significant difference between the indoor and outdoor biting preference of *An. aconitus* and *An. sundaicus*, suggesting that these species can be both endophagic and exophagic.

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Biting time is essential to be understanding the underlying biological properties of mosquitoes and to avoid *Anopheles* bites to control malaria infection. The data obtained suggest different biting times of *Anopheles* in Jambi and Sumba. Early evening (18.00-20.00) is most likely to be the mosquito feeding time in Jambi, when most people are undertaking activities and are unprotected. However, in the late evening (21.00-02.00), more people in Sumba may get *Anopheles* bites, reflecting sleeping time, when Sumbanese people may be vulnerable to infection with malaria parasites. This suggests the importance of ITNs for evading malaria infection in Sumba. The biting time of *Anopheles* in Jambi is similar to that in Halmahera, Maluku Island (303). However, the finding from Sumba Island is different from other parts of Indonesia, which shows a gradual increase or decrease in the number of *Anopheles* mosquitoes in accordance with its biting time (303). Limited studies have tried to describe mosquito biting patterns in relation to the selection of malaria control strategies (319, 320). This finding strengthens the previous report that effective malaria prevention depends on local *Anopheles* vector biting behavior. *Anopheles* vectors in Jambi share the same behavior as those in Burkina Faso, where bed net protection may not be effective for preventing biting exposure as *Anopheles* species in the area are dominant in the early evening (320). In contrast, similar to Uganda, intensive use of ITNs combined with indoor residual spraying is the most effective protection approach for Sumba Island for avoiding malaria infection (319).

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Biting preference has previously been known to have an underlying genetic background (321). For instance, chromosome inversions of *2Rbc*, *2Ra* and *3Ra* and circadian clock genes are associated with exophagic and endophagic behavior in some *Anopheles* species (322-324). However, genetic background may vary within the genus

and among mosquitoes within the same species in different locations (324). The finding also suggests that differences in *Anopheles* biting time may be an effect of different genetic backgrounds. Further research might explore this aspect.

5.2.Risk factor of malaria incidence in Indonesia

Based on Indonesia basic health profile, Jambi was categorized to have low cumulative incidence, while Sumba as part of NTT province has high cumulative incidence (311). By transforming the total number of collected cases in each location, Jambi and Sumba, then all the site has a high cumulative incidence of malaria (5.4 and 15.7, respectively). There is a discrepancy of classifying endemicity level between national data and the collected data from the current study. This phenomenon partly can be explained by the different denominator of the data, as national data considers a total number of populations in a provincial level rather than each sub-district level. Considering the fact that malaria varies greatly between sub-district and district and is not uniformly distributed, this is may be the case (312, 313).

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One of the interesting findings of the current study is the different pattern of the source of infection between Sumba and Jambi. Night activity outdoor, history of visiting forest areas from the previous month and working place is inside forest are protective factors in Jambi, while the history of visiting forest areas and requiring overnight stay inside a forest are being risk factors in Sumba. This finding suggests that most of the case from Jambi was infected in the residential areas and forest area were the source of malaria infection from Sumba. This finding underlines that visiting forestry areas is not always being a risk for malaria infection as previous research has found (290, 300, 301). The phenomenon may be explained by the relationship of human

and mosquito infection over which may be caused by uneven distribution of mosquito bites across the human population. For example, a study showed that in several areas a core group of the human population receive a substantial proportion of mosquito bites (325). Additionally, another finding indicates that a group with more individuals experiences a lower rate of mosquito bites (326).

Sumba and Jambi share the same individual risk factors namely no possession of ITNs, low education level and ever contacted with malaria infected patient. It is common that ITNs and low education level being a risk factor for malaria as previously discovered (286, 290, 327). The effective impact of ITNs has been extensively described in previous studies (328, 329). Although defining the coverage of the ITNs use is problematic (330, 331). The effect of education on malaria infection has also been found from the previous study (286). Studies have demonstrated that the poor performance of children at school is the risk of malaria, and if knowledge of prevention is adequately elevated will lower the incidence of malaria (332, 333). While other researchers found that the performance of education may be temporary and not be prolonged (334). Additionally, most of the case had contact with the other cases compared to control suggesting that they may have an infection during the interaction process. Cases may sleep in the same house or involved in a late conversation or any way of interaction in which mosquito could bite them simultaneously.

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There are several differences in individual risk factors between Sumba and Jambi. Occupation is statistically significant to correlate with malaria in Jambi. This finding is in line with the previous discussion where most of the controls are a farmer that require them to go to the forestry areas. Most of the case has an occupation that

needs them to reside in the housing area such as a midwife, workshop worker or odd jobs. In the other hand, in Sumba, ever consumed antimalarial drug and traveling outside the residential area are the risk factors of malaria infection. Considering the effectiveness of the current antimalarial drugs, the odd of antimalarial drug consumption is since the majority of the case may have re-infection that require them to have frequent antimalarial drug prescription. It was described that re-infection is a common situation in a high transmission area (335). The risk of traveling outside residential areas with malaria infection is in line with previous study (290). The participant of the current study may have traveled in neighboring villages in which infection rate are high. There are several studies that have demonstrated the risk of traveling into a high infection rate area (336, 337). A reporting system need to be established to identify import cases from neighboring villages or areas.

There are several same environmental factors between Sumba and Jambi, i.e., the existence of shrubs and puddle/stagnant water surrounding the household area and the presence of livestock nearby house area. Studies have found that "bush" is a risk factor in a densely forested area and can promote mosquito to breed (289, 338, 339). Since case and control resided in a densely forested, basic biological attribute of the vector may play a role. It was shown that shrub promotes the malaria transmission capacity by providing an abundant source of sugar for the male while lack of sugar source contributes to lower insemination rate to females (340, 341). Moreover, as observed in *An. gambiae*, *Anopheles* mosquito is distributed among dense growth of bush (342, 343). They may rest in the shrub prior to get ready to bite. The existence of puddle has found to be a potential breeding place where *Anopheles* mosquito could oviposit (344-347). Although evidence suggested that no or low *Anopheles* larvae

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density found when water is identified as turbid as puddles, drains or swamps (348). As previously described, the existence of livestock nearby house area increases the chance of mosquito contact (275). It was previously found that the presence of livestock at the household level can significantly alter the local species composition, feeding and resting behavior of malaria vector (349). However, the net impact of livestock-associated variation in malaria vector ecology on malaria exposure risk was unknown (349). In addition, the pattern of host attraction and biting behavior of *Anopheles* mosquito in Indonesia has not been yet extensively studied and only limited to one locality (350). *Anopheles* mosquito can be attracted to livestock even the primary vector of malaria, besides of their biting preference as zoo-anthropophilic species (351). Furthermore, placing of livestock inside the house is also significantly correlated with malaria in Sumba, suggesting a different cultural behavior of tethering livestock. Our study demonstrates the importance of controlling malaria using livestock-based intervention or using any zoo-prophylactic agent as described elsewhere (352, 353).

House construction has been associated with malaria in Sumba such as non-permanent house wall, non-permanent floor construction and not having a ceiling of the rooftop. Such factors are in line with the previous report regarding the associated demographic factor of malaria infection underlying the importance of human dwelling construction (1, 275). However, this finding may not be the case since there is a difference with entomological finding as discussed below. Additionally, the proximity of the house to forestry areas and rice filed are the risk factor for malaria in Jambi and Sumba, respectively. As discussed previously, housing location in proximity to lower vegetation cover is the protective factor for malaria (290). Jambi and Sumba have different agricultural activity. Most of the people from Jambi work on rubber and palm

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plantation that require a large area of land, a person could handle 5-10 hectares. On the contrary, Sumbanese people are mostly working on cashew or rice which requires a relatively limited space of land. Agricultural activities have been shown to be a predisposing factor for malaria in which suitable habitat of the vector may take place (276, 284). It was previously described that rice field agro-ecosystem contributed significant vector populations (354, 355). However, with the same densely forested areas, the source of infection is different between the two sites as above-mentioned discussion.

Previous studies have found differences in associated variables between low and high-risk countries (292). As well as environmental factors are also varied across spatially different regions (356). In order to strengthen such fact, in the current study, we selected a different API area for comparison. Based on GLM, there is a significant effect of spatial heterogeneity in the risk factor variable. The GLM analysis was set to equate starting from the number of case and control and the only associated variables that presence in both sites. It suggests that the risk factor variable on malaria infection is influenced by area that may reflect the differences in API. Additionally, considering the fact of the different number of associated individual and environmental variables between Sumba and Jambi suggests that a high API area like Sumba has more diverse risk factors than low API area.

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Finding from the current study and the other indicates that housing construction is associated with malaria infection (357). Even others recommended that improved housing is a promising intervention for malaria (358, 359). However, our entomological observation found that housing construction does not necessarily lead to increased risk

of *Anopheles* bites. Only MH found to be significantly different with NH or PH and NH. PH is always in a higher mean number of collected *Anopheles* mosquito than NH regardless of the sites suggesting no protective effect of housing on malaria infection. This finding is also supported by malaria infection rate of the house types that MH is higher than the two types of houses while NH and PH share the same number of infection rate. This phenomenon can be best explained by the existence of risk factors other than only housing types such as the presence of livestock, shrub, puddle, ceiling in the rooftop or housing localities. Additionally, modern cement type house (PH) in both localities were still found an open hole in the eaves or cracked in the wall or door where the mosquito could enter. With the condition of PH, the warmer temperature may be suitable to attract mosquito compared to NH. As long as the other environmental risk factors and complete closure of potential gaps do not be controlled, the housing improvement program may not be effective as stated in the previous findings (358, 359).

5.3.Population adherence to ACT and primaquine medication in Indonesia

5.3.1. Dihydroartemisinin+piperaquine

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It was widely known that poor adherence of a population to antimalarial medication will lead to the development of treatment failure prior to the spread of parasite resistance genetically (360-362). There are several risk factors that known to promote the emergence of malaria parasite i.e. population coverage of antimalaria medication, half-lives of the selected drug, the residue of the drug inside host body, a high mutation rate of the parasite, population fitness of early developed resistant parasite, declining transmission intensity and a low coverage of other preventive

measure (363). Interestingly, by maintaining a good quality ACT whether pharmacokinetically or satisfying population adherence, it is possible to eliminate malaria even in the area where ACT resistance have been spread (364). It was also previously described that a higher treatment failure achieved when adherence level was lower compared to optimal adherence level (365). It was shown that approximately 4 times higher probability of treatment failure may be achieved as each dosing time is not taken by the patient (365). In fact, although full adherence has been achieved, it still leads to ~5% of treatment failure of the patient (365).

Our study shows that the level of community adherence to ACT medication in Indonesia are 66% (Jambi) and 79.1% (Sumba). If several studies of ACT adherence gathered, the average adherence level is 75.2% (53, 366-393). Therefore, population adherence in Jambi is below the average of worldwide adherence level of ACT (66% vs 75.2%), while in Sumba is only slightly higher than the average (79.1% vs 75.2%). However, none of the studies have discovered a community adherence to DHP medication (53, 366-393). Only one study that have described the community adherence to DHP medication in Northern Ghana, which the result is 50.9% of adherence lower than our present study (394). Although, it is difficult to have conclusive finding of global community level of adherence to ACT medication due to the fact of varying study design, study protocol and ACT prescription type. Taken together, the community adherence to ACT medication in our study setting is considered to be less than 80% which needs to be elevated to avoid the growing trend of treatment failure as seen globally (395-403).

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The main reason of correct intake of ACT from our study is similar to the previous study which is a correct instruction has been given in the clinic/local health facility (304). The other patients claimed that they have taken the same pills before as recognition of the drug is an imminent factor to adhere the treatment regimen. The reason of non-adherence behavior of our study is seemingly similar to the other findings. The reason of certain non-adherence (pills remaining) which are either patient felt cured or forgot to take the medication may be an usual reason to not take a proper medication compared to other findings (53, 368, 372, 404). Similarly, the main reason of probable non-adherence is the patients claimed an incorrect instruction has been given in the clinic/local health facility may be due the fact of lack understanding of the patients to the prescribed drug (368, 404). This particular type of patient needs to be given a more detailed explanation of the drug and how to take it properly by the pharmacist. Additionally, some patients explained the reason of being probable non-adherence is due to their thought of taking all the pills in the first or second day of treatment will cure faster, or patients could not swallow the pills or other reason. Such reasons are generally found throughout studies (53, 372, 374, 383) and it emphasized the importance of targeted health promotion to improve patient's awareness of the impact of improper adherence behavior to the treatment regimen.

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Factors associated with adherence to ACT is varied between studies (53, 367-369, 372, 373, 375, 377, 380-383, 386, 387, 392). The factors associated with adherence in current study were under 5 years children (OR= 2.79 [95%CI; 1.188-6.552]) and patient knowledge of malaria prevention (OR= 0.29 [95%CI; 0.09-0.9]). Age has been known to be associated with adherence to ACT. Our finding is similar to Mace *et al* (373) where the younger the person the more likely to be non-adherent to ACT

medication. As opposed, Lawford *et al* (375) found that the older the person the more likely to be non-adherent. It was postulated that the older the patient, the better the understanding of ACT administration and may have had prior experience in taking the treatment (375, 405). It has been also previously found that lack of appropriate dose formulation may lead to improper adherence behavior to such group of age (406). It is one of the concerned problem that the development of parasite resistance is more higher in the children due to higher parasite biomass inside them increasing the possibility of developing *de novo* resistance of the parasite (381, 406, 407). The other risk factor from our study is patient understanding to malaria prevention strategy (the use of bed nets). A slightly different finding has been discovered by Gerstl *et al* (383) where adherence to ACT has been associated with patient's recognition of malaria cause (malaria transmitted by mosquito bites) rather than malaria prevention strategy. Taken together, it is imperative to monitor adherence especially in the infant and young children where such vulnerable age group is intensifying the development of parasite resistance. Additionally, public policy maker needs to consider escalating a better understanding of malaria cause and prevention starting from the local health workers and eventually down to local society.

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5.3.2. Primaquine

Preserving a high adherence behavior toward antimalarial drugs, especially primaquine, is the key to achieve malaria elimination and to prevent maintenance of malaria foci and importation (408-411). Primaquine is the only known and licensed drug against hypnozoite form of *Plasmodium vivax*. A standard 14 days course of

treatment have been described by WHO. However, with such a long time of treatment, it would be a poor adherence of the community.

In our study, we have demonstrated a relatively low level of adherence to primaquine medication in our study setting. Our study is in agreement with the previous study in Peruvian Amazon (412). The worst situation has been found from Thailand community (413). A variety of risk factors underlying non-adherence behavior has been observed between study localities. In our study setting, the risk of being non-adherent is a complementary prescription of primaquine with other drugs. Non-adherence behavior related to treatment complexity has been observed from an anti-hypertensive drug (414) and cardiovascular medication (415). The complexity of medication has proven to lower adherence level in a community, thus instead of prescribing cascade by giving other drug, health professionals should give only primaquine tablets for identified *Plasmodium vivax* infection. Additionally, giving the community knowledge regarding malaria etiology, sign and symptom and general medication may help to elevate adherence level (413).

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The discrepancy of measuring adherence has been observed that may include social desirability bias (416). More advanced measurement including blood metabolite has been reviewed. A carboxy-primaquine concentration of 80 ng/ml at day-4 may help to measure precisely adherence in a community (416). As shown by Cheoymung *et al* as they measured community primaquine adherence based on drug metabolite which shows a 100% adherence (417). This high adherence level may due to the study used a strict protocol that requested the patients to return to the clinic at day-3. In contrast, our study and others (412, 413) used a non-strict protocol that did not request the patient to

return to the clinic instead of visiting them at home at the completion day of the drug medication time. Further research needs to combine structured questionnaires and drug metabolite with non-strict protocol to avoid social desirability bias.

5.4.Efficacy of dihydroartemisinin+piperaquine

As mentioned in the background, previous antimalarial drugs have been discovered to be ineffective due to resistance development of the parasite. Artemisinin, newly developed drug, has managed to tackle such problem by providing a high efficacious effect on the parasite. However, as time passed by, malaria parasite that develops resistance to this peculiar drug has begun to appear. In Indonesia, such resistance phenomenon has not been reported yet. Therefore, the information herein will help to complete such information gap.

As discussed in a review, the growing trend of ACTs resistance in South-East Asian countries is worsening (418). The review included all combination of artemisinin and its partner drug. The review found that dihydroartemisinin+piperaquine outperformed the other artemisinin combination drugs due to its prolonged half-life of piperaquine inside host body (419, 420). Although, after the introduction of piperaquine resistance in South-East Asia (SEA), the performance of DHP is believed to be non-efficacious (421, 422). Even, in Vietnam, due to decreasing effectivity of piperaquine, the country has considered to revert to AS+MQ to combat the rising treatment failure of DHP (422, 423). The reason of ACTs failure is hypothesized to be: (1) the rising artemisinin failure has resulted in overburdening the partner drug causing a complete failure of the combined therapy (424) or (2) the failure of ACTs is suggested to be a cause of simultaneous failure effect of artemisinin and its partner drug in the region

(421, 423). Interestingly, the review has only found an ACTs failure only in SEA countries (418). Some of SEA countries have reported resistance to ACT drugs genotypically and phenotypically such as Thailand, Myanmar, Cambodia, Vietnam, etc. (418). However, it is not in line with our finding that found DHP treatment can be considered as efficacious drug against malaria parasite in Indonesia. It seems that Indonesia has independent lineage or no population cross-breeding between SEA countries and Indonesia as seen in the genetic flow of resistance lineage in the greater Mekong subregion (425). Secondly, the reason of a high efficacy of DHP in Indonesia is this peculiar drug has never been introduced previously as single or combination with other type of artemisinin. Therefore, no prior exposure has been experienced by the parasite from its first introduction until current.

Our study found that the combination of dihydroartemisinin+piperaquine is still adequate to be used as first line treatment against malaria parasite in Indonesia despite almost a decade of utilization. The cumulative success of the treatment against *Plasmodium falciparum* is 0.982 (PCR-adjusted), while 100% efficacious for *Plasmodium vivax* (PCR-adjusted). Our finding is in line with other similar researches in Papua New Guinea (PNG) and Nigeria. The study that was conducted in PNG showed a cumulative success of DHP treatment of 0.977 by microscopy and 100% as corrected with PCR (426). While in Nigeria, the cumulative incidence of success was 0.981 (PCR-adjusted) (427). However, our study is different with the other studies conducted in South East Asia. In Cambodia, the risk of artemisinin resistance has been seen since 2008-2010 with a study reported the cumulative incidence of success of DHP medication of 0.892 (419). Ten years later in 2016, DHP medication has been considered inefficacious by the discovery of a significant reduced effectivity of DHP

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with cumulative success of only 0.54 with a higher piperaquine IC_{50} and correlated significantly with a view markers of resistance (424). While in Myanmar and its border with china, currently, DHP is considered to be efficacious with a hundred percent of cumulative success in Myanmar and in range of 99.2-100% efficacy in the China-Myanmar border (428-430). However, other study suggested that a careful monitoring need to be taken in Myanmar due to its finding that found the prolonged median of parasite clearance and a higher clearance half-life (431). The same situation was also discovered in Vietnam where DHA-PPQ is considered to be satisfactory, but the parasite clearance time and rate are indicative of emerging artemisinin resistance (402).

5.5.Distribution of the sequence of K13 gene and *Plasmepsin II* copy numbers

As mentioned in the background section, K13 is currently believed to be responsible for artemisinin resistance of malaria parasite. While *Plasmepsin II* is a newly discovered gene that its multicopy is an indicative of higher parasite fitness against piperaquine drug. The information of the distribution of K13 and *Plasmepsin II* copy number is very limited in Indonesia. Therefore, our finding will complete such gap of information in Indonesia.

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Putative gene (PfKelch13) for artemisinin resistance have been described by Ariey *et al* in 2014. These researchers have been managed to produce artemisinin-resistant parasite from a 5 years study through 125 repeated cycle of artemisinin selection generated from the artemisinin-susceptible F32 Tanzania clone. Subsequently, the produced resistant parasite and its germinal line recognized eight SNPs in seven genes by whole genome sequencing. Causative agent of artemisinin resistance, among these SNPs, was concluded to the M476I mutation in PfKelch13. Significant correlation

further confirmed between four mutation of PfKelch13 (Y493H, R539T, I543T and C580Y) were closely associated with elevated RSA survival rate (34).

Afterwards, straight evidence of relationship between PfKelch13 alteration with artemisinin resistance was noticed (148, 149). These report explained a single mutational alteration (M476I, Y493H, R539T, I543T or C580Y) of PfKelch13 gene in an artemisinin-susceptible *Plasmodium falciparum* clone (Dd2) enhanced RSA survival rates, likewise revertant of relevant mutational sites (R539T, I543T and C580Y) affected a significant decrease in the RSA survival rates (149).

Recently, there are 124 mutation points in K13 gene over 6 domains. However, currently, only 20 out of 124 mutations that have been associated with artemisinin failure: P441L, F446I, G449A, N458Y, C469Y, A481V, Y493H, S522C, G538V, R539T, I543T, P553L, R561H, V568G, P574L, C580Y, D584V, F673I, A675V, and H719N (309). Although, only 4 mutation points that have been validated both *in vivo* and *in vitro*: Y493H, R539T, I543T, and C580Y (309). In Indonesia, we found 20 out of 124 mutation points. Of them, three mutational sites are associated previously with artemisinin resistance: G449S, N458I and C469Y. However, only a single sample that has mutation on the three mutational sites we found. We found no relationship between the efficacy study result with the K13 genotyping by comparing independent codon or the allele. This may imply that Indonesian falciparum has developed a genetic background of K13 gene in response to artemisinin utilization. However, this genetic background may not affect their susceptibility to artemisinin drug, yet.

Derived from Cambodian isolates, in the Greater Mekong subregion where clinically treatment failure of concurrent artemisinin along with partner drugs have been

noticed (162-166, 432), *Plasmepsin II* and *plasmepsin 3* gene amplifications on chromosome 14 successfully described in accordance to piperaquine resistance. Additionally, another marker also has been reported which resides on chromosome 13 (*exo-E415G*) to be associated to piperaquine that undergo significant linkage disequilibrium with *Plasmepsin II-3* (36). Genome wide study was then used to achieve greater degree of association, which resulting in significant correlation between resistant parsites in vitro with *Plasmepsin II*. Strengthening the fact, dihydroartemisinin-piperaquine failure clinically was associated with *Plasmepsin II* multi copy (35). However, thought the invention of *plasmespin 2-3* as clinically and in vitro associated marker, *exo-E415G* also came with identical evidence of piperaquine resistance (36).

These reports evidenced that amplification of *Plasmepsin II-3* becoming such a robust marker for piperaquine. Transfection study of *Plasmepsin II-3* through laboratory strains will enhance current knowledge of exploring underlying mechanism of piperaquine resistance. A plausible explanation of emergence of piperaquine resistance laid on the fact that previous emerging resistant strain to relatively short half-life (3 days) artemisinin have led to a greater residual parasite biomass exposed to the partner drugs (167) which eventually allowing drug resistant strain selection (432). This also due to the pharmacokinetics properties of piperaquine that has an initial relatively rapid plasma clearance followed by a long terminal half-life (2-4 weeks) (168), these sequence of exposure by which minimal increase of parasiticidal concentrations come after initial concentrations will yield in a much shorter period of time parasites undergoing adequate parasiticidal concentrations, and then eventually to selection of resistant.

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In Indonesia, we found 2 samples (13.3%) that have multi-copy of *Plasmepsin-2*. These samples exhibited a treatment failure against DHP drug combination. Although, we could not find multi-copy of *Plasmepsin II* out from the other 5 samples that also exhibited a treatment failure against DHP. Similar to K13, this may suggest that Indonesian falciparum started to develop a resistance stage against piperaquine drug. However, the parasite has not spread the mutation to all of the population. Therefore, a close monitoring of the use of DHP drug need to be ascertained to prevent the parasite to spread the resistance genotypically or in the worst situation to reach a linkage disequilibrium.

5.6.Conclusion

Based on the result, it can be concluded that:

5.6.1. Biting time may suggest the use a different prevention approach in each area; for example, people in Jambi may need to use mosquito repellent during activities in the early evening, while ITNs combined with indoor residual spraying may need to be deployed to protect malaria infection during sleeping hours in Sumba.

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5.6.2. [1] visiting forestry area or agricultural activity is not always a risk factor for malaria as a source of infection may differ between location. [2] livestock-based intervention or using any zoo-prophylactic agent is inevitably effective to avoid mosquito attraction regardless of the area. [3] improving dwelling strategy may not be successful before controlling other environmental factors. [4] risk factors are site-dependent suggesting that applying risk factor management need to consider the endemicity status of an area.

5.6.3. Our study found non-satisfying level of adherence in the localities. The factors associated with adherence to DHP in our study setting were age and understanding of malaria prevention strategy. While, identified associated factor related to non-adherence behavior of primaquine medication in our setting is primaquine prescription with another drug. The present study clearly demonstrated the need of more careful monitoring of adherence level in a population, essentially those who are under-aged where they could develop parasite fitness faster in the population. In addition, more attention is needed to escalate community awareness of malaria cause and prevention, thus could prevent malaria foci in the localities. Moreover, simplifying primaquine medication therapy by not adding another non-related drug may elevate the adherence level.

5.6.4. Our efficacy study concludes that the combination of dihydroartemisinin+piperaquine drug is still effective to be used as the first line treatment against *Plasmodium falciparum* and *Plasmodium vivax* in Indonesia. Although, due to a minor treatment failure has been detected, careful monitoring needs to be done to prevent any further development of the parasite from its current state.

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5.6.5. Our result show that a present of mutation in several mutational sites of K13 as well as multi copy of *Plasmepsin II* gene. It indicates that the parasite has already developed a genetic background of resistance gene in Indonesia. Although, we are unable to produce a relationship between the mutation and the efficacy result, but it necessitates to conduct a continuing monitoring of artemisinin resistance in Indonesia. Our finding also suggest that Indonesia have

not been affected in any way of crossbreeding to neighboring South-East Asian countries where the parasite already developed resistance to artemisinin.

5.6.6. Finally, taken together, to prevent a drug resistance problem is not just about the treatment. However, many factors that involve the human, parasite and environment need to be considered. Our result clearly demonstrates that to prevent the growing trend of artemisinin resistance, all above-mentioned variables need to be controlled in order to achieve a long-lasting efficacious artemisinin therapy.

5.7.Strengths and limitations

The general strength of our study is we discovered a broad factor relating to the parasite intensity leading to a resistance development. Each of our study section have not been conducted in Indonesia previously, so it will complete such an information gap for public policy maker as well as a novelty for the researcher. Although we have also limitation(s) which is no conclusive statement from the molecular finding. Additionally, the comparison presented in this study only limited to low endemicity and high endemicity.

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5.8.Recommendation for further research

We strongly recommend for further research as follows:

1. A wide discovery of vector behavior of *Anopheles* species including biting preferences and behavior. It is also in need for further study to assess the blood feeding pattern of the species to better understand the blood seeking behavior whether to bite human or nonhuman or both.

2. Further study needs to consider a comparison of wider scale not only limited to low and high endemicity but as well as the comparison between low to low and high to high endemicity. It is to understand the net impact of the difference in associated variables measured.
3. Finally, for molecular study of K13 and *Plasmepsin II* markers, further study needs to add more sample to gain a clearer picture of the prevalence of such marker in the population of *Plasmodium falciparum* in Indonesia.

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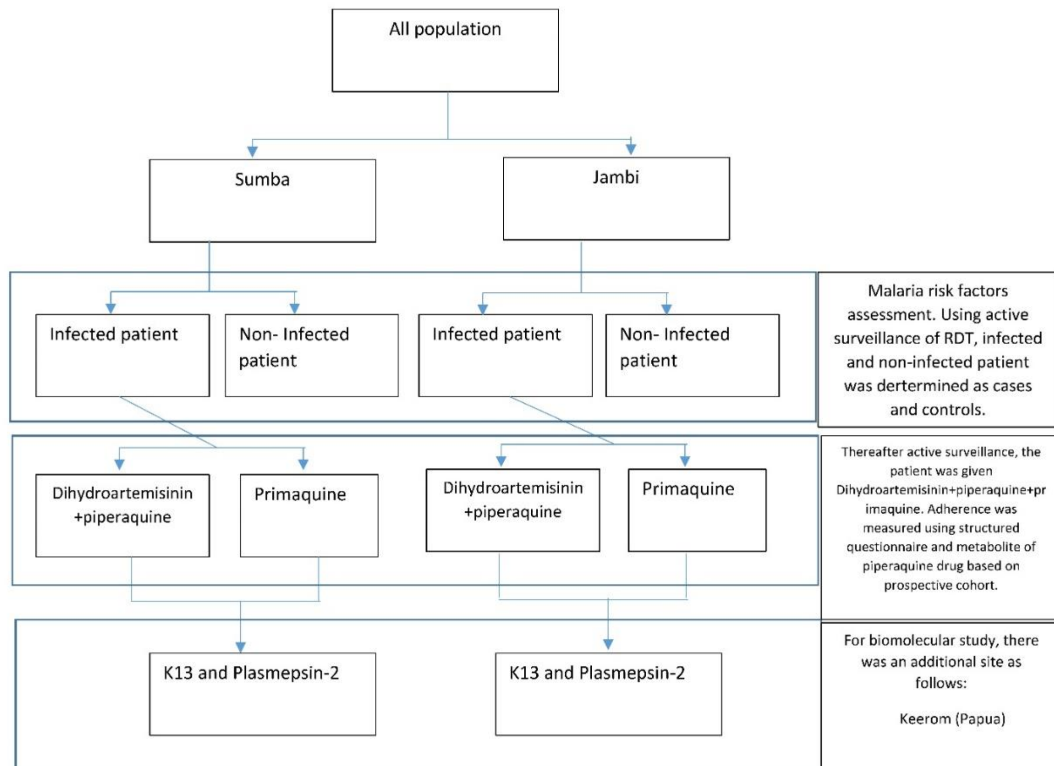

Figure 15. Research flowchart

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APPENDIX 1

Code of questionnaire:

Research questionnaire

Risk factor assessment of malaria

(*Subjected to community in the sampled location*)

A. General information

1. Name :
2. Age :
3. Sex :
4. Address :
5. Position in society :
6. Coordinate of house :
    a. Latitude :
    b. Longitude :
    c. Elevation :
7. Elevation :
8. Date of entry :

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B. Individual information of the residents of the house

1. Do you have night activity outdoor?
    a. Yes
    b. No
2. Is this activity frequently?
    a. Yes
    b. No
3. When is the last night outdoor activity you have been doing it?
    a. Yesterday
    b. In this week
    c. In this month
    d. Others, Specify…..
4. Do you have bed net?
    a. Yes
    b. No
5. Do you have mosquito repellant?

a. Yes
b. No

6. Do you frequently use mosquito repellant?
   a. Yes
   b. No
7. What is the type of mosquito repellant you use?
   a. Spray
   b. Mosquito coils
   c. Lotion
   d. Others, Specify…..
8. Do you use pesticide?
   a. Yes
   b. No
9. What is the type of pesticide you use?
   Specify…………….
10. What is the last education level you took?
    a. Primary school
    b. Junior high school
    c. Senior high school
    d. Bachelor
    e. Master
    f. PhD
11. Have you taken any antimalarial drugs?
    a. Yes
    b. No
12. What is the type of antimalarial drugs?
    Please, specify…….

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13. How much is your salary per month (in IDR)?
    a. < 1.000.000
    b. 1000.000-2.000.000
    c. 2.000.000-3.000.000
    d. 3.000.000-4.000.000
    e. 4.000.000-5.000.000
    f. >5.000.000
14. What is your job?
    a. Farmer
    b. Miner
    c. Teacher
    d. Government employees
    e. Others, specify
15. Have you ever contacted to malaria patient?
    a. Yes
    b. No

16. Do you often travel outside your place?
    a. Yes
    b. No
17. If yes, how often?
    a. Once a day
    b. Once a week
    c. Once a month
    d. More than once a day
    e. More than once a week
    f. More than specified option, specify
18. Have you visited a forest in previous month?
    a. Yes
    b. No
19. Is your working place in the forest?
    a. Yes
    b. No
20. Is it requiring overnight stay?
    a. Yes
    b. No

C. Observational investigation of the researcher

1. Is there any gauze or barrier on the ventilation?
   a. Yes
   b. No
2. Is there any shrubs?
   a. Yes
   b. No
3. Is there any stagnant or puddle water?
   a. Yes
   b. No
4. Is there any predator fish in the stagnant or puddle water?
   a. Yes
   b. No
5. Is there any Livestock inside house?
   a. Yes
   b. No
6. Is there any Livestock nearby house?
   a. Yes
   b. No
7. Is hygiene and sanitation of the house appropriate enough?
   a. Yes
   b. No

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8. What is the type of household wall?
    a. Made by wood
    b. Made by cement
    c. Made by bamboo
    d. Others, specify…..
9. Is the house near with rice field?
    a. Yes
    b. No
10. What is the floor of house made of?
    a. Permanent construction
    b. Non-permanent construction
11. Is there any ceiling on the rooftop of the house?
    a. Yes
    b. No
12. Is there any water pool nearby the house?
    a. Yes
    b. No
13. What is the wall of the house made of?
    a. Wood
    b. Permanent construction
    c. Bamboo
    d. Others, Specify…
14. Is there any hanged clothes
    a. Yes
    b. No
15. Is there any breeding place?
    a. Yes
    b. No
16. What is the type of breeding place?
    a. A river
    b. A stagnant water
    c. Others, specify………….
17. Coordinates of the breeing place (if any)

    Specify
    Latitude :
    Longitude :
    Elevation :
18. Is there any livestock cage?
    a. Yes
    b. No
19. Coordinates of the livestock cage

    Specify
    Latitude :

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Longitude :
Elevation :

20. Is there any forest?
    a. Yes
    b. No
21. If any, go to sampling coordinates section

Thank you for your willingness to be respondent

(…………………………………….)

Interviewer

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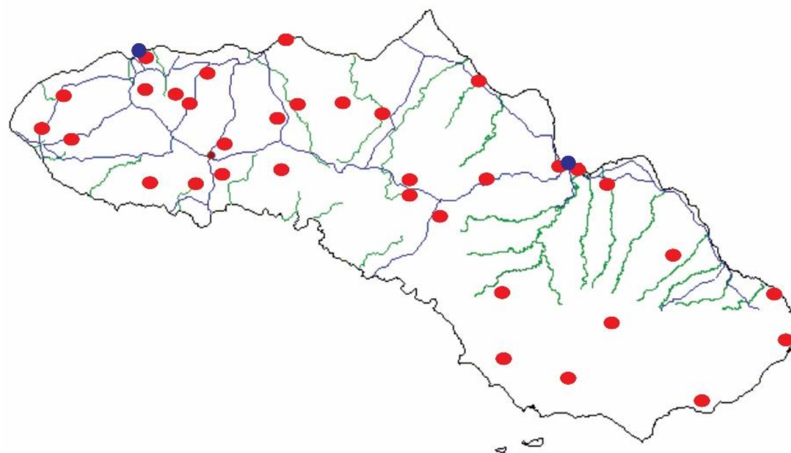
APPENDIX 2

MODIFIED MAP OF SUMBA IN RELATION TO HEALTH FACILITIES

MAP OF HOSPITAL AND PRIMARY HEALTH FACILITIES
SUMBA, NUSA TENGGARA TIMUR



Red circle denotes primary health facilities, while blue denotes hospitals. Blue line denotes provincial border, while green line denotes river

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APPENDIX 3

DHP-PQ AND PRIMAQUINE ADHERENCE STUDY, INDONESIA

CENTRE QUESTIONNAIRE at health Centre

Date: |__|__| (dd) / |__|__| (mm) / 2018

Inclusion Nr: HC - |__|__|__|__¦

Note: first digit is always the unique number of the data collector

Name of data collector: ____________________________________

PATIENT'S IDENTIFICATION

Family name: _________________________ Given name: ___________________________________

Gender: M / F Age: |__|__| *year(s) or if < 1 year |__/__/ month(s)* Weight: _____ kg

PATIENT'S ADDRESS *(if needed, you can make a drawing on the back of this page)*

Family and given name parent/caretaker *(if patient is a child)*: _________________________________

Village name: ____

Section of quartier: ____

Name of house owner: ____

How many minutes did you walk from home to the health clinic? |__|__|__| *minutes*

Other indicators: ____

BEFORE PATIENT CAME TO THE HEALTH CENTRE

How many days ago did the patient's symptoms start? |__|__| *days*

Did the patient take any treatment since then? Yes / No

If yes, which one/s *(specify)*: ____

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CARETAKER'S IDENTIFICATION (If only the patient was unable to take the drug by him-/her-self)

Relation to patient		
	Patient is respondent (Adult)	1
	Parent (Mother/Father)	2
	Grandparent (Grandfather/Grandmother)	3
	Sibling (Brother/Sister)	4
	Aunt/Uncle	5
	Other (*specify below*)	6
	______________________________________	

Caretaker: Gender: M / F Age |__|__| years Adult patient, no caretaker ☐

Highest level of education		
	Unable to read and write	1
Ask if able to read / write first:	Primary but incomplete	2
- *if "no" circle 1 and continue to next question,*	Primary completed	3
- *if "yes" determine what level.*	Secondary but incomplete	4
	Secondary completed	5
	Higher level but incomplete	6
	Higher level completed	7

QUESTIONS TO PARENT / CARETAKER

Q1	Can you tell me what disease you / your child/ your relative has at that time?	No (Don't know)	0
		Yes – name is Malaria	1
		Signs and symptoms (malaria) only, does not know name	2
		Other *(specify below)*	3
		______________________________	
Q2	*Ask to see medicines given by the clinic.* You / your child/ your relative has malaria. Of the treatments you have here, can you show me which one(s) is for malaria?	Yes – Shows ACT only	1
		Shows ACT with other drugs	2
		Shows other drugs only	3
	If answers (1) or (4) skip to Q4 *If answers (2) or (3) continue to Q3*	Doesn't know	4
Q3	Which other drugs shown?	Paracetamol (PCM)	1
		Folic acid (FA)	2
	Multiple answers possible	Other unrelated (*specify below*)	3
		______________________________	

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Q4	Indicating the ACT tablets Have you or your child/ your relative taken this treatment before? If yes, how many times?	Yes, ____________________times	1
		No, this is the first time	0

Q5 Indicating ACT tablets again:

Can you tell me how did you take / to give this treatment?

Let patient/caretaker enough time to answer, don't push.

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____
____
____
____
____
____
____
____
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____
____

Clarification of treatment instructions:

Q6	Did the person who gave you this treatment ask if you understood how to give it?	Yes	1
		No	0
Q7	Did they ask you to repeat back the instructions to you?	Yes	1
		No	0

Q8	Did they give you any other information or advice about the treatment?	Yes *(specify below)* ____________________________ ________	1
Q9	Did you/your child already/ your relative take one dose of this treatment at the clinic?	Yes No	1 0
Q10	Did you/your child/ your relative take any other treatment in the clinic? *If YES, go to Q11* *If NO, go to Q12*	Yes No	1 0
Q11	If yes, which one?	Paracetamol (PCM) Folic Acid (FA) Other/s *(specify below)*	1 2 3
Q12	Show the ACT tablets again How many time you need to give/take this treatment after came to the clinic? *Possible answers: 1 time, 2 times, 3 times, don't know etc.*	________________________________ _____ ________________________________	
Q13	How many tablets you need to give / take?	|__|__|tablets	
Q14	*If balance remaining:* What will you do with the remaining tablets?	Keep for next time household member unwell	1 2
Q15	Will you still give/take the treatment after your child/ your relative/you felt better? *(Day 1)*	Yes No	1 0
Q16	What you need to do If you / your child/ your relative is not better in 3 days?	________________________________ ____ ________________________________ _____	

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TREATMENT PRESCRIBED TO PATIENT AT THE HEALTH CENTRE

Nr.	Name of treatment prescribed	Nr. of tablets	Times per day	Nr. of days
1.	....	|__|__|	OD / BD / TDS	for |__|__| days
2.	....	|__|__|	OD / BD / TDS	for |__|__| days
3.	....	|__|__|	OD / BD / TDS	for |__|__| days
4.	....	|__|__|	OD / BD / TDS	for |__|__| days
5.	....	|__|__|	OD / BD / TDS	for |__|__| days
6.	....	|__|__|	OD / BD / TDS	for |__|__| days
7.	....	|__|__|	OD / BD / TDS	for |__|__| days
8.	....	|__|__|	OD / BD / TDS	for |__|__| days
9.	....	|__|__|	OD / BD / TDS	for |__|__| days
10.	....	|__|__|	OD / BD / TDS	for |__|__| days

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APPENDIX 4

DHP-PQ AND PRIMAQUINE STUDY, INDONESIA

Home-Questionnaire

Date: |__|__| (dd) / |__|__| (mm) / 2013	**UNIQUE INCLUSION NUMBER C-|__|__|__|**
INTERVIEWER NAME: __________/ ________/ ________	

Interviews need to be conducted ONLY with the patient or caretakers/parents who gave the treatment to the patient (child).

If patient or caretakers/parents are not present at your home visit and cannot be traced in a reasonable amount of time consider them as "lost to follow-up". Select the next home to perform an interview.

LOST TO FOLLOW-UP *(circle)*: Yes / No

Please use the introduction sheet to explain the purpose of your visit and ask for written consent (separate sheet).

If the patient or caretakers/parents refuse to participate, never insist on their participation. Leave the home and select the next home to perform an interview.

WRITTEN CONSENT OBTAINED *(circle)*: Yes / No

I. DEMOGRAPHIC AND SOCIOECONOMIC INFORMATION OF HOUSEHOLD

Q1	Respondent relationship to patient	Respondent is the patient Parent (Mother/Father) Grandparent (Grandfather/Grandmother) Sibling (Brother/Sister) Aunt/Uncle Other (*specify below*) ________________________________ _________________	1 2 3 4 5 6
Q2	Age of patient	|__|__|	*years*
Q3	Weight of patient	4.5-8kg (infant) 9-17kg (young children) 18-35kg (adolescent) >=36kg (adult) <4.5kg (infant)	1 2 3 4 5
Q4	Sex of patient	Male Female	1 2
Q5	Respondent him/herself present at the clinic to receive treatment *If YES, go to Q6* *If NO, go to Q5*	Yes No	1 0
Q6	*If respondent was not present at clinic:* Who went to the clinic?	Parent (Mother/Father) Grandparent (Grandfather/Grandmother) Sibling (Brother/Sister) Aunt/Uncle Other (*specify below*)	1 2 3 4 5

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		______________________________ ____________________	
Q7	Patient: highest level of education	Unable to read and write Primary but incomplete Primary completed Secondary but incomplete Secondary completed Higher level but incomplete Higher level completed	1 2 3 4 5 6 7
Q8	How many people live in your household currently? *Including respondent*	Household members	___ ___
Q9	How many children under 5 years of age live in your household?	Children under 5 years	___
Q10	What is the profession of the head of household? *Person who has **responsibility** for whole **household***	Subsistence farmer Farmer for trading Trader Workman (e.g. joiner, mechanic etc) Daily worker No work Other *(specify below)* ______________________________ ____________________	1 2 3 4 5 6 7

II. TREATMENT			
Q11	How is the patient feeling at the time? *If cured, go to Q12* *If not cured, go to Q11*	Cured / OK Better but not cured Still unwell	1 2 3
Q12	If patient not cured: Has patient been back to the clinic in the last 2 days?	Yes No	1 0
Q13	Is respondent happy with the treatment received at the clinic?	Yes No	1 0

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II.a. TREATMENT INTAKE DAY 0			
Show tablet samples to		ASAQ tablets	
Q14	While still **at the clinic**, did the patient take any of these tablets?	Yes No	1 0
Q15	When the patient came **home** from the clinic, did they take any of these tablets before going to sleep? *If NO, go to Q19* *If YES, go to Q16*	Yes No	1 0

<table>
<tr><td>Q16</td><td>How many times total?</td><td colspan="6">|__|__| *times*</td></tr>
<tr><td>Q17</td><td>How many tablets each time?
Make an additional note at the back of the paper if more than 3 times</td><td colspan="2">1st time</td><td colspan="2">2nd time</td><td colspan="2">3rd time</td></tr>
<tr><td></td><td></td><td colspan="2">|__|__|</td><td colspan="2">|__|__|</td><td colspan="2">|__|__|</td></tr>
<tr><td>Q18</td><td>When?</td><td>Morn
Aft
Eve</td><td>1
2
3</td><td>Morn
Aft
Eve</td><td>Morn
Aft
Eve</td><td>1
2
3</td><td>Morn
Aft
Eve</td></tr>
<tr><td>Q19</td><td>Spat out or vomited tablets?</td><td>Yes
No</td><td>1
0</td><td>Yes
No</td><td>Yes
No</td><td>1
0</td><td>Yes
No</td></tr>
</table>

II.b. TREATMENT INTAKE DAY 1

<table>
<tr><td></td><td colspan="3"></td><td colspan="4">ASAQ tablets</td></tr>
<tr><td>Q20</td><td colspan="3">The day after the patient went to the clinic did the patient take any tablets?
If NO, go to Q24
If YES, go to Q21</td><td colspan="2">Yes
No</td><td colspan="2">1
0</td></tr>
<tr><td>Q21</td><td>How many times total?</td><td colspan="6">|__|__| *times*</td></tr>
<tr><td>Q22</td><td>How many tablets each time?
Make an additional note at the back of the paper if more than 3 times</td><td colspan="2">1st time</td><td colspan="2">2nd time</td><td colspan="2">3rd time</td></tr>
<tr><td></td><td></td><td colspan="2">|_|_|</td><td colspan="2">|_|_|</td><td>|_|_|</td><td></td></tr>
<tr><td>Q23</td><td>When?</td><td>Morn
Aft
Eve</td><td>1
2
3</td><td>Morn
Aft
Eve</td><td>1
2
3</td><td>Morn
Aft
Eve</td><td>1
2
3</td></tr>
<tr><td>Q24</td><td>Spat out or vomited tablets?</td><td>Yes
No</td><td>1
0</td><td>Yes
No</td><td>1
0</td><td>Yes
No</td><td>1
0</td></tr>
</table>

II.c. TREATMENT INTAKE DAY 2

<table>
<tr><td></td><td></td><td></td><td colspan="2">ASAQ tablets</td></tr>
<tr><td>Q25</td><td colspan="2">What about the following day, did the patient take any tablets?
If NO, go to Q29
If YES, go to Q26</td><td>Yes
No</td><td>1
0</td></tr>
<tr><td>Q26</td><td>How many times total?</td><td colspan="3">|__|__| *times*</td></tr>
<tr><td>Q27</td><td>How many tablets each time?</td><td>1st time</td><td>2nd time</td><td>3rd time</td></tr>
</table>

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	Make an additional note at the back of the paper if more than 3 times						
		|__|__|		|__|__|		|__|__|	
Q28	When?	Morn Aft Eve	1 2 3	Morn Aft Eve	1 2 3	Morn Aft Eve	1 2 3
Q29	Spat out or vomited tablets?	Yes No	1 0	Yes No	1 0	Yes No	1 0

III. PRESENCE OF MEDICINE			
Q30	Ask to see the medicine/ medicine packaging given to them at the clinic	Empty ACT or primaquine blister / labelled drug bag seen Blister / drug bag with tablets seen No blister / bag seen	1 2 0
		If tablets still present *If all tablets taken but incorrectly* *If all tablets taken correctly* *If blister / bag was NOT seen:* *Check if tablets were taken incorrectly* *Check if tablets are taken correctly*	*Go to Q31* *Go to Q33* *Go to Q34* *Go to Q33* *Go to Q34*
Q31	How many tablets are remaining? *Must be **seen** by interviewer*	Number of tablets seen:	|__|__|
Q32	Can you tell me why there are tablets remaining? *Multiple answers possible, please circle **all** responses given* *Now go to Q34*	Patient got cured, so no need to continue the treatment Patient got cured, so I kept the remaining tablets for next time Household poor, so kept some tablets for next time Patient forgot to take some tablets / Caretaker forgot to give some tablets Patient did not feel better, the treatment was not working Patient / Caretaker claims that wrong instructions were given in the clinic Other reasons/s (*specify below)*	1 2 3 4 5 6 7

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		______________________________ ___________ ______________________________ ___________	
Q33	The patient has taken the tablets incorrectly, why? *Multiple answers possible, please circle **all** responses given* *Now go to Q34*	Patient / Caretaker thought patient would cure faster Patient / Caretaker claims that wrong instructions were given in the clinic Tablets made patient feel sick Patient could not swallow tablets Patient was vomiting Other reason/s (*specify below)* ______________________________ ___________ ______________________________ ___________	1 2 3 4 5 6
Q34	The tablets have been taken correctly, well done! Why do you think it was done correctly? *Multiple answers possible, please circle **all** responses given*	Patient / Caretaker / household member previsouly taken / given the same medicine before and knows how to take it Right instructions were given at the clinic Was helped by the community health volunteer (CHV) Other reason/s (*specify below)* ______________________________ ___________ ______________________________ ___________	1 2 3 4
Q35	In the past 3 days, did the patient take any other medication to treat malaria? *If YES go to Q35* *If NO go to Q37*	Yes No	1 0
Q36	If yes, what was taken? *Show respondent tablet samples* *Multiple answers possible, please circle **all** responses given*	Paracetamol (PCM) Folic Acid (FA) Herbal *(specify below)* ______________________________ ___________ Other/s *(specify below)* ______________________________ ___________	1 2 3 4

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Q37	If yes, where did this medicine come from? *Multiple answers possible, please circle **all** responses given*	Clinic during same consultation Other public health structure *(specify i.e. Gov. hospital, PHU, CHV, GRC)* ______________________ __________ Home / Relative / Friend Bought on market Traditional healer Other place/s *(specify below)* ______________________ __________	1 2 3 4 5 6
IV. PERCEPTION OF MALARIA			
Q38	The patient had malaria. Do you know how you get sick with malaria? *Multiple answers possible, please circle **all** responses given*	Don't know Standing/ working/ playing in hot sun Bad spirit Contaminated water Problem with food Mosquito bite Other reason/s *(specify below)* ______________________ __________ ______________________ __________ ______________________ __________	1 2 3 4 5 6 7
Q39	How do you think you can protect yourself from getting malaria? *Multiple answers possible, please circle **all** responses given*	Don't know Sleep under mosquito net Amulettes or magic Taking medication Cleaning around the house Good cooking habits Spraying inside the house Digging a latrine Other protection/s (*specify below)* ______________________ __________ ______________________ __________ ______________________ __________	1 2 3 4 5 6 7 8 9
Q40	How many bednets are present in your house? *If none, please enter '0'*	|__|__| bednets	
Q41	Do you know what ACT use for? *Multiple answers*	Don't know To treat malaria To cure fever and headache To make the body feel better	

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	*possible, please circle **all** responses given*	Other protection/s (*specify below)* ____ ________________ ____ ________________ ____ ________________
Q42	Do you know what primaquine use for? *Multiple answers possible, please circle **all** responses given*	Don't know To treat malaria To cure fever and headache To make the body feel better To prevent malaria transmission Other protection/s (*specify below)* ____ ________________ ____ ________________ ____ ________________

Please thank the respondent very much for his/her participation and help.

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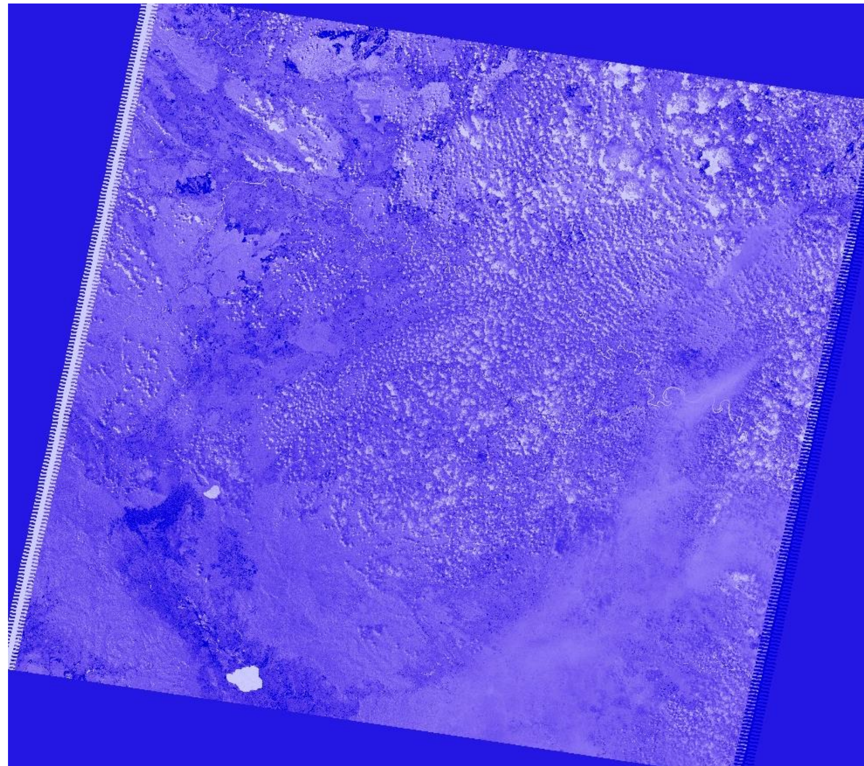
APPENDIX 5

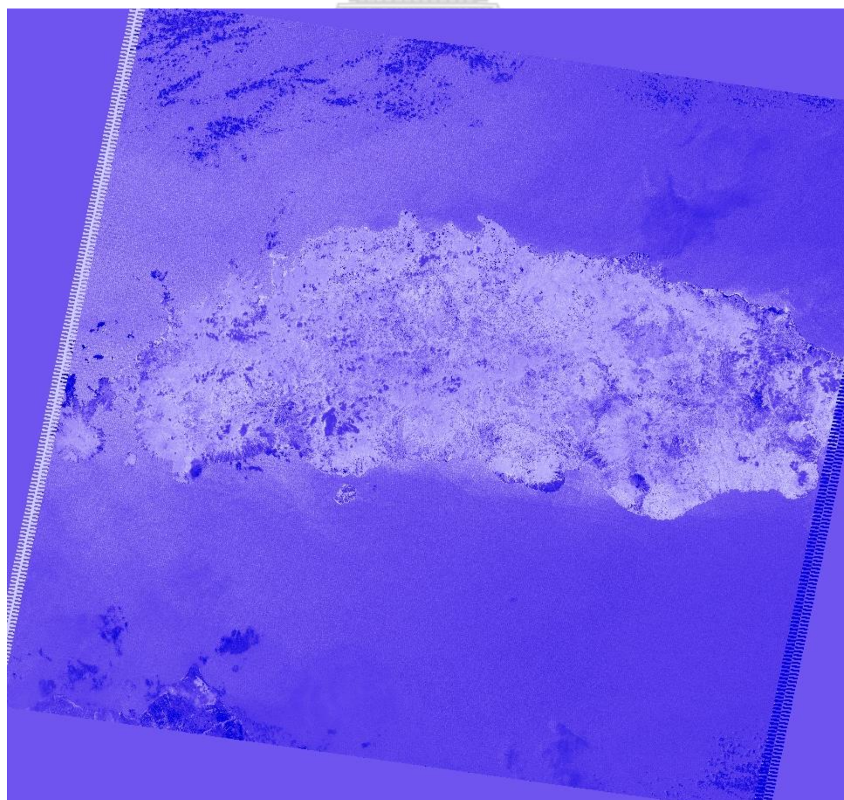
MODIFIED SIMULATED NDVI (NROMALISED DIFFERENCE VEGETATION INDEX) MAP OF EACH SAMPLED ISLAND



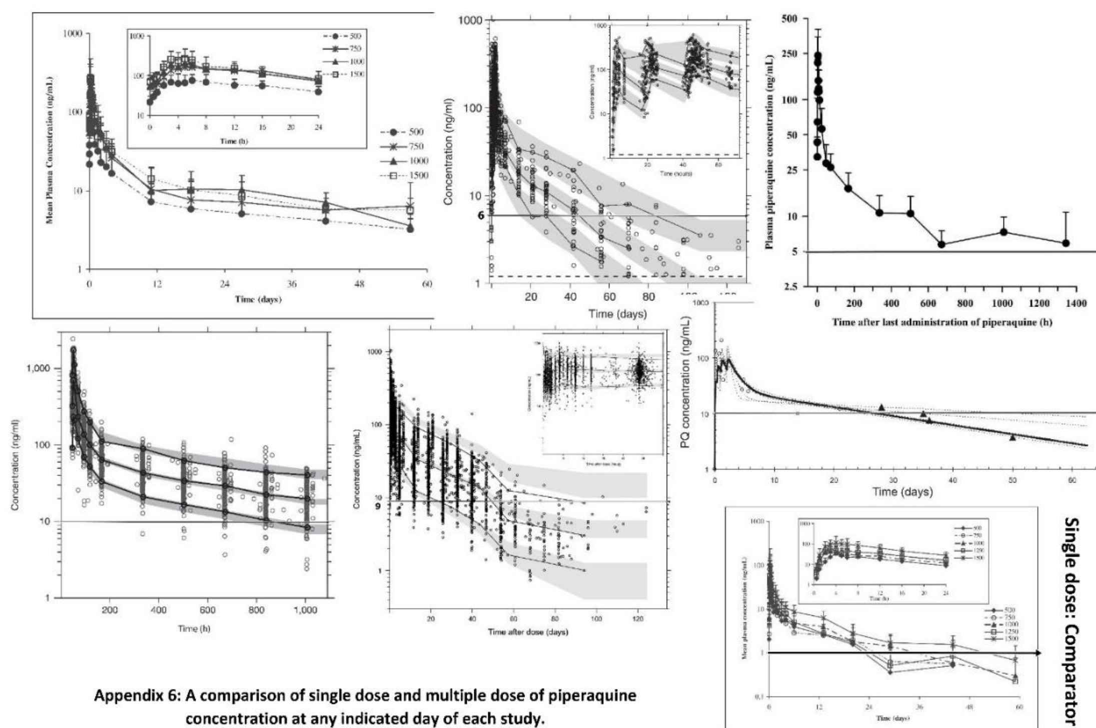
High : 0.384615

Low : -0.741935

Jambi



Sumba



Appendix 6: A comparison of single dose and multiple dose of piperaquine concentration at any indicated day of each study.

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Appendix 7: Ethical approval

KEMENTERIAN RISET, TEKNOLOGI DAN PENDIDIKAN TINGGI
KOMITE ETIK PENELITIAN KESEHATAN
Fakultas Kedokteran Universitas Hasanuddin
RSPTN Universitas Hasanuddin
RSUP dr. Wahidin Sudirohusodo Makassar
Sekretariat : Lantai 2 Gedung Laboratorium Terpadu FKUH
JL.PERINTIS KEMERDEKAAN KAMPUS TAMALANREA KM.10 MAKASSAR 90245
Contact Person: dr. Agussalim Bukhari, MMed, PhD,SpGK Telp. 081241850858, Fax : 0411-581431

REKOMENDASI PERSETUJUAN ETIK
Nomor : 663 /H4.8.4.5.31/PP36-KOMETIK/2016

Komisi Etik Penelitian Kesehatan Fakultas Kedokteran Universitas Hasanuddin, RSPTN UH, RSUP dr. Wahidin Sudirohusodo setelah melalui pembahasan dan penilaian, memutuskan penelitian berjudul:

Efikasi dan Keamanan Dihidroartemisinin-Piperakuin pada Pengobatan Malaria Plasmodium Falciparum dan Plasmodium Vivax Tanpa Komplikasi di 3 Daerah Sentinel di Indonesia

dengan Peneliti Utama: **Prof. dr. Syafruddin, PhD**

No. Register	U	H	1	6	0	4	0	4	2	6

Yang diterima pada tanggal : **19 Mei 2016**

Perbaikan diterima pada tanggal : **25 Mei 2016**

dapat disetujui untuk dilaksanakan di **Propvinsi Papua, Provinsi Jambi dan Provinsi Bengkulu.**
Persetujuan Etik ini berlaku satu tahun sejak tanggal ditetapkan. Laporan perkembangan penelitian diserahkan kepada KEPK FKUH, RSPTN UH dan RSWS Makassar setiap ~~tiga bulan/enam bulan~~/satu tahun.
Pada akhir penelitian, **laporan akhir penelitian** harus diserahkan kepada KEPK FKUH, RSPTN UH dan RSWS Makasar paling lambat **27 Mei 2017 .** Jika ada perubahan protokol dan /atau perpanjangan penelitian, harus mengajukan kembali permohonan kajian etik penelitian (amandemen protokol).

Makassar, *27 Mei 2016*
Komisi Etik Penelitian Kesehatan Fak. Kedokteran Unhas

Ketua

Prof.Dr.dr.Suryani As'ad,M.Sc,Sp.GK
NIP 19600504 1986 01 2 002

Sekretaris

dr. Agussalim Bukhari,PhD ,SpGK
NIP 19700821 1999 03 1 001

KEMENTERIAN RISET, TEKNOLOGI DAN PENDIDIKAN TINGGI
UNIVERSITAS HASANUDDIN
FAKULTAS KEDOKTERAN
RSPTN UNIVERSITAS HASANUDDIN
RSUP Dr. WAHIDIN SUDIROHUSODO MAKASSAR
KOMITE ETIK PENELITIAN KESEHATAN

Sekretariat : Lantai 3 Gedung Laboratorium Terpadu
JL.PERINTIS KEMERDEKAAN KAMPUS TAMALANREA KM.10 MAKASSAR 90245.
Contact Person: dr. Agussalim Bukhari.,MMed,PhD, SpGK TELP. 081241850858, 0411 5780103, Fax : 0411-581431

REKOMENDASI PERSETUJUAN ETIK
Nomor : 356 / H4.8.4.5.31 / PP36-KOMETIK / 2017

Tanggal: 31 Mei 2017

Dengan ini Menyatakan Perpanjangan Protokol dan Dokumen yang Berhubungan Dengan Protokol berikut ini telah mendapatkan Persetujuan Etik :

No Protokol	**UH16050426**	No Sponsor Protokol	
Peneliti Utama	**Prof. dr. Syafruddin, PhD**	Sponsor	**WHO**
Judul Penelitian	**Efikasi dan Keamanan Dihidroartemisinin-Piperakuin pada Pengobatan Malaria Plasmodium Falciparum dan Plasmodium Vivax Tanpa Komplikasi di 3 Daerah Sentinel di Indonesia**		
No Versi Protokol	**3**	Tanggal Versi	**22 Mei 2017**
No Versi PSP	**3**	Tanggal Versi	**22 Mei 2017**
Tempat Penelitian	**Provinsi Papua (Kabupaten Jayapura, Kota Jayapura Dan Kabupaten Kerom), Provinsi Jambi (Kabupaten Sarolangun), dan Provinsi Bengkulu (Kabupaten Bengkulu Utara)**		
Dengan Nomor Rekomendasi Persetujuan Etik Lama	Nomor: 663/H4.8.4.5.31/PP36-KOMETIK/2016		
Jenis Review	☐ Exempted ☒ Expedited ☐ Fullboard Tanggal	Masa Berlaku **31 Mei 2017** sampai **31 Mei 2018**	Frekuensi review lanjutan **1 Minggu**
Ketua Komisi Etik Penelitian	Nama **Prof.Dr.dr. Suryani As'ad, M.Sc.,Sp.GK (K)**	Tanda tangan	Tanggal
Sekretaris Komisi Etik Penelitian	Nama **dr. Agussalim Bukhari, M.Med.,Ph.D.,Sp.GK (K)**	Tanda tangan	Tanggal

Kewajiban Peneliti Utama:

- Menyerahkan Amandemen Protokol untuk persetujuan sebelum di implementasikan
- Menyerahkan Laporan SAE ke Komisi Etik dalam 24 Jam dan dilengkapi dalam 7 hari dan Lapor SUSAR dalam 72 Jam setelah Peneliti Utama menerima laporan
- Menyerahkan Laporan Kemajuan (progress report) setiap 6 bulan untuk penelitian resiko tinggi dan setiap setahun untuk penelitian resiko rendah
- Menyerahkan laporan akhir setelah Penelitian berakhir
- Melaporkan penyimpangan dari prokol yang disetujui (protocol deviation / violation)
- Mematuhi semua peraturan yang ditentukan

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