The detection of Plasmodium in mosquitoes from Sumba and Sorong districts, Indonesia

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Short Communication: The detection of *Plasmodium* in mosquitoes from Sumba and Sorong districts, Indonesia

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Abstract. *Munirah M, Wahid I, Hamid F, Wahyuni S. 2021. Short Communication: The detection of* Plasmodium *in mosquitoes from Sumba and Sorong districts, Indonesia. Biodiversitas 22: 2680-2684.* Malaria is heading for elimination in 2030, but the disease remains prevalent in Indonesia. Therefore, this study aimed to determine a vector other than *Anopheles* that is potentially responsible for malaria transmission in the country's endemic areas. The mosquitoes were trapped by Kelambu trap, collected using a mouth aspirator, and stored in a tube containing silica gel. They were also examined microscopically following O'Connor identification keys, and Nested PCR was used to detect *Plasmodium* DNA. The number of mosquitoes collected from two areas was 1.336. In Sumba (Gaura village) district, 493 mosquitoes were captured, and the dominant genus was *Anopheles* (58.6%), followed by *Culex* (31%), *Armigeres* (9.9%), *Aedes* (0.2%), and *Lutzia* (0.2%). Among 843 mosquitoes collected from Sorong (Aimas), *Culex* was 98.9%, followed by *Aedes* (0.8%) and *Anopheles* (0.2%). The result of nested PCR found two *An. sundaicus* from Sumba carried *Plasmodium* which belongs to *P. falciparum* and *P. vivax* species (0.14%). Although we did not detect the presence of *Plasmodium* in mosquitoes other than the genus *Anopheles*, in this study, we found *Anopheles* species that have never previously been reported from the area, namely *An. bailey* and *An. barbirostris* from Papua and *An. nivipes* in West Sumba.

Keyword: *Anopheles,* malaria, nested PCR, *Plasmodium*, vector

INTRODUCTION

Malaria is caused by *Plasmodium* protozoan, and the total cases have reached 228 million globally, with 405,000 deaths in 2018 (WHO 2019). East Nusa Tenggara (Sumba) and West Papua are the areas having the highest prevalence, with 7.04% and 31.29% Annual Parasites Incidence (API), respectively (Indonesian Ministry of Health 2016). Attempts have been made to eliminate the parasite, including mass treatment, breeding site suppression, application of insecticide net (PATH Malaria Centre 2014), and vector surveillance (Indonesian Ministry of Health 2014). However, API's change is still not satisfactory and those methods need expansion to ensure malaria is eliminated.

The high prevalence is inseparable from the existence of *Anopheles,* and the species responsible for spreading malaria is not the same in all areas. In America, the typical vector is *An. freeborni* Aitken, but it is *An. atroparvus van Thiel* in the Middle East and Europe, *An. arabiensis Patton* in Africa and *An. barbirostris* van der Wulp in Asia. In Indonesia, the dominant *Plasmodium* vector differs across all Islands (Sinka et al. 2012), for instance, it is *An. farauti* in Jayapura (Elyazar 2013), *An. balabacensis* in Kalimantan (St. Laurent et al. 2016) and *An. barbirostris* in Sulawesi (Nurdin et al. 2003; Veridiana et al. 2019). Importantly, *Culex* is the mosquito genus first known as an intermediate host of lymphatic filariasis (Ogoma et al. 2010). Afterward, *Aedes*, *Anopheles*, and *Mansonia* also transmit it (WHO 2013), while malaria cases likely likewise encounter a related situation.

Mutations are changes that occur in the genetic material and it can be passed to heredity (Campbell et al. 2014; Campbell et al. 2015). The mutation allows the parasite to adapt to the host, and it tends to also benefit either or both of them (M'Gonigle 2009). In terms of their interaction, the mutation rate is determined by genome size which is shorter in parasites than the host, therefore causing higher mutation frequency (McDew-White et al. 2019). This condition makes the parasite, known as a pathogenic microorganism, adapt to a new host when exposure occurs continuously (Mackinnon and Marsh 2010). Global warming also plays a role in adding a new host for the parasite (Mills et al. 2010). Stress due to heating causes a mutation in both unicellular or multicellular organisms, which allows them to survive and pass their ability to the next generation for adaptation to the new host environment (Berger et al. 2017). Based on the personal discussion with the head of the communication disease from Sorong Health Office, the survey conducted in one of the district's villages during the malaria outbreak found no *Anopheles* larvae. The theory and case raise the query of whether there is

another genus that potentially carries the *Plasmodium* parasite. This study aims to detect *Plasmodium* as a vector of malaria in female mosquitoes from various genera in malaria-endemic areas.

MATERIALS AND METHODS

Mosquitos collection

Female mosquitoes were collected from the residential area of Gaura Village, South West Sumba District of East Nusa Tenggara, Indonesia (October 2018), and Aimas, Sorong District of West Papua, Indonesia (August 2019). Provincial health offices determined those two areas have the highest API in the districts. Due to the absence of electricity to make a light trap, mosquitoes were collected using Kelambu and the cow available in the area were used as the bait. Kelambu modified from previous studies (Davidson et al. 2019). The mosquito collection in Gaura and Aimas was carried out at 7 different locations, respectively, in one-night collection was done only in one location. Mosquitoes in the Kelambu were collected every hour, from 7 p.m until 1 a.m, using a mouth aspirator. They were placed in 1.5 ml tubes containing silica gel to prevent mold growth and structural damage of the vector, which absorbs moisture to reduce the tube's humidity. Microscopic mosquito identification was performed by following O'Connor's key (O'Connor et al., 1981). Especially for *Anopheles*, the species were validated by PCR using ITS2 primer (Weeraratne et al. 2017), and the product was sent to Genetica Science, Singapore, for sequencing. For Plasmodium DNA identification, the head-thorax was separated from the abdomen by a fine needle, and both parts were stored in different tubes.

DNA Extraction

One to five head-thorax parts of mosquitoes from the same species were pooled in a 1.5 ml microtube and macerated using disposable Teflon pestles, while the DNA was extracted using the TIANamp Genomic kit. The head-thorax was smashed in a 1.5 ml tube, 20 ml proteinase K was added, then vortexed and incubated at 56°C (3 hours). Two hundred μl of buffer GB was added to the tube, mixed thoroughly, vortexed before incubation at 70°C (10 minutes) and then centrifuged. Another 200 μl ethanol (96-100%) was poured into the 1.5 tubes, mixed thoroughly, vortexed, and then centrifuged. The mixture from the 1.5 tubes was moved to the spin column which was then placed onto the collection tube. Five hundred μl of buffer GD was added to the spin column, centrifuged at 12000 rpm (30 seconds), discarded the flow-through, and then placed onto the collection tube. Six hundred μl of buffer PW was added and processed as before and repeated once again, which was centrifuged at 12000 rpm for 2 minutes to dry the membrane completely. The spin-column was put into a 1.5 ml tube and 100 μl of buffer TE was pipetted to the membrane's center. It was incubated at room temperature for 5 minutes and then centrifuged as the previous, followed by storing the purified DNA at -20°C. When the PCR product indicated *Plasmodium* in the head-thorax pool, the corresponding mosquito's DNA from the abdomen pools was extracted separately to determine the number of positive species and then stored at -20° for further analysis.

DNA Amplification

Polymerase chain reaction (PCR) Thermociclador T100 Biorad was used to detect *Plasmodium* in the mosquitoes. Nested PCR was performed using three pairs of primers, namely rPLU5: 5'-CCTGTTGTTGCCTTAAACTTC-3' and rPLU6: 5'-TTAAAATTGTTGCAGTTAAAACG-3' for *Plasmodium* species in the nested 1. Furthermore, the other two were rVIV1: 5'-CGCTTCTAGCTTAATCCA CATAACTGATAC-3' and rVIV2: 5'-ACTTCCAAGCCG AAGCAAAGAAAGTCCTTA-3' for *Pl. vivax* in nested 2, rFAL1: 5'-TTAAACTGGTTTGGGAAAACCAAATATATT-3' and rFAL2: 5'-ACACAATGAACTCAATCATGACTA CCCGTC-3' for *Pl. falciparum* in nested 2, as previously described (Snounou et al. 1993; Singh et al. 2013).

Figure 1. Map of Indonesia. The sampling areas in this study in: 1. Gaura Village, South West Sumba District of East Nusa Tenggara, Indonesia (October 2018); 2. Aimas, Sorong District of West Papua, Indonesia (August 2019)

The thermal profile of 35 cycles for nested 1 was 94°C denaturation, 55°C annealings, and 72°C extensions, all at 1 minute each. That of nested 2 was 94°C denaturation (30 seconds), 55°C annealings (1 minute), and 72°C extensions (30 seconds). The entire reactions had 12.5 µl volumes and contained 7 µl PCR enzymes (2×Taq plus PCR Mix, Tiangen Biotech), reverse and forward primers at 1 µl each, 2 µl template, with 2.5 µl ddH_2O. The template for Nested 1 was the DNA, while that of 2 was its PCR results which were then run in 2% agarose gel for electrophoresis. After the *Anopheles* species were identified microscopically, their DNA was extracted and amplified by PCR ITS2 primer and then sequenced for confirmations.

RESULTS AND DISCUSSION

Results

A total of 1,336 mosquitoes were trapped using the *Kelambu* in two different islands, where the highest number of females was 843 in Aimas, but 493 in Gaura village. Furthermore, the total species in the samples was 28, 13 species of *Anopheles* and it was the largest species, followed by *Culex* (10), *Armigeres* (2), *Aedes* (2), and *Lutzia* (1).

In Gaura village, the commonest species was found is *Anopheles* (289), dominated by *An. vagus*, followed by *Culex* (153), *Armigeres* (49), *Aedes* (1), and *Lutzia* (1). The *Culex* genus dominated the mosquitoes trapped in the Aimas region, Sorong district (834 out of the 843), and the most numerous species was *C. quinquefasciatus,* while for *Aedes* and *Anopheles*, only 7 and 2 were caught, respectively.

From 285 mosquito pools (each containing 1-5 mosquitoes of the same species), one was positive for *Pl. falciparum*, another for *Pl. vivax,* and both were from the *Anopheles sundaicus* pools. The display of PCR electrophoresis result contained positive *Pl. falciparum* and *Pl. vivax* can be seen in figure 2. From this study, it was found that one species of mosquito that acts as a malaria vector in Gaura Village, namely *An. sundaicus*, while in Aimas, Sorong District, malaria vectors were not found in all of 843 mosquitoes. This study was the first research conducted in malaria-endemic areas. In addition, after the *Anopheles* species sequencing process, we reported the first time that *A. bailey* species was found in Indonesia.

Discussion

One perspective regarding malaria transmission is accruing the number of vectors from other species, causing the disease's elimination to become difficult. Therefore this study was conducted to determine the vector of *Plasmodium* in malaria-endemic areas. It was known that mutation occurs in living organisms frequently due to the replication, transcription, and translation process (Lynch 2010; Campbell et al. 2015), and this affects the capability to widen or narrow the symbiosis between mosquito and its borne microbes. Particularly, there is the possibility that *Anopheles* is not the only host for *Plasmodium*. To ascertain whether a mosquito species is a vector, laboratory experiments were carried out by transfecting it with a certain microbe. However, the host's capacity to accept microorganisms is dependent on the evolution of these microbes (Duffy et al. 2012; Sheppard et al. 2018). The finding of new species that acts as a vector for *Plasmodium* explains why this disease remains prevalent and also creates the opportunity to control the transmission. As best known, identifying mosquito species other than *Anopheles* in malaria-endemic areas in Indonesia has never been done.

Table 1. The mosquito genera and species collected by *Kelambu* trap in housing residents and the total number of pools tested from Sumba and Sorong districts, Indonesia

Area	Genera	Species	No. of mosquitoes collected	No. of pools positive/ no. of pools tested
Gaura, Sumba	*Anopheles*	*An. vagus**	101	0/21
		*An. sundaicus**	53	2/11
		*An. aconitus**	18	0/5
		An. kochi	15	0/3
		*An. flavirostris**	6	0/2
		An. indefinitus	13	0/3
		*An. maculatus**	10	0/2
		*An. minimus**	7	0/2
		*An. annularis**	55	0/13
		*An. nivipes**	1	0/1
		An. subpictus	10	0/2
		Sub total	289	2/65
	Culex	*C. tritaeniorhyncus*	36	0/8
		C. fuscocephalus	19	0/4
		C. sitiens sitiens	4	0/1
		C. vishnui	8	0/2
		C. gellidus	1	0/1
		C. quinquefasciatus	1	0/1
		C. whitei	1	0/1
		C. hutchinsoni	24	0/5
		C. pseudovishnui	59	0/12
		Sub total	153	0/35
	Armigeres	*Arm. kesseli*	22	0/5
		Arm. subalbatus	27	0/6
		Sub total	49	0/11
	Aedes	*Aedes sp*	1	0/1
	Lutzia	*Lutzia sp*	1	0/1
Aimas, Sorong	*Anopheles*	*An. baileyi* *	1	0/1
		*An. barbirostris**	1	0/1
		Sub total	2	0/2
	Aedes	*Ae. aegypti*	2	0/1
		Ae. Albopictus	5	0/1
		Subtotal	7	0/2
	Culex	*C. quinquefasciatus*	740	0/148
		C. bitaeniorhynchus	63	0/13
		C. tritaeniorhynchus	28	0/6
		C. gellidus	3	0/1
		Subtotal	834	0/168
		Totally	1336	Total: 2/285

Note: **Anopheles* validated by PCR using ITS2 primer (Weeraratne et al. 2017) and sequenced to clarify the species

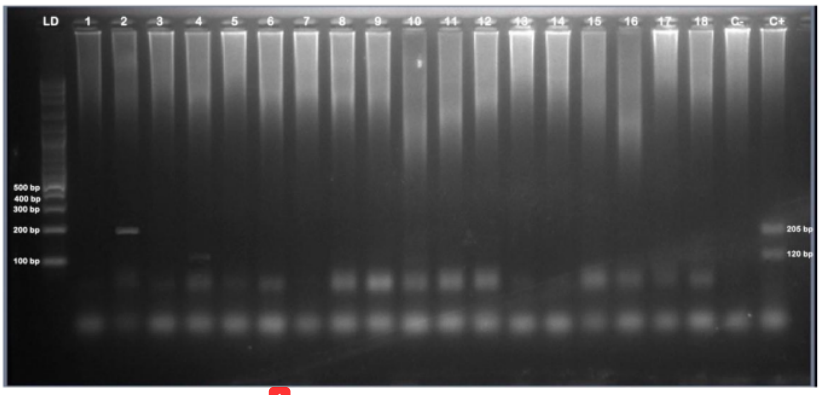

Figure 2. The result was based on electrophoresis of nested PCR mosquito samples. (LD: DNA ladder, C-: control negative, C+: control positive, 2: *An. sundaicus* for positive *Plasmodium falciparum* and 4: *An. sundaicus* for positive *P. vivax*). Also, there were 120 bp (base pairs) for positive *Plasmodium vivax* and 205 bp for positive *P. falciparum*

This study found that *An. sundaicus* was the vector of malaria in Gaura. As we know that *An. sundaicus* is an important vector for spreading malaria in Indonesia (Elyazar et al. 2013; Sugiarto et al. 2016). It has a habit of biting at dusk and dawn (Chakim and Pumpaibool 2019) and is generally found in coastal areas (Surendran et al. 2010). Furthermore, it sucks blood from humans (anthropophilic) and animals (Arifianto 2015), which allows them to breed quickly. In the last decades, *An. sundaicus* has been reported as resistant to several insecticides (Silva et al. 2014; Raghavendra et al. 2017), which causes this species to survive longer. The contribution of mosquitoes to malaria transmission is influenced by environmental factors, blood-feeding, vector competence, season, and others. Overall, no vector other than *Anopheles* was detected as a *Plasmodium* host in this study. However, we also found differences between Gaura and Aimas (24 and 8 species, respectively). Environmental characteristics between the two villages seem responsible for those conditions. Compared to Aimas, people living in Gaura are more diverse because some live in Inland, coastal areas, and hills.

The Abundance of *An. vagus* found in Gaura village requires investigation into its possible contribution to malaria transmission in this village, considering *An. vagus* has been reported as a malaria vector in the South Sumatra region (Budiyanto et al. 2017). In this study *An. vagus* was captured outdoor. This allows the species to be protected from vector control programs such as Indoor Residual Spraying (IRS). Testing of *Anopheles*, *Culex*, *Armigeres*, *Aedes* vectors caught in malaria-endemic areas for the presence of *Plasmodium* may be helpful to confirm their role in malaria transmission.

Apart from COI, ITS2 primers are often used to differentiate *Anopheles* species because of their excellent accuracy. In this study, *An. nivipes* was found in West Sumba while *An. baileyi* and *An. barbirostris* were found in West Papua. From reports on the distribution and bionomics of *Anopheles* as a malaria vector, there are no reports of this species in that area. *Anopheles nivipes* has been reported in Sumatera while *An. bailey* in Thailand (Somboon et al. 2020). Furthermore, *An. barbirostris* is an essential vector for the spread of malaria in the country. However, this species has never been reported in West Papua, but only in Kalimantan (Indriyati et al. 2017), Sumatra (Yulidar 2017; Yahya et al. 2020), Java (Ndoen et al. 2010; Hastuti 2011) and Sulawesi (Pinontoan et al. 2017).

The detection of *Plasmodium* in mosquitoes was performed by placing 1-5 head-thorax from the same species in one pool for DNA analysis that allowed more amplification. Once it was detected, each abdomen of the vectors was molecularly examined to determine the number of species carrying parasites in the pool. This method reduces time and budget consumption, especially in a developing country where malaria is usually endemic.

Anopheles is the most suitable host for *Plasmodium*. However, the larvae's absence found in the survey during the malaria outbreak in Sorong district requires an explanation of whether the samples taken were less representative. Also, this is potentially due to other reasons, such as the existence of alternative vectors. The number of mosquitoes collected in this study indirectly affects the results. The more samples in detection, the better it will be in the future. Also, the inability to carry the nitrogen tank to the plane caused ten days collection time in each area to prevent mosquitoes' damage because they were placed at room temperature only.

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