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Original Article

Microscopic and submicroscopic analysis using polymerase chain reaction of asymptomatic malaria in Nunkurus village, Kupang district, Indonesia

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ABSTRACT

Malaria is a parasitic infection disease that is still a world health problem, especially in tropical countries including Indonesia. In malaria endemic areas, the largest proportion of malaria is asymptomatic malaria. Most people with asymptomatic malaria are not diagnosed and do not receive therapy. Asymptomatic malaria sufferers carry gametocytes which play an important role in the transmission of malaria, this situation will result in carrier sufferers or malaria sufferers without clinical symptoms (asymptomatic) at any time can transmit parasites to other people, thus new cases and even extraordinary events (outbreaks) of malaria can occur at unexpected times. This research is an interventional descriptive study with the aim of detecting the presence of asymptomatic malaria in the people of Nun Kurus Village, Kupang Regency, East Nusa Tenggara through the mass blood survey (MBS) work program from Naibonat Public Health Centre, Kupang Regency, and East Nusa Tenggara. The sample in this study was obtained using a total sampling method, namely, all subjects who participated in the MBS activity who were more than 5 years old, with the total of 68 people. Microscopic examination results were compared with submicroscopic examination results using the polymerase chain reaction (PCR) method. Before carrying out PCR, DNA extraction from dried blood spot was carried out using Qiagen Kit followed by detection of *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale* genes in samples based on the nested PCR method from Snounou and continued with electrophoresis. The results of the study found no cases of asymptomatic malaria were detected microscopically, but 13.23% of asymptomatic malaria were detected by PCR. PCR examination results from 68 samples showed 5.88% positive for *P. vivax* and 7.35% positive for *P. vivax* and *P. falciparum* genes (mixed infection), no *P. malariae* and *P. ovale* genes were found.

Keywords: Asymptomatic, malaria, microscopic, submicroscopic

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INTRODUCTION

Malaria is a parasitic infection disease that is still a world health problem, especially in tropical countries including Indonesia. Malaria is still one of the causes of death in both adults and children. According to the World Health Organization (WHO) malaria report (2019), the estimated number of malaria cases in the world has reached 228 million cases. The majority of cases occurred is in the African region (93%), followed by Southeast Asia including Indonesia (3.4%), then the East Mediterranean region (2.1%).[1]

The incidence of malaria decreased globally between 2010 and 2018, from 71 to 51 cases per 1000 population at risk. For

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the Southeast Asia region, the WHO data show a reduction in incidence of 70% from 17 cases per population at risk in 2010, down to five cases in 2018. The number of deaths due to malaria globally in 2018 reached 405,000 deaths, a decrease compared to 2017 of 416,000 and 585,000 in 2010. Children under 5 years of age are the most vulnerable to malaria. In 2018, child deaths due to malaria accounted for 67% of the total deaths from malaria worldwide.[1]

Data from the Indonesian Ministry of Health regarding the achievements of District or City Elimination of Malaria and annual parasite incidence (API) reported that <1 per Province in 2019 while there are three provinces where all districts and cities have achieved malaria elimination, namely, Jakarta, Bali and East Java. East Nusa Tenggara Province is one of the five provinces where there are no districts/cities that have achieved elimination of malaria. The five provinces are Papua, West Papua, Maluku, North Maluku, and East Nusa Tenggara. 86% of cases originated in Papua Province.[2] Final data for the Malaria Program in 2019 from the East Nusa Tenggara Provincial Health Office show that in 2019, the number of positive cases of malaria in East Nusa Tenggara Province was 12,909 cases with API of 2.43 per 1000 population and there were no deaths due to malaria. Based on malaria endemicity data, the NTT Provincial Health Office in 2019, there are three districts classified as high endemicity, namely, West Sumba (API 34.68), East Sumba (API 6.68), and Southwest Sumba (10.37). Kupang Regency is one of the districts with low endemicity with API <1 per 1000 population, namely, 0.23 per 1000 population.[3]

In malaria endemic areas, the largest proportion of malaria is asymptomatic malaria. Asymptomatic malaria infection is poorly understood by most people. Most people with asymptomatic malaria are not diagnosed and do not receive therapy. Asymptomatic malaria sufferers carry gametocytes which play an important role in the transmission of malaria; therefore, new cases and even extraordinary events (Outbreaks) of malaria can occur at unexpected times. Research in malaria endemic areas such as Bangladesh found malaria prevalence of 30.7% with the proportion of asymptomatic malaria cases confirmed by polymerase chain reaction (PCR) was 77.0%, and malaria cases with symptoms was only 19.8%.[4] Research conducted by Santoso, 2010 in West Southeast Maluku showed that the parasite *Plasmodium* sp was found not only in patients with clinical malaria but also in patients without clinical symptoms of malaria (asymptomatic).[5] Research by Arwati *et al*., 2013 in a hypoendemic area in Trenggalek Regency, East Java using the single step PCR method also found asymptomatic malaria, although in active case detection there were no clinical subjects with malaria.[6] The high risk of anemia is also significantly increased in individuals with asymptomatic malaria, as shown in the asymptomatic malaria study in Papua.[7] Microscopic malaria diagnosis is the gold standard recommended by the WHO. However, because parasitemia in asymptomatic malaria is very low and difficult to examine microscopically, the molecular diagnosis by single step PCR in this study was used to confirm the validity of the microscopic diagnosis. Nunkurus Village is one of the villages in the working area of the Naibonat Community Health Center. The area of Nunkurus Village is mostly in the form of rice fields, fields, and forests with several stagnant water which allows it to be a breeding ground for the malaria parasite transmission vector, namely, the *Anopheles* mosquito. Most of the people of Nunkurus Village are rice field farmers. Naibonat Community Health Centre is located in Kupang Regency. Data on Malaria at the Naibonat Community Health Center show that the number of malaria in 2014 was 12 vivax malaria patients and 11 falciparum malaria patients, in 2015 there were three vivax malaria patients and two falciparum malaria patients, in 2016 there were three vivax malaria patients, and two falciparum malaria patients and in 2017, there were six vivax malaria patients and four *Plasmodium falciparum* patients. All sufferers come from the village of Nunkurus.[8] Therefore, in this study, we are interested in detecting asymptomatic malaria in Nunkurus Village, Kupang Regency microscopically, and confirming these results at the submicroscopic level by molecular analysis with PCR.

MATERIALS AND METHODS

This was conducted in Nunkurus Village from July to August 2018. Total sampling method was used which are all subjects who participated in the mass blood survey (MBS) activity, with the age of above 5 years old at the Health Center Naibonat in Nun Kurus Village. Subjects with fever, or had a history of fever in the last 24 h, with or without clinical symptoms of malaria, were examined which included anamneses, physical examination and thick preparations of malaria peripheral blood examination, and Hb examination. Previously, patients were asked to fill out a consent form signed by the subjects or parents for children under 14 years old.

The parasitological examination was in the form of thick and thin blood stained with Giemsa 3% solution. In addition to being dropped on a glass object for making malaria blood smears, the subjects' blood was also dripped on filter paper for making dried blood spots (DBS) for PCR examination. If a *Plasmodium* is found on microscopic examination, the subject will be given DHP treatment in accordance with the malaria case management guidelines issued by the Ministry of Health. The slides were examined microscopically at the Naibonat Health Center. The results of the microscopic examination were compared with the results of submicroscopic examination using the PCR method. Before carrying out PCR, DNA extraction from DBS was carried out using the Qiagen Kit, followed

by detection of the *P. falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale* genes in samples based on the nested PCR method from Snounou and continued with electrophoresis.

This study has received ethical approval from the Health Research Ethics Commission of the Faculty of Medicine, University of Nusa Cendana.

RESULTS

The research sample consisted of 69 slides of malaria blood smear and 68 filter paper containing DBS of subjects who participated in the MBS activity in Nunkurus Village. The basic characteristics of research subjects based on gender were male as much as 34.8% and female as much as 65.2%. The basic characteristics of research subjects based on the age group, namely, the age group <17 years old were 4.3%, the 18–35 years old age group was 42%, the 36–55 years old age group was 34.8%, and the age group of above 56 years old as much as 18.8%. The basic characteristics of research subjects based on age were not normally distributed with $P = 0.036$.

Microscopic Examination Results

Making thick and thin blood smears were done using methanol and then stained with Giemsa 3% for 45 min after the MBS activities were completed at the Naibonat Health Center. The slides were examined microscopically and all slides tested negative for malaria.

PCR Examination Results

In Figure 1a-g, it can be seen that all negative controls appear blank indicating that there is no contamination and all samples

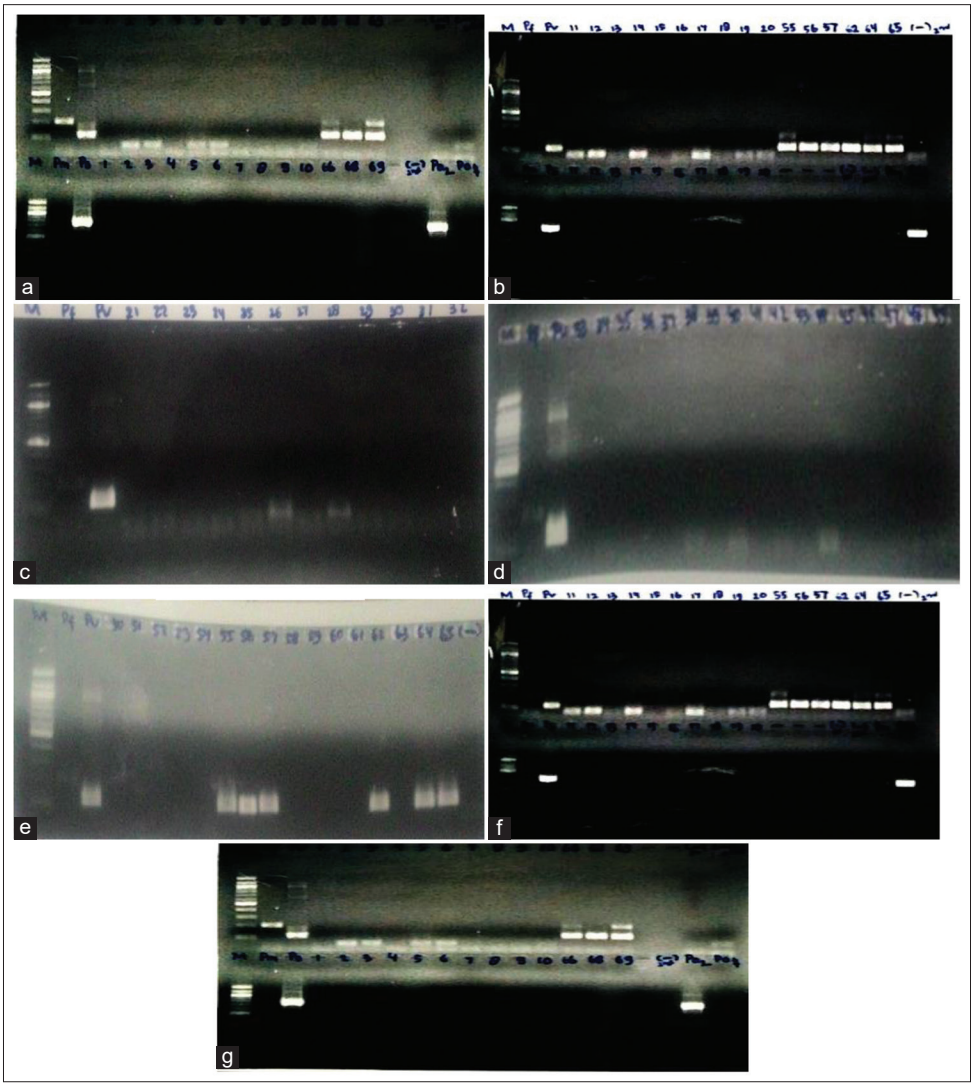

Figure 1: Results of polymerase chain reaction examination of all samples NK01-NK69. (a). Samples NK01-NK10. (b). Samples NK11-NK20. (c). Samples NK11-NK30. (d). Samples NK31-NK40. (e). Samples NK41-NK50. (f). Samples NK51-NK60. (g). Samples NK61-NK69

are negative for malaria. Figure 1f, sample NK 55, NK 56, NK 57, NK 62, NK 64, and NK 65 showed positive for Mix infection of *P. falciparum* and *P. vivax*, *P. vivax*, *P. vivax*, mix infection of *P. falciparum* and *P. vivax* and Mix infection of *P. falciparum* and *P. vivax*, respectively. In addition, Figure 1g depicted that samples NK 66 were positive for both *P. falciparum* and positive for *P. vivax,* while sample NK 68 was positive for *P. vivax*. NK 69 samples were also positive for both *P. falciparum* and *P. vivax*.Furthermore, PCR examination results from 68 samples showed 5.88% positive for *P. vivax* and 7.35% positive for *P. vivax* and *P. falciparum* genes, no *P. malariae* and *P. ovale* genes were found [Table 1].

Comparison of Microscopic and PCR Examination Results

The results of the microscopic examination were compared with the results of PCR examination, which showed a significant difference. All the results of microscopic examination did not identify malaria parasites in all blood smears, while the submicroscopic examination using the PCR method found 13.23% positive samples containing the malaria gene [Table 2].

DISCUSSION

The Health Ministry of Indonesia stated that the highest malaria prevalence rate based in Indonesia was recorded in Eastern Indonesia, namely in West Papua (10.6%), Papua (10.1%), and East Nusa Tenggara (4.4%). Kupang Regency is one of the districts with a fairly high number of malaria cases in East Nusa Tenggara Province. In this study, of the 69 slides examined, none of them were positive when examined microscopically. However, submicroscopically using PCR it was found that 13.23% were positive for malaria. As in other places in Indonesia, Kupang Regency is only known for two seasons, namely, the dry season and the rainy season. In the months of June to September, the wind currents originate from Australia and do not contain much water vapor resulting in a dry season. On the other hand, in December to March, the wind currents contain a lot of water vapor originating from Asia and the Pacific Ocean thus the rainy season occurs. This situation changes every half year after passing the transitional period in April to May and October to November. Considering that Kupang Regency is close to Australia, the wind currents that contain a lot of water vapor from Asia and the Pacific Ocean to the Kupang Regency area have reduced water vapor content which results in less rainy days in Kupang Regency compared to areas close to Asia. This makes Kupang Regency a relatively dry area where only 5 months (January to April and December) are relatively wet and the remaining 7 months are relatively dry.[9] Sampling was conducted on August 2018, which was during the summer. In the summer, the mobility of the population is higher than during the rainy season, both for working in the fields, in the garden, looking for wood in the forest, selling to the market, traveling outside the city, or other activities outside the home. This allows the risk of malaria transmission to occur in the summer. Research conducted by Budarja (2001) in West Sumba Regency states that the high transmission of malaria is partly due to geographical conditions that allow malaria vectors to reproduce, large areas of rice fields that are breeding grounds for malaria vectors, the habit of sleeping at night without using mosquito nets, the habit of being outside the house at night, and the habit of putting animal or livestock pens near housing.[10] These factors facilitate the transmission of malaria vectors. Natural conditions allow many breeding places for *Anopheles* sp. such as forests, rice fields, and abandoned ditches or ditches also support the occurrence of malaria in a place.[10] Nunkurus village, which is the location of this research, is a rice field and forest area that allows *Anopheles* sp. mosquito larvae to thrive in this environment and is very potential as a breeding ground for *Anopheles* mosquitoes. The residents also have livestock in their cages next to the houses. This causes malaria to develop well in this area. Apart from that, people's habits and attitudes toward treatment are also closely related to malaria transmission. In Indonesia, it is common to diagnose and treat yourself for malaria. In fact, people have become accustomed to consuming medicines that can be bought in small shops easily without a doctor's prescription.[11]

Malaria transmission is common in most areas where people have gametocytes in their blood so that the *Anopheles* mosquito

Table 1: Results of PCR examination

PCR result	*P. vivax*	*P. falciparum*	*P. malariae*	*P. ovale*	Mix infection
Positive malaria n/N (%)	4/68 (5.88)	0/68 (0)	0/68 (0)	0/68 (0)	5/68 (7.35)

PCR: Polymerase chain reaction; *P. vivax*: *Plasmodium vivax*; *P. falciparum*: *Plasmodium falciparum*; *P. malariae*: *Plasmodium malariae*; *P. ovale*: *Plasmodium ovale*

Table 2: Comparison of microscopic and PCR examination results

Microscopic (*n*=69)		PCR (*n*=68)	
Positive Malaria, n/N (%)	Negative Malaria, n/N (%)	Positive Malaria, n/N (%)	Negative Malaria, n/N (%)
0	69/69 (100)	9/68 (13.23)	58/68 (85.29)

PCR: Polymerase chain reaction

becomes infected and transmits to healthy people.[12] In the blood smears of all subjects in this study, no malaria parasites were found. However, on submicroscopic examination by PCR, it was found that 13.43% contained the malaria gene. This shows that these subjects are still at risk for transmitting malaria even though clinically the signs and symptoms of malaria are not found/are asymptomatic. In malaria endemic areas, most malaria infections are asymptomatic/asymptomatic. This is because people in endemic areas have a higher immune system against malaria than those in non-endemic areas.

The high prevalence of asymptomatic malaria in endemic areas requires a serious attention because asymptomatic malaria can also be transmitted. Those with asymptomatic malaria do not seek treatment to treat the infection, so they become a reservoir for the parasite. Asymptomatic infection is also responsible for its ill-defined health effects. The previous studies have found a correlation between asymptomatic infection and anemia, thrombocytopenia, and inhibited cognitive function, while the association with nutritional status has yielded conflicting results. Asymptomatic malaria is difficult to diagnose due to the lack of clarity of clinical manifestations and is often not detected by microscope and most of it can only be confirmed by PCR.[11-13]

Starzengruber *et al*. (2014) studied malaria in endemic areas such as Bangladesh, which found malaria prevalence of 30.7% with the proportion of asymptomatic malaria cases confirmed by PCR was 77.0%, and malaria cases with symptoms was only 19.8%. The high risk of anemia is also significantly increased in individuals with asymptomatic malaria, as shown in the asymptomatic malaria study in Papua. There are currently no guidelines regarding the management of asymptomatic malaria in Indonesia. Further research is needed to analyze the causes of asymptomatic malaria in Kupang Regency, especially in Nunkurus Village, so that the target of malaria elimination by 2030 in Indonesia, especially in East Nusa Tengga, can be achieved.

CONCLUSION

In this study, of the 69 slides examined, none of them were positive for malaria when examined microscopically. However, submicroscopically using PCR it was found that 13.23% were positive for malaria.

ACKNOWLEGMENTS

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CONFLICT OF INTERESTS

There is no conflict of interests found during this study.

ETHICS

This study has received ethical approval from the Health Research Ethics Commission of the Faculty of Medicine, University of Nusa Cendana.

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