ABSTRACT

Trenggalek district is one of hypoendemic areas of malaria in East Java Province. Most of the malaria cases are imported from endemic areas outside of Java Island, as the destination of the seasonal migratory workers. When they return to Trenggalek district usually they are infected with malaria. Blood examination has been done to the patients who have recovered from malaria infection in order to find out the asymptomatic malaria in this region. The examination of Giemsa-stained blood smear is a gold standard method recommended by WHO. However, due to the very low parasitemia in asymptomatic malaria makes difficult in microscopic examination, the molecular diagnosis by single step PCR in this research is aimed to confirm the results of microscopic examination of asymptomatic malaria. The objective of this paper was to find out malaria asymptomatic in Dongko sub-district, Kabupaten Trenggalek district by microscopic examination and single step PCR methods. Blood samples were collected during May and September 2012 from the patients who have recovered from malaria infection. The number of sample was according to the list of patients hospitalized in Pandean public health service (Puskesmas Pandean), Dongko sub-district, Pande district, East Java province. Microscopic diagnosis was done on Giemsa-stained thick and thin smears at 1000x magnification. Molecular diagnosis was done by single step PCR method according to Patsoula et al (2002). During May and September 2012, asymptomatic malaria have been found, where of the 18 samples, 5 samples were positive by microscopic examination two for P. falciparum, two for P. vivax, one for both of species, and 13 samples were negative. Single step PCR diagnosed two samples P. falciparum, two samples as P. vivax, 6 samples as mix infection and 6 as negative samples. In conclusion, single step PCR provides a sensitive tool for detection extremely low level of parasitemia in asymptomatic malaria. Mass screening of asymptomatic malaria is needed to be performed simultaneously in collaboration with research institute or research hospital prior to mapping the cases and early medication. The presence of asymptomatic malaria cases is a challenge for the management of malaria elimination program in any endemic area including Trenggalek District. (FMI 2013;49:150-154)

Keywords: Asymptomatic malaria, single step PCR, Trenggalek district

Correspondence: Heny Arwati, Department of Parasitology, Faculty of Medicine, Airlangga University, Surabaya, Indonesia.
INTRODUCTION

Malaria remains a serious global health burden, with an annual incidence of 247 million cases and nearly one million deaths in Africa (WHO 2008). Of the four human malaria parasites species, *Plasmodium falciparum* is reported to cause the highest morbidity and mortality. Young children and pregnant women are particularly vulnerable to this disease and considered to be the highest risk populations for malaria-related death (UNICEF 2000). However, malaria mortality rates have been reduced by 45% globally, and by 49% in Africa since 2000 (WHO 2013). In countries where access to malaria control intervention has improved most significantly, overall child mortality rates have fallen by approximately 20% (WHO 2012). The severity of *P. falciparum* infection ranges from severe and complicated, to mild and uncomplicated, to asymptomatic (Marsh et al 1995). Understanding the impact of malaria on human host is critical to improve the management of the disease.

Malaria in Indonesia remains a major health problem. Although malaria control program has successfully decreased malaria incidence in some areas, however, due to rapid people movement from Java Island to hyper and mesoendemic areas of malaria in outside of Java Island, and vice versa, cause malaria reemerge in Java Island. Trenggalek district is one of hypendemic areas of malaria in East Java Province, where most of the malaria cases are imported from outside of Java Island. According to the data of Pandean Public Health Service (PHC), the people of this district accustomed to work in Kalimantant and Sumatra Islands and return to the district infected with malaria, and mostly caused by *P. falciparum*, *P. vivax*, and mix of both species (Ministry of Health, District Trenggalek 2012).

Commonly, people seek for malaria medication when clinically develop the symptoms of malaria, such as fever, chill, headache and splenomegali, and revealed by the finding of parasites on Giemsa-stained thick and thin smears of peripheral blood. During May and September 2012, in remote areas of Dongko Sub District, Trenggalek District, active case detection found no people with clinical symptoms of malaria, however, surprisingly when microscopic examination was done malaria parasites were found (asymptomatic malaria). This finding was then confirmed by single step PCR method using a modification of technique originally described by Patsoula et al (2003) with slight modification.

Detection of malaria by light microscopy of Giemsa-stained thick and thin smears is the gold standard of laboratory method for the diagnosis of malaria. Microscopic examination is low cost, requires much labor by well trained-personnel, and able to detect malaria as long as parasitemia level is high, but very low parasitemia level is undetectable by microscopic examination. Polymerase chain reaction (PCR) is a sensitive method for the detection and identification of parasites in malaria patients. Numerous experiments on malaria diagnosis and identification of four species of *Plasmodium* based on PCR using various primers have been reported, including conventional PCR, nested PCR (Johnston et al 2006) and real time PCR (Coleman et al 2006). Single step PCR developed by Patsoula (2003) is a kind of multiplex PCR which used genomic DNA and several pairs of primers within a tube to differentiate *P. falciparum* and *P. vivax*. Based on this method, the asymptomatic malaria cases in the people of Dongko Sub District, Trenggalek District in East Java Province have been detected.

MATERIALS AND METHODS

Blood samples were collected from respondents who have 3-6 months recovered from malaria infection by vena puncture in tube containing EDTA to prepare thick and thin smears, and blood spot on filter paper. Sampling areas were villages within Pandean PHC in Dongko Sub District, Trenggalek District, East Java Province, Indonesia. Respondents in this research were the residences with histories in malaria infections, in traveling to malaria endemic areas in outside of Java Island based on the data collected by Pandean PHC during 2011 and 2012. Giemsa stained-thin and thick blood smears were examined to detect and identify the species and stage of malaria parasites at magnification of x1000 (Olympus CX21, Tokyo, Japan)). Examination of blood smears were done in longitudinal fields before they were stated as negative sample.

Parasites DNA was isolated by chelex-100. Blood spots were cut from filter papers and soaked in 0.5% Saponin for 30 minutes, washed in PBS and then 200 µl of 50% chelex-100 were added. After centrifugation at 12,000 rpm for 30 second, the supernatant containing DNA were then collected in a fresh tube and used as template in PCR. Single step PCR used in this study was based on Patsoula et al (2003). Primers used in this PCR were based on the sequences of small-sub unit ribosomal RNA (ssu-rRNA) of *P. falciparum* and *P. vivax*. Three primers were used in a single tube of PCR mixture. The complete sequences of primers used in single step PCR were as follow:

PL3 5ATG GCC GTT TTT AGT TCG TG3
PL4 5GGA AAC GGT ACG ATA AGC CA3
PL5 5ACG CGT GCA GCC TAGTTT AT3
PL3 primer amplifies sequence common to rRNA of *P. falciparum* and *P. vivax*. PL4 primer is specific for *P. vivax* and PL5 is specific for *P. falciparum*. Together, PL3 and PL4 produced a 266 bp product specific for *P. vivax*, whereas PL4 and PL5 produced a 346 bp product specific for *P. falciparum*. This PCR was performed to detect the species of Plasmodium. Total volume of PCR mixture was 20 µl containing 12.5 µl of 2x PCR mixture (Intron, Singapore), 5 µl of DNA template and 20 pmol of each primer. The single step PCR was run with an initial denaturation at 94°C for 5 minutes was followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension was at 72°C for 30 seconds. PCR products were subjected to electrophoresis on 2% gel agarose, the bands were then visualized under UV illuminator and documented using gel doc (Biostep, Germany).

**RESULTS**

Of the 18 samples, 5 samples were positive by microscopic examination: one for *P. falciparum*, three for *P. vivax*, one for mix of both species, and 13 samples were negative. The *P. falciparum* banana-shape gametocyte stage was detectable easily in peripheral blood by microscopic examination. Ring form stage of *P. vivax* on thick and thin smears was similar to that of *P. falciparum* and often misdiagnosed, however, single step PCR was able to distinguish ring form stage of these two species. Single step PCR diagnosed two of *P. vivax* infections as mix infection, and of the 13 negative samples, three were mix infection.

**Table 1. Comparison of microscopic examination and single step PCR for detection of Plasmodium species in asymptomatic malaria**

<table>
<thead>
<tr>
<th>Species</th>
<th>Microscopy</th>
<th>Single step PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pf</td>
<td>Pv</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mix</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>13</td>
<td>2</td>
</tr>
</tbody>
</table>

The representative picture of electrophoresis result of PCR products is shown on Figure 1. Single step PCR resulting in a 346 bp band for *P. falciparum* and as 266 bp for *P. vivax*. Mix infection of both species as shown as two bands of both 346 bp and 266 bp. However, the band of *P. vivax* was very faint, therefore, could not be shown in this picture (Table 1).

**DISCUSSION**

In malaria endemic areas, a significant proportion of children without presenting clinical signs of malaria are considered as asymptomatic cases (Greenwood 1987). In remote areas such Dongko sub District in Trenggalek District where individuals have difficulty in reaching malaria clinic, active surveillance provides a tool for detection of asymptomatic malaria. Parasitemia rates in asymptomatic patients are often quite low and often undetectable by microscopic examination. When symptomatic patients show a relatively high parasitemia rates microscopy examination can provide an accurate diagnosis prior to medication. However, microscopy accuracy can decrease significantly at low parasitemia level as seen in asymptomatic patients. In this case, PCR provides a sensitive tool for detection extremely low level of parasitemia.

Interestingly, by this single step PCR mix infection was detected in one tube reaction without any further step. The single step PCR was able to differentiate *P. falciparum* and *P. vivax* as shown as two bands of 346 bp for *P. falciparum* and 266 bp for *P. vivax* (Figure 1). This single step PCR proved a more sensitive and faster detection method to detect asymptomatic malaria in Dongko Sub District, Trenggalek District. Single step PCR method described by Patsoula et al (2003) is relatively simple, accurate and can be used to process many samples concurrently, gives results within 4 hours, and has low detection threshold particularly for *P. falciparum*. Its reasonable performance when used to test dry blood spots collected on filter paper is encouraging, indicating its potential usefulness in large-scale field studies.

Comparison of PCR and microscopic examination for detection of asymptomatic malaria in Thailand showed that PCR is a less subjective test than microscopy and PCR is a viable method for conducting active malaria surveillance (Coleman et al 2006). However, PCR is too expensive for daily use in a remote area. A collaboration work with a research institute or research hospital is suggested for the successful detection and mass screening of asymptomatic malaria in a remote area where malaria is endemic.

Asymptomatic malaria were frequently described in high and middle transmission in Ghana (Crookston et al 2010), Kenya (Laishram et al 2012, Coleman et al 2002) and Burma (Richards et al 2007). In recent years asymptomatic malaria have been found in low transmission areas in Colombia (Roper et al 2000) and Solomon Island (Gray et al 2013). In high transmission areas, continuous exposure to malaria parasites leads to partial immunity and consequently creates
Asymptomatic malaria in a given population (Staalsoe & Hviid 1998). Asymptomatic malaria provides a fundamental reservoir of parasites and they become gametocyte carriers, contributing the persistence of malaria transmission (Bousema et al 2004). Specific immunity to genetic variant is one of the key components to explain the low level parasitemia during the extended periods without clinical symptoms (Staalsoe & Hviid 1998). Hosts genetic variants of mannose binding lectin (MBL), tumor necrosis factor α (TNF-α) and inducible nitric oxide synthase 2 (NOS2) loci have been associated with resistance/susceptibility to clinical malaria (Luty et al 1998). Furthermore, it has been suggested that, long term asymptomatic malaria could lead to anemia (Nagel & Roth 1989).

In areas of seasonal malaria transmission, malaria cases usually increase after beginning of rainy season and rare during dry season (Males et al 2012). But, in case of Trenggalek district, fluctuation of malaria cases depends on the seasonal people movement to and from endemic areas in outside of Java Island. Temporary jobs in Kalimantan, Papua, Maluku, Sumatra and other islands, are not only increasing their income but also increasing the malaria cases in their home town, because they return to home town usually are infected with malaria. Malaria cases increase in the district short after they return and very rare after several months until this finding of asymptomatic malaria cases.

The symptomless but infected individuals could keep malaria spreading. On this basis, epidemiological studies of alternative control measures, which may even contemplate treatment of entire populations in remote, isolated areas are urgently needed (Alves et al 2005). The treatment of asymptomatic malaria cases in Brazil, gave the effect in decreasing malaria incidence when no clinical cases were observe in the locality, indicating that the local parasites pool was only represented by the parasites circulating in the asymptomatic parasites carriers (Tada et al 2012). Therefore, the presence of asymptomatic malaria cases is a challenge for the management of malaria elimination program in any endemic area (Zoghi et al 2012) including Trenggalek District of East Java Province in Indonesia. Mass screening of asymptomatic malaria is needed to be performed simultaneously in collaboration with research institute or research hospital prior to mapping the cases and early medication. The presence of asymptomatic malaria cases is a challenge for the management of malaria elimination program in any endemic area including Trenggalek District of East Java Province in Indonesia.

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